Published in final edited form as:

Breast Cancer Res Treat. 2012 June ; 133(2): 417–426. doi:10.1007/s10549-011-1755-0.

Effect of anti-DR5 and chemotherapy on basal-like breast cancer

Patsy G. Oliver

Department of Radiation Oncology, Comprehensive Cancer Center, University of Alabama at Birmingham, 1825 University Blvd., SHEL 701, Birmingham, AL 35294-2182, USA

Albert F. LoBuglio, Tong Zhou, and Andres Forero

Department of Medicine, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL, USA

Hyunki Kim, Kurt R. Zinn, and Guihua Zhai

Department of Radiology, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL, USA

Yufeng Li and Choo H. Lee

Department of Medicine, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL, USA

Donald J. Buchsbaum

Department of Radiation Oncology, Comprehensive Cancer Center, University of Alabama at Birmingham, 1825 University Blvd., SHEL 701, Birmingham, AL 35294-2182, USA

Abstract

The purpose is to evaluate sensitivity of basal-like breast cancer to treatment with anti-DR5 alone and in combination with chemotherapy. Cytotoxicity of TRA-8 anti-DR5 alone and in combination with doxorubicin or paclitaxel was examined. The role of a DR5-associated molecule (DDX3) in the regulation of apoptosis by recruitment of cIAP1 to the DR5/DDX3 complex was studied. SUM159 and 2LMP orthotopic xenografts were treated with TRA-8 alone and in combination with Abraxane or doxorubicin, and tumor growth inhibition determined. Diffusion-weighted magnetic resonance imaging was used to monitor early tumor response. The majority (12/15) of basal-like cell lines were very sensitive to TRA-8-induced cytotoxicity (IC_{50} values of 1.0–49 ng/ml). In contrast, 8/11 luminal or HER2-positive cell lines were resistant ($IC_{50} > 1,000$ ng/ml). Enhanced killing of basal-like cell lines was produced by combination treatment with TRA-8 and doxorubicin. Majority of basal cell lines expressed lower levels of DR5-associated DDX3 and cIAP1 than luminal and HER2-positive cell lines. TRA-8 inhibited growth of basal xenografts and produced 20% complete 2LMP tumor regressions. TRA-8 and chemotherapy produced greater 2LMP growth inhibition than either alone. An increase in apparent diffusion coefficient in 2LMP tumors was measured in a week of therapy with TRA-8 and Abraxane. Basal-like cell lines were more sensitive to TRA-8-mediated cytotoxicity than HER2-over-expressing and luminal cell lines, and chemotherapy enhanced cytotoxicity. High sensitivity of basal cells to TRA-8 correlated with low expression of DR5/DDX3/cIAP1 complex. Treatment with TRA-8 and chemotherapy may be an effective therapy for basal-like breast cancer.

Keywords

Basal-like breast cancer; Anti-DR5 antibody; Chemotherapy

Introduction

Breast cancer is the leading cause of cancer and second in cancer caused mortality in American women with an estimated 209,000 cases diagnosed and 40,000 deaths in 2010 [1]. The disease is heterogeneous, and treatment strategies are increasingly subtype specific. Gene expression profiles were used to characterize luminal, HER2-amplified, basal-like, and normal-like breast cancer subtypes [2–6]. In general, luminal subtypes are ER positive (PR variable), HER2-amplified may be ER positive or negative, and basal-like are primarily ER/PR/HER2 negative (triple-negative breast cancer, TNBC) [7]. The term “targeted” therapy includes the use of anti-estrogen therapy in luminal breast cancer and anti-HER2 therapy in HER2-amplified tumors. Unfortunately, patients with basal-like or TNBC have no successful “targeted” treatment strategy and represent a priority for novel therapies [8].

TRAIL is a cytokine that binds to pro-apoptotic cell surface death receptors DR4 and DR5, anti-apoptotic decoy receptors DcR1 and DcR2, and soluble osteoprotegerin [9]. TRAIL preferentially induces apoptosis in many human tumor cell lines compared with normal cells, and agonistic monoclonal antibodies targeting DR4 or DR5 also induce apoptosis in a variety of human cancer cell lines [10]. The potential utility of TRAIL or death receptor antibodies is limited by intrinsic resistance of many tumor cell lines and tumors to these agents [11], and clinical development of TRAIL or antibodies targeting DR4 or DR5 would be facilitated by the identification of patients most likely to respond to these agents [12].

It was reported that basal B breast cancer cell lines have substantial in vitro sensitivity to TRAIL-mediated cytotoxicity in contrast to TRAIL resistance by basal A, luminal, and HER2-amplified cell lines [13]. Our previous report [14] of monoclonal anti-DR5 (TRA-8)-mediated cytotoxicity to breast cancer cell lines included sensitivity by three basal-like and resistance by five HER2-amplified or luminal cell lines. Combining chemotherapy agents (doxorubicin or paclitaxel) with anti-DR5 enhanced in vitro cytotoxicity and in vivo antitumor efficacy in a sub-cutaneous 2LMP (basal B) xenograft model [14].

The present studies examined monoclonal anti-DR5-mediated cytotoxicity alone and combined with chemotherapy in a large panel of breast cancer cell lines ($n = 26$) and two basal-like orthotopic xenograft murine models and included mechanistic studies of these in vitro and in vivo observations.

Materials and methods

Cells and reagents

Details on breast cancer cell lines and culture conditions are presented in the Supplementary material. TRA-8 antibody (mouse IgG1 isotype) was prepared at the University of Alabama at Birmingham [15]. Doxorubicin was obtained from Polymed Therapeutics (Houston, TX). Paclitaxel was from Sigma Aldrich Chemical Co. (St. Louis, MO). Abraxane was from the University of Alabama at Birmingham Hospital Pharmacy.

Cell viability assays using ATPLite

Cell viability assays were performed as described previously [14, 16]. Briefly, cell viability was assessed after 24 h of exposure to TRA-8 by measuring cellular ATP levels (ATPLite, Perkin Elmer Biosciences, Meriden, CT). For combination treatments, cells were pretreated

with chemotherapy drugs for 24 h before adding TRA-8 antibody, then ATP levels were determined 24 h later. ATP values are the mean $\pm$ SE from at least three independent experiments with a minimum of four replicates each.

Co-immunoprecipitation and western blot detection of DR5/DDX3/cIAP1 complex

Cell lysates (2 mg total protein in 2 ml 0.5% NP40 lysis buffer) were incubated overnight with 50 μ l humanized TRA-8 (Tigatuzumab)-conjugated Sepharose 4B at 4°C. Beads were washed five times with lysis buffer and resuspended in SDS-PAGE loading buffer. Co-immunoprecipitated proteins were separated using SDS-PAGE to detect DDX3, cIAP1, and DR5 (40, 40, and 20% of immunoprecipitate, respectively), then blotted onto nitrocellulose membranes, and probed overnight at 4°C with murine monoclonal anti-DDX3 (3E4) or anti-cIAP1 (4H6) antibodies or rabbit polyclonal anti-DR5 antibody. HRP-conjugated goat anti-murine or anti-rabbit IgG secondary antibodies and chemiluminescent substrate (Thermo Scientific, Cincinnati, OH) were used to reveal proteins.

Quantitative measurement of DR5/DDX3/cIAP1 complex with chemiluminescent ELISA

Chemiluminescent ELISA plates (NUNC, Naperville, IL) were coated with 10 μ g/ml TRA-8 or murine IgG1 (Southern Biotech, Birmingham, AL) in PBS for 1 h at 37°C, blocked with SuperBlock Blocking Buffer (Thermo Scientific), and incubated with 50 μ g total cell protein in lysis buffer/5% BSA for 1 h at room temperature. Plates were washed five times with 0.1% Tween 20 in PBS, incubated with HRP-conjugated anti-DDX3 (3E4), anti-cIAP1 (4H6) or anti-DR5 (2B9) for 1 h at room temperature, and washed five times. Chemiluminescent substrate (KPL) was added, and plates were counted in a TopCount plate reader (Packard, Hartford, CT). Specific binding was determined by subtracting counts per second bound to control IgG-coated wells from binding to anti-DR5-coated wells.

In vivo therapy studies using orthotopic xenograft models of TNBC

Female athymic nude mice were obtained at 4–6 weeks of age from Harlan Laboratories (Indianapolis, IN). Orthotopic breast cancer xenografts were established by implanting 4×10^6 2LMP or SUM159 cells in a 1:1 mixture with Matrigel (BD Biosciences, San Jose, CA) into the mammary fat pad. Mice were randomized into six treatment groups of 10 mice. Therapy was initiated 14 days after implantation when tumors were 5–7 mm in diameter. Mice were treated with 200 μ g TRA-8 given by i.p. injection on days 14, 17, 21, 24, 28, and 31; 6 mg/kg doxorubicin given by i.v. injection on days 15, 19, and 23; 20 mg/kg Abraxane given by i.v. injection on days 15, 19, 23, 27, and 31; TRA-8 plus doxorubicin or Abraxane; or were left untreated. Tumor growth was monitored twice weekly by measuring tumor diameter in the two largest dimensions with calipers. Mean tumor size was calculated from the product of individual tumor diameters and reported relative to tumor size at the start of treatments. Tumor growth, tumor doubling time (TDT), and tumor regression rates were determined. All studies were conducted in accordance with University of Alabama at Birmingham Institutional Animal Care and Use Committee regulations. Mice were examined daily for physical and behavioral changes and weighed twice weekly to assess toxicity of treatments. Tumor growth was monitored until the mean tumor size for each group at least doubled in size or until the study was terminated.

Imaging studies

Female nude mice (4–6 weeks old) were used for in vivo magnetic resonance imaging (MRI) study. 1×10^6 2LMP (groups 1–4) or 4×10^6 SUM159 (groups 5–8) cells were implanted subcutaneously into the left flank ($n = 5$ for groups 2–4, 6, 7; $n = 4$ for group 1; $n = 6$ for groups 5 and 8). Therapy started when tumors were about 5–10 mm in diameter. Mice were treated with 200 μ g TRA-8 given by i.p. injection on days 0 and 3 (groups 3 and

7); 20 mg/kg Abraxane given by i.v. injection on days 1 and 5 after therapy initiation (groups 2 and 6); TRA-8 plus Abraxane (groups 4 and 8); or were left untreated (groups 1 and 5). Anatomical MRI and diffusion-weighted imaging (DWI) were performed at days 0 (prior to dosing), 3, and 7 (details presented in Supplementary materials). A total of 3–7 slices (1 mm thick) were used to cover tumor regions of interest. Apparent diffusion coefficient (ADC) of tumor regions and tumor volume measurements were made among groups 1–8 over 7 days and analyzed using two-way repeated measures analysis of variance (RM ANOVA).

Statistical analysis

IC₅₀ values were estimated using the Hill Equation [17, 18]. The combination index (CI) for dose–effect relationships of TRA-8 and drugs in combination was calculated based on the multiple drug–effect equation of Chou–Talalay [19]. $CI = D_A/IC_{X,A} + D_B/IC_{X,B}$ where IC_{X,A} and IC_{X,B} are concentrations of drugs producing X% inhibition for each respective drug alone, and D_A and D_B are concentrations of each drug in the mixture that yield X% inhibition. The CI curve or modified isobologram is generated by plotting CI vs. X, ranging from 0 to 100%. Drug interactions are readily identified at any level of inhibition. The resulting CI theorem offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations [19]. The quantitative diagnostic plot was generated with Statistical Analysis Software (SAS) version 9.1. The synergism effect was further confirmed with concentration–effect curve with nonlinear regression method [20] and isobologram methods (data not shown). Experimental animal treatment groups were composed of 10 animals each to provide evidence of substantial tumor sensitivity to TRA-8 therapy with or without chemotherapy. For xenograft models, TDT was estimated for each animal using empirical distribution, and median TDT between treatment groups was compared using Kruskal–Wallis nonparametric statistical test.

Results

Breast cancer cell line sensitivity to monoclonal anti-DR5-mediated cytotoxicity

Table 1 lists the origin and genotype of 26 breast cancer cell lines examined in this study, including 12/15 (80%) basal-like TNBC cell lines that were highly sensitive to anti-DR5-mediated cytotoxicity (IC₅₀ values < 50 ng/ml). The basal B subtype were all highly sensitive (9/9) with IC₅₀ values ranging from 1.0 to 18.2 ng/ml while 3/6 of basal A cell lines were sensitive with IC₅₀ values of 12.8–48.8 ng/ml. In contrast, all luminal and HER2-over-expressing cell lines (8/11) were totally resistant (IC₅₀ > 1,000 ng/ml) or moderately sensitive (IC₅₀ = 304–388 ng/ml). Sensitivity or resistance did not correlate with tumor origin of cell lines.

Effect of chemotherapy on anti-DR5-mediated cytotoxicity to breast cancer cell lines

We previously reported [14, 16, 21–25] that chemotherapy agents can enhance anti-DR5-mediated cytotoxicity to various tumor types. Treatment with TRA-8 and doxorubicin or paclitaxel produced synergistic cytotoxicity against 12/14 or 10/14 basal-like cell lines, respectively (Fig. 1; Table 2). In general, stronger synergistic effects occurred in combination with TRA-8 in cell lines resistant to TRA-8 cytotoxicity compared to TRA-8 sensitive cell lines, as dramatic synergy occurred with TRA-8 and doxorubicin in T-47D and HCC1937 but only additive effects occurred with 2LMP.

Correlation of DR5-associated DDX3/cIAP1 and sensitivity to anti-DR5-mediated cytotoxicity

Our previous studies identified a novel regulatory mechanism of DR5-mediated apoptosis [26, 27]. In this model, DDX3, a Helicard protein, associates with DR5 and recruits cIAP1 to form a protein complex at the DR5 death domain, which may inhibit death domain signal transduction initiated by caspase 8/10. To determine whether sensitivity of basal-like cell lines to DR5-mediated apoptosis correlates with DR5/DDX3/cIAP1 protein complex formation, we examined DR5/DDX3/cIAP1 complexes in 22 human breast cancer cell lines with defined TRA-8 sensitivity by co-immunoprecipitation and chemiluminescent ELISA. The majority of non-TNBC cell lines were resistant to TRA-8-induced apoptosis ($IC_{50} > 1,000$ ng/ml), except HCC1569 and HCC1954 (Table 1). All cell lines showed positive DR5 immunoprecipitation (Fig. 2a, b, lower). All TRA-8-resistant non-TNBC cell lines except T-47D expressed higher levels of DDX3 and cIAP1 (Fig. 2a) co-immunoprecipitated with DR5, while two moderately sensitive HER2-amplified basal (ER^{-}) cell lines (HCC1569 and HCC1954) had lower levels of DDX3 and cIAP1. TRA-8-resistant HCC1937 and HCC1143 basal A cells expressed high levels of DR5-associated DDX3 and cIAP1. In contrast, the majority of TRA-8-sensitive TNBC cell lines, particularly the basal B type, expressed nondetectable or lower levels of DR5-associated DDX3 and cIAP1 (Fig. 2b). Levels of DR5, DDX3 and cIAP1 in the complex were quantitatively measured using chemiluminescent ELISA. The ratio of DDX3 or cIAP1 to DR5 in the majority of DR5-resistant non-TNBC cell lines was greater than 1, except two moderately sensitive cell lines (HCC1569 and HCC1954) (Fig. 2c), but far less than 1 in the majority of DR5-sensitive basal-like cell lines (Fig. 2d). These results suggest that levels of DR5-associated DDX3 and cIAP1 may account for the exceptional sensitivity of basal-like cell lines to DR5-mediated apoptosis.

Anti-tumor efficacy of anti-DR5 alone or combined with Abraxane or doxorubicin in basal B xenografts

Single-agent TRA-8, Abraxane, and doxorubicin all produced significant inhibition of 2LMP tumor growth compared with untreated tumors (Fig. 3a) with TDT of 58, 46, and 51 days, respectively, compared with control of 9 days ($P < 0.0001$ each). The combination of TRA-8/Abraxane or TRA-8/doxorubicin produced substantial tumor regression to $<50\%$ of original tumor size and tumor growth inhibition with TDTs of 87 and 78 days, respectively. TRA-8/Abraxane was more effective than single-agent TRA-8 ($P < 0.0029$) or Abraxane ($P < 0.0001$), while TRA-8/doxorubicin was more effective than TRA-8 ($P < 0.032$) or doxorubicin ($P < 0.005$). Two complete tumor regressions without recurrence over 100 days occurred with TRA-8 alone and TRA-8/Abraxane as well as one complete regression with single-agent Abraxane.

A similar study was carried out in the slower-growing SUM159 orthotopic model (Fig. 3b) where single-agent TRA-8 and both combination regimens produced comparable efficacy (TDT = 81, 75, and 85 days) that was significantly better than control TDT of 18 days ($P < 0.0001$) or Abraxane (TDT = 49 days; $P < 0.012$) or doxorubicin (TDT = 42 days; $P < 0.0001$). In this model, the striking efficacy of TRA-8 prevented observation of any benefit from combination therapy.

MRI assessment of anti-tumor efficacy

We have previously shown that increases in the ADC at early time points post-therapy in mouse xenograft models correlate with tumor cell apoptosis and anti-tumor efficacy [28]. Figure 4a illustrates the percent change in ADC of 2LMP tumors at day 3 and 7 post-therapy. At day 3, the three treatment groups have a rising ADC as compared to the falling control group ADC, which reaches statistical significance by day 7 for the TRA-8/Abraxane

($P < 0.001$) and Abraxane ($P = 0.047$) groups. Figure 4b provides ADC data for SUM159 tumors, illustrating a similar pattern that did not reach statistical significance. In both models, MRI assessment of tumor volumes was unchanged over 7 days of observations. Thus, alterations in ADC occurred prior to any demonstrable anti-tumor efficacy as assessed by tumor size.

Discussion

Identification of specific tumor subtypes that are sensitive to TRAIL or death receptor antibodies would be very beneficial to the clinical development of these agents. Buchsbaum et al. identified breast cancer cell lines of low, intermediate, and high sensitivity to TRA-8 anti-DR5 antibody in vitro and reported anti-tumor efficacy of TRA-8 against 2LMP (metastatic subclone of MDA-MB-231) basal xenografts, an effect that was enhanced by chemotherapy, radiation, or combination treatment with anti-DR4 antibody [14, 29]. However, the observation that the basal subtype might be particularly sensitive to strategies targeting death receptors was not appreciated until Rahman et al. reported that TRAIL produced significant in vitro cytotoxicity against a subset of breast cancer cell lines, particularly TN basal-like lines exhibiting a mesenchymal phenotype, whereas luminal breast cancer cell lines were resistant to TRAIL [13].

The present studies examined 26 breast cancer cell lines of TN basal-like, HER2-amplified basal and luminal subtypes, and luminal cell lines with normal HER2 levels to evaluate sensitivity to TRA-8-mediated cytotoxicity alone or in combination with chemotherapy. TRA-8 preferentially induced killing of TN basal-like cell lines with IC_{50} values of 1.0–4.8 ng/ml obtained for 12/15 cell lines, as compared to IC_{50} values of 304–325 ng/ml for 2 HER2 expressing basal and >1,000 ng/ml for 8/9 luminal cell lines; 25 ng/ml TRA-8 reduced viability by ~50% in some resistant basal, HER2-positive, and luminal cell lines (HCC1143, HCC1937, T-47D, and SK-BR-3), but higher doses of TRA-8 had little additional effect.

Rahman et al. reported that TNBC cell lines of mesenchymal or basal B subtype were sensitive to TRAIL ($IC_{50} < 250$ ng/ml) [13], while we found that basal B cell lines were very sensitive to TRA-8 ($IC_{50} < 18$ ng/ml). Rahman et al. also found that 3 of 3 cell lines that express a TN epithelial or basal A phenotype were resistant to TRAIL ($IC_{50} > 1,000$ ng/ml) [13], whereas we found that 3 of 6 basal A cell lines were sensitive to both TRA-8 and TRAIL ($IC_{50} < 50$ ng/ml). This apparent discrepancy may be related to differences in TRAIL preparations used in the two studies, as we used SuperKiller™ TRAIL instead of the GST-TRAIL fusion protein employed by them.

The TRA-8-sensitive TN basal-like cell lines included inflammatory (SUM149) and medullary (MDA-MB-157) subtypes. BRCA1 function did not directly correlate with TRA-8 sensitivity in three basal-like cell lines with *BRCA1* mutations [30, 31]. TRA-8-sensitive TN basal-like cell lines included 7/12 derived from primary tumors and 5/12 from metastatic tumors. Three HER2-expressing cell lines were derived from primary tumors, and 8/9 luminal cell lines were isolated from metastatic tumors, including pleural effusions, ascitic fluids, and one brain metastasis. It is encouraging that all five TN basal-like cell lines isolated from metastatic tumors were sensitive to TRA-8, suggesting that TRA-8 may affect both primary and metastatic tumors.

Doxorubicin and taxanes are frequently used in first-line therapy of TNBC (adjuvant or metastatic). Therefore, we examined the interaction of these drugs with TRA-8 in combination therapy of TN basal, HER2-positive and luminal cell lines. The majority of TN basal-like cell lines (11/14) were sensitive to doxorubicin (IC_{50} 500 nM). Combination

treatment with TRA-8 and doxorubicin produced synergistic killing of 85% of TN basal-like cell lines, including *BRCA-1* mutant cell lines (Table 2). Combination treatment with TRA-8 and doxorubicin also produced synergistic killing of HER2-positive and luminal cell lines (Table 2 and Ref. [14]). Although paclitaxel produced mainly additive cytotoxicity with TRA-8 against TNBC cell lines, this combination may be more effective in a therapeutic setting in which tumors are exposed to both agents for a longer period.

DDX3 was identified as a novel DR5-associated adaptor protein that mediates apoptosis resistance at the DR5 death domain by recruiting inhibitor of apoptosis proteins (IAPs) to form DR5/DDX3/cIAP1 complexes [26, 27]. We hypothesize that crucial post-translational modifications of components in the complex, such as phosphorylation and/or cleavage, lead to differential function of DR5 in sensitive and resistant cells. Expression levels of each component in the complex may influence signal transduction or, alternatively, the presence of DDX3 with absent CARD region would function as a naturally occurring dominant negative molecule and impair cIAP1 binding, resulting in high sensitivity to TRA-8-mediated apoptosis. This model predicts that the DDX3/cIAP1 complex is dominant over the death domain complex, producing resistance to DR5-mediated apoptosis. Li et al. [26] described DDX3 binding to DR5, which correlated with reduced caspase-8 activation after TRA-8 treatment in MDA-MB-231 cells with induced resistance to TRA-8. Doxorubicin treatment reduced DDX3 binding to DR5 in these cells, producing caspase activation and TRA-8 sensitization, suggesting that DR5/DDX3/cIAP1 complex modulation by doxorubicin overcomes DDX3-mediated inhibition of TRA-8-induced apoptosis [26]. Thus, DR5-associated DDX3 and cIAP1 may serve as a biomarker for predicting response to anti-DR5 (TRA-8)-mediated apoptosis, and these observations represent an initial investigation of this potential biomarker to help select patients in a clinical trial.

The anti-tumor efficacy of TRA-8 and doxorubicin or Abraxane was evaluated in two orthotopic xenograft models of basal breast cancer in athymic nude mice. SUM159 tumor growth was significantly inhibited by TRA-8, but no complete tumor regressions occurred. The mean tumor growth inhibition was less pronounced in 2LMP xenografts treated with TRA-8 alone, but 2/10 complete tumor regressions occurred. Combination treatment with TRA-8 and Abraxane or doxorubicin produced tumor growth inhibition and significant delays in TDT of 2LMP and SUM159 orthotopic tumors (Fig. 2). Combination treatment with TRA-8 and doxorubicin produced 1/10 complete tumor regressions in mice bearing SUM159 tumors, whereas 2/9 complete tumor regressions occurred in 2LMP tumors treated with TRA-8 and Abraxane. DWI measured effective therapy early with combined TRA-8 and Abraxane for TNBC xenografts at 7 days post-treatment. Thus, combination treatment with either Abraxane or doxorubicin enhanced the anti-tumor efficacy of TRA-8 against basal-like orthotopic xenografts.

The observation that basal-like breast cancers generally exhibit high sensitivity to TRA-8 or TRAIL [13] suggests that selecting such patients for DR5 therapy is likely to improve the odds of obtaining clinical benefit from these agents, similar to selecting patients with HER2-over-expressing tumors for Herceptin therapy [32]. Tigatuzumab, the humanized version of TRA-8, has completed Phase I studies with no dose-limiting toxicity detected [33]. A randomized Phase II trial of Abraxane ± Tigatuzumab treatment for patients with metastatic TNBC is being performed by the Translational Breast Cancer Research Consortium (ClinicalTrials.gov NCT01307891). Tissue samples will be analyzed for DR5-associated DDX3 and cIAP1, and the results correlated with efficacy.

The availability of an effective targeted therapy for aggressive basal-like cancer subtypes would be important, as the relapse rate from chemotherapy is significant and other therapeutic options are limited [8]. The finding that combination treatment with TRA-8 and

doxorubicin or paclitaxel produced enhanced cytotoxicity against the majority of TRA-8-sensitive and TRA-8-resistant breast cancer cell lines suggests that concurrently targeting more than one cytotoxic pathway may increase TRA-8 efficacy and reduce the probability of developing resistance. The lack of overlapping toxicities between TRA-8 or TRAIL and chemotherapy drugs is another important factor in designing clinical trials with death receptor agonists. The generally high initial chemosensitivity of primary basal-like breast tumors suggests that neoadjuvant treatment with death receptor agonists and combination chemotherapy might inhibit both primary and metastatic tumor growth to decrease relapse rates and produce the greatest clinical response.

In summary, the current studies suggest that TN basal-like breast tumors may be susceptible to the therapeutic effects of an agonistic antibody to DR5 and justify an approach targeting TRAIL death receptors in combination with chemotherapy in clinical trials with the basal-like breast cancer subtype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Sally Lagan for help in preparing the manuscript. Supported in part by Komen for the Cure Promise Grant KG090969 and the UAB SPORE in Breast Cancer 5P50 CA089019-08.

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Fig. 1.

In vitro cytotoxicity of TRA-8 in combination with doxorubicin. Breast cancer cells were pretreated with drug for 24 h followed by treatment with drug alone, TRA-8 alone, or drug plus TRA-8 for an additional 24 h, and then cell viability was evaluated by measuring ATP levels. Values are mean and SE determined from at least three independent experiments with a minimum of four replicates in each

Fig. 2.

Analysis of DR5/DDX3/cIAP1 protein complex in breast cancer cell lines. Co-immunoprecipitation and western blot analysis of DR5/DDX3/cIAP1 complex in a panel of non-TNBC (a) and TNBC (b) cell lines. Total cell lysates were prepared and immunoprecipitated with Tigatuzumab-conjugated Sepharose 4B. The immunoprecipitated proteins were separated in SDS-PAGE and western blotted. The blots were probed with anti-DDX3, anti-cIAP1, and anti-DR5 antibodies. The representative results of three experiments are shown. **c, d** Quantitative measurement of DR5-associated DDX3 and cIAP1 by chemiluminescent ELISA. Total cell lysates were incubated in the anti-DR5 antibody and isotype control antibody-coated ELISA plates. The DR5-associated DDX3 and cIAP1 were detected with HRP-conjugated anti-DDX3 and anti-cIAP1 antibody, and the DR5 levels were detected with rabbit polyclonal antibody. The results are shown as the average counts per second of duplicates. The specific binding was calculated by subtracting the isotype control background counts

Fig. 3. In vivo efficacy of TRA-8 alone or in combination with Abraxane or doxorubicin against orthotopic basal B xenografts in athymic nude mice. 2LMP (a) or SUM159 (b) cells were implanted in the mammary fat pad, and treatments began 14 days later when tumors were well established. The *bars* indicate the interval during which treatment was administered, and the *numbers in parentheses*, the fraction of animals in which tumors completely regressed ($n = 9-10$ mice/group)

Fig. 4. Analysis of 2LMP and SUM159 subcutaneous tumor response to treatment. **a, b** Intratumoral apparent diffusion coefficient (ADC) change of **a** 2LMP and **b** SUM159 tumors, measured at 0 (baseline), 3, and 7 days after initiation of therapy, when mice were untreated or treated with Abraxane (20 mg/kg at days 1 and 5, i.v.), TRA-8 (200 μ g at days 0 and 3, i.p.), and combined therapy. Statistical difference between control and each treated group is indicated by *asterisk* (two *asterisks* indicate that $P < 0.001$)

Table 1

TRA-8 cytotoxicity against breast cancer cell lines

Phenotype	Cell line	IC ₅₀ TRA-8 (ng/ml)	Tumor origin
Luminal	MCF-7	>1,000	Met AC, PE
	T-47D	>1,000	IDC, PE
	ZR-75-1	388	IDC, AF
	MDA-MB-134	>1,000	IDC, PE
HER2-amplified luminal (ER ⁺)	BT-474	>1,000	IDC, 1°
	DY36T2 (subclone MDA-MB-361)	>1,000	Met AC, BR
	ZR-75-30	>1,000	IDC, AF
HER2-amplified luminal (ER ⁻)	MDA-MB-453	>1,000	MC, PE
	SK-BR-3	>1,000	AC, PE
HER2-amplified basal (ER ⁻)	HCC1569	304	Met C, 1°
	HCC1954	325	DC, 1°
TNBC (basal A)	MDA-MB-468	12.8	Met AC, PE
	HCC1187	24.1	DC, 1°
	BT-20	48.8	AC, 1°
	HCC1937	>1,000	DC, 1°
	HCC1143	>1,000	DC, 1°
TNBC (basal B)	HCC1599	>1,000	DC, 1°
	SUM149	1.0	Inf DC, 1°
	HCC38	1.0	DC, 1°
	2LMP (subclone MDA-MB-231)	2.1	LM
	SUM159	1.9	AnCa, 1°
	MDA-MB-436	3.3	AC, PE
	SUM102	6.5	Int DC, 1°
	MDA-MB-157	11.8	Med, PE
	BT-549	13.4	IDCp, 1°
	MDA-MB-231	18.2	Met AC, PE

Tumor type: *AC* adenocarcinoma, *AnCa* anaplastic carcinoma, *DC* ductal carcinoma, *IDC* invasive ductal carcinoma, *IDCp* invasive ductal carcinoma, papillary, *Inf DC* inflammatory ductal carcinoma, *Int DC* intraductal carcinoma, *Med* medullary carcinoma, *Met AC* metastatic adenocarcinoma, *Met C* metaplastic carcinoma, *MC* metastatic carcinoma

Source: 1° primary tumor, *AF* ascitic fluid, *BR* brain metastasis, *LM* lung metastasis, *PE* pleural effusion, *PF* pericardial effusion

Table 2

In vitro cytotoxicity of TRA-8 and chemotherapy drugs against breast cancer cell lines

Phenotype	Cell line	Doxorubicin			Paclitaxel		
		Synergy	Dose (nM)	CI ^a	Synergy	Dose (nM)	CI
Luminal	T-47D	Yes	100–1,000	0.033–0.006	Yes	2.5–200	0.570–0.053
	MDA-MB-134	Yes	250–1,000	0.020–0.005	No	2.5–200	2.5–64.3
HER2-amplified luminal (ER ⁺)	BT-474	Yes	250–5,000	0.007–0.001	Yes	2.5–200	0.009–0.048
	ZR-75-30	Yes	500–1,000	0.011–0.007	No	5–200	12.6–29.4
HER2-amplified basal (ER ⁻)	HCC1569	Yes	100–1,000	0.254–0.002	Yes	2.5–200	0.377–0.050
	HCC1954	Yes	250–1,000	0.709–0.002	Yes	5–200	0.300–0.101
Basal A	MDA-MB-468	Yes	50–1,000	0.790–0.065	Yes	2.5–5, 20	0.874–0.656
	HCC1187	Yes	100–1,000	0.452–0.020	Yes	20–200	0.662–0.640
	BT-20	Yes	100–500	0.450–0.004	Yes	5–200	0.882–0.254
	HCC1937	Yes	250–1,000	0.046–0.001	Yes	2.5–200	0.960–0.022
	HCC1143	Yes	100–1,000	0.230–0.002	Yes	2.5–200	0.743–0.009
Basal B	SUM149	Yes	375–1,000	0.675–0.067	No	2.5–200	2.1–17.0
	HCC38	No	100–375	10.3–90.0	No	2.5–200	3.6–23.1
	2LMP	No	25–250	3.2–19.5	Yes	5–200	0.989–0.152
	SUM159	Yes	250–500	0.752–0.496	Yes	20–200	0.591–0.348
	MDA-MB-436	Yes	250–1,000	0.730–0.264	Yes	10–200	0.898–0.607
	SUM102	Yes	375–500	0.097–0.037	No	2.5–200	3.9–118.7
	MDA-MB-157	Yes	50–1,000	0.740–0.070	No	2.5–10, 200	1.2–1.6, 188.0
	BT-549	Yes	100–1,000	0.815–0.036	Yes	2.5–200	0.760–0.087
	MDA-MB-231	Yes	25–500	0.651–0.088	Yes	5–200	0.497–0.104

^aCI combination index as determined at 50% effect level