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Award Number: DAMD17-02-1-0264

TITLE: Treatment of Breast Cancer with Antibodies Against DR4 and DR5 Receptors in Combination with Chemotherapy

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REPORT DATE: June 2005

TYPE OF REPORT: Final

20060125 005

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE		Form Approved OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-06-2005	Final	1 Jun 2002 – 31 May 2005

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Treatment of Breast Cancer with Antibodies Against DR4 and DR5 Receptors in Combination with Chemotherapy	5b. GRANT NUMBER DAMD17-02-1-0264
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Donald J. Buchsbaum, Ph.D.	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
University of Alabama at Birmingham Birmingham, AL 35294-0109	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT-SEE ATTACHED PAGE

15. SUBJECT TERMS
Breast cancer, immunotherapy, chemotherapy, death receptors

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	62	19b. TELEPHONE NUMBER (include area code)

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Abstract

The overall goal of this proposal is to determine the therapeutic potential of apoptosis-inducing anti-human DR5 and DR4 antibodies, alone or together, in combination with chemotherapeutic drugs with activity against breast cancer, for the treatment of metastatic breast cancer. Aim 1 was to determine the expression profile in human breast cancer cell lines of DR5 and DR4 before and after treatment with anti-DR5 and -DR4 MAb alone, together, and in combination with chemotherapy drugs. Aim 2 was to determine the expression profile of DR5 and DR4 during the progression of breast cancer. Aim 3 was to determine the cytotoxicity of anti-DR5 and -DR4 antibodies against human breast cancer cells alone, together, and in combination with adriamycin or paclitaxel. Aim 4 was to determine the therapeutic efficacy and toxicity of anti-DR5 and -DR4 antibodies against human breast cancer xenografts alone, together, and combined with adriamycin or paclitaxel. We investigated mTRA-8 anti-DR5 antibody cytotoxicity alone and in combination with several chemotherapy and biomodulating drugs and identified which combinations resulted in synergistic cytotoxicity. These combination treatments were then tested in nude mice with subcutaneous breast cancer xenografts. We also showed that bioluminescence imaging can be used to evaluate tumor response in mammary fat pad and disseminated breast cancer models. Mechanistic studies of combination treatment were carried out both *in vitro* and *in vivo*. Expression of DR5 was determined by immunohistochemistry to be present in human biopsy specimens of breast cancer and in adjacent normal breast epithelium.

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1.   Buchsbaum DJ, Zhou T, Grizzle WE, Oliver PG, Hammond CJ, Zhang S, Carpenter M, LoBuglio AF: 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. *Clin Cancer Res* 9:3731-3741, 2003.
2.   Ohtsuka T, Buchsbaum D, Oliver P, Makhija S, Kimberly R, Zhou T: Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway. *Oncogene* 22: 2034-2044, 2003.

INTRODUCTION

The purpose of the work carried out during the three years of this project was to evaluate the expression of DR4 and DR5 antigens in a panel of human breast cancer cell lines, and to evaluate the cytotoxicity of TRA-8 (anti-DR5) and 2E12 (anti-DR4) antibodies against the panel of cell lines alone or in combination with adriamycin, paclitaxel, or other chemotherapeutic or biomodulating agents. Furthermore, we were interested whether exposure of human breast cancer cells to chemotherapy drugs changed the expression of DR4 or DR5. Most importantly, we wanted to evaluate whether combination treatment of breast cancer xenografts with TRA-8 or 2E12 and chemotherapy or biomodulating drugs resulted in increased therapeutic efficacy compared to antibody or drug treatment alone, and to elucidate the mechanisms involved. In addition, we evaluated the use of bioluminescence imaging to monitor tumor response to treatment in both mammary fat pad and disseminated breast cancer models. Expression of DR5 was determined in human breast cancer biopsy specimens and adjacent normal tissue.

BODY

1.   DR5 expression and TRA-8 induced cytotoxicity in breast cancer cell lines. As illustrated in **Figure 1A**, all nine breast cancer cell lines were DR5 positive with varying degrees of expression from strongly positive (LCC6 and MDA-MB-453) to weakly positive (MDA-MB-468 and SK-BR-3). **Figure 1B** illustrates the TRA-8 induced cytotoxicity of the nine cell lines. Four cell lines were sensitive to TRA-8 induced cytotoxicity with IC_{50} concentrations of 17 to 299 ng/ml (LCC6, 2LMP, MDA-MB-231, MDA-MB-468), while others were quite resistant (DY36T2, BT-474, 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.

TRA-8 effects on chemotherapy-induced cytotoxicity were then examined with adriamycin **(Figure 2A)** and paclitaxel **(Figure 2B)**. An analysis to test for interaction between antibody and drug effects 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.

Table 1. *In Vitro* Interaction Effects for Combination Treatments

Cell Line	TRA-8 + adriamycin		TRA-8 + paclitaxel	
	Interaction	p-value[a]	Interaction	p-value[a]
LCC6	Synergistic	<0.001	Additive	0.624
MDA-MB-453	Synergistic	<0.001	No response[c]	0.615
2LMP	Additive	0.153	Additive	0.937
MDA-MB-231	Additive	0.663	Additive	0.064
BT-474	Synergistic	<0.001	ND[b]	0.992
ZR-75-1	Synergistic	0.013	Additive	0.172
DY36T2	ND[b]	0.808	ND[b]	0.798
MDA-MB-468	Additive	0.184	Additive	0.724
SK-BR-3	Additive	0.361	No response[c]	0.871

[a] p-value 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-value is not < 0.05 then the combination effects were considered additive.
[b] Not determined because the TRA-8 effect was not significant, but the adriamycin/paclitaxel effect was significant.
[c] There was no significant dose response for either agent.

2.   DR4 expression and cytotoxicity of 2E12 and adriamycin against breast cancer cell lines. We detected DR4 expression on the surface of nine human breast cancer cell lines using 2E12 anti-DR4 monoclonal antibody and flow cytometry, as illustrated in **Figure 3**. DR4

levels ranged from strongly positive on 2LMP to weakly positive on SK-BR-3 cells. We investigated the cytotoxic effects of 2E12 in combination with chemotherapeutic drugs against the panel of breast cancer cell lines. In the presence of cross-linking secondary antibody, treatment with 2E12 in combination with adriamycin produced additive cytotoxicity against 2E12 sensitive 2LMP, and LCC6, MDA-MB-231, and MDA-MB-468 cells, which were moderately sensitive to 2E12 alone, as shown in **Figure 4**. Combination treatment with 2E12 and adriamycin produced synergistic killing of three 2E12 resistant cell lines (**Figure 4**), resulting in 85 to 95% killing of BT-474, DY36T2 and MDA-MB-453 cells. However, three other breast cancer cell lines, MCF-7, SK-BR-3 and ZR-75-1 cells, remained resistant to 2E12 following treatment with 2E12 and adriamycin. These results demonstrate that DR4 expression is not sufficient for 2E12-mediated cytotoxicity, alone or in combination with adriamycin treatment. We previously demonstrated that adriamycin and mTRA-8 combination treatment produced synergistic cytotoxicity against ZR-75-1 and additive cytotoxicity against SK-BR-3 cells (Buchsbaum et al., 2003). Taken together, these findings indicate that individual breast cancer cell lines may differ in sensitivity to DR4 (2E12) and DR5 (mTRA-8) antibodies, despite cell surface expression of both death receptors, and that adriamycin-dependent sensitization to one death receptor antibody does not predict response to the other antibody. The mechanisms responsible for the differential sensitization to mTRA-8 and 2E12 by adriamycin have not yet been determined.

3.   **Cytotoxicity of mTRA-8 in combination with other chemotherapeutic and biomodulating drugs against breast cancer cell lines.** We investigated several other drugs in combination with mTRA-8 against human breast cancer cell lines, in an effort to identify which agents produce synergistic killing with mTRA-8. The most effective combinations were evaluated in animal model studies. mTRA-8 treatment in combination with adriamycin and drugs with COX-2 inhibitory activity, including sulindac sulfide (SS) and indomethacin, resulted in increased cell killing of some breast cancer cell lines. Sulindac sulfone (SSF), a sulindac metabolite that does not inhibit COX-2 enzyme activity but may reduce COX-2 enzyme levels, exhibited a similar spectrum of activity compared to SS and indomethacin in combination treatments with mTRA8 and/or adriamycin against DY36T2 breast cancer cells (**Figure 5**). However, SSF and indomethacin were less effective than SS in the triple combinations using BT-474 breast cancer cells (**Figure 6**). Other drugs with more specific COX-2 inhibitory activity, such as NS-398 and nimesulide, exhibited similar cytotoxic activity compared to SS and indomethacin in the triple combinations with DY36T2 cells, but were less effective against BT-474 cells. These findings suggest that both COX-2 dependent and independent mechanisms may contribute to the increased sensitivity to mTRA-8 in cells treated with these agents, and the results provide mechanistic information on mTRA-8-mediated cell killing.

        We investigated the cytotoxic effects of mTRA-8 in combination with velcade against 2LMP, LCC6 and BT-474 breast cancer cells, which are very sensitive, moderately sensitive or resistant to mTRA-8, respectively. Treatment for 48 h of 2LMP and LCC6 cells with 5 nM velcade resulted in ~50% cytotoxicity, whereas ≥90% of the cells were killed at 10 nM doses. The addition of 1 ng/ml (2LMP) or 5 ng/ml (LCC6) mTRA-8 to cells during the last 24 h of velcade exposure resulted in modestly increased cell killing (**Figure 7**). In contrast, BT-474 cells were relatively resistant to velcade, but there was significantly increased killing of BT-474 cells treated with 50-100 nM velcade for 24 h followed by 24 h exposure to 25 ng/ml mTRA-8 and drug (**Figure 7**). These findings support the concept that mTRA-8 resistant cells may become sensitized to the antibody treatment following proteasome inhibition.

        Concurrent treatment of BT-474 cells with mTRA-8 and 100 µM troglitazone or 25 µM 15d-PGJ$_2$ for 24 h resulted in highly synergistic cell killing (**Figure 8**). Particularly striking results were obtained using 25 µM 15d-PGJ$_2$, which was nontoxic as a single agent, but in combination with 25 ng/ml mTRA-8 resulted in ≥90% cytotoxicity. Combination treatment of LCC6 cells with 50-100 µM troglitazone and mTRA-8 resulted in additive increases in cell killing

(data not shown). In contrast, combination treatment of DY36T2 cells, a mTRA-8 resistant cell line, with 75-100 µM troglitazone and mTRA-8 resulted in synergistic cytotoxicity and ~90-95% cell killing at doses of 25 ng/ml mTRA-8 and 100 µM troglitazone (data not shown). Combination treatment of LCC6 or DY36T2 cells with ciglitazone and mTRA-8 produced less cytotoxicity than that obtained with troglitazone and mTRA-8 (data not shown). Addition of adriamycin to combination regimens with mTRA-8 and troglitazone produced less than additive cytotoxicity (data not shown).

We investigated other biomodulating agents, including lovastatin, pravastatin, simvastatin, resveratrol, pentoxifylline and nitrolinoleate, based on reports that these drugs may increase sensitivity to TRAIL, induce alterations in cell membranes, or affect pathways that may influence cell survival. Cells were exposed to combinations of these drugs and mTRA-8, both with and without chemotherapy, in regimens that involved 24 h drug pretreatment, followed by 24 h treatment with mTRA-8 and drug, or 24 h concurrent treatment with drug and antibody. Modestly increased cytotoxicity occurred against BT-474 cells following treatment with triple combinations of mTRA-8, adriamycin and lovastatin, resveratrol or nitrolinoleate (data not shown). However, drug and antibody combinations resulted in less than additive killing. Increasing concentrations of chemotherapeutic or biomodulating drugs increased drug-specific killing but did not significantly enhance cytotoxicity in combination with mTRA-8, suggesting that increased drug toxicity did not translate to more efficient killing mediated by the death receptor antibody (data not shown).

We investigated the cytotoxic effects of gemcitabine and mTRA-8 against 9 human breast cancer cell lines. **Figure 9** shows that the combination treatment produced greater than additive killing of LCC6 cells, which were very sensitive to gemcitabine alone, and gemcitabine resistant 2LMP cells. Combination treatment with gemcitabine and mTRA-8 produced additive cytotoxicity against MDA-MB-468 cells, which were moderately sensitive to gemcitabine alone, but gemcitabine had no effect on TRA-8 cytotoxicity against 6 other breast cancer cell lines (BT-474, DY36T2, MDA-MB-453, MDA-MB-231, SK-BR-3, and ZR-75-1) which were resistant to the drug (data not shown). Thus, it is not possible to predict which combinations of chemotherapeutic drugs and death receptor antibodies will produce additive or synergistic cytotoxicity against breast cancer, and therefore specific antibody and drug combinations must be identified for individual cell lines to maximize cell killing. Detailed mechanistic studies will be required to explain synergistic cytotoxicity of death receptor antibodies and chemotherapeutic agents against breast cancer cells. In addition, these findings suggest that tailoring specific combination treatment regimens to particular patients may significantly improve the therapeutic response to mTRA-8 or 2E12 and chemotherapy in future clinical trials.

The results of *in vitro* cytotoxicity assays described above demonstrated that chemotherapy enhanced mTRA-8 killing of mTRA-8 sensitive and resistant breast cancer cells. One possible mechanism for this enhancement involves drug-induced elevation in ceramide levels by chemotherapeutic drugs, including adriamycin, gemcitabine, paclitaxel and sulindac sulfide. Therefore, we investigated the effects of combination treatment of mTRA-8 resistant breast cancer cells with mTRA-8 and additional drugs known to alter ceramide production or metabolism, including 4-hydroxyfenretinimide (4-HPR), tamoxifen, exogenous short chain ceramide, and inhibitors of ceramidase and glucosylceramide synthase. BT-474 cells were also treated with mTRA-8 and combinations of drugs known to affect different ceramide pathways, such as 4-HPR and tamoxifen, to target *de novo* ceramide production and block ceramide conversion to non-cytotoxic derivatives, respectively. Combination treatment with 4-HPR and mTRA-8 produced greater than additive cytotoxicity, with 47% killing of BT-474 cells treated for 24 h with 10 µM 4-HPR and 1 µg/ml mTRA-8, as illustrated in **Figure 10A**. Treatment with 10 µM tamoxifen produced 25% killing alone and 46% in combination with mTRA-8, whereas treatment with 20 µM tamoxifen alone resulted in 80% cell death. Combination treatment of BT-474 cells with 10 µM 4-HPR, 10 µM tamoxifen and 1 µg/ml mTRA-8 resulted in 68% cytotoxicity.

In comparison, combinations of mTRA-8, the ceramidase inhibitor LCL-102, and 4-HPR, tamoxifen or exogenous C6-ceramide resulted in 61 to 95% cell killing of BT-474 cells following 24 h treatment (**Figures 10A** and **10B**). Taken together, these results demonstrate that mTRA-8 resistant breast cancer cells may be sensitized to mTRA-8-induced killing by drug combinations that target ceramide metabolism.

In summary, additive to synergistic cytotoxicity was produced by combination therapies against cells that are moderately sensitive to resistant to mTRA-8. In contrast, combination therapies resulted in less than additive *in vitro* cytotoxicity against mTRA-8 sensitive cells. Taken together, these findings suggest that combinations of death receptor antibody, standard chemotherapeutic drugs, and biomodulating drugs to target multiple cell signaling pathways may have potential utility in developing more effective antitumor therapies *in vivo*. Future studies will investigate the molecular mechanisms involved in the induction of apoptosis by these combination regimens.

4. ***In vitro* synergistic mechanisms of combination cytotoxicity with mTRA-8 and adriamycin or cisplatin.** We found that combination treatment with mTRA-8 and adriamycin or cisplatin resulted in enhanced cytotoxicity against MDA-MB-231 breast cancer cells compared to single agent therapy. Elevated activity of MKK4 was observed following treatment with mTRA-8 and adriamycin or cisplatin, resulting in increased phosphorylation and synergistic activation of JNK and p38 MAP kinase but not ERK1/2. Enhanced cleavage of caspases 8, 9 and 3 occurred in cells with activated JNK and p38 and resulted in increased cleavage of PARP and Bid. The combination treatment increased activation of the mitochondrial apoptosis pathway relative to single agent therapy as demonstrated by enhanced release of cytochrome c and Smac/DIABLO from mitochondria and loss of mitochondrial membrane potential. These findings suggest that combinations of death receptor antibody and chemotherapy may synergistically activate pro-apoptotic signaling pathways. It appears that the death receptor and mitochondrial apoptosis signaling pathways may converge at the level of JNK and p38 and thus provide a positive feedback loop amplifying caspase 8 and 9 signals initiated by mTRA-8 and chemotherapeutic drugs.

In additional studies, TRA-8 induced caspase-3, 8 and 9 cleavage in 2LMP and LCC6 cells at 2-3 h, but no activation of caspases was seen in BT-474 cells at 3 h (**Figure 11A**). Caspase activation correlated with TRA-8 cytotoxicity in each line. When cells were pretreated with adriamycin or velcade for 24 h, activation of caspases-3, 8 and 9 was seen in all three cell lines, with a greater induction in combination with TRA-8 treatment. Cleaved Bid, a product of caspase-8 activity, and poly (ADP-ribose) polymerase (PARP), a product of caspase-3 activity, were seen in 2LMP and LCC6 cells treated with TRA-8 alone and combination with either drug (data not shown). BT-474 cells showed Bid and PARP cleavage only with the combination treatment. These results show mechanistic differences between cell types of varying sensitivity to mTRA-8 and synergistic enhancement of caspases, especially in resistant cells.

XIAP, a member of the IAP apoptosis inhibitor family of proteins, strongly inhibits caspase-3 and caspase-9 activity. XIAP levels in cells treated with mTRA-8 alone or in combination with chemotherapy were determined using Western blot analysis. XIAP remained unmodulated in 2LMP and LCC6 cells, but was decreased by combination treatment with TRA-8 and adriamycin or velcade in BT-474 cells. This indicates XIAP may be significant in the resistance of the BT-474 cells to individual therapies and the synergistic induction of apoptosis with combination therapy (**Figure 11B**).

The Bcl-2 family of proteins is important in the activation of the mitochondrial cell death pathway, and certain members of the Bcl-2 family of proteins are important in drug resistance. We examined the expression of the pro-apoptotic Bax and the anti-apoptotic Bcl-2 and Bcl-XL. No change was detected in these proteins with any treatments. The basal levels of expression may still be important in predicting treatment response, but their modulation is not important in the synergistic induction of apoptosis in these cell lines.

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Smac/DIABLO is a pro-apoptotic protein released from the mitochondria following certain apoptotic stimuli. No change was detected in Smac/DIABLO levels following TRA-8, adriamycin or combination treatment in TRA-8 sensitive or resistant cells. However, examining in cytosolic versus mitochondrial fractions may determine a difference in localization of existing Smac/DIABLO in cells following treatment.

To determine the effect of duration and sequence of treatment, we used Western blot analysis to detect caspase-3 levels in 2LMP cells following TRA-8 and velcade treatment. Cells were exposed to three concentrations of velcade for 21 h prior to TRA-8 treatment that lasted 3 h. Another set of cells received concurrent TRA-8, velcade or combination therapy for 24h. 2LMP cells which received sequential treatment showed a greater induction of caspase-3 cleavage products. Concurrently treated cells showed little caspase cleavage, and no active products were seen.

5. *In vivo* **anti-tumor effects of TRA-8 alone or in combination with chemotherapy and/or radiation.** TRA-8 at doses of 200 μg and 600 μg twice a week for 6 doses produced a similar inhibition of tumor growth for well-established 2LMP *s.c.* tumors (**Figure 12**). In three additional independent experiments, the 200 μg dose/schedule produced statistically significant inhibition of tumor growth (p<0.004, Kruskal-Wallis test on tumor doubling times) compared to untreated controls, and this dose and schedule was selected for further studies. **Figure 13** illustrates the effects of TRA-8, adriamycin, or a combination of TRA-8 and adriamycin on anti-tumor efficacy. As compared to untreated controls, therapy with TRA-8 alone or TRA-8 plus adriamycin produced significant inhibition of tumor growth (p=0.002 Kruskal-Wallis test), while 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 which produced no complete regressions (p<0.001, Fisher exact test). *In vivo* TRA-8 and adriamycin synergism was evaluated using an early growth curve analysis. 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 (**Figure 14**). As compared to 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 than paclitaxel alone (p=0.008) and produced 3/8 complete regressions as compared to 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}Co radiation as single agents and in various combinations as illustrated in **Figure 15**. 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}Co produced tumor growth inhibition that was significantly different from all other treated groups, while both dual therapy groups (adriamycin plus TRA-8 or ^{60}Co plus TRA-8) were different from either single agent group (p < 0.001). The ^{60}Co animals treated with radiation alone did not differ from untreated controls (p=0.926). All two-way treatment combinations had significant synergistic effects (p<0.001). Complete regressions were seen in 6/8 animals receiving triple therapy and 4 animals did not have tumor recurrence over 180 days of follow-up.

The *in vivo* anti-tumor studies in aggregate 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}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 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 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 achieved complete regression, and 28 of these received TRA-8 alone or in combination with other modalities. Complete regression occurred in 1/42 control animals: 1/54 animals receiving chemotherapy, radiation, or a combination; and 28/68 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, 14/68 animals receiving TRA-8 or TRA-8 combinations did not have tumor re-growth compared to 1/42 controls and 0/52 animals treated with chemotherapy and/or radiation. The relapse-free regressions had observation periods of 99 to 171 days (146 ± 24 days).

Table 2. Aggregate Results of Doubling Time and Complete Regression of 2LMP Tumors

Treatment	# of Animals	Tumor Doubling Time (days) (mean/median)	Complete Regressions		
			Total (%)	No relapse (%)	Mean Observation Period (days)
Untreated Controls	44 (42)[a]	12/8	1 (2%)	1 (2%)	177
[60]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]Co	8 (8)	39/36	1 (13%)	0	197
TRA-8	30 (26)	47/23	6 (20%)	5 (17%)	159
TRA-8 + [60]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]Co	8 (6)	>140/179	6 (75%)	4 (50%)	192

[a] The numbers in parentheses are the number of uncensored animals.

We have begun to evaluate the effect of TRA-8 treatment alone or in combination with chemotherapy against an intermediate sensitivity breast cancer tumor model. LCC6 cells express DR5 and show a lower degree of sensitivity to TRA-8 treatment *in vitro* as compared to 2LMP cells (**Figure 1B**). **Figure 16** shows the tumor growth curve for animals treated with TRA-8 + adriamycin and radiation, TRA-8 + adriamycin + [60]Co, and adriamycin + [60]Co, which produced the greatest reduction in time to tumor doubling (p <0.001). The anti-tumor effect on the LCC6 tumors was less than that obtained with the more sensitive 2LMP tumor model. We investigated whether higher doses of mTRA-8 (600 µg 2x/wk x 3 wk) and adriamycin (6 mg/kg q4dx3) or adriamycin (10 mg/kg q7dx3) plus mTRA-8 (200 µg 2x/wk x 3 wk) produced increased inhibition of tumor growth. As shown in **Figure 17**, both high dose treatment groups produced a greater inhibition of tumor growth than previously seen. These results suggest that further investigation of higher dose regimens are warranted. In the next study, animals bearing LCC6 xenografts received high dose mTRA-8 + high dose adriamycin treatment. The results shown in **Figure 18** illustrate good inhibition of LCC6 tumor growth. However, when only two high doses of mTRA-8 (800 µg + 400 µg) were combined with a single high dose of adriamycin (10 mg/kg), there was an absence of tumor growth inhibition (**Figure 19**).

Treatment of nude mice bearing well established 2LMP or LCC6 tumors with mTRA-8 and gemcitabine resulted in the reduction of tumor size and tumor growth delay, as illustrated in

Figures 20A and **B**. Treatment with mTRA-8 and gemcitabine produced 6 of 7 2LMP tumor regressions compared to 4 of 7 regressions for gemcitabine alone. The combination treatment produced 2 of 7 LCC6 tumor regressions, whereas gemcitabine alone produced no LCC6 regressions. However, the addition of mTRA-8 to gemcitabine treatments did not significantly retard tumor regrowth relative to animals treated with gemcitabine alone. The possibility remains that treatment with gemcitabine and higher doses of mTRA-8 or extending the treatment interval may increase the *in vivo* therapeutic efficacy of this combination against breast cancer.

6.   **Apoptosis in treated tumors.** The induction of apoptosis in 2LMP xenografts following treatment with TRA-8, adriamycin, paclitaxel, TRA-8 + adriamycin, and TRA-8 + paclitaxel was assessed using the TUNEL technique **(Figure 21)**. In untreated animals, tumors had 4% stained cells (1% intense), while 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. DR5 was detected in both treated and untreated tumors, but expression levels appeared unchanged (data not shown)

7.   **Effect of a second course of treatment with TRA-8 and adriamycin.** We initiated an experiment to determine whether a second course of treatment with TRA-8 and adriamycin would inhibit 2LMP *s.c.* breast cancer xenograft beyond that achieved with a single course of treatment. TRA-8 (200 µg) was injected *i.p.* into a group of athymic nude mice on day 8 after tumor cell injection, and additional doses of 200 µg TRA-8 were administered on days 12, 15, 19, 22, and 26. Adriamycin (6 mg/kg) was injected *i.v.* on days 9, 13, and 17. A second group of 8 animals received a second course of treatment with TRA-8 and adriamycin starting at 7 days after the last injection of TRA-8 in the first course of treatment (*i.e.* 200 µg TRA-8 injected *i.p.* on days 33, 36, 40, 43, 47, and 50 and 6 mg/kg adriamycin *i.v.* on days 34, 38, and 42). Other groups of animals received 1 or 2 courses of TRA-8 alone, adriamycin alone, or were untreated. The results of this study are shown in **Figure 22**. There were 3/8 complete regressions in the group treated with two courses of TRA-8, 1/8 in the group receiving one course of TRA-8 + adriamycin, and 2/8 in the group treated with two courses of TRA-8 + adriamycin. The results indicate that the growth of 2LMP xenografts was further inhibited by a second course of treatment with TRA-8 alone, adriamycin alone, or TRA-8 plus adriamycin, and show that the tumors that survive the first course of therapy with TRA-8, adriamycin, or the combination of these two agents are responsive to a second course of therapy with each agent. In addition, the most effective therapy was combined treatment with TRA-8 and adriamycin. Furthermore, the tumors appear to continue to express DR5, since two courses of TRA-8 inhibited tumor growth to a greater extent than a single course of treatment.

8.   **Anti-tumor efficacy of 2E12 alone or in combination with adriamycin.** We examined the effect of 2E12 alone and in combination with adriamycin in athymic nude mice bearing 2LMP breast cancer xenografts **(Figure 23** and **Table 3)**. Seven animals were untreated controls, 8 animals were treated with adriamycin alone, 8 animals were treated with 2E12 alone, and 8 were treated with the adriamycin + 2E12 combination. The median times to tumor doubling are given for each therapy group in **Table 3**. From the Kruskal-Wallis test, there were significant differences in growth inhibition in terms of time to tumor doubling (p<0.001). Multiple comparisons indicated that 2E12 in combination with adriamycin significantly increased the time to tumor doubling over either therapy alone, and both single therapies had significant increases over controls, but the single therapy groups were not significantly different from each other. Moreover, 2E12 in combination with adriamycin produced significant increases in tumor regression and recurrence-free tumor regression rates (p = 0.008 and p=0.045, respectively), as compared to either agent alone or untreated controls. While no tumor regressions occurred in the untreated controls or the single therapy groups, 4 out of 8 tumors in the adriamycin + 2E12 group regressed. On average these regression occurred within 20 days after start of therapy.

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Three out of the 4 regressions were recurrence-free, with an average follow-up period of 41 ± 13 days. The treatment groups were also compared with respect to growth inhibition. From ANOVA, there were significant differences in growth inhibition between the treatment groups (p<0.001), with multiple comparisons indicating that all treatment groups had significantly inhibited tumor growth as compared to controls (mean time to tumor doubling was 7 days). The single modality, 2E12 alone and adriamycin alone mean time to tumor doubling (29 and 21 days, respectively), were not significantly different from one another but achieved significantly less growth inhibition than the 2E12 + adriamycin combination (mean time to tumor doubling was over 97 days). Therefore, adriamycin + 2E12 treatment combination produced a significant amount of tumor growth inhibition, as compared to either therapy alone or no treatment.

Table 3. Anti-tumor Efficacy for 2E12 alone and in Combination with Adriamycin

Treatment	# of Animals	Tumor Doubling Time (mean/median)	Complete Regressions		
			Total (%)	No relapse (%)	Mean Recurrence
Untreated Controls	7	7/7	0	0	-
Adriamycin	8	21/23	0	0	-
2E12	8	29/23	0	0	-
2E12 + Adriamycin	8	> 97/65	4 (50%)	3 (38%)	5 (n=1)

9.   Combination treatment with TRA-8 plus 2E12 with or without adriamycin in nude mice bearing 2LMP xenografts. We next examined the effect of combining TRA-8 and 2E12 (same dose and schedule), with or without adriamycin (**Figure 24** and **Table 4**). Thirty-two animals were randomly assigned into groups of 8 animals, untreated controls, adriamycin, 2E12 + TRA-8, and 2E12 + TRA-8 + adriamycin. The mean and median time to tumor doubling for each group are given in **Table 4**. From the Kruskal-Wallis test, there were significant differences in the tumor growth inhibition with respect to tumor doubling times, on average (p<0.001). Multiple comparisons indicated that both the 2E12 + TRA-8 and 2E12 + TRA-8 + adriamycin therapy groups significantly increased the time to tumor doubling over all other groups, but these two regimens were not significantly different from each other. Adriamycin treatment was not significantly different than the controls. Moreover, the combination of the two antibodies produced significant increases in tumor regression and recurrence-free regression rates, as compared to untreated controls and single-agent therapy groups (p < 0.001). No tumor regressions occurred in the untreated controls or in the adriamycin treated animals, but a striking number, 7 out of 8 of the 2E12 + TRA-8 and 8 out of 8 of the 2E12 + TRA-8 + adriamycin treated animals, had complete tumor regressions, occurring on average just 6 days ± 1.5 days after start of therapy. Even more striking is the fact that 7 out of 8 of the 2E12 + TRA-8 + adriamycin tumor regressions were recurrence-free, with an average follow up of 176 ± 2 days. The treatment groups were also compared with respect to growth inhibition. From ANOVA, there were significant differences in growth inhibition between the treatment groups (p<0.001), with multiple comparisons indicating that the 2E12 + TRA-8 and 2E12 + TRA-8 + adriamycin treated animals (mean times to tumor doubling were over 131 and 158 days, respectively) significantly inhibited tumor growth as compared to the untreated controls and adriamycin treated animals (mean time to tumor doubling, 9 and 13 days, respectively). Although not statistically different from 2E12 + TRA-8, the 2E12 + TRA-8 + adriamycin treated animals did achieve the greatest observed inhibition of tumor growth with 100% complete tumor regressions and 88% were relapse-free. In addition, this finding for 2E12 + TRA-8 + adriamycin demonstrated improvement over the results from the experiment involving the TRA-8 and adriamycin combinations, described above.

Table 4. Anti-tumor Efficacy for 2E12 and TRA-8 in Combination with Adriamycin

Treatment	# of Animals	Tumor Doubling Time (mean/median)	Complete Regressions		
			Total (%)	No relapse (%)	Mean Recurrence
Untreated Controls	8	9/8	0	0	-
Adriamycin	8	13/14	0	0	-
2E12 + TRA-8	8	> 131/182	7 (88%)	5 (63%)	22 (n=2)
2E12 + TRA-8 + Adriamycin	8	> 158/185	8 (100%)	7 (88%)	97 (n=1)

10.   Upregulation of DR5 by adriamycin or radiation in LCC6 cells. Figure 25 shows that exposure of LCC6 breast cancer cells to adriamycin or radiation results in increased expression of DR5.

11.   Bioluminescence imaging of non-palpable breast cancer xenografts during treatment with mTRA-8 and chemotherapy. While TRA-8, an anti-DR5 antibody, induces apoptosis in TRAIL-sensitive tumor cells, no sensitive non-invasive methods are available to monitor the treatment effects on minimal disease in animal models. Bioluminescence imaging allows quantitative assessment of a small number of cells and monitoring the tumor growth and treatment response by detecting the light emitted from tumor cells expressing the firefly luciferase (Luc) as a reporter gene. The human breast tumor cell line 2LMP was transfected with adeno-associated virus encoding firefly Luc gene, and a stable Luc-positive 2LMP (Luc-2LMP) cell line was established by screening clones in 96-well plates using a Xenogen IVIS-100 imaging system. *In vitro* studies established that the Luc-2LMP cell line responded identically to treatments as compared to the parent cell line. For *in vivo* studies, four groups (4/group) of athymic female nude mice were implanted with Luc-2LMP cells (1×10^6/mouse) in the mammary fat pad. Treatments included combined mTRA-8 (100-200 µg/mouse) + adriamycin (6 mg/kg) (Gr 1), mTRA-8 (Gr 2), adriamycin (Gr 3), and untreated controls (Gr 4). All treatments were given intravenously (2X/week for 4 wks). Mice were imaged over time with the IVIS-100, and tumor mass was determined by measuring light transmitted from the Luc-positive tumors. Bioluminescence imaging demonstrated high sensitivity for non-invasive detection and treatment monitoring of non-palpable breast tumors in mice (**Figure 26**). The measurement of light emission from stable Luc-2LMP cells in mice allowed tracking the tumor regression during therapy. Real time imaging data revealed significant inhibition of tumor growth in all treatment groups relative to controls, while the combined treatment with TRA-8 + adriamycin was most effective (**Figure 27**). About 95% of tumor cells were killed after the first two doses of combination therapy.

12.   Detection of disseminated breast cancer growth and treatment response using non-invasive bioluminescence imaging. Two groups (5/group) of athymic female nude mice were used. Four sites (liver, spleen, chest cavity and peritoneum) of each mouse were injected with Luc-positive 2LMP cells (0.25×10^6/site). After 7 days, bioluminescence imaging revealed disseminated tumor sites; one group of mice was injected *i.v.* with 150 µg mTRA-8 and 6 mg/kg adriamycin, and the second group of mice did not receive any treatment. All treatments were given 2X/week for 3 wks. Mice were imaged over time with an IVIS-100 Xenogen imaging system and tumor mass was estimated from ventral, dorsal, left and right lateral views by measuring light transmitted from the luciferase positive tumors. All untreated mice died by day 21 with 4-6 fold increases in bioluminescence and extensive metastasis, including bone. Treated mice had a dramatic decrease in bioluminescence to 15-20% of day 7 values that persisted for 45 days followed by a progressive regrowth of tumor (increasing bioluminescence) in multiple sites. Dissemination of breast cancer was detected by bioluminescence imaging and confirmed by dissection. In all untreated mice, numerous tumor nodules were detected in lungs, heart, pleural space, bones including ribs and spine, diaphragm, esophagus and pericardium, liver, spleen, stomach, kidneys, ovaries, uterus, and peritoneal membrane. Bioluminescence imaging demonstrated high sensitivity for non-invasive detection and treatment monitoring of

disseminated breast tumors in mice. Combination therapy with TRA-8 and adriamycin was highly effective in the regression of disseminated breast cancer in this animal model.

13. *In vivo* **synergistic mechanisms of combination therapy with anti-death receptor antibodies and adriamycin.** Although the synergistic effect of TRAIL and chemotherapy in tumor cell death has been well established, the molecular mechanisms, particularly regarding *in vivo* apoptosis signal transduction, have not been examined. The striking anti-tumor efficacy of combination therapy with TRA-8, 2E12 and chemotherapy in the 2LMP human breast cancer xenograft model provides a useful *in vivo* model to investigate the molecular mechanisms by which such a potent synergistic tumoricidal activity is achieved. We hypothesized that the initial signal for triggering apoptosis by the death receptor antibody and chemotherapy agents may be different. For example, the death receptors primarily utilize caspase 8 to transduce an apoptosis signal whereas most chemotherapy agents cause DNA damage and trigger apoptosis through the mitochondrial cell death pathway. The regulatory mechanisms occurring proximally in both pathways could be different although both pathways merge distally at the level of caspase 3. Thus, we examined the kinetic alterations of several key apoptosis-regulatory proteins in the 2LMP tumor tissues that were undergoing significant tumor regression *in vivo* after treatment with antibodies and adriamycin. Our results led to our hypothesis that the death receptor and adriamycin may utilize different pathways to trigger apoptosis. This may be complementary by removal of the apoptosis inhibitors in each pathway, thereby facilitating tumor regression.

Nude mice were *s.c.* inoculated with the human breast cancer line, 2LMP. Seven days later when tumor growth was visible, mice were treated with TRA-8 and 2E12 alone, adriamycin alone or antibodies and adriamycin as indicated in **Figure 28**. Tumor tissues were collected at one day after each cycle of the treatment (day 10, 15 and 18). Tumor growth was arrested after one dose of treatment and tumor regression occurred after the second dose of treatment. In contrast, untreated animals and adriamycin-treated mice showed a continuous growth of tumor (**Figure 28**). The activity of caspase 8, 9 and 3 in tumor tissues lysates was determined by fluorescent matrix analysis and presented as percent of control tissue activity. The expression of Bax, Bcl-xL, and XIAP as protein levels in tumor tissues was determined by Western blot analysis and quantitated with a densitometer.

a.   Differential utilization of caspase pathways by the death receptors and adriamycin. To determine the utilization of the caspase pathway in combination therapy, the activity of three major caspases was examined. High levels of caspase 8 activity were observed in the antibody treated tumors, regardless of the presence of adriamycin. However, the combination of anti-death receptor antibodies and adriamycin led to consistently higher levels of activity of caspase 8 (**Figure 29A**). These results indicate that the death receptors primarily utilize caspase 8 as an initiator caspase to trigger apoptosis, and the activity of caspase 8 can be further increased by adriamycin. In contrast, adriamycin alone was able to induce caspase 9 activation, which was significantly enhanced by the combination with anti-death receptor antibodies. The death receptor antibodies alone only induced a minimum activation of caspase 9 (**Figure 29B**). These results indicate that adriamycin *in vivo* can activate the caspase 9 pathway, whereas the death receptors do not utilize caspase 9 unless in combination with adriamycin. As anticipated, the activity of caspase 3 was synergistically enhanced by the combination (**Figure 29C**), suggesting that two upstream caspase pathways merge at caspase 3. An interesting finding is that adriamycin alone was unable to activate caspase 3 although it strongly induced caspase 9 activation (**Figure 29B, C**). These results suggest that the activation from caspase 9 to caspase 3 might be a limiting step for adriamycin-induced apoptosis, and the presence of inhibitors at this step might lead to an adriamycin-resistant status as demonstrated by the low efficacy of adriamycin treatment alone. However, this potential limiting factor could be removed by the combined presence of the death receptor-associated signal.

b.   Adriamycin induces Bax expression. Because the Bcl-2 family proteins and mitochondrial cell death pathway play a significant role in the regulation of the death receptor-mediated apoptosis signal transduction, we examined the expression of a number of the proteins in this family, including pro-apoptosis proteins: Bax, Bad, and Bak; and anti-apoptosis proteins: Bcl-2 and Bcl-XL. The 2LMP xenografts expressed high levels of Bad, which was not altered by the treatment (data not shown). In contrast, the 2LMP tumors did not express detectable levels of Bcl-2 regardless of the treatment (data not shown). These results rule out a role for Bad or Bcl-2 in the synergistic induction of apoptosis of 2LMP tumor cells by combination treatment. However, a striking effect of antibody and adriamycin treatment was a bi-directional alteration of the pro-apoptosis protein, Bax. First, anti-death receptor antibody treatment resulted in a decrease in Bax expression (**Figure 30A**), suggesting that down-modulation of Bax might be associated with the development of the resistance to death receptor-induced apoptosis, which is consistent with a recently published observation indicating that loss of Bax expression is associated with the resistance of tumor cells to TRAIL-mediated apoptosis. As previously reported, adriamycin was able to increase Bax expression. Importantly, the decreased Bax expression induced by the death receptor antibodies was prevented by combination with adriamycin. These results suggest that the combination of anti-death receptor antibodies with adriamycin could block the development of the resistance of tumor cells to death receptor-mediated apoptosis by increasing Bax expression.

Bcl-xL, an anti-apoptosis protein of the Bcl-2 family, was also significantly altered during treatment. First, while anti-death receptor antibodies induced a dramatic down-modulation of Bax, they also slightly increased the expression of Bcl-xL (**Figure 30B**). Because the ratio of Bax to Bcl-xL is critical for regulation of apoptosis, the elevated Bcl-xL expression induced by anti-death receptor antibodies further increases the resistance of tumor cells to death receptor-mediated apoptosis. Adriamycin-treated tumors exhibited initially decreased Bcl-xL expression after one dose of treatment but gradually increased to control levels. Combination treatment with anti-death receptor antibodies and adriamycin resulted in the lowest expression of Bcl-xL after two doses of treatment. These results indicate that down-modulation of Bcl-xL by the combination treatment plays a role in the synergistic induction of apoptosis of tumor cells *in vivo*.

Taken together, the decreased ratio of Bax to Bcl-xL is likely involved in the development of resistance of tumor cells to death receptor-mediated apoptosis whereas adriamycin could reverse this process, thereby maintaining the susceptibility of tumor cells to death receptor-mediated apoptosis.

c.   Death receptors signal down-modulation of XIAP. XIAP is a member of the IAP apoptosis inhibitor superfamily, which primarily inhibits caspase 9. The untreated tumor xenograft tissue expressed high levels of XIAP, which may explain the adriamycin resistance in this model and the failure of caspase 3 activation by caspase 9. However, treatment with TRA-8 and 2E12 alone led to significantly decreased expression of XIAP (**Figure 31**). Thus, the death receptors signal a pro-apoptotic pathway to adriamycin by down-modulation of XIAP, which synergistically enhances apoptosis response in the combination by activating the pathway from caspase 9 to caspase 3. These results are also consistent with previously published results showing that the death receptor-mediated caspase 8 cleaves XIAP, thereby inactivating its inhibitory activity.

Our results suggest several key control points for modulation of signal transduction by death receptor and chemotherapeutic agent. 1) The triggering caspases for death receptor and chemotherapy agent-mediated apoptosis are different *in vivo*: the death receptor-mediated apoptosis is triggered by caspase 8 while chemotherapy agent is initiated by caspase 9. The combination of two up-stream caspases results in a more complete activation of the key down-stream caspase 3. 2) Cancer cells may utilize different anti-apoptosis mechanisms to protect themselves from death receptor or chemotherapy agent-induced apoptosis. The decreased

expression ratio of Bax/Bcl-xL appears to be associated with the development of resistance to death receptor-mediated apoptosis, which can be reversed or prevented by adriamycin. On the other hand, XIAP is likely a key inhibitor for adriamycin-induced apoptosis, which prevents the activation of caspase 3 mediated by caspase 9. This anti-apoptosis mechanism can be down-modulated by the death receptor antibody.

14.   Expression of DR5 in human breast cancer biopsy specimens. The expression of DR5 in 3 human breast cancer specimens and uninvolved epithelium of the breast, determined by immunohistochemical staining with mTRA-8, is shown in **Figure 32**. The immunostaining score for 18 cases is shown in **Figure 33**. The level of membrane DR5 expression was slightly higher in the tumor specimens.

KEY RESEARCH ACCOMPLISHMENTS
- Demonstrated DR4 and DR5 expression on a large panel of human breast cancer cell lines.
- Demonstrated variable cytotoxicity of human breast cancer cell lines to TRA-8 and 2E12 alone or in combination with adriamycin or paclitaxel.
- Identified combinations of mTRA-8 anti-DR5 or 2E12 anti-DR4 antibody and chemotherapy or biomodulating drugs that produced synergistic toxicity.
- Identified mechanisms contributing to synergistic cytotoxicity.
- Showed that DR5 cell surface expression is increased by exposure to adriamycin or radiation *in vitro*.
- Demonstrated increased antitumor efficacy *in vivo* of TRA-8 and 2E12 in combination with adriamycin, paclitaxel, and radiation.
- Showed that high doses of mTRA-8 and adriamycin inhibited the growth of intermediate sensitivity breast cancer xenografts.
- Identified mechanisms contributing to enhanced therapeutic effect of mTR-8 anti-DR5 and 2E12 anti-DR4 antibody plus adriamycin in subcutaneous breast cancer xenografts.
- Demonstrated that bioluminescence imaging can be used to monitor the response of mammary fat pad and disseminated breast cancer xenografts.

REPORTABLE OUTCOMES
Developed two monoclonal antibodies that interact synergistically with chemotherapy drugs and radiation both *in vitro* and *in vivo*. Demonstrated that bioluminescence imaging can be used to monitor response to treatment.

CONCLUSIONS
These studies indicate that targeting either DR4 or DR5 can produce anti-tumor effects, that both monoclonal antibodies can enhance drug (adriamycin) anti-tumor effects and that the combination of both antibodies has a striking anti-tumor efficacy. 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. Co-incubation of TRA-8 and adriamycin or paclitaxel produced enhancement of cytotoxicity compared to either agent alone in TRA-8 sensitive breast cancer cell lines. The enhancement was synergistic in certain cell lines and additive in others. Cytotoxicity studies with mTRA-8, adriamycin, and COX-2 inhibitors suggest that both COX-2 dependent and independent mechanisms contribute to the increased sensitivity to mTRA-8 in cells treated with these agents, and the results provide mechanistic information on mTRA-8-mediated cell killing. Combination treatment with mTRA-8 and PPARγ ligands (troglitazone, 15d-PGJ2) produced additive or synergistic cytotoxicity *in vitro*. Additive to synergistic cytotoxicity was produced by combination therapies against cells that are moderately sensitive to resistant to mTRA-8. In contrast, combination therapies resulted in less than additive *in vitro* cytotoxicity against mTRA-

8 sensitive cells. Taken together, these findings suggest that combinations of death receptor antibody, standard chemotherapeutic drugs, and biomodulating drugs to target multiple cell signaling pathways may have potential utility in developing more effective antitumor therapies *in vivo*. Combinations of death receptor antibody and chemotherapy may synergistically activate pro-apoptotic signaling pathways. It appears that the death receptor and mitochondrial apoptosis signaling pathways may converge at the level of JNK and p38 and thus provide a positive feedback loop amplifying caspase 8 and 9 signals initiated by mTRA-8 and chemotherapeutic drugs.

For the *in vivo* studies, we utilized the 2LMP breast cancer cell line that was developed as a more aggressive sub-clone derived from MDA-MB-231. This cell line had moderate expression of DR5 and was sensitive to TRA-8 induced cytotoxicity *in vitro*. The 2LMP cell line had a dose dependent *in vitro* cytotoxicity with either adriamycin or paclitaxel, and the combination of TRA-8 and adriamycin or paclitaxel produced additive enhancement of cytotoxicity. Neither adriamycin nor paclitaxel produced significant tumor growth inhibition *in vivo* compared to controls, while producing striking tumor inhibition and tumor regression when combined with TRA-8. The combination of adriamycin and TRA-8 fulfilled the criteria for *in vivo* synergism (p <0.001) and produced 4 out of 8 complete regressions of tumor. The combination of paclitaxel and TRA-8 produced similar effects, although the interaction met criteria for additive effects and included 3 of 8 complete regressions. Forty-one percent of animals receiving TRA-8 alone or in combination with chemotherapy and/or radiation had complete regressions (28 of 68 animals). Further, 21% of these animals had no evidence of tumor recurrence over 148-192 days of observation. The TRA-8 regimens complete regression rate, rate of recurrence-free complete regressions, and effects on tumor doubling time were all statistically different than single agents or non-TRA-8 combinations. Higher dose regimens of mTRA-8 and/or adriamycin resulted in increased tumor growth inhibition of moderately resistant breast cancer xenografts. Bioluminescence imaging demonstrated high sensitivity for non-invasive detection and treatment monitoring of non-palpable breast tumors in mice. Bioluminescence imaging demonstrated high sensitivity for non-invasive detection and treatment monitoring of disseminated breast tumors in mice. Combination therapy with TRA-8 and adriamycin was highly effective in the regression of disseminated breast cancer in this animal model. Our results suggest several key control points for modulation of signal transduction by death receptor and chemotherapeutic agent. 1) The triggering caspases for death receptor and chemotherapy agent-mediated apoptosis are different *in vivo*: the death receptor-mediated apoptosis is triggered by caspase 8 while chemotherapy agent is initiated by caspase 9. The combination of two up-stream caspases results in a more complete activation of the key down-stream caspase 3. 2) Cancer cells may utilize different anti-apoptosis mechanisms to protect themselves from death receptor or chemotherapy agent-induced apoptosis. The decreased expression ratio of Bax/Bcl-xL appears to be associated with the development of resistance to death receptor-mediated apoptosis, which can be reversed or prevented by adriamycin. On the other hand, XIAP is likely a key inhibitor for adriamycin-induced apoptosis, which prevents the activation of caspase 3 mediated by caspase 9. This anti-apoptosis mechanism can be down-modulated by the death receptor antibody.

Statement of Work

SPECIFIC AIM #1. To determine the expression profile in human breast cancer cell lines of DR5 and DR4 before and after treatment with anti-DR5 and -DR4 MAb alone, together, and in combination with chemotherapy drugs.

Task 1: *Months 1-3. To determine cell surface expression of DR5 and DR4 in untreated breast cancer cells by flow cytometry analysis.*

Task 2: *Months 1-6. To determine whether there is a change in DR5 and DR4 expression in human breast cancer cells after exposure to anti-DR5 and -DR4 MAb alone or in combination with chemotherapy drugs.*

These tasks were completed in year 1.

SPECIFIC AIM #2. To determine the expression profile of DR5 and DR4 during the progression of breast cancer.

Task 1: *Months 6-36. To determine DR5 and DR4 expression in breast cancer tissues by immunohistochemistry staining.*

Task 2: *Months 6-36. To determine whether there is a correlation of increased DR5 and DR4 expression with the progression of breast cancer and other tumor markers.*

These studies were completed in years 2 and 3.

SPECIFIC AIM #3. To determine the cytotoxicity of anti-DR5 and -DR4 antibodies against human breast cancer cells alone, together, and in combination with adriamycin or paclitaxel.

Task 1: *Months 6-18. To examine DR5 and DR4 positive breast cancer cell lines for their susceptibility to anti-DR5 and -DR4 antibody-mediated apoptosis in the presence or absence of chemotherapy drugs.*

These studies have been completed.

Task 2: *Months 12-24. To determine the effect of timing of drug exposure, before, at the same time, or following antibody exposure to identify the optimum regimen for the induction of apoptosis.*

These studies have been completed.

SPECIFIC AIM #4. To determine the therapeutic efficacy and toxicity of anti-DR5 and -DR4 antibodies against human breast cancer xenografts alone, together, and in combination with adriamycin or paclitaxel.

Task 1: *Months 6-24. To examine the in vivo therapeutic potential of TRA-8 and anti-DR4 antibodies alone, together, and in combination with chemotherapy drugs in nude mice with localized breast cancer xenografts.*

These studies have been completed.

Task 2: *Months 18-26.* To examine the in vivo therapeutic potential of TRA-8 and anti-DR4 antibodies alone, together, and in combination with chemotherapy drugs in nude mice with metastatic breast cancer xenografts.

These studies have been completed.

Publications
Manuscripts
3. Buchsbaum DJ, Zhou T, Grizzle WE, Oliver PG, Hammond CJ, Zhang S, Carpenter M, LoBuglio AF: 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. *Clin Cancer Res* 9:3731-3741, 2003.
4. Ohtsuka T, Buchsbaum D, Oliver P, Makhija S, Kimberly R, Zhou T: Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway. *Oncogene* 22: 2034-2044, 2003.

Abstracts

1. Chaudhuri TR, Cao Z, Ponnazhagan S, Stargel A, Simhadri PL, Zhou T, LoBuglio AF, Buchsbaum DJ, Zinn KR: Bioluminescence imaging of non-palpable breast cancer xenografts during treatment with TRA-8, an anti-DR5 antibody and chemotherapy. *Proc Am Assoc Cancer Res* 45, March 2004.
2. Chaudhuri TR, Cao Z, Ponnazhagan S, Stargel A, Simhadri PL, Zhou T, LoBuglio AF, Buchsbaum DJ, Zinn KR: Detection of disseminated breast cancer growth and treatment response using non-invasive bioluminescence imaging (BI). *Proc ASCO* 23:62, 2004.
3. Oliver PG, Amm HM, Nan L, Zhou T, LoBuglio AG, Buchsbaum DJ: Antitumor efficacy of mTRA-8 anti-DR5 monoclonal antibody against human breast cancer is enhanced by combination chemotherapy. *Proc the 12th SPORE Investigators Workshop*, 307, 2004.
4. Amm HM, Oliver PG, Buchsbaum DJ: TRA-8 anti-DR5 antibody and chemotherapy agents produce cytotoxicity and activate apoptotic pathways in breast cancer cells. *The 96th Annual Meeting of the American Association for Cancer Research*, Anaheim, CA, April 16-20, 2005.
5. Buchsbaum DJ, Zhou T, Chaudhuri TR, Zinn KR, LoBuglio AF, Grizzle WE, Oliver PG: Treatment of breast cancer with antibodies against tumor necrosis factor-related apoptosis-inducing ligand death receptors in combination with chemotherapy. *Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting*, Philadelphia, PA, June 8-11, 2005.
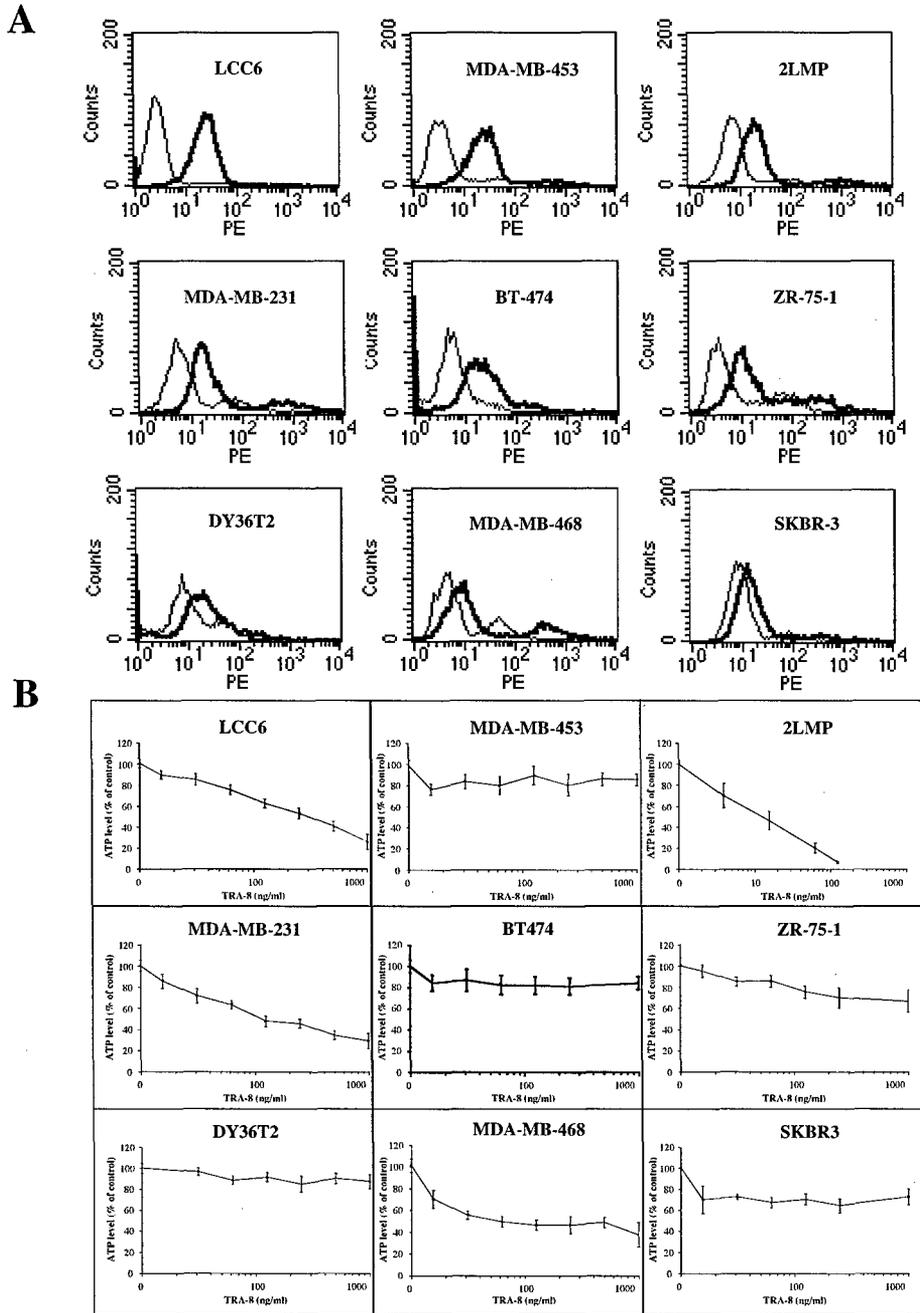
Figure 1. **A)** Flow cytometry analysis of DR5 cell surface expression in a panel of human breast cancer cell lines. Breast cancer cells were harvested using EDTA and stained with 10 µg/ml TRA-8 mAb for 1 h at 4°C followed by PE-conjugated goat anti-mouse IgG1, then analyzed using FACScan and CellQuest software. Thick histograms indicate TRA-8 staining and thin histograms indicate 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 1,000 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 2-3 independent experiments, each done in triplicate.
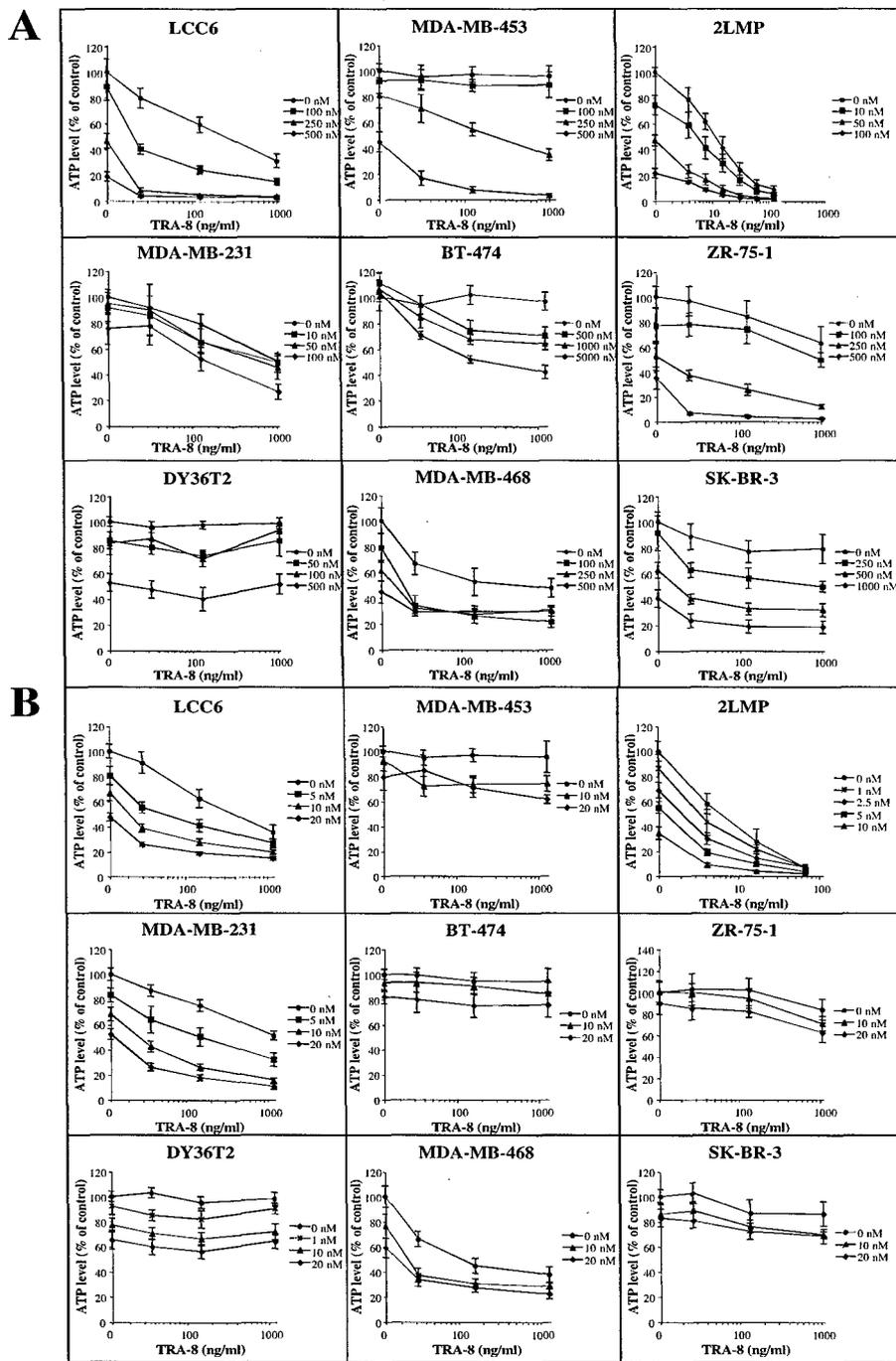
Figure 2. A) Cytotoxicity of TRA-8 and adriamycin combination treatment of human breast cancer cell lines. Cells (1,000/well) were exposed to various concentrations of adriamycin for 24 h at 37°C beginning 24 h after plating cells. TRA-8 was added 24 h after adriamycin addition, and ATP levels were determined 24 h later. Values represent the mean and SE of triplicate determinations from 2-4 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 (1,000/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 2-4 independent experiments each done in triplicate, and are reported relative to untreated control cells.

Figure 3. Flow cytometry analysis of DR4 cell surface expression in a panel of human breast cancer cell lines. Breast cancer cells were harvested with CellStripper (Mediatech, Herndon, VA) and stained with 5 µg/ml 2E12 mAb or IgG1 isotype control antibody for 1 h at 4°C followed by PE-conjugated goat anti-mouse IgG1, then analyzed using FACScan and CellQuest software. Thick histograms indicate 2E12 staining and thin histograms indicate staining with mouse IgG1 isotype control antibody.
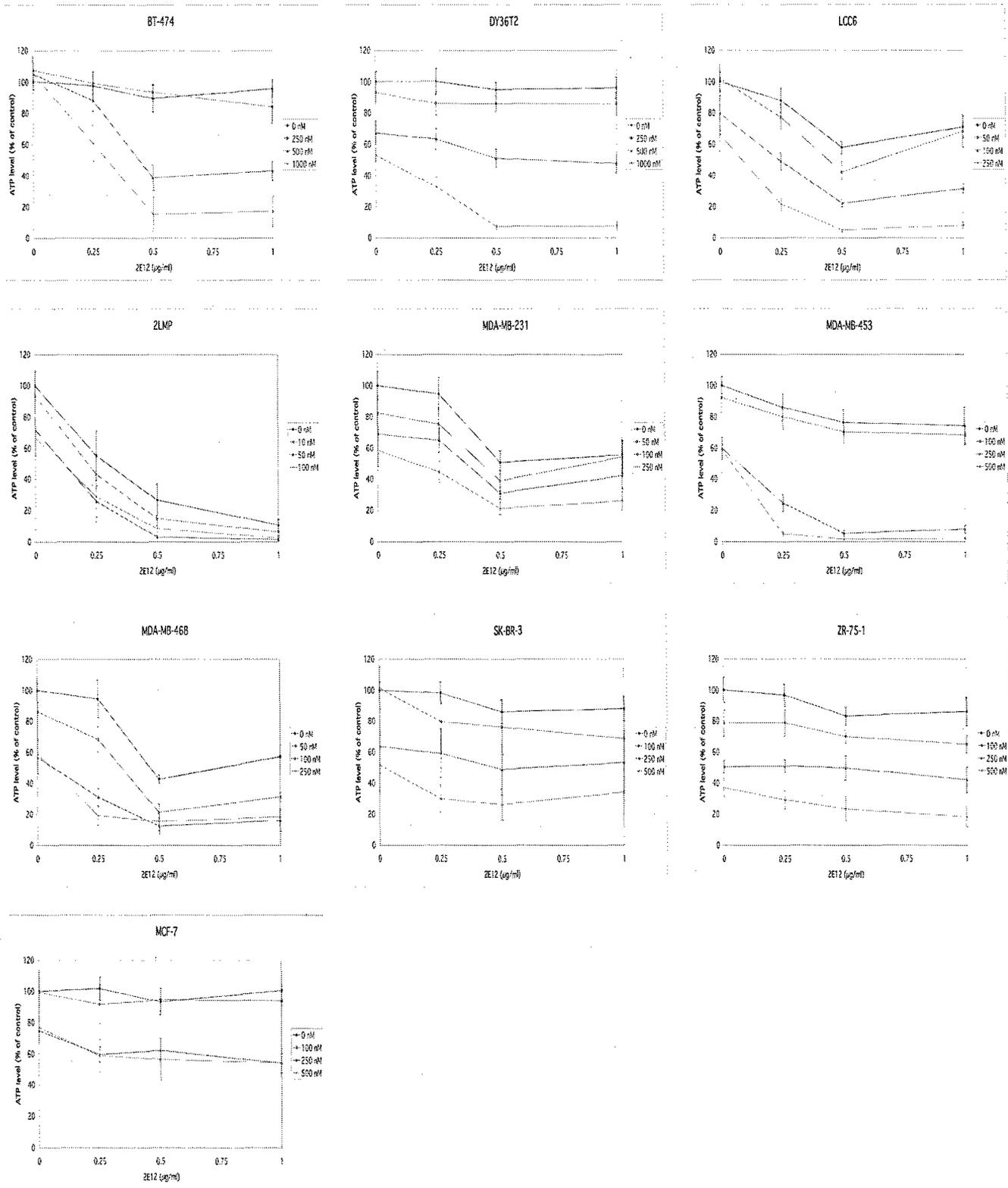
Figure 4. Cytotoxicity of 2E12 and Adriamycin combination treatment of human breast cancer cell lines. Cells were trypsinized and replated at a density of 1,000 cells/well in 96-well plates. Cells were exposed to various concentrations of Adriamycin for 24 h at 37°C beginning 24 h after plating cells. 2E12 and 2 μg/ml goat anti-mouse IgG1 cross-linking antibody were added 24 h after Adriamycin addition, and ATP levels were determined 24 h later. Values represent the mean and SE of quadruplicate determinations from 2-4 independent experiments and are reported relative to untreated control cells.
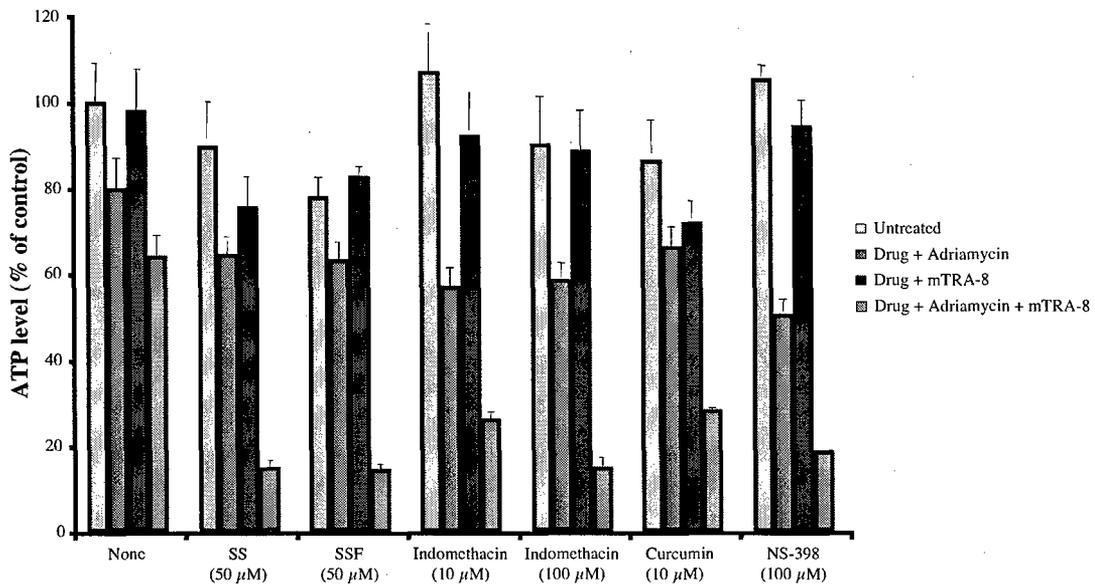
Figure 5. Cytotoxicity of mTRA-8, Adriamycin, and NSAIDs against DY36T2 cells. Cells were treated with test drugs and/or Adriamycin (500 nM) for 24 h before adding mTRA-8. ATP levels were determined 24 h after adding Ab. Plotted values are for 25 ng/ml mTRA-8.
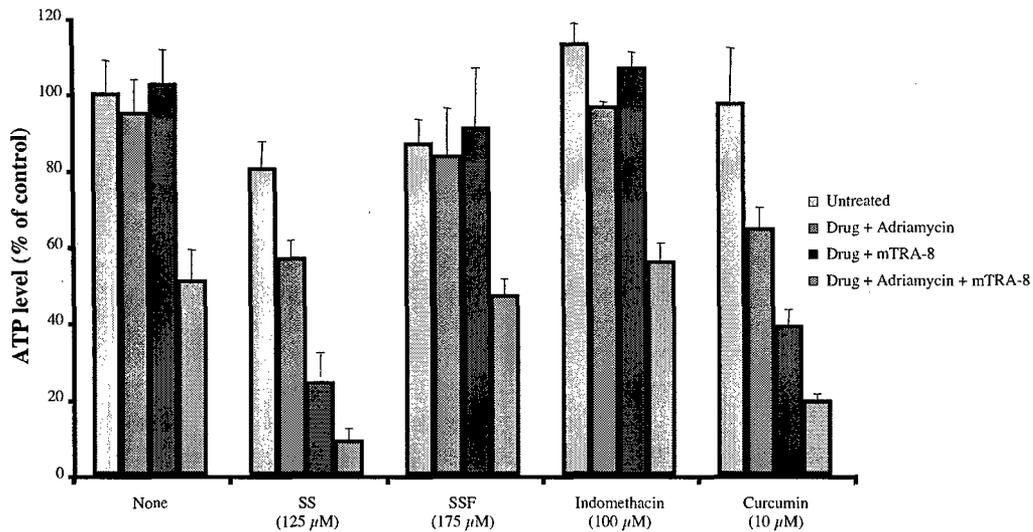
Figure 6. Cytotoxicity of mTRA-8, Adriamycin, and biological modifiers against BT-474 breast cancer cells. Cells were treated with test drugs and/or Adriamycin (1 μM) for 24 h before adding mTRA-8. ATP levels were determined 24 h later. Plotted values are for 1000 ng/ml mTRA-8.
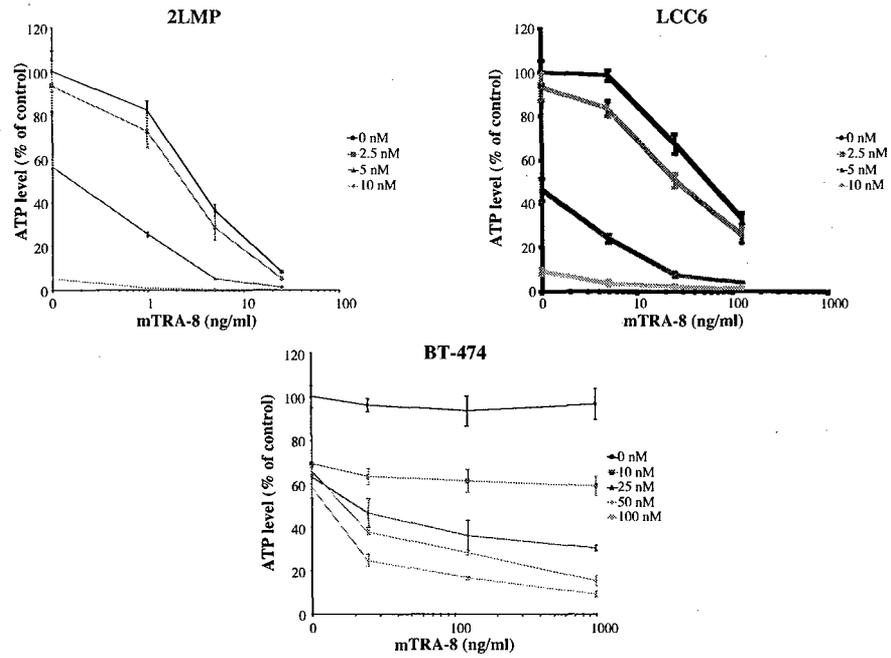
Figure 7. Cytotoxicity of mTRA-8 and Velcade against breast cancer cells. Cells (1,000 per well) were incubated with 2.5-100 nM Velcade for 24 h at 37°C followed by the addition of mTRA-8. Cell viability was determined by ATPLite assay 24 h after mTRA-8 addition and expressed as a percentage of untreated control cells. There were at 4 replicates in each assay. The mean ± SD is shown.
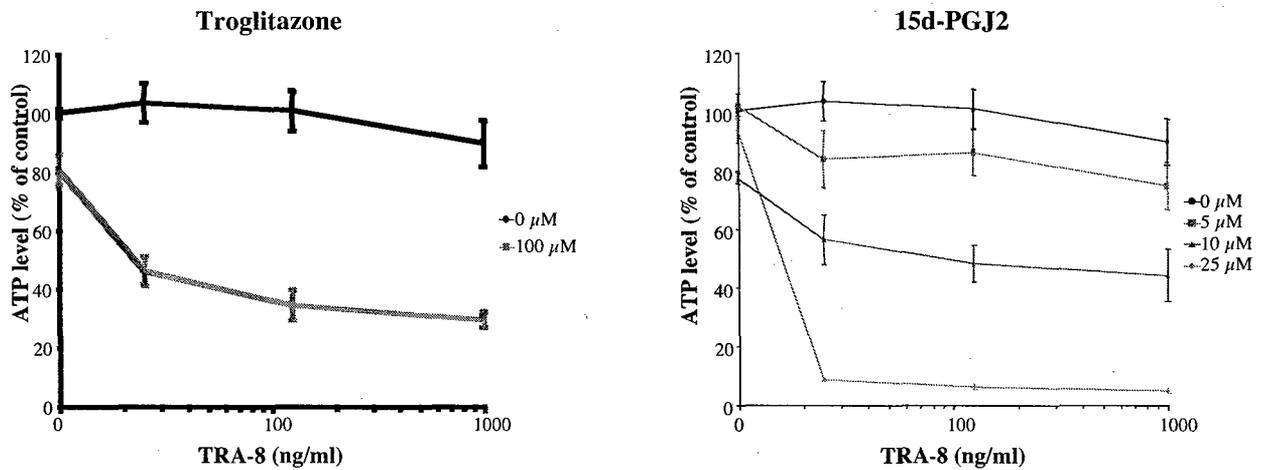
Figure 8. Cytotoxicity of mTRA-8 and Velcade against breast cancer cells. Cells (1,000 per well) were incubated with 2.5-100 nM Velcade for 24 h at 37°C followed by the addition of mTRA-8. Cell viability was determined by ATPLite assay 24 h after mTRA-8 addition and expressed as a percentage of untreated control cells. There were at 4 replicates in each assay. The mean ± SD is shown.
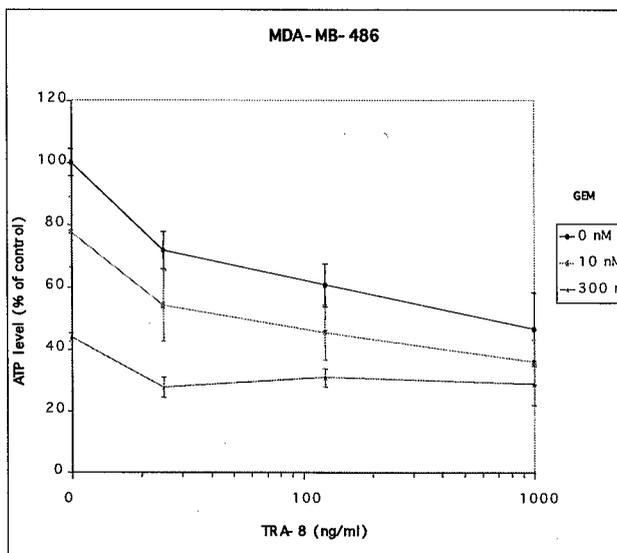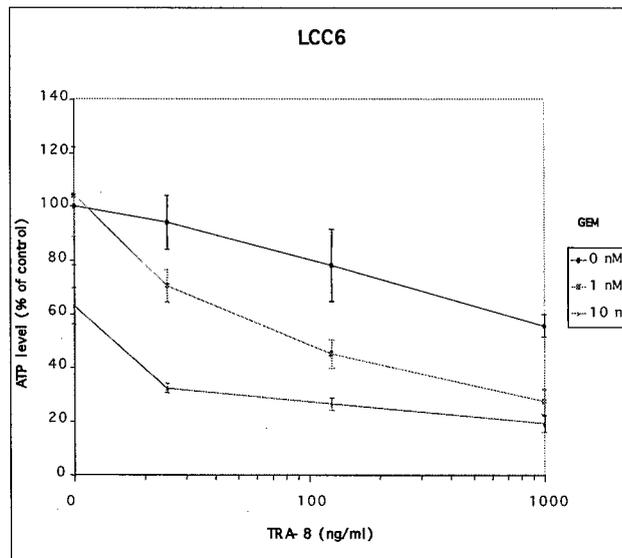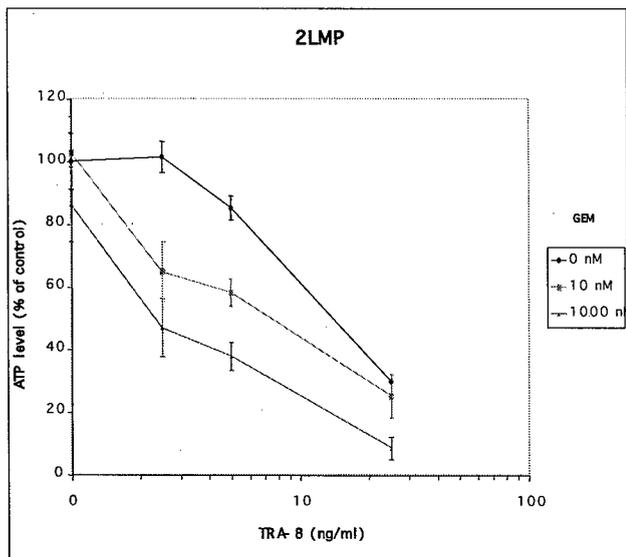
Figure 9. Cytotoxicity of mTRA-8 and gemcitabine combination treatment of human breast cancer cell lines. Cells were trypsinized and replated at a density of 1,000 cells/well in 96-well plates. Cells were exposed to various concentrations of gemcitabine for 24 h at 37°C beginning 24 h after plating cells. mTRA-8 was added 24 h after gemcitabine addition, and ATP levels were determined 24 h later. Values represent the mean and SD of quadruplicate determinations from a single representative experiment, which was repeated at least once, and are reported relative to untreated control cells.
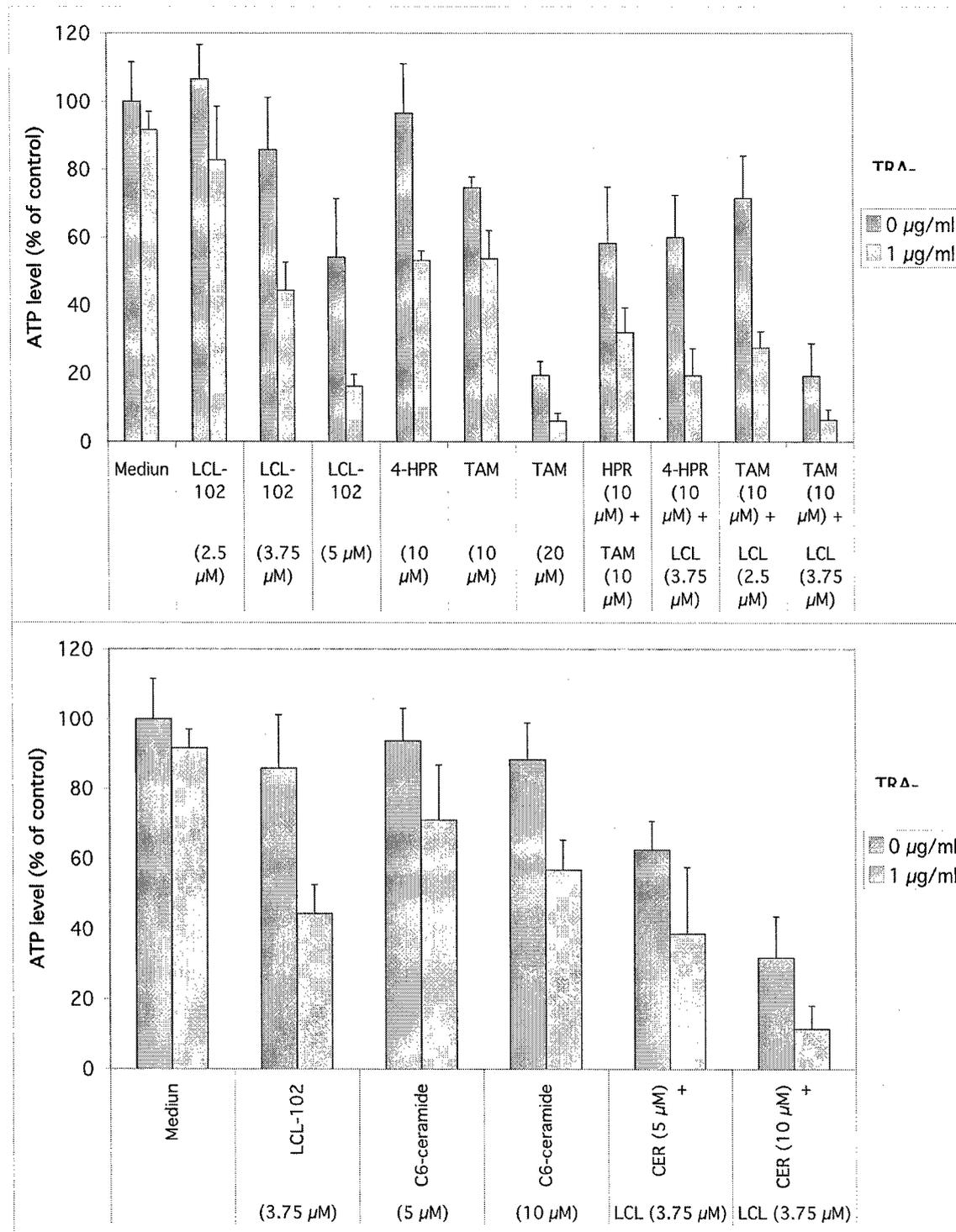
Figure 10. Cytotoxicity of mTRA-8 and ceramide modulating drugs against BT-474 breast cancer cells. Cells were trypsinized and replated at a density of 1,000 cells/well in 96-well plates, and all treatments began 24 h later. Cells were treated concurrently with single drugs or combinations of drugs and mTRA-8 (1 µg/ml) for 24 h at 37°C, then ATP levels were determined. Ceramide modulating drug treatments and purported mechanisms included LCL-102, to inhibit acid ceramidase; 4-hydroxyfenretinimide (fenretinide, 4-HPR), to stimulate *de novo* ceramide synthesis; tamoxifen, to block ceramide conversion to glucosylceramide; and exogenous short chain (C6) ceramide. Values are mean and SD of quadruplicate determinations from a single representative experiment and are reported relative to untreated control cells.
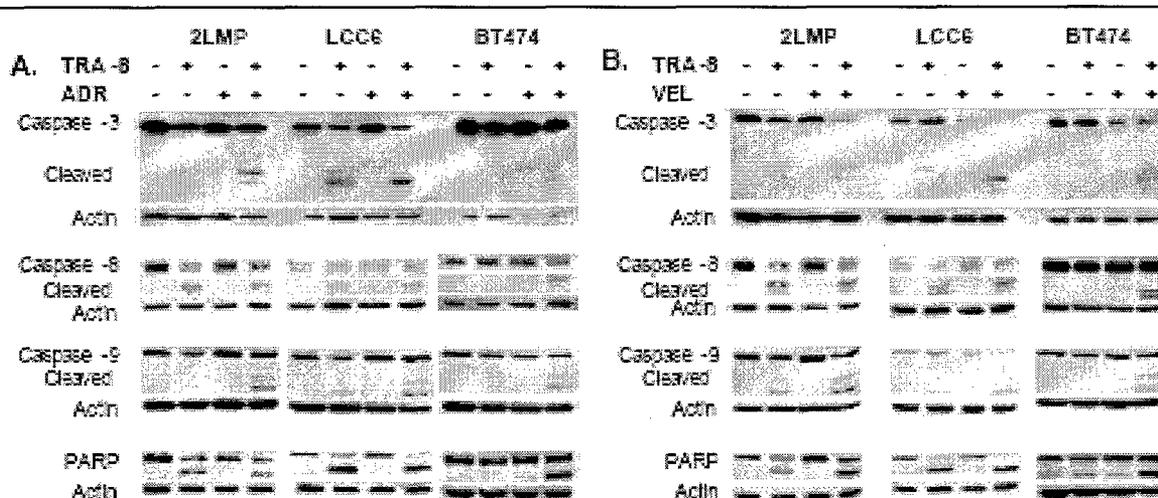
Figure 11A Caspase and PARP cleavage was induced by TRA-8 and adriamycin or velcade treatment. 2LMP, LCC6, and BT-474 cells were retreated with **(A)** ADR (50 nM, 240 nM, and 1000 nM, respectively) or **(B)** VEL (5 nM, 10 nM, and 100 nM) before the addition of TRA-8 for a total time of 24 h. 2LMP and BT-475 cells received 3 h of TRA-8 treatment (125 ng/ml and 1000 ng/ml), whereas LCC6 were treated with TRA-8 for 2 h (500 µg/ml).
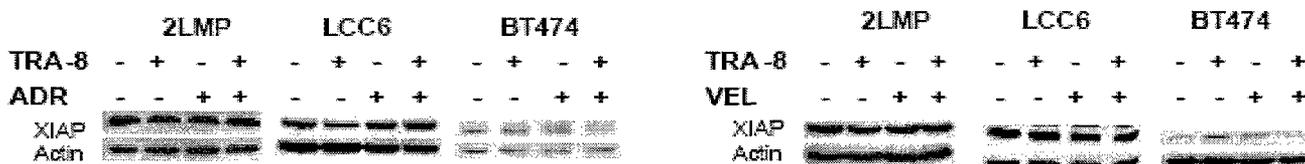
Figure 11B. TRA-8 and combination treatment modified the expression of XIAP. Cell lysates were collected after 24 h treatment with ADR or VEL and 2-3 h TRA-8 treatment and analyzed by Western blot.
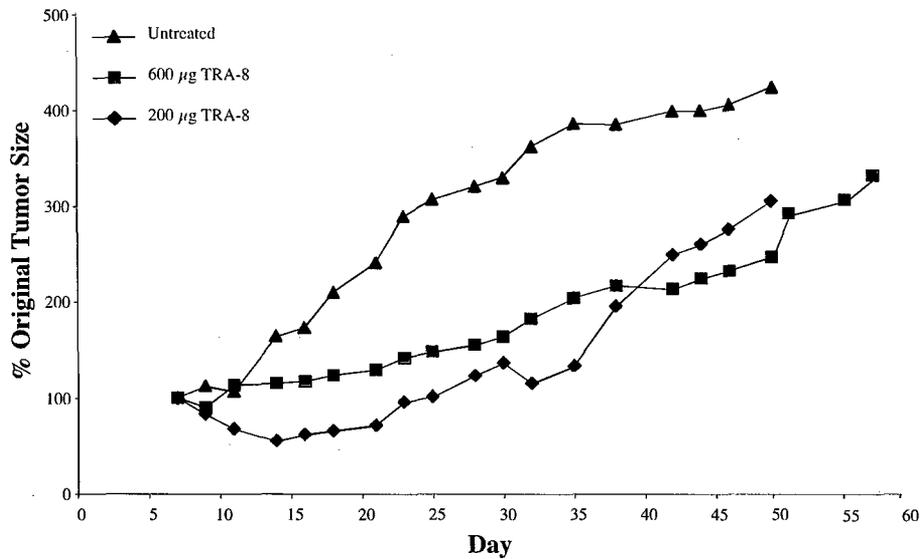
Figure 12. The effect of TRA-8 on tumor growth in athymic nude mice bearing established 2LMP human breast cancer xenografts. 2LMP cells (3×10^7) were injected *s.c.* on day 0. Two groups of mice were injected *i.p.* with 200 µg or 600 µg TRA-8 on days 7, 10, 14, 17, 21, and 24. One group of mice received 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).
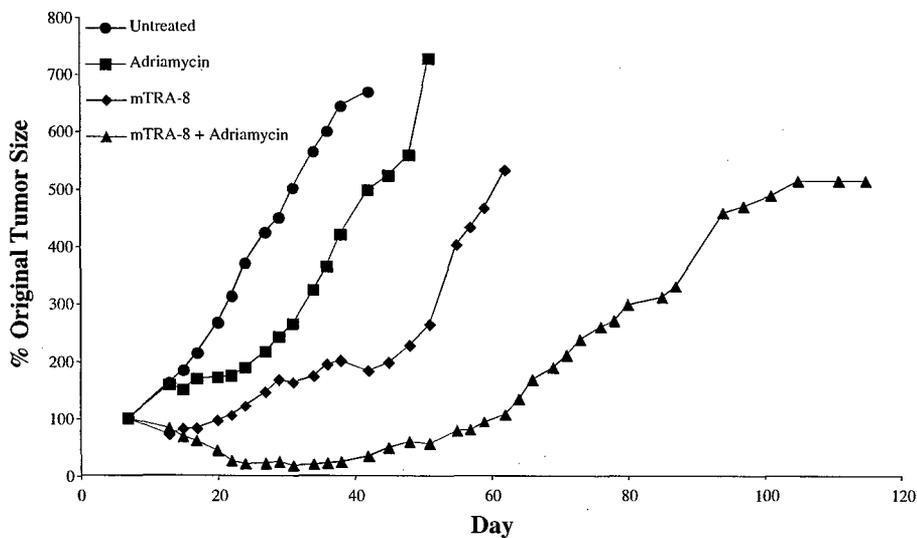

Figure 13. The effect of TRA-8 and adriamycin on tumor growth in athymic nude mice bearing breast cancer xenografts. 2LMP cells (3×10^7) were injected *s.c.* into athymic nude mice on day 0. Two groups of mice were injected *i.p.* with 200 µg TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice 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).
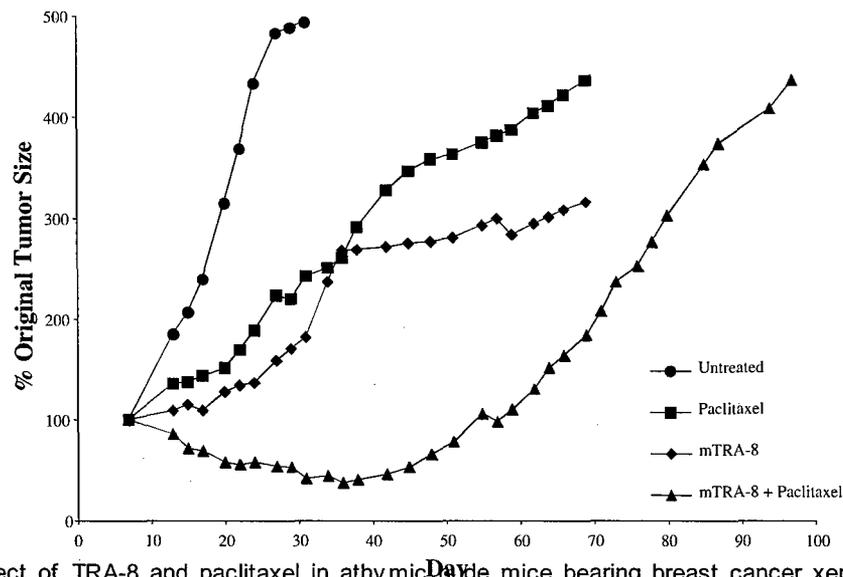
Figure 14. The effect of TRA-8 and paclitaxel in athymic nude mice bearing breast cancer xenografts. 2LMP cells (3x10⁷) were injected *s.c.* into athymic nude mice on day 0. Two groups of mice were injected *i.p.* with 200 μg TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received *i.v.* 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).
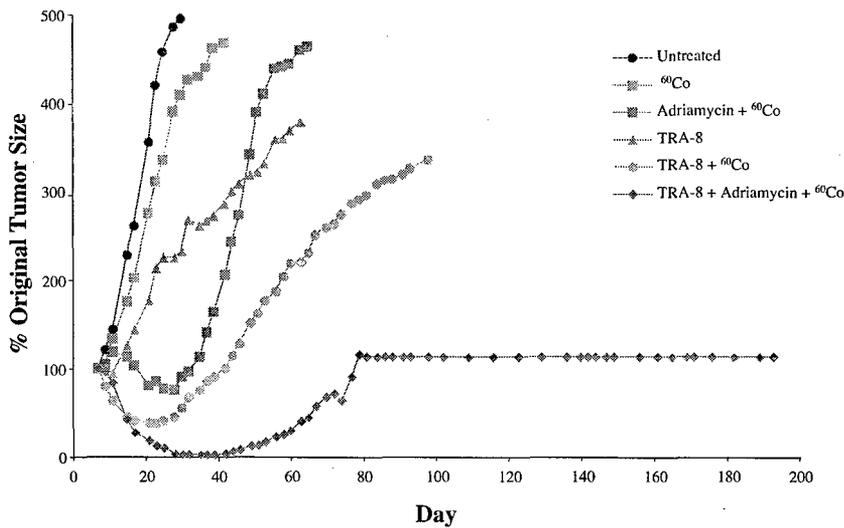
Figure 15. The effect of TRA-8, adriamycin, and ⁶⁰Co radiation on tumor growth in athymic nude mice bearing breast cancer xenografts. 2LMP cells (3x10⁷) were injected *s.c.* into athymic nude mice on day 0. Three groups of mice were injected *i.p.* with 200 μg TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received *i.v.* adriamycin (6 mg/kg) on days 8, 12, and 16. Four groups of mice received 3 Gy ⁶⁰Co 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).
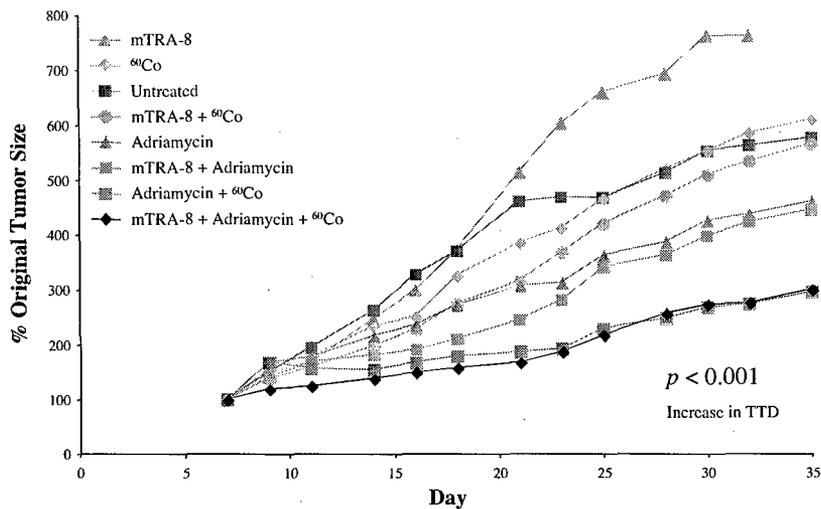
Figure 16. The effect of TRA-8, adriamycin, and radiation on growth of LCC6 breast cancer xenografts. See **Figure 15** legend for details.
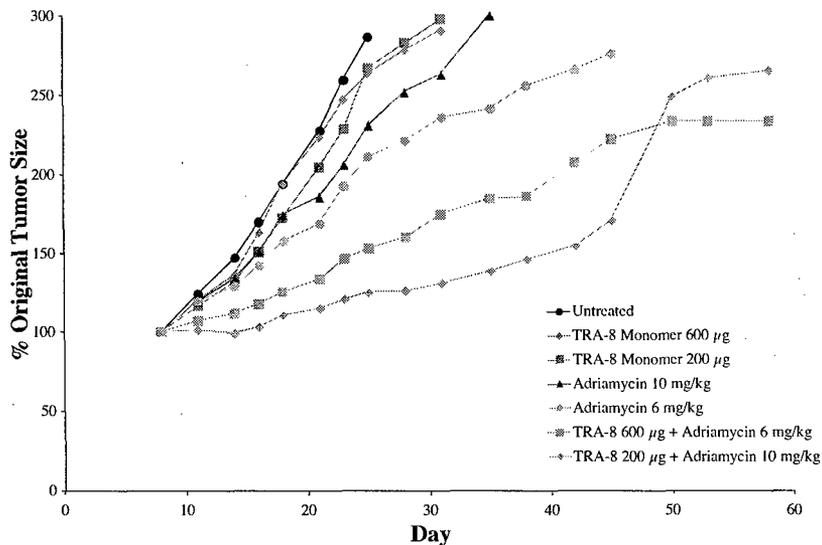

Figure 17. The effect of mTRA-8 and Adriamycin administered at high doses against LCC6 breast cancer xenografts. Cells (3×10^7) were injected s.c. on day 0. Groups of mice were injected i.p. with 200 or 600 μg monomeric mTRA-8 on days 8, 11, 15, 18, 22, and 25. Groups of mice received Adriamycin (6 mg/kg) i.v. on days 9, 13, and 17 or (10 mg/kg) on days 9, 16, and 23. One group of mice received no treatment. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 8. (n=8 mice/group).
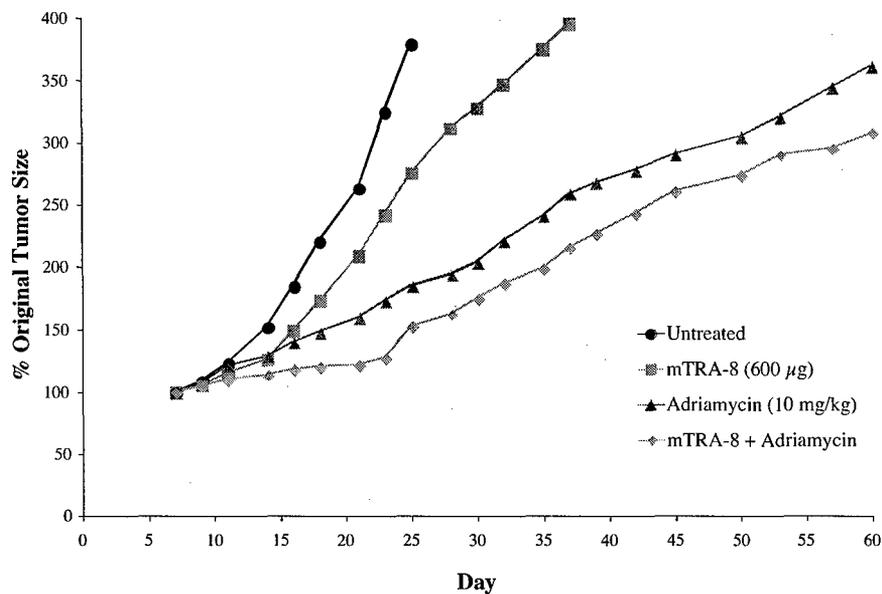
Figure 18. The effect of mTRA-8 and Adriamycin administered at high doses against LCC6 breast cancer xenografts. Cells (3×10^7) were injected *s.c.* on day 0. Groups of mice were injected *i.p.* with 600 µg monomeric mTRA-8 on days 7, 10, 14, and 17. Groups of mice received Adriamycin (10 mg/kg) *i.v.* on days 8 and 15. One group of mice received no treatment. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 7. (n=8 mice/group).
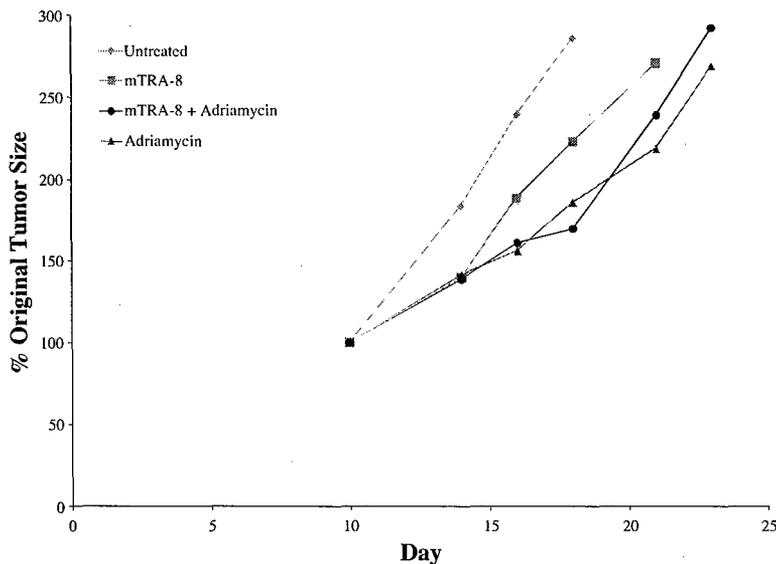

Figure 19. The effect of mTRA-8 and Adriamycin administered at high doses against LCC6 breast cancer xenografts. Cells (3×10^7) were injected *s.c.* on day 0. Groups of mice were injected *i.p.* with 800 µg monomeric mTRA-8 on day 11 and 400 µg on day 14. Groups of mice received Adriamycin (10 mg/kg) *i.v.* on day 10. One group of mice received no treatment. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 10. (n=8 mice/group).
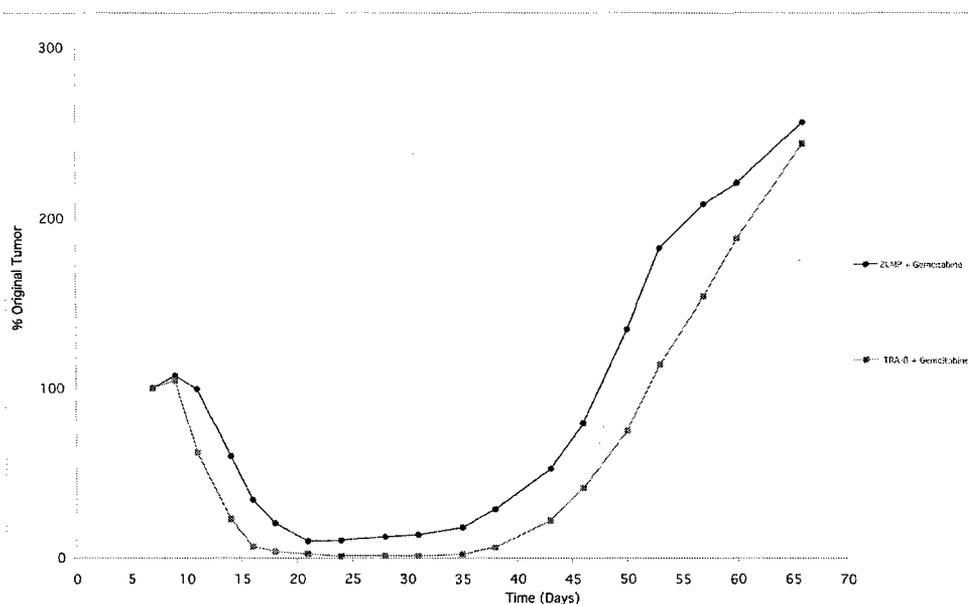
Figure 20A. The effect of mTRA-8 and Adriamycin administered at high doses against LCC6 breast cancer xenografts. Cells (3x10⁷) were injected *s.c.* on day 0. Groups of mice were injected *i.p.* with 600 µg monomeric mTRA-8 on days 7, 10, 14, and 17. Groups of mice received Adriamycin (10 mg/kg) *i.v.* on days 8 and 15. One group of mice received no treatment. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 7. (n=8 mice/group).
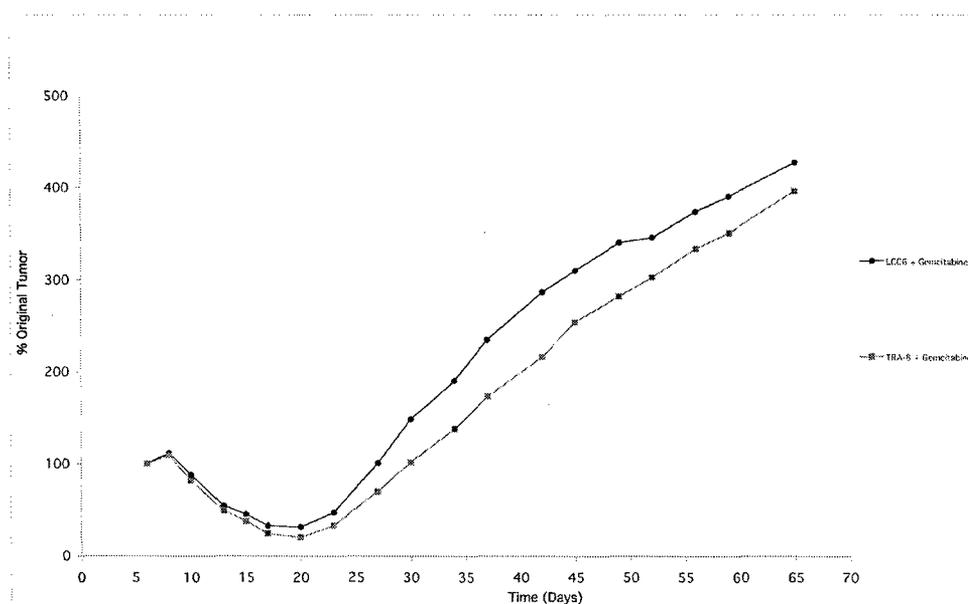

Figure 20B. The effect of mTRA-8 and Adriamycin administered at high doses against LCC6 breast cancer xenografts. Cells (3x10⁷) were injected *s.c.* on day 0. Groups of mice were injected *i.p.* with 800 µg monomeric mTRA-8 on day 11 and 400 µg on day 14. Groups of mice received Adriamycin (10 mg/kg) *i.v.* on day 10. One group of mice received no treatment. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 10. (n=8 mice/group).
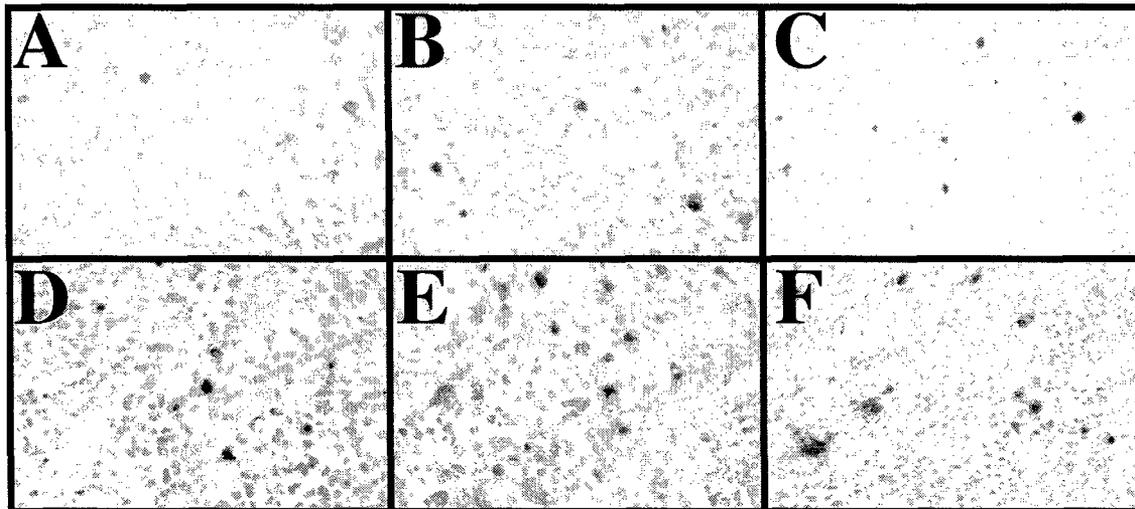
Figure 21. A) Demonstrates an apparently viable area of a 2LMP tumor xenograft from an untreated nude mouse; **B)** demonstrates a viable area of a 2LMP xenograft tumor from an animal treated with adriamycin only. Like the untreated tumor, few cells exhibited apoptosis (dark brown); **C)** demonstrates 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)** demonstrates a characteristic area of tumors from animals treated only with TRA-8 antibody at a dose of 100 μg administered twice with a 3 day interval between injections. A high percentage of cells demonstrate apoptosis; **E)** demonstrates 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)** demonstrates 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 400X
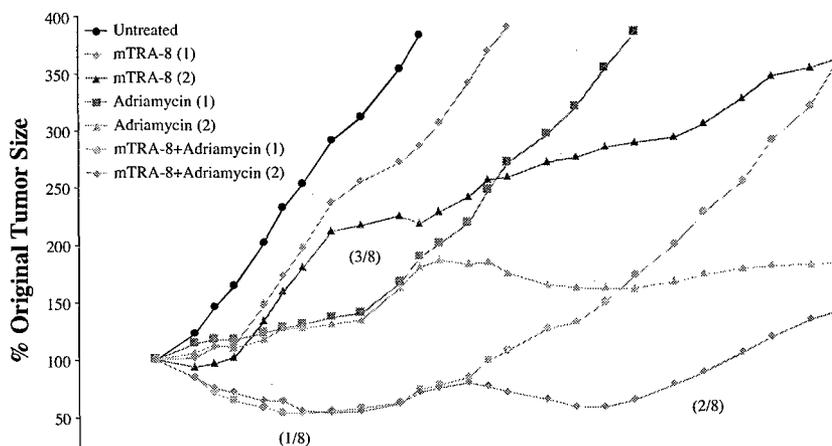

Figure 22. Treatment of 2LMP s.c. xenografts with one or two courses of TRA-8 and adriamycin. Groups of 8 mice were injected s.c. with 3×10⁷ 2LMP cells on day 0. Course 1 began on day 8 and lasted until day 26. Four groups of mice were injected i.v. with 200 μg TRA-8 on days 8, 12, 15, 19, 22, and 26. Four groups of mice were injected i.v. with 6 mg/kg adriamycin on days 9, 13, and 17. Course 2 began on day 33 and ended on day 50. Groups of mice previously treated with TRA-8 received 200 μg TRA-8 i.v. on days 33, 36, 40, 43, 47, and 50. Groups of mice previously treated with CPT-11 received 6 mg/kg CPT-11 i.v. on days 34, 38, and 42. Data are expressed as the average change in tumor size (surface area equal to product of two largest diameters) relative to size on day 8. Complete regressions are shown in parentheses. Dashed line indicates only 3/8 surviving mice.
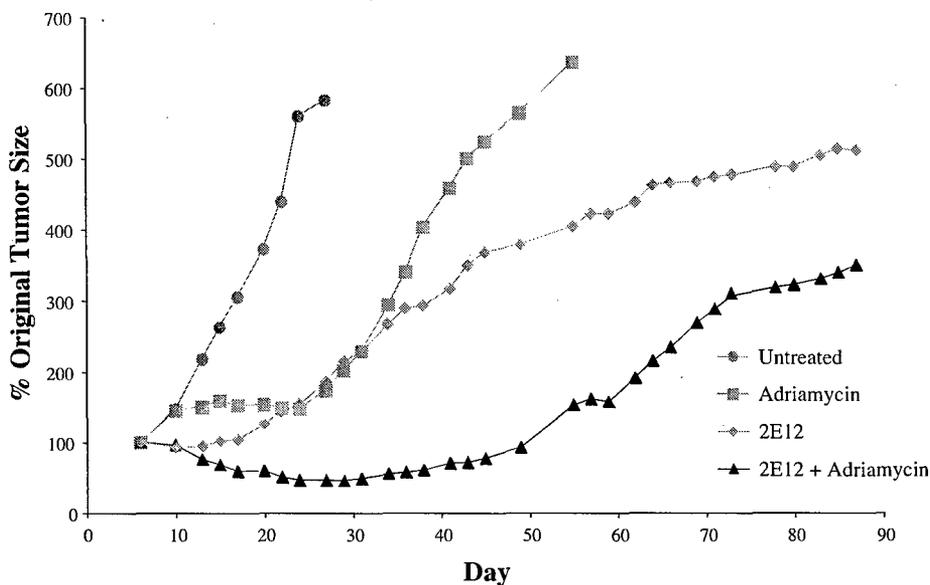
Figure 23. The effect of 2E12 and adriamycin in athymic nude mice bearing breast cancer xenografts. 2LMP cells ($3x10^7$) were injected *s.c.* into athymic nude mice on day 0. Two groups of mice were injected *i.p.* with 200 µg 2E12 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received *i.v.* 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 relative to size on day 7 (n=8 mice/ group).
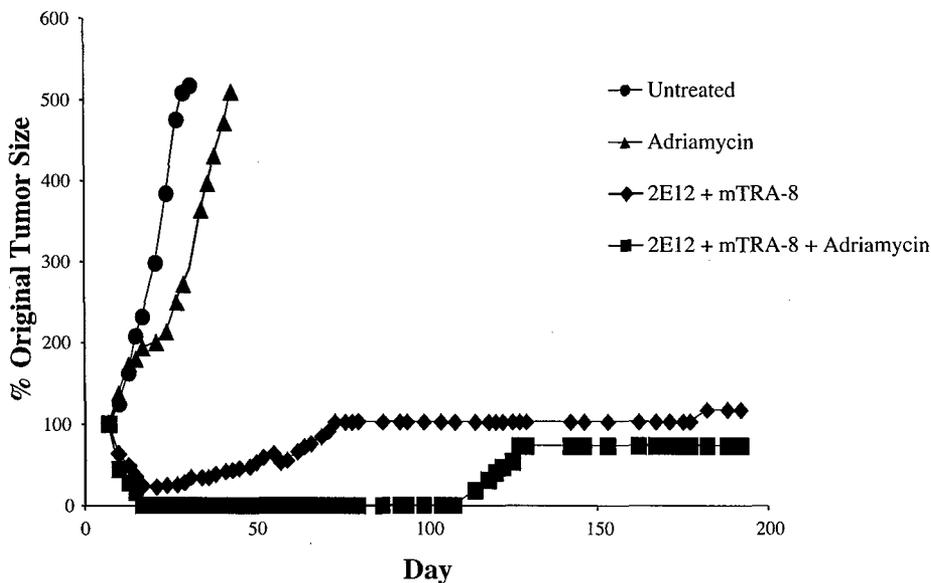

Figure 24. The effect of TRA-8, 2E12 and adriamycin in athymic nude mice bearing breast cancer xenografts. 2LMP cells ($3x10^7$) were injected *s.c.* into athymic nude mice on day 0. Two groups of mice were injected *i.p.* with 200 µg TRA-8 and 2E12 on days 7, 10, 14, 17, 21, and 24. Two groups of mice 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 relative to size on day 7 (n=8 mice/ group).

Adriamycin

RT
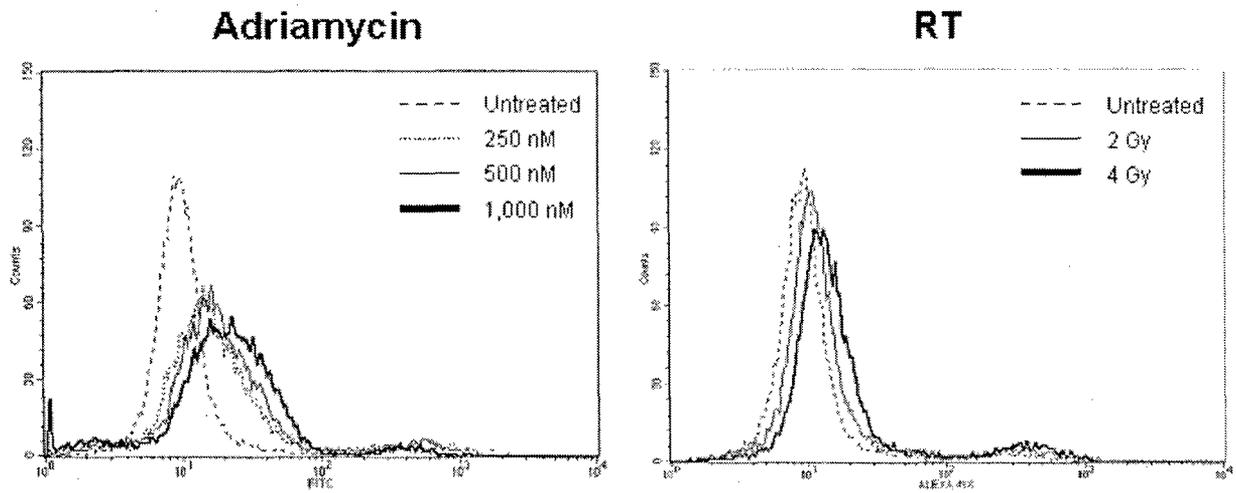
Figure 25. Upregulation of DR5 by adriamycin or radiation in LCC6 cells.
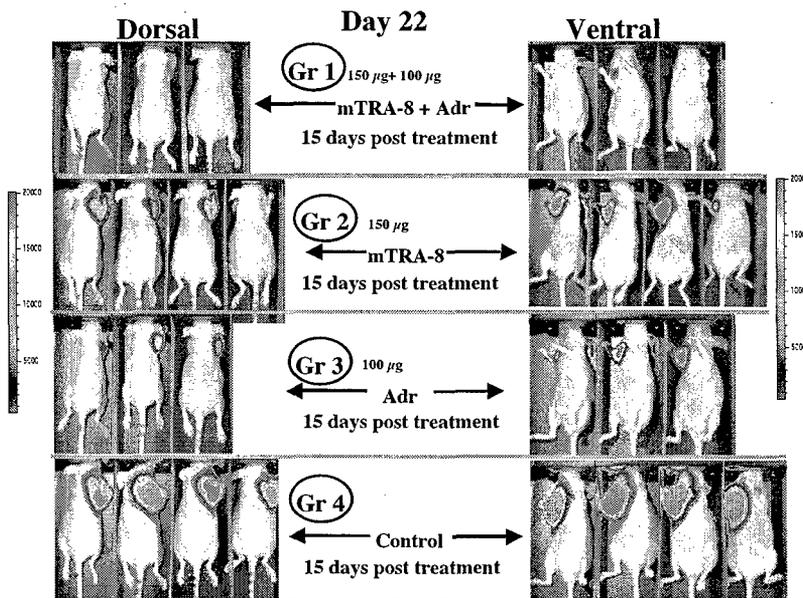

**Figure 26.** Bioluminescence images of mammary fat pad 2LMP-Luc after treatment.
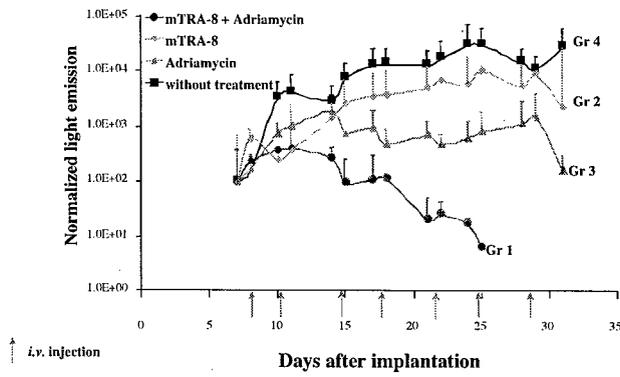
Figure 27. Measurement of luciferase in groups of treated animals
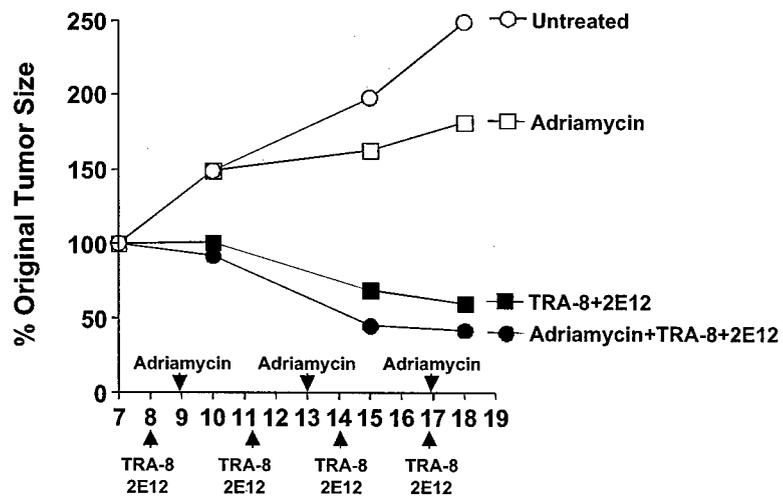
Figure 28. Tumor regression after combination therapy.
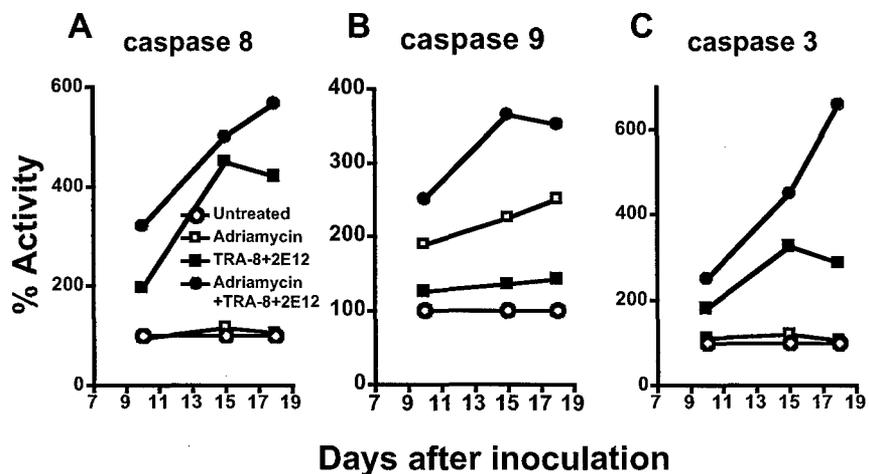
Figure 29. Caspase activity in tumor tissues after treatment. Caspase activity in the tumor tissues was measured by fluorescent caspase substrate kits. The results are presented as % of untreated control.
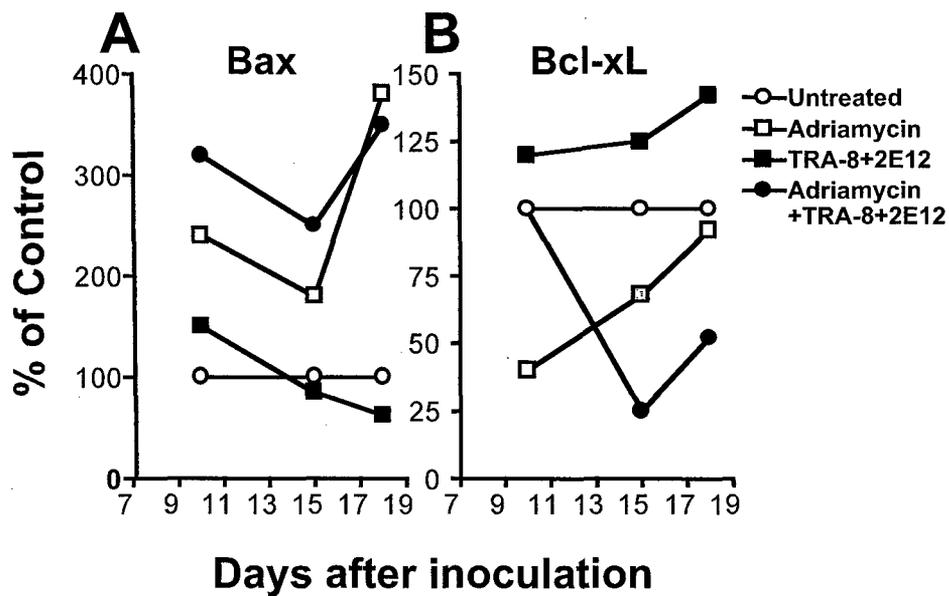
Figure 30. Expression of Bax and Bcl-xL in tumor tissues after treatment. The protein levels of Bax and Bcl-xL were measured by Western blot analysis and determined by densitometry. The results are presented as % of untreated control.
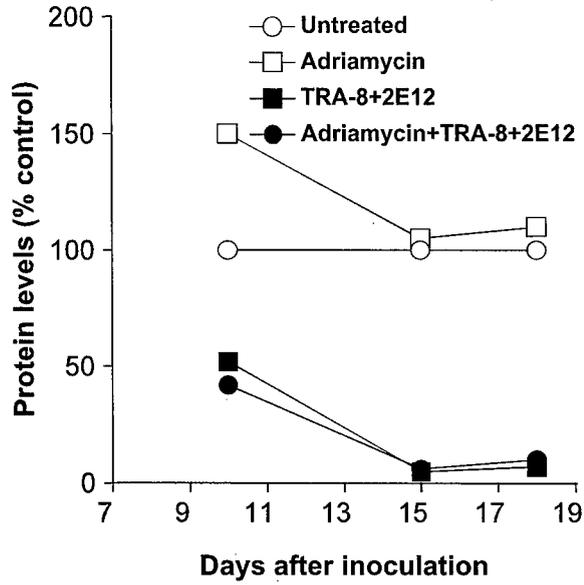
Figure 31. Down-modulation of XIAP by death antibody treatment. XIAP was measured by Western blot. The results are presented as % of untreated control.
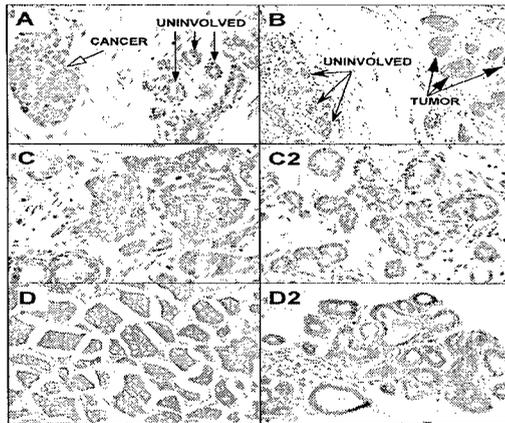
Figure 32. DR5 expression in human breast cancer specimens (A-D) and uninvolved epithelium (B, C2, D2).
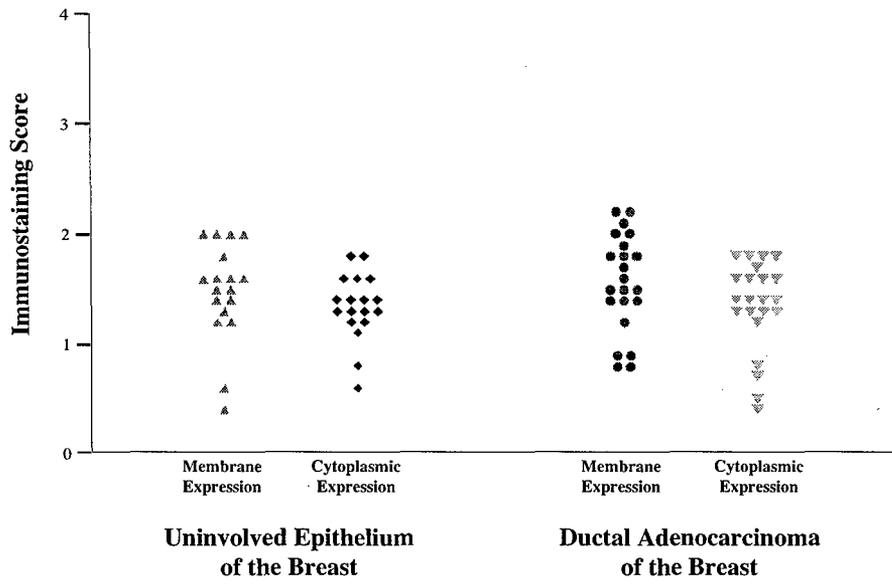
**Figure 33.** Immunostaining score of DR5 in human breast cancer specimens and uninvolved epithelium of the breast.

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[1]

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William E. Grizzle, Patsy G. Oliver,
Charlotte J. Hammond, Sijian Zhang,
Mark Carpenter, and Albert F. LoBuglio

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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 ^{60}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_{50} 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,[3] 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-

[3] 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 μg/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_2 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_2O 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^{2+} and Mg^{2+} 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 μg/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 μg/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 ≤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 ± 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 × 10⁷ 2LMP cells. At 7 days after tumor cell injection, 200 or 600 μg (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 μg 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}Co irradiation of 2LMP xenografts on days 9 and 17. The mean ± SD baseline tumor size (surface area equal to product of two largest diameters) for all studies at the start of treatment was 61 ± 16 mm^2.

Analysis of Apoptosis in Xenografts. Athymic nude mice injected s.c. with 3 × 10^7 2LMP cells on day 0 received 100 μg 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-μm paraffin sections of tissue were mounted on Superfrost/Plus slides and heated at 58°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 μg/ml in distilled deionized H$_2$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°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 H$_2$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® (39).

RESULTS

DR5 Expression and TRA-8 Induced Cytotoxicity in Breast Cancer Cell Lines. As illustrated in Fig. 1*A*, 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. 1*B* illustrates the TRA-8-induced cytotoxicity of the nine cell lines. Four cell lines were sensitive to TRA-8-induced cytotoxicity with 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.
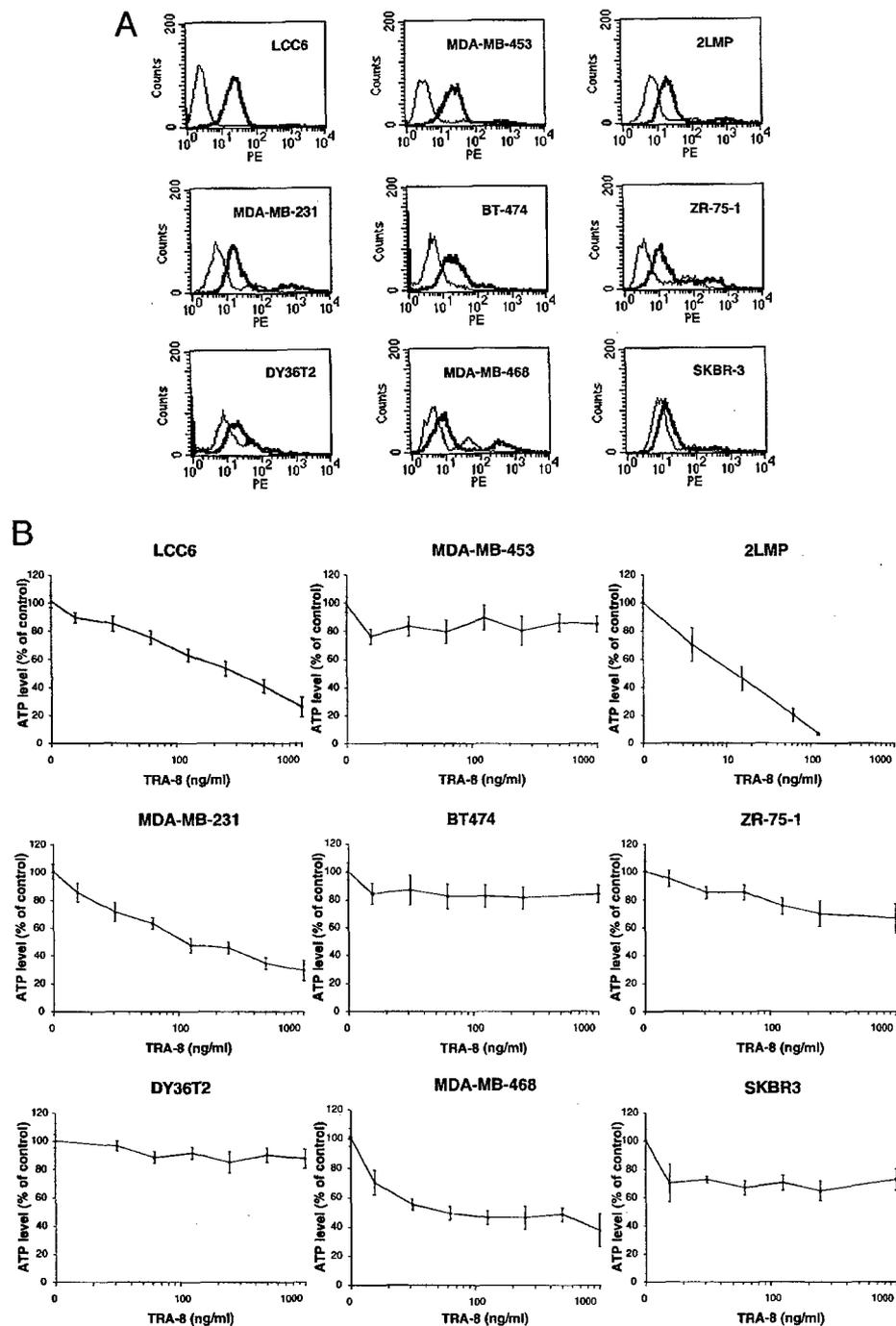
Fig. 1 *A,* flow cytometry analysis of DR5 cell surface expression in a panel of human breast cancer cell lines. Breast cancer cells were harvested using EDTA and stained with 10 μg/ml TRA-8 mAb for 1 h at 4°C followed by PE-conjugated goat antimouse IgG1, then analyzed using FACScan and Cell-Quest software. *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. 2*A*) and paclitaxel (Fig. 2*B*). 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 μg 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[b]	0.615
2LMP	Additive	0.153	Additive	0.937
MDA-MB-231	Additive	·0.663	Additive	0.064
BT-474	Synergistic	<0.001	ND[c]	0.992
ZR-75-1	Synergistic	0.013	Additive	0.172
DY36T2	ND[c] ·	0.808	ND[c]	0.798
MDA-MB-468	Additive	0.184	Additive	0.724
SK-BR-3	Additive	0.361	No response[b]	0.871

[a] 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.
[b] There was no significant dose response for either agent.
[c] Not determined because the TRA-8 effect was not significant, but the Adriamycin/paclitaxel effect was significant.
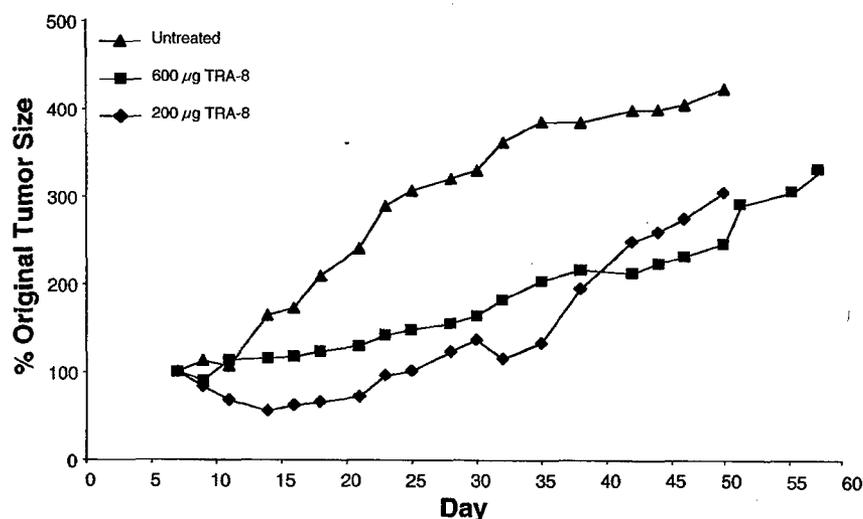

Fig. 3 The effect of TRA-8 on tumor growth in athymic nude mice bearing established 2LMP human breast cancer xenografts. 2LMP cells (3×10^7) were injected s.c. on day 0. Two groups of mice were injected i.p. with 200 or 600 μg of TRA-8 on days 7, 10, 14, 17, 21, and 24. One group of mice received no 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}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}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}Co plus TRA-8) were different from either single agent group (P < 0.001). The ^{60}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 × 10⁷) were injected s.c. into athymic nude mice on day 0. Two groups of mice were injected i.p. with 200 μg 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).
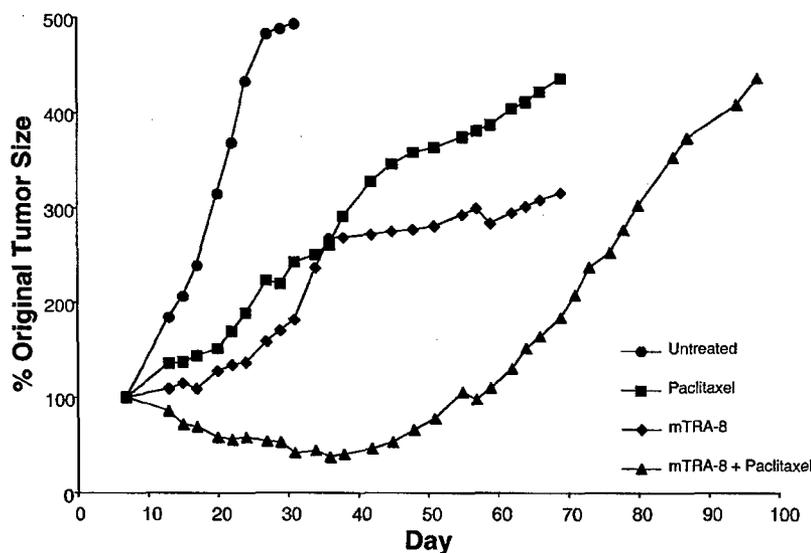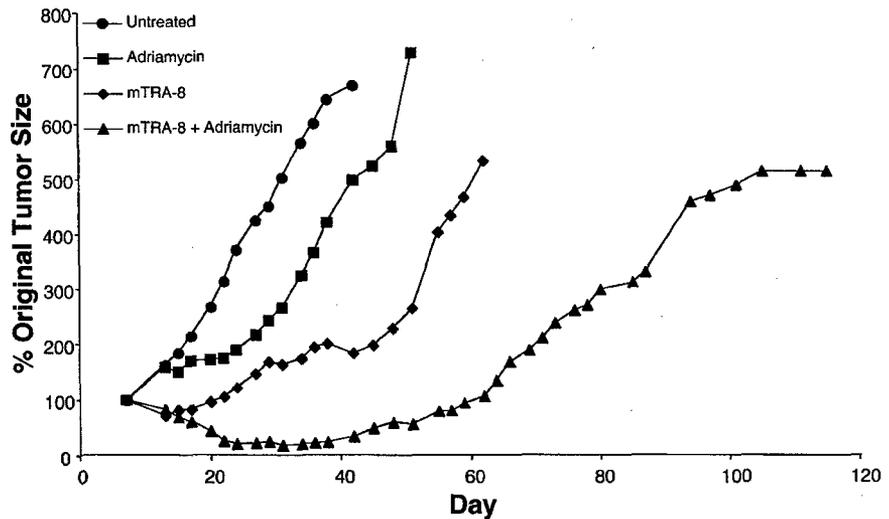
Fig. 5 The effect of TRA-8 and paclitaxel in athymic nude mice bearing breast cancer xenografts. 2LMP cells (3 × 10⁷) were injected s.c. into athymic nude mice on day 0. Two groups of mice were injected i.p. with 200 μg of TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received i.v. paclitaxel (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 + ⁶⁰Co 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
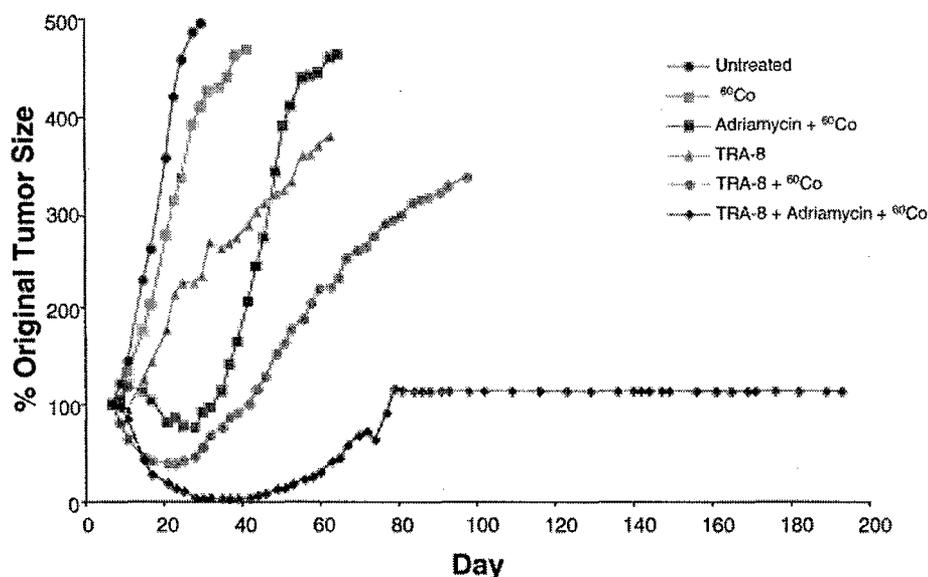
Fig. 6 The effect of TRA-8, Adriamycin, and ^{60}Co radiation on tumor growth in athymic nude mice bearing breast cancer xenografts. 2LMP cells (3×10^7) were injected s.c. into athymic nude mice on day 0. Three groups of mice were injected i.p. with 200 μg of TRA-8 on days 7, 10, 14, 17, 21, and 24. 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}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)[a]	12/8	1 (2%)	1 (2%)	177
^{60}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}Co	8 (8)	39/36	1 (13%)	0	197
TRA-8	30 (26)	47/23	6 (20%)	5 (17%)	159
TRA-8 + ^{60}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}Co	8 (6)	>140/179	6 (75%)	4 (50%)	192

[a] 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 ± 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
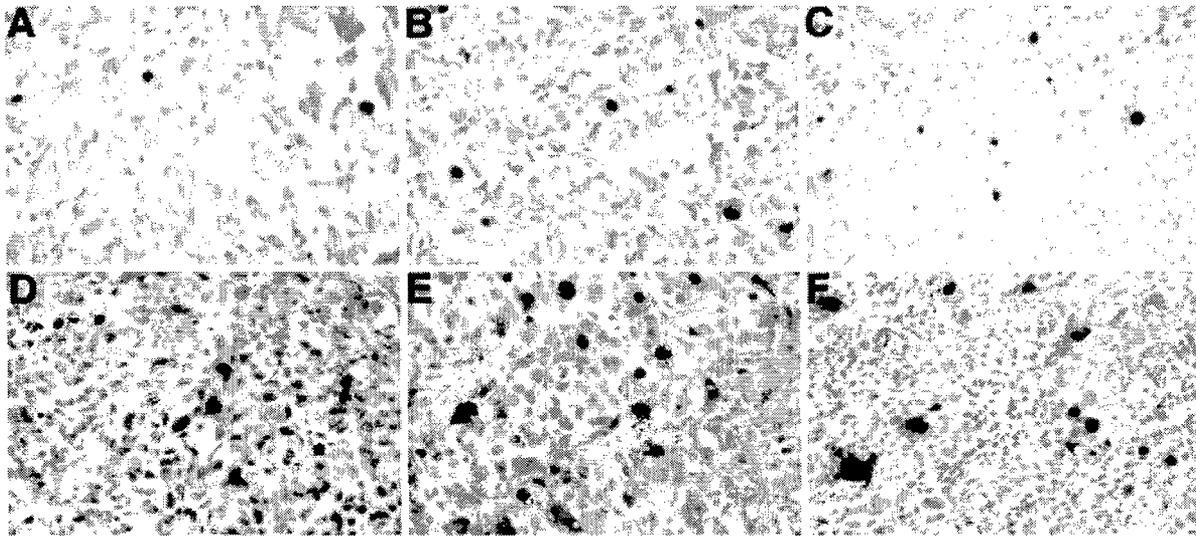
Fig. 7 A, an apparently viable area of a 2LMP tumor xenograft from an untreated nude mouse; *B,* a viable area of a 2LMP xenograft tumor from an animal treated with Adriamycin only. Like the untreated tumor, few cells exhibited apoptosis (*dark brown*); *C,* a viable area of a 2LMP xenograft tumor from 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 μg 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: ×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.[4]

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. 1*A*) and was sensitive to TRA-8-induced cytotoxicity *in vitro* (Fig. 1*B*). The 2LMP cell line had a dose-dependent cytotoxicity with either Adriamycin (Fig. 2*A*)

or paclitaxel (Fig. 2*B*), 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 ~60 mm² 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 μg (8 mg/kg) given six times over 3 weeks. A dose of 600 μg × 6 (24 mg/kg) was no better (Fig. 3), and lower doses had less efficacy.[5]

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

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[4] D. J. Buchsbaum and P. G. Oliver, unpublished observations.

[5] 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 ≥ 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.[6] 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.[7] 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.

ACKNOWLEDGMENTS

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Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway

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Using two agonistic monoclonal antibodies specific for each death receptor of TRAIL, 2E12 (anti-human DR4) and TRA-8 (anti-human DR5), we examined the signal transduction of the death receptors in combination with or without chemotherapy agents such as Adriamycin (doxorubicin hydrochloride) and Cisplatin. Our results demonstrated that chemotherapy agents were able to enhance apoptosis-inducing activity of these antibodies against several different types of tumor cell lines through enhanced caspase activation. The combination of the antibodies and chemotherapy agents led to a synergistical activation of the JNK/p38 MAP kinase, which was mediated by MKK4. The combination also caused an increased release of cytochrome c and Smac/DIABLO from mitochondria in parallel with the profound loss of mitochondrial membrane potential. These results suggest that the enhanced activation of the JNK/p38 kinase and the mitochondrial apoptosis pathways play a crucial role in synergistic induction of the death receptor-mediated apoptosis by chemotherapy agents. Thus, the simultaneous targeting of cell surface death receptors with agonistic antibodies and the intracellular JNK/p38 and the mitochondrial death pathways with chemotherapy agents would enhance the efficacy and selectivity of both agents in cancer therapy.
Oncogene (2003) **22**, 2034–2044. doi:10.1038/sj.onc.1206290

Keywords: apoptosis; TRAIL receptor; chemotherapy; JNK; mitochondria

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is an apoptosis-inducing member of the TNF gene superfamily (Wiley *et al.*, 1995; Griffith and Lynch, 1998). TRAIL interacts with several distinct cell surface receptors including the death receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), and the decoy receptors, DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), as well as osteoprotegerin, which serve as an inhibitor of TRAIL-mediated apoptosis (Pan *et al.*, 1997a, b; Emery *et al.*, 1998). Similar to TNF-R1 and Fas, DR4 and DR5 contain the intracellular death domain that is essential for the induction of apoptosis. TRAIL-induced activation of DR4 and DR5 involves the receptor multimerization with subsequent recruitment of several intracellular adaptor molecules to the death domain including Fas-associated death domain (FADD) and the initiator procaspase-8, which triggers cell death (Chaudhary *et al.*, 1997; Schneider *et al.*, 1997; Bodmer *et al.*, 2000; Kischkel *et al.*, 2000; Kuang *et al.*, 2000; Sprick *et al.*, 2000). The activation of initiator caspase-8 can induce activation of the effector caspases in the cell death receptor pathway (Ashkenazi and Dixit, 1998). The signaling events leading to apoptosis through the death receptors also involve the mitochondrial pathway (Green and Reed, 1998), in which death signals lead to changes in mitochondrial membrane potential and membrane permeability, and the subsequent release of proapoptotic factors including cytochrome c (Liu *et al.*, 1996) and second mitochondria-derived activator of caspase (Smac/DIABLO) (Du *et al.*, 2000; Verhagen *et al.*, 2000). The cytosolic cytochrome c promotes caspase-9 activation and Smac/DIABLO downmodulates the apoptosis-inhibitory function of the proteins of the inhibitor of apoptosis (IAP) family such as X-linked IAP (XIAP) (Liu *et al.*, 1996; Du *et al.*, 2000; Verhagen *et al.*, 2000). Members of the Bcl-2 family, including antiapoptotic Bcl-2 and Bcl-X_L, or proapoptotic Bax and Bid, are major regulators in the mitochondrial apoptosis pathway (Gross *et al.*, 1999; Kroemer and Reed, 2000). The interaction between pro- and antiapoptotic proteins may determine the susceptibility of cells to a death signal (Gross *et al.*, 1999; Zhang *et al.*, 2000).

Unlike TNF-α and FasL, TRAIL appears to selectively induce apoptosis of transformed tumor cells (French and Tschopp, 1999). The potential and safety of soluble TRAIL as an anticancer therapeutic agent has been demonstrated in mice and non-human primates (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999). However,

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it has also been reported that certain forms of soluble TRAIL are able to induce apoptosis of normal human hepatocytes *in vitro*, which highlights concerns of potential toxicity in humans (Jo *et al.*, 2000). Recently, we have generated a novel agonistic anti-human DR5 monoclonal antibody, TRA-8, which induces apoptosis of most TRAIL-sensitive tumor cells both *in vitro* and *in vivo* (Ichikawa *et al.*, 2001). Unlike TRAIL, TRA-8 does not induce hepatocellular toxicity, indicating that specific targeting of DR5 with TRA-8 might be a safer and more effective strategy for cancer therapy than TRAIL.

There is increasing evidence showing that many chemotherapeutic agents have the ability to synergize TRAIL-mediated apoptosis of cancer cells both *in vitro* and *in vivo* (Gliniak and Le, 1999; Keane *et al.*, 1999; Mizutani *et al.*, 1999, 2001; Gibson *et al.*, 2000; Yamanaka *et al.*, 2000; Cuello *et al.*, 2001; Hernandez *et al.*, 2001; Lacour *et al.*, 2001; Nagane *et al.*, 2001; Frese *et al.*, 2002). However, the signaling mechanisms leading to synergistic induction of tumor cell apoptosis by such a combination are poorly understood. Recently, we found that bisindolylmaleimide VIII (Bis VIII), a death receptor signaling enhancer (Zhou *et al.*, 1999), enhances DR5-mediated apoptosis through the synergistic activation of the c-Jun N-terminal protein kinase (JNK)/p38 mitogen-activated protein kinase (MAPK) pathway and the mitochondrial death signaling pathway (Ohtsuka and Zhou, 2002). Because the JNK/p38 signaling and the mitochondrial apoptosis pathways are crucially involved in cell death induced by many chemotherapeutic agents, we further examine the role of these two signaling pathway in synergistic induction of apoptosis of tumor cells with agonistic antibodies against DR4 and DR5 and chemotherapy agents. Our results demonstrate that the chemotherapy agents with the capability of inducing DNA damage, such as Adriamycin and Cisplatin, are able to synergistically enhance both anti-DR5 and DR4 antibody-induced apoptosis of several types of human tumor cells regardless of their pre-existing susceptibility to anti-death receptor antibody-mediated apoptosis. Similar to those mediated by Bis VIII, the enhanced activation of the JNK/p38 kinase pathway and the mitochondrial apoptosis pathway appears to be a critical mechanism leading to synergistic induction of apoptosis by the combination of anti-death receptor antibody and chemotherapy agent.

Results

Enhanced cell death of tumor cells by combination of anti-death receptor antibody and chemotherapy agent

DR4 and DR5 dual-positive MDA-MB-231-KS cells, a human breast cancer cell line, were examined for their susceptibility to TRAIL-, TRA-8- and 2E12-mediated apoptosis in the absence or presence of three commonly used chemotherapy agents, Adriamycin, Cisplatin and Taxol. In the absence of chemotherapy agents, the

MDA-231 cells exhibited a different dose-dependent apoptotic response to TRAIL, TRA-8 and 2E12. Their susceptibility to apoptosis was high for TRA-8, intermediate for TRAIL and relatively low for 2E12 (Figure 1a). The chemotherapy agent alone did not induce significant cell death at the indicated dose and period of time. However, among three tested chemotherapy agents, Adriamycin and Cisplatin but not Taxol significantly enhanced cell death induced by TRAIL, TRA-8 or 2E12. The synergistic effect of Adriamycin and Cisplatin on TRAIL receptor-mediated apoptosis was very similar to that induced by Bis VIII as previously shown (Ohtsuka and Zhou, 2002). More complete cell death could occur with low doses of TRAIL (100 ng/ml) and TRA-8 (10 ng/ml) in the presence of 3 μM Adriamycin or 100 μM Cisplatin. While 100 ng/ml of 2E12 alone was unable to induce significant cell death, more than 60–70% cell death was induced by the same dose in the presence of Adriamycin or Cisplatin. Adriamycin appeared to be strong in the combination with TRA-8 as the susceptibility of cells to TRA-8-mediated apoptosis was increased more than 50-fold. The synergistic effect of Adriamycin and Cisplatin on TRA-8-induced apoptosis was dose-dependent (Figure 1b). Enhanced apoptosis of tumor cells by the combination treatment was evident as indicated by increased DNA fragmentation (Figure 1c). When the cells were treated with chemotherapy agent (Adriamycin, Cisplatin, or Taxol) alone for long period of times (48–72 h), significant loss of cell viability was induced at lower concentrations of the agents (data not shown). Importantly, TRA-8 could still enhance the cell death induced by the chemotherapy agent in a synergistical manner (data not shown).

The synergistic effect of Adriamycin and Cisplatin on TRA-8- or 2E12-induced apoptosis was also examined in several other types of tumor cell lines including 2LMP (breast), HeLa (cervix), UL-3B (ovary), 1321N1 (astrocytoma) (Figure 2), and two colon (Widr and HT29) and two prostate cell lines (Du145 and PC-3) (Table 1). All tested tumor cells became very susceptible to TRA-8-mediated apoptosis in the presence of either Adriamycin or Cisplatin regardless of their pre-existing sus-ceptibility to TRA-8 and chemotherapy agents. Although 2E12 was less effective in induction of apoptosis of tumor cells, the apoptotic response in two susceptible lines (2LMP and HeLa) was significantly enhanced by Adriamycin or Cisplatin. Taken together, these results indicate that Adriamycin and Cisplatin can synergistically enhance the death receptor-mediated apoptosis by enhancing the rate of cell death.

Enhanced caspase activation by combination of anti-death receptor antibody and chemotherapy agent

To determine whether the synergistic induction of apoptosis by combination of anti-death receptor antibody and chemotherapy agent is through caspase pathway, the activation of three key caspases was examined by Western blot analysis. Treatment of MDA-MB-231-KS cells with low concentration of

a

b

c
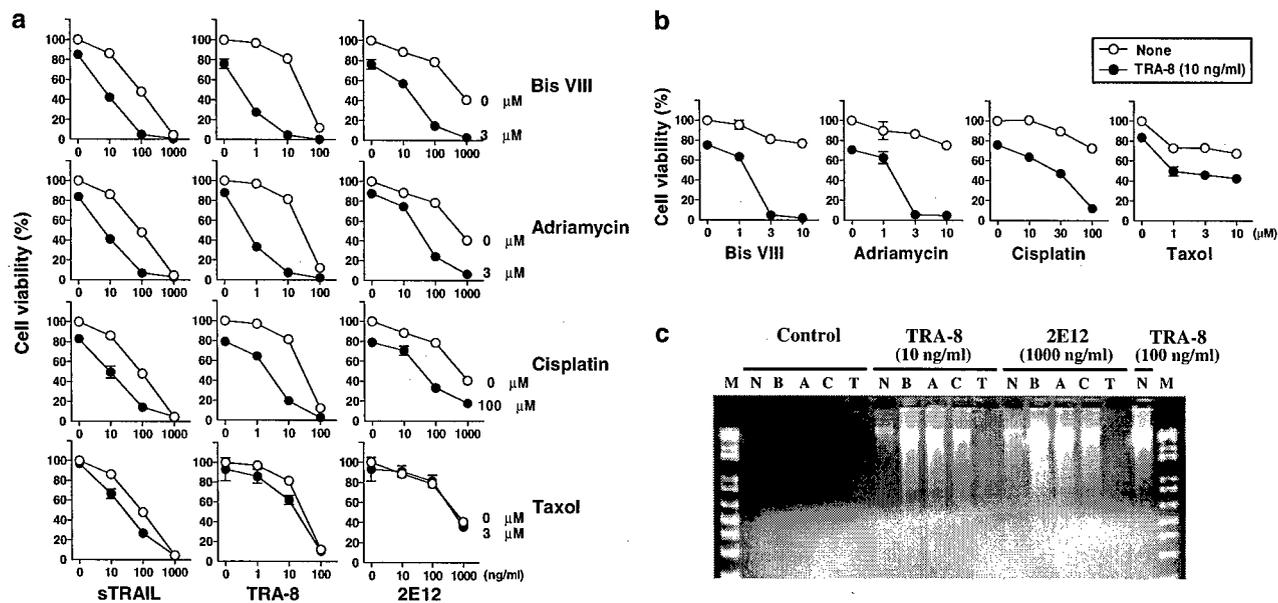
Figure 1 Chemotherapy agents enhanced DR4- and DR5-induced cell death of breast cancer MDA-MB-231-KS cells. (a) Apoptosis-enhancing effect of several chemotherapy agents in combination with DR4 or DR5 receptor agonist. MDA-MB-231-KS cells were incubated with indicated concentrations of Bis VIII, Adriamycin, Cisplatin, or Taxol in combination with either soluble TRAIL (sTRAIL) plus 2 µg/ml enhancer, TRA-8, or 2E12 plus 2 µg/ml crosslinker for 14 h, and cell viability was determined by the ATPLite assay. (b) Dose dependencies of chemotherapy agents on potentiation of apoptosis induced by TRA-8. MDA-MB-231-KS cells were incubated with indicated concentrations of chemotherapy agents or Bis VIII in the absence or presence of 10 ng/ml TRA-8 for 14 h, and cell viability was determined by the ATPLite assay. (c) Detection of DNA ladder formation induced by 2E12 or TRA-8 in combination with chemotherapy agents. MDA-MB-231-KS cells were left untreated (N) or incubated with 3 µM Bis VIII (B), 3 µM Adriamycin (A), 100 µM Cisplatin (C), or 3 µM Taxol (T) in combination with either TRA-8 or 2E12 plus 2 µg/ml crosslinker for 14 h. After the incubation, cellular DNA was extracted and analysed by electrophoresis on 2% agarose gel to detect DNA fragmentation. M, size marker DNA (1 kb Plus DNA Ladder, from Life Technologies)

TRA-8 (10 ng/ml) or 2E12 (1000 ng/ml) did not induce strong cleavage of caspases-8, -9, and -3 (Figure 3a). As previously demonstrated, Bis VIII alone had little effect on the activation of caspases and cleavage of PARP. Although Adriamycin or Cisplatin alone induced a weak cleavage of caspase-9 and -3, the strong activation of all three caspases was only observed in the combination treatment with the anti-death receptor antibody (TRA-8 or 2E12) and the chemotherapy agent (Adriamycin or Cisplatin). The enhanced activation of caspases is indicated by decreased procaspase-8 and increased cleavage of caspase-9 and -3. The cleavage of PARP, a substrate for caspase-3, was also enhanced by the combination treatment. The synergistically induced cell death by the combination is also caspase-dependent as a caspase inhibitor, Z-VAD-FMK, significantly inhibited cell death (Figure 3b). The similar caspase activation and dependence was also observed in 1321N1 and UL-3B cells treated with the combination of anti-death receptor antibody and chemotherapy agent (data not shown). Taken together, these results indicate that synergistic induction of apoptosis of tumor cells by anti-death receptor antibody and chemotherapy agent is through caspase pathway. The strong activation of caspase-8 and -9 suggests that both the initiator caspases are involved in the synergistic induction of apoptosis.

Synergistic activation of JNK/p38 pathway by combination of anti-death receptor antibody and chemotherapy agents

Our previous study has shown that Bis VIII enhances DR5-induced JNK/p38 through MKK4 activation (Ohtsuka and Zhou, 2002). To examine the effect of chemotherapy agents on the activation of JNK/p38 during TRA-8- or 2E12-induced apoptosis, activation of these MAPK was determined by Western blot analysis with antibodies that recognize activated phosphorylated forms of these kinases. Treatment of MDA-MB-231-KS cells with Adriamycin or Cisplatin alone resulted in a mild activation of JNK and p38, similar to that with TRA-8 or 2E12 alone, which was significantly enhanced by the combination of either antibody with Adriamycin or Cisplatin (Figure 4a). These effects resulted from increased phosphorylation of JNK and p38 rather than increased total amount of the protein (data not shown). In addition, these effects appear to be selective for JNK and p38, as the activation of a third member of this kinase family, ERK1/2, was not affected (Figure 4b). These results indicate that the enhancement of apoptosis by Adriamycin or Cisplatin is associated with the synergistic activation of JNK/p38. To identify upstream kinase(s) for JNK and p38 activation, we examined the activation of three MAPK kinases, MKK4, which
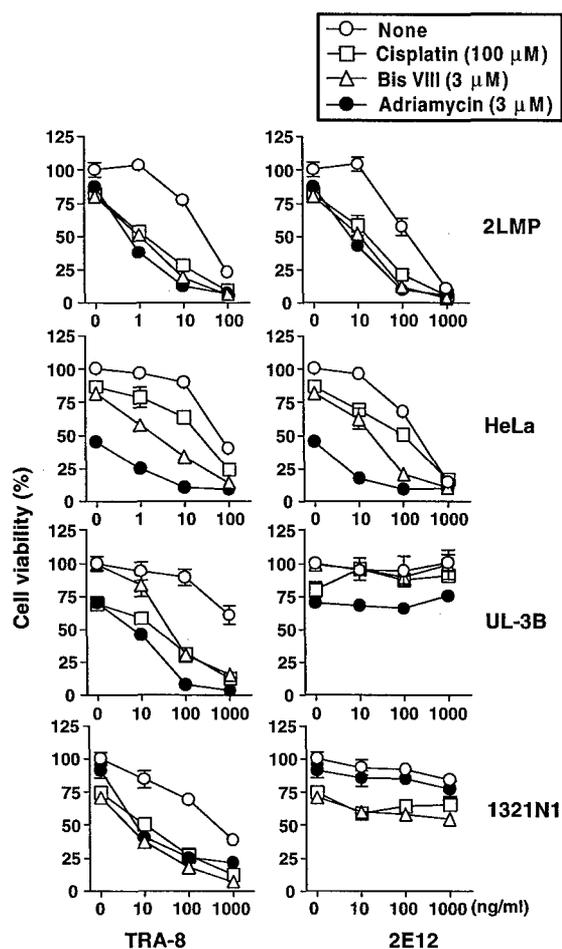
Figure 2 Chemotherapy agents enhanced DR4- and DR5-induced cell death of several cell lines. 2LMP, HeLa, UL-3B, or 1321N1 cells were incubated with indicated concentrations of chemotherapy agents or Bis VIII in combination with indicated concentrations of either TRA-8 or 2E12 plus 2 μg/ml crosslinker for 15 h, and cell viability was determined by the ATPLite assay

activates both JNK and p38, and MKK3 and -6, which only activate p38, during the treatment with TRA-8, 2E12, and chemotherapy agent. We found that only MKK4 and not MKK3/6 was activated by TRA-8, 2E12, or chemotherapy agents including Adriamycin and Cisplatin (Figure 4a and data not shown). The combined treatment with the antibody and the chemotherapy agent resulted in a great increase in the activated MKK4 (Figure 4a), suggesting that MKK4 is an upstream kinase responsible for DR4-, DR5-induced and chemotherapy agent-enhanced activation of JNK/p38.

The synergistic activation of JNK/p38 by TRA-8 and chemotherapy agents was also observed in other types of tumor cells including astrocytoma, cervix, colon, and ovary (Table 1). Similar to that observed in MDA-MB-231-KS cells, Adriamycin, Cisplatin, or Bis VIII alone was able to induce a mild activation of JNK/p38 in these cells (data not shown). However, the TRA-8-induced JNK/p38 activation was greatly enhanced in the presence of Adriamycin or Cisplatin. Importantly, the

enhanced activation of JNK/p38 appeared to be associated with the increased susceptibility of tumor cells to anti-death receptor-mediated apoptosis. These results suggest that the JNK/p38 pathway might be a convergence point in the death receptor and chemotherapeutic agents-induced signal transduction leading to apoptosis.

To access the role of JNK/p38 in death process induced by death receptor antibody and chemotherapy agent, we used *N*-acetyl-L-cysteine (NAC) as an inhibitor for the JNK/p38 pathway as recent reports show that death receptor-mediated sustained activation of JNK and p38 is regulated by ASK1 (Matsuzawa and Ichijo, 2001), one of the MAPK kinase kinase family members, which can be inhibited by antioxidant such as NAC (Tobiume *et al.*, 2001). As we expected, pretreatment with NAC effectively suppressed activation of JNK/p38, which was induced by either TRA-8 or chemotherapy agent alone or in combination (Figure 5a). Correlated with the inhibitory ability to the JNK/p38 pathway, NAC was able to significantly inhibit cell death caspase-9 and -3 activation, and processing of Bid and PARP (Figure 5c) induced by TRA-8 alone or a TRA-8 and chemotherapy agent combination. Since NAC was not able to or only partially inhibit caspase-8 processing induced by TRA-8 alone or a TRA-8 and chemotherapy agent combination (Figure 5c), the caspase activation process was interrupted by NAC after activation of caspase-8 initiated by death receptor activation. Similar results were obtained in other cell lines including 1321N1 cells (data not shown). These results further support the notion that activation of JNK/p38 is involved in death receptor-mediated caspase activation and cell death.

Increased activation of mitochondrial apoptosis pathway by combination of anti-death receptor antibody and chemotherapy agent

Many apoptosis stimuli including the death-inducing ligands and chemotherapy agents can activate the mitochondrial apoptosis pathway. The loss of the mitochondrial membrane potential and the release of cytochrome *c* and Smac/DIABLO are the markers for the activation of mitochondrial pathway, which are regulated by the pro- and anti-apoptotic proteins of the Bcl-2 family (Green and Reed, 1998; Gross *et al.*, 1999; Kroemer and Reed, 2000; Zhang *et al.*, 2000). To determine whether the mitochondrial pathway is involved in the enhancement of DR4- or DR5-induced apoptosis by chemotherapy agents, we first examined the alteration of mitochondrial membrane potential using a fluorescent dye, JC-1. Treatment of MDA-MB-231-KS cells with a low dose of TRA-8 or a higher dose of 2E12 alone led to a moderate loss of mitochondrial membrane potential (Figure 6a). The chemotherapy agents alone including Adriamycin and Cisplatin, as well as Bis VIII, could induce a significant loss of mitochondrial membrane potential at a dose that was not sufficient to induce apoptosis. The complete loss of the mitochondrial membrane potential only occurred in

Table 1 Synergistic induction of DR5-mediated apoptosis with chemotherapeutic agents through JNK/p38

Cells	Tumor	TRA-8 alone		TRA-8 + BIS VIII		TRA-8 + Adriamycin		TRA-8 + Cisplatin	
		Cell death[a]	JNK/p38[b]	Cell death	JNK/p38	Cell death	JNK/p38	Cell death	JNK/p38
MDA-MB-231-KS	Breast	84.6	2.4/2.6	99.5	13.8/17.9	97.1	17.6/21.0	96.7	22.3/28.3
MDA-MB-231-PO	Breast	23.0	5.3/5.8	86.2	15.8/12.1	83.0	11.3/11.5	63.2	ND
132INI	Astrocytoma	37.2	0.6/3.7	94.6	12.6/12.2	93.7	4.9/8.4	94.0	20.2/15.1
HeLa	Cervix	58.2	2.5/4.7	90.5	8.0/25.1	90.0	9.0/28.6	87.7	32.6/47.4
Widr	Colon	47.3	2.3/8.2	88.9	3.9/14.1	90.5	13.2/16.7	58.5	40.0/25.1
HT29	Colon	19.3	ND	64.9	ND	81.8	ND	72.0	ND
UL-3B	Ovary	4.0	1.1/1.2	44.3	6.8/13.3	77.8	13.9/15.3	55.0	4.7/9.6
UL-3C	Ovary	42.9	3.6/8.0	91.8	4.3/10.4	92.5	5.2/10.6	80.5	20.4/21.8
DU145	Prostate	26.6	ND	77.8	ND	67.8	ND	43.1	ND
PC-3	Prostate	17.4	ND	69.8	ND	80.2	ND	57.6	ND

[a]Tumor cells were cultured with 15 ng/ml TRA-8 alone or with 5 μM Bis VIII, 3 μM Adriamycin or 100 μM Cisplatin overnight. Cell death was determined by ATPLite assay. The data are presented as percent cell death of the control with medium or drug alone
[b]Tumor cells were cultured with TRA-8 alone (1000 ng/ml for MDA-MB-231-PO and UL-3B cells, or 100 ng/ml for other cells) or with 3 μM Bis VIII, 3 μM Adriamycin or 100 μM Cisplatin for 4 h. Activation of JNK and p38 (JNK/p38) was determined by Western blot analysis of the phosphorylated JNK and the phosphorylated p38, and quantitated by densitometry. The data are presented as fold increase of medium control. ND, not determined
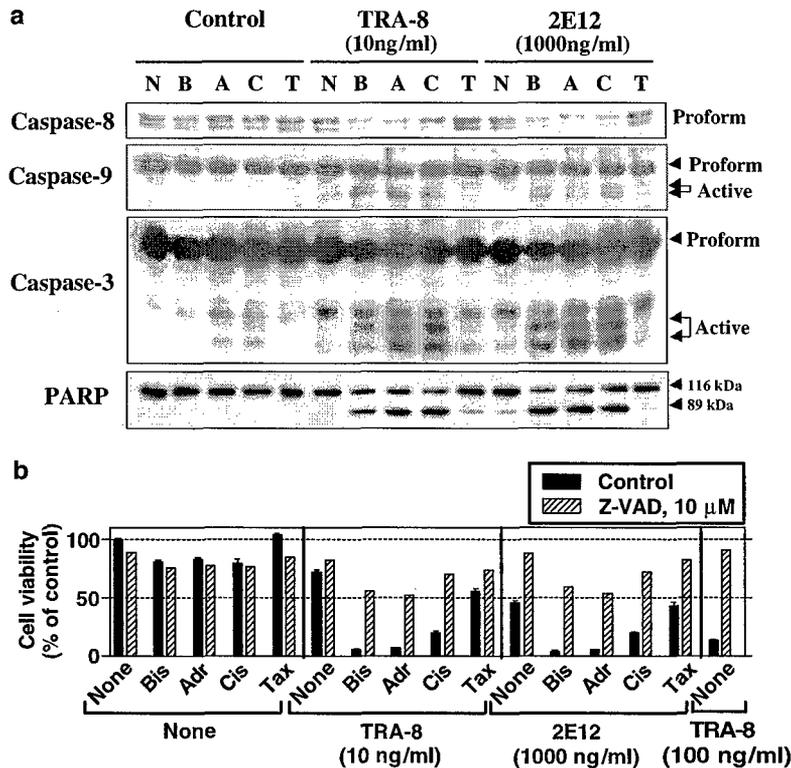

Figure 3 Chemotherapy agents enhanced DR4- and DR5-induced caspase activation of breast cancer MDA-MB-231-KS cells. (a) MDA-MB-231-KS cells were left untreated (N) or incubated with 3 μM Bis VIII (B), 3 μM Adriamycin (A), 100 μM Cisplatin (C), or 3 μM Taxol (T) in combination with 10 ng/ml TRA-8 or 1 μg/ml 2E12 plus 2 μg/ml crosslinker for 9 h. Whole-cell lysates were prepared and subjected to Western blot analysis using the respective antibodies as indicated. (b) MDA-MB-231-KS cells were preincubated with or without 10 μM Z-VAD-FMK for 1 h and then left untreated (None) or incubated with 3 μM Bis VIII (Bis), 3 μM Adriamycin (Adr), 100 μM Cisplatin (Cis), or 3 μM Taxol (Tax) in combination with indicated concentrations of either TRA-8 or 2E12 plus 2 μg/ml crosslinker for 14 h. Cell viability was determined by the ATPLite assay

the combination of either antibody with any single chemotherapy agent. These results suggest that the decreased mitochondrial membrane potential by chemotherapy agents might lead to increased susceptibility of tumor cells to death receptor-mediated apoptosis.

In the mitochondrial pathway, the death signals lead to the release of proapoptotic factors including cytochrome *c* and Smac/DIABLO, which results in activation of caspase-9 and inactivation of IAPs, particularly XIAP, respectively (Liu *et al.*, 1996; Du
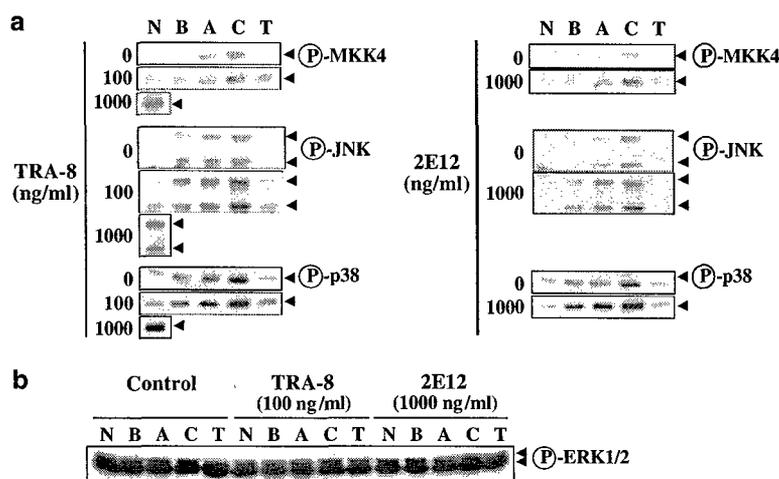
Figure 4 Chemotherapy agents enhanced DR4- and DR5-induced MKK4/JNK/p38 activation of breast cancer MDA-MB-231-KS cells. (a) MDA-MB-231-KS cells were left untreated (N) or incubated with 3 μM Bis VIII (B), 3 μM Adriamycin (A), or 100 μM Cisplatin (C), or 3 μM Taxol (T) in combination with indicated concentration of TRA-8 or 2E12 plus 2 μg/ml crosslinker for 4 h. Whole-cell lysates were prepared and subjected to Western blot analysis using antibodies to phospho-MKK4 (℗-MKK4), phospho-JNK46/54 (℗-JNK), phospho-p38 (℗-p38). (b) The same whole-cell lysates prepared in Panel a were subjected to Western blot analysis using antibodies to phospho-ERK42/44 (℗-ERK 1/2)

et al., 2000; Verhagen et al., 2000). To further evaluate the effect of chemotherapy agents on mitochondrial apoptosis signaling pathway, we examined the release of cytochrome c and Smac/DIABLO into cytosol in the presence or absence of death receptor signaling. The combination of TRA-8 and Adriamycin induced more release of cyto-chrome c and Smac/DIABLO than any single treatment, which was well correlated with the loss of mitochondrial membrane potential (Figure 6b). The expression and cleavage of several key proteins related to the mitochondrial death pathway was examined by Western blot analysis. The cleavage of Bid, a substrate of caspase-8, was enhanced by the combination of a low dose of TRA-8 with Adriamycin or Cisplatin (Figure 6c), sugg-esting that the death pathway from caspase to mitochondria was activated by the combination. The Bcl-X$_L$, an antiapoptosis protein was cleaved from 30 kDa into 16-kDa fragment in a caspase-dependent fashion, which was blocked by Z-VAD-FMK (Figure 6d). The 16 kDa cleavage product of Bcl-X$_L$ has been shown to involve acceleration of cell death (Clem et al., 1998). The activation of caspase-9 was enhanced as well (Figure 6c). In contrast, the levels of XIAP and Bax did not significantly change by the treatment. These results indicate that the combination treatment leads to a shift from antiapoptosis to proapoptosis by altering the function of the proteins in the Bcl-2 family, which contributes to enhanced release of cytochrome c and Smac/DIABLO from mitochondria.

Discussion

There is accumulating evidence suggesting that syner-gistic induction of apoptosis with TRAIL and chemo-

therapy agents might be a potent anticancer therapy in the future (Gliniak and Le, 1999; Gibson et al., 2000; Yamanaka et al., 2000; Lacour et al., 2001; Mizutani et al., 2001; Nagane et al., 2001). However, the synergistic mechanisms by which chemotherapy agents enhance the death receptor-mediated apoptosis are not fully understood. It has been difficult to examine specifically the death receptor-mediated signal transduc-tion using TRAIL because many receptors for TRAIL complicate the signal transduction. The availability of TRA-8 and 2E12 allows us to determine the susceptibility of tumor cells to DR5- and DR4-mediated apoptosis without the interference of the decoy receptors. Our previous study demonstrates that the expression levels of DR5 are not necessarily correlated with its apoptosis-inducing function (Ichikawa et al., 2001). Although many cancer cells can express high levels of cell surface DR4 and DR5, their susceptibility to the death receptor-mediated apoptosis varies largely, suggesting that the intracellular control of the death receptor signal transduction is likely a key to induction of apoptosis by anti-death receptor anti-body. Thus, identification of the synergistic strategy and mechanisms would be important for the future development of TRAIL and anti-death receptor antibody based anticancer therapy. In the present study, we utilized two agonistic monoclonal antibodies against DR4 and DR5 to demonstrate the death signal transduction by DR4 and DR5 and the manip-ulation by chemotherapy agents. Our results indicate that Adriamycin and Cisplatin can significantly enhance apoptosis induced by anti-death receptor antibodies, and the synergistic activation of the JNK/p38 kinase pathway and the mitochondrial death pathway are crucial mechanisms for enhanced apoptosis.
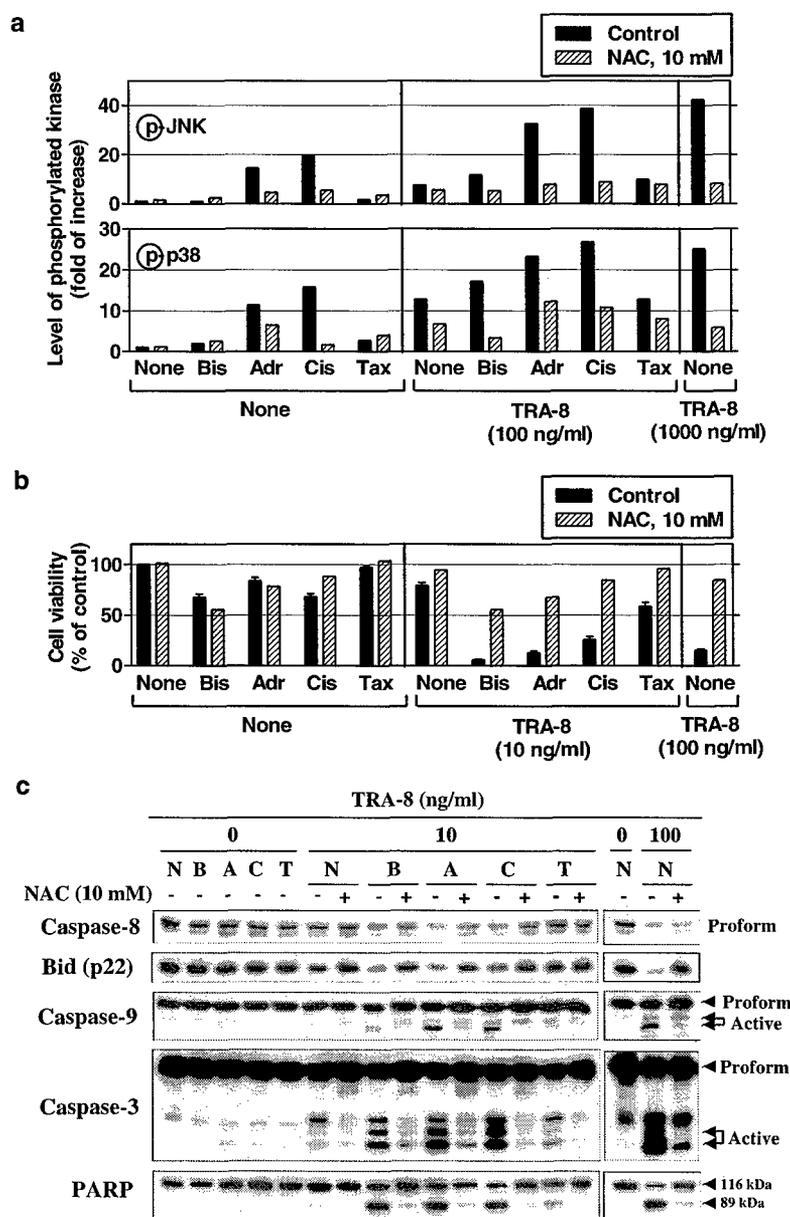
Figure 5 NAC inhibited DR5-induced chemotherapy agent-enhanced JNK/p38 activation and apoptosis of breast cancer MDA-MB-231-KS cells. (a) MDA-MB-231-KS cells were preincubated with or without 10 mM NAC for 1 h and then left untreated (*None*) or incubated with 3 μM Bis VIII (*Bis*), 3 μM Adriamycin (*Adr*), or 100 μM Cisplatin (*Cis*), or 3 μM Taxol (*Tax*) in combination with indicated concentration of TRA-8 for 4 h. Whole-cell lysates were prepared and subjected to Western blot analysis using antibodies to phospho-JNK46/54 ((p)-JNK), or phospho-p-38 ((p)-p38). Fold stimulation was determined by densitometric scanning of respective bands on the blots and plotted as graphs. (b) MDA-MB-231-KS cells were preincubated with or without 10 mM NAC for 1 h and then left untreated (*None*) or incubated with 3 μM Bis VIII (*Bis*), 3 μM Adriamycin (*Adr*), 100 μM Cisplatin (*Cis*), or 3 μM Taxol (*Tax*) in combination with indicated concentrations of TRA-8 for 14 h. Cell viability was determined by the ATPLite assay. (c) MDA-MB-231-KS cells were preincubated with or without 10 mM NAC for 1 h and then left untreated (*N*) or incubated with 3 μM Bis VIII (*B*), 3 μM Adriamycin (*A*), 100 μM Cisplatin (*C*), or 3 μM Taxol (*T*) in combination with indicated concentrations of TRA-8 for 9 h. Whole-cell lysates were prepared and subjected to Western blot analysis using the respective antibodies as indicated

In the previous studies, we have demonstrated that the synergistic activation of both the JNK/p38 kinase pathway and the mitochondrial death pathway plays a crucial role in the synergistic induction of apoptosis of tumor cells by combination of TRA-8 and Bis VIII, a death receptor signaling enhancer (Ohtsuka and Zhou, 2002). These results promoted us to examine the synergistic effect of chemotherapy agents on DR5 and DR4-mediated apoptosis. Our results suggest that in the presence of DR4 or DR5 signaling, the activation of JNK/p38 controls the process of apoptosis. 2E12- or TRA-8-induced apoptosis was accompanied by
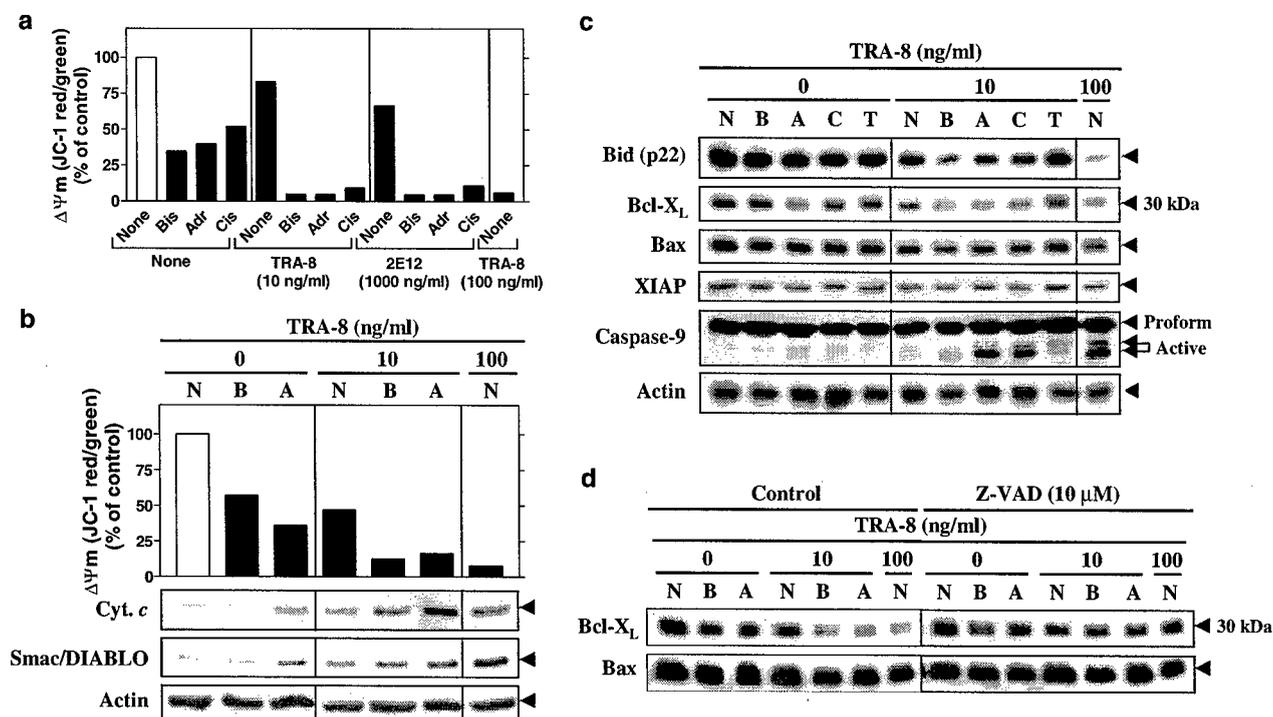
Figure 6 Chemotherapy agents enhanced DR4- and DR5-induced activation of mitochondrial apoptosis signaling pathway. (a) Induction of loss of mitochondrial membrane potential ($\Delta\Psi$m) induced by either TRA-8 or 2E12 in combination with chemotherapy agents or Bis VIII. MDA-MB-231-KS cells were left untreated (*None*) or incubated with 3 μM Bis VIII (*Bis*), 3 μM Adriamycin (*Adr*), or 100 μM Cisplatin (*Cis*) in combination with indicated concentrations of either TRA-8 or 2E12 plus 2 μg/ml crosslinker for 14 h. Alteration in mitochondrial membrane potential was measured by flow cytometry using JC-1 staining as described under 'Materials and methods'. One of three independent experiments is shown. (a) Cytochrome c and Smac/DIABLO release in response to Adriamycin or Bis VIII in the presence or absence of TRA-8. MDA-MB-231-KS cells were left untreated (*N*) or incubated with 3 μM Bis VIII (*B*) or 3 μM Adriamycin (*A*) in combination with indicated concentrations of TRA-8 for 8 h. Alteration in mitochondrial membrane potential was measured by flow cytometry as in panel a. Cytochrome c and Smac/DIABLO release into cytosol were measured by Western blotting. Cytosolic actin was used as the loading control. (c and d) Processing of Bid, Bcl-X$_L$, and caspase-9 in response to chemotherapy agents or Bis VIII in the presence or absence of TRA-8. MDA-MB-231-KS cells were preincubated with or without 10 μM Z-VAD-FMK for 1 h and then left untreated (*N*) or incubated with 3 μM Bis VIII (*B*), 3 μM Adriamycin (*A*) 100 μM Cisplatin (*C*), or 3 μM Taxol (*T*) in combination with indicated concentrations of TRA-8 for 9 h. Whole-cell lysates were prepared and subjected to Western blot analysis using the respective antibodies as indicated

induction of caspase processing (Figure 3a) and activation of JNK/p38 (Figure 4a and Table 1), which was mediated by an upstream kinase MKK4 (Figure 4a). Adriamycin or Cisplatin clearly enhanced 2E12- or TRA-8-induced cell death (Figures 1 and 2, and Table 1) by facilitating the caspase activation (Figure 3) in parallel with an increase in the activation of the MKK4/JNK/p38 pathway (Figure 4a and Table 1). Since 2E12- or TRA-8-induced activation of JNK/p38 was strongly inhibited by caspase-8 inhibitor (Ohtsuka and Zhou, 2002), there might be a positive interaction between caspase-8 and the JNK/p38, which triggers an amplification loop between caspase cascade and the JNK/p38 pathway. DR4 or DR5 signaling initiates a weak activation of MKK4/JNK/p38 through caspase-8, and in this event, enhanced activation of JNK/p38 by chemotherapy agents further leads to activation of caspase-8 and downstream caspases and then enhancement of apoptosis. The inhibition of the JNK/p38 pathway by NAC antagonizes both the enhancing effect of chemotherapy agent on death-receptor-induced cas-

pase activation and cell death (Figure 5). Since NAC was able to inhibit strongly activation of both JNK/p38, downstream caspases and Bid processing but not caspase-8 processing induced by TRA-8 or a TRA-8 and chemotherapy agent combination, prolonged activation of JNK/p38 activation may play a role for enhancement of caspase activation process and Bid cleavage after processing of caspase-8 initiated by death receptor activation. Close correlation between enhanced activation of JNK/p38 and the synergistic induction of DR4- or DR5-mediated caspase activation and apoptosis by chemotherapy agents as well as Bis VIII suggests that the signal transduction triggered by these agents might merge at JNK/p38.

In the present study, we also demonstrated the significance of mitochondrial apoptosis signaling in enhancement of death receptor-mediated apoptosis by chemotherapy agents. Similar to Bis VIII, treatment of cells with chemotherapy agents including Adriamycin or Cisplatin alone resulted in loss of mitochondrial membrane potentials (Figure 6a and b). In addition,

Adriamycin alone induced a weak release of cytochrome *c* and Smac/DIABLO, suggesting that mitochondria is likely one of the targets of chemotherapy agents as reported previously (Decaudin *et al.*, 1997; Kidd *et al.*, 2002; Sartorius and Krammer, 2002). Thus, the mitochondrial apoptosis pathway through cytochrome *c* and Smac/DIABLO releases is a crucial convergence point triggered by death receptors and chemotherapy agents such as Adriamycin and Cisplatin as well as Bis VIII. The loss of mitochondrial membrane potential by Bis VIII or chemotherapy agents might be able to sensitize the cells to death receptor-mediated apoptosis by enhancing utilization of the mitochondrial apoptosis pathway. Activated JNK/p38 might be able to regulate mitochondrial pathway through cleavage of Bid by enhanced activation of caspase-8, phosphorylation of Bcl-2 and Bcl-X_L for functional inactivation (Maundrell *et al.*, 1997; Yamamoto *et al.*, 1999; Kharbanda *et al.*, 2000), and/or direct activation of mitochondrial death machinery (Aoki *et al.*, 2002).

In conclusion, we propose a model in which a positive interaction between the JNK/p38 pathway and caspase activation cascade though caspase-8 and mitochondrial pathway leads to enhanced apoptosis signal transduction. Two death signal pathways initiated by death receptors and chemotherapy agents merged at the JNK/p38 kinases and mitochondria, which in turn synergistically enhances the caspase-8 and mitochondrial death signal transduction, leading to more complete cell death in tumor cells. Thus, the simultaneous targeting of cell surface death receptors with agonistic monoclonal antibodies and the intracellular JNK/p38 and mitochondrial pathways with chemotherapy agents would be an important strategy for more effective anticancer therapy.

Materials and methods

Reagents

An agonistic anti-human DR4 monoclonal antibody, 2E12, was generated by Ichikawa *et al.* (TRAIL R2 (DR5) is a novel, selective therapeutic target for rheumatoid arthritis, submitted for publication). An agonistic anti-human DR5 monoclonal antibody, TRA-8, was prepared as described (Ichikawa *et al.*, 2001). Bis VIII, Adriamycin, Cisplatin, Taxol, and recombinant soluble TRAIL were purchased from Alexis Biochemicals (San Diego, CA, USA). The caspase inhibitor Z-VAD-FMK was from R&D Systems, Inc. (Minneapolis, MN, USA), *N*-acetyl-L-cysteine (NAC) was from Sigma, and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were from Molecular Probes, Inc. (Eugene, OR, USA). Anti-phospho-JNK (Thr183/Tyr185), anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-extracellular signal-regulated protein kinase (ERK) 1/2 (Thr202/Tyr204), anti-phospho-MAPK kinase 4 (MKK4) (Thr261), anti-Bid, anti-Bax, anti-Bcl-X_L, anti-XIAP, and anti-poly (ADP-ribose) polymerase (PARP) antibodies, and horseradish peroxidase-linked anti-rabbit IgG were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-caspase-3, -8, -9 and anti-cytochrome *c* antibodies were from PharMingen (San Diego, CA, USA), and horseradish peroxidase-linked anti-mouse IgG

was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Anti-Smac/DIABLO, antiactin, and horseradish peroxidase-linked goat anti-mouse IgM were from Oncogene (Boston, MA, USA).

Cell culture and cell viability assay

MDA-MB-231-KS, MDA-MB-231-PO, and 2LMP cells were isolated by subcloning from parental MDA-MB-231 cell line purchased from the American Tissue Culture Collection (ATCC) (Manassas, VA, USA), and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air in DMEM medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (FCS), 50 μg/ml streptomycin, and 50 U/ml penicillin (all from Cellgro, Mediatec, Inc., Herndon, VA, USA). Human 1321N1 astrocytoma cells were kindly provided by Dr Richard Jope (University of Alabama at Birmingham), and maintained in DMEM medium supplemented with 5% heat-inactivated FCS, and antibiotics as described above. Human ovarian cancer cell lines, UL-3B and UL-3C, were kindly provided by Dr Cicek Gercel Taylor (University of Louisville) and maintained in RPMI1640 medium supplemented with 10% heat-inactivated FCS. All other cell lines were purchased from ATCC (Manassas, VA, USA) and grown in culture according to the instructions provided with them.

For cell viability assay, cells (1–2 × 10³ cells/well) were seeded onto 96-well plate in a volume of 50 μl. The caspase inhibitors were added 1 h before addition of stimulants. After incubating the cells for indicated periods, cell viability was determined using the ATPLite kit according to the manufacture's instructions (Packard Instruments, Meriden, CT, USA).

Western blot analysis

After the required treatments, cells (1–3 × 10⁶) were washed once with phosphate-buffered saline and lysed in the sample buffer (100–120 μl) for SDS–polyacrylamide gel electrophoresis (PAGE) and immediately boiled for 4 min. To measure the release of cytochrome *c* and Smac/DIABLO from mitochondria, cytosolic fraction was prepared using the Mitochondrial/Cytosol Fractionation Kit according to the manufacturer's instructions (Alexis Biochemicals, San Diego, CA, USA). Each sample was subjected to 8 or 12.5% SDS–PAGE, and the proteins separated in the gel were subsequently electrotransferred onto a polybinylidene difluoride membrane (MILLIPORE, Bedford, MA, USA). The membrane was blocked with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl (pH 7.4), 8 g/l NaCl, and 0.1% Tween 20) for 1–2 h at room temperature. The membrane was then incubated with indicated primary antibodies in TBS-T containing either 5% nonfat dry milk or 5% bovine serum albumin at 4°C overnight. The membrane was washed three times with TBS-T and probed with peroxidase-conjugated secondary antibodies at room temperature for 1.5 h. After washing four times with TBS-T, the protein was visualized using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Proteins were quantified by densitometric analysis using Quantify One program (Bio-Rad Laboratories, Hercules, CA, USA).

DNA fragmentation assay

After the required treatments of cells (1 × 10⁶), both adherent and detached cells were collected as described above, washed once with phosphate-buffered saline, lysed in 100 μl of TE-T buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100. Lysates were centrifuged at 14 000 r.p.m.

for 5 min at 4°C and supernatants were then subjected to digestion with ribonuclease A (0.2 mg/ml) for 1 h at 37°C followed by incubation with proteinase K (0.2 mg/ml) for 1 h at 37°C. DNA in the sample was precipitated by centrifugation at 14 000 r.p.m. for 15 min at 4°C after treatment with 50% isopropanol and 0.5 M NaCl overnight at −20°C. DNA was resuspended in 30 μl of TE buffer and analysed by electrophoresis on 2% agarose gel in the presence of 0.2 μg/ml ethidium bromide.

Determination of mitochondrial membrane potential

The mitochondrial membrane potential was assessed by using JC-1, a lipophilic cation that can selectively enter into mitochondria (Reers *et al.*, 1991). JC-1 was dissolved in dimethylsulfoxide to give a 1 mg/ml solution. This was further diluted to 20 μg/ml in a FACS buffer containing 5% FCS and 0.1% NaN₃ in phosphate-buffered saline, and filtered using 0.45-μm filter. After the required treatments of cells (2 × 10⁵), both adherent and detached cells were collected as described above and resuspended in 125 μl of the FACS buffer. The cell suspension was incubated for 20 min at room temperature with 250 μl of the filtered working solution of JC-1. Both red and green fluorescence emissions were analysed with a flow cytometer (FACScan, Becton Dickinson, Sunnyvale, CA, USA). A minimum of 10 000 cells per sample was acquired in list mode and analysed using Winmdi software. The decrease in mitochondrial membrane potential was determined by a decrease in the ratio of red to green fluorescence intensities.

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