Enhanced Metastasis Suppression by Targeting TRAIL Receptor 2 in a Murine Model of Triple-Negative Breast Cancer

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Abstract

Purpose: Metastatic breast cancer is a deadly disease which requires new therapeutic strategies. Endogenous tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) functions as a metastasis suppressor by activating proapoptotic TRAIL receptors (TRAIL-R1/DR4 and/or TRAIL-R2/DR5) in transformed cells, making it an attractive pathway for antimetastatic therapies. However, it is unclear whether TRAIL-R1 or TRAIL-R2 is a better therapeutic target in metastatic breast cancer.

Experimental Design: Several metastatic, triple (estrogen receptor, progesterone receptor and HER2)-negative cancer cell lines were treated with human agonistic monoclonal antibodies targeting TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab). The effects on cell viability, apoptosis, and caspase-8 activation were determined. An orthotopic model of triple-negative breast cancer in which fluorescently labeled breast cancer cells metastasize from the mammary gland to lymph nodes and lung was utilized to evaluate the effects of mapatumumab, lexatumumab or doxorubicin on primary and metastatic tumor burden in vivo.

Results: Lexatumumab was more effective than mapatumumab in activating caspase-8, inducing apoptosis and inhibiting long-term survival of metastatic cancer cells, which expressed both TRAIL-R1 and TRAIL-R2. Human mammary epithelial cells transformed by oncogenic Ras were more sensitive to lexatumumab than non-transformed cells. Lexatumumab inhibited lymph node and lung metastases more robustly than mapatumumab in an orthotopic model of triple-negative breast cancer; both agents inhibited mammary tumor growth. Additionally, lexatumumab was more effective than doxorubicin at suppressing metastases at doses of doxorubicin that were associated with toxicity, even though doxorubicin reduced primary tumor burden more robustly than lexatumumab.
Conclusion: Targeting TRAIL-R2 receptor may be an effective therapeutic strategy for metastatic breast cancer.

Translational Relevance

The tumor-selectivity and limited toxicity of TRAIL receptor agonists in preclinical models has accelerated their clinical translation. Several TRAIL receptor agonists, including recombinant human TRAIL and human agonistic monoclonal antibodies (mAbs) targeting TRAIL-R1/DR4 or TRAIL-R2/DR5 are in clinical trials in patients with diverse malignancies. Although TRAIL receptor mAbs have several potential advantages over TRAIL, including improved pharmacokinetics and lack of decoy receptor binding, it is unclear whether TRAIL-R1 or TRAIL-R2 is a better therapeutic target for metastatic breast cancer, a largely fatal disease with few effective therapeutic options. We found that the TRAIL-R2 agonist lexatumumab is more effective than the TRAIL-R1 agonist mapatumumab at (1) initiating apoptosis in several metastatic, triple (estrogen receptor, progesterone receptor and HER2)-negative cancer cell lines and (2) reducing lymph node and lung metastases in an orthotopic model of triple-negative breast cancer. Our findings provide robust preclinical evidence for targeting TRAIL-R2 in metastatic breast cancer.
Introduction

Breast carcinoma is the most prevalent cancer and the leading cause of cancer deaths in woman in the world today (1). Mortality from breast cancer is invariably due to metastasis, the dissemination of cancer cells from the primary tumor to distant organs, such as the lungs, bones, brain and liver, via lymphatic and/or hematogenous routes. Metastasis is a complex, step-wise process whereby tumor cells migrate and invade the surrounding stroma, intravasate into the circulation, extravasate into distant organs, and colonize the organ microenvironment (2). At each step, tumor cells must escape apoptosis in order to survive detachment from the extracellular matrix of the primary tumor (“anoikis”), in the circulation and in the organ microenvironment (3). Current adjuvant therapies for breast cancer, including radiation and chemotherapy, are often ineffective against metastases (4). Thus, new therapeutic agents for advanced breast cancer are greatly needed to improve the prognosis for patients with this largely incurable stage of the disease.

Therapeutic targeting of the apoptotic machinery in cancer cells has emerged as a promising approach with the potential to overcome many of the shortcomings of non-selective cytotoxic agents. Indeed, (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) is a proapoptotic cytokine in the TNF-α family that has emerged as a promising cancer therapeutic agent because of its relative tumor-specificity and limited toxicity (5, 6). TRAIL initiates apoptosis by binding to its proapoptotic receptors, TRAIL receptor-1 (TRAIL-R1/DR4) and TRAIL receptor-2 (TRAIL-R2/DR5), which recruit the adaptor protein FADD to their cytoplasmic death domains. FADD then recruits procaspases-8 and 10 to ligand-activated TRAIL-R1 and TRAIL-R2, resulting in dimerization-mediated activation of caspases-8 and 10, the initiator caspases in the extrinsic or death receptor apoptotic pathway. Other TRAIL
receptors, including DcR1/TRAIL-R3, DcR2/TRAIL-R4 and osteoprotegerin, lack functional death domains and act as decoy receptors to antagonize the proapoptotic actions of TRAIL by competing with proapoptotic TRAIL-R1 and TRAIL-R2 for ligand binding. Endogenous TRAIL plays a critical role in innate immune surveillance of primary and metastatic tumors by natural killer cells as evidenced by increased tumor burden in TRAIL-deficient mice or mice treated with neutralizing anti-TRAIL antibodies in several tumor models (7, 8). Genetic deletion of the single proapoptotic TRAIL receptor in mice promotes c-myc-mediated lymphomas and visceral metastases to the lungs and liver (9). Furthermore, TRAIL-R-deficient mice are no more susceptible to carcinogen-induced skin papillomas and squamous cell carcinomas than wild-type mice, but TRAIL-R-deficient mice develop more lymph node metastases (10). Collectively, these findings suggest that TRAIL may function primarily as a metastasis suppressor. Consistent with this idea, matrix-detachment induced apoptosis or anoikis, which functions as a barrier against metastasis, is mediated by TRAIL-induced activation of TRAIL-R2 in colorectal carcinoma cells (11).

Extensive preclinical studies have demonstrated that TRAIL and agonistic monoclonal antibodies (mAbs) targeting TRAIL-R1 or TRAIL-R2 preferentially activate apoptosis in cancer cells versus non-transformed cells, reduce tumor burden in vivo, and have little systemic toxicity (12-17). Recently, several proapoptotic death receptor agonists, including recombinant human TRAIL (dulanermin) and fully humanized agonistic monoclonal antibodies (mAbs) targeting TRAIL-R1/DR4 (mapatumumab) or TRAIL-R2/DR5 (lexatumumab, PRO95780, conatumumab and CS-1008) have been evaluated as single agents or in combination with chemotherapy in early clinical trials in patients with advanced malignancies (18-25). Although all of these agents appear to be well tolerated in patients, TRAIL receptor mAbs have several potential therapeutic
advantages over TRAIL: (1) the mAbs do not bind to TRAIL decoy receptors; (2) the mAbs have a half-life of 6-21 days in the circulation versus 30-60 minutes for TRAIL; and (3) mAbs may initiate antibody-dependent cellular cytotoxicity (18). Despite these potential advantages, it is unclear whether TRAIL-R1 or TRAIL-R2 is a better therapeutic target for metastatic breast cancer. Importantly, both TRAIL-R1 and TRAIL-R2 are expressed in the vast majority of human breast cancer cell lines and human breast tumors (26-28). Recent studies suggest that TRAIL-induced apoptosis in different tumor types may be mediated by distinct TRAIL receptors (29-31). One study reported that TRAIL-induced apoptosis in a subset of human breast cancer cell lines, which are “triple negative” for the estrogen receptor, the progesterone receptor and HER2 gene amplification, is mediated by TRAIL-R2 in vitro (32). However, it remains to be determined whether TRAIL-R2 is the principal proapoptotic death receptor in breast tumors in vivo and whether targeting TRAIL-R2 confers greater antimetastatic activity in vivo than targeting TRAIL-R1.

To this end, we examined the sensitivity of several human metastatic, triple-negative cancer cell lines to apoptosis induction by fully human agonistic mAbs targeting TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab). Here we report that lexatumumab is more effective than mapatumumab in activating caspase-8, inducing apoptosis and inhibiting long-term survival of these metastatic cancer cell lines in vitro. Importantly, transformed human breast epithelial cells are more sensitive to lexatumumab than non-transformed cells, confirming the preferential proapoptotic activity of this agent against cancer cells. To evaluate the antimetastatic activity of these agents in vivo, we developed an orthotopic model that utilizes mCherry-labeled human MDA-MB-231 triple negative breast cancer to quantitate lymph node and lung metastases in vivo. Both lexatumumab and mapatumumab inhibited mammary tumor

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growth and lung metastases in vivo, although lexatumumab was more effective than mapatumumab at reducing lymph node and lung metastases. Intriguingly, doxorubicin was more effective at inhibiting mammary tumor growth than either TRAIL receptor agonist, but was less effective than lexatumumab at reducing lung metastases. Taken together, our findings strongly suggest that TRAIL-R2 may be a promising therapeutic target in metastatic breast cancer.

**Material and Methods**

**Cell culture and reagents.** Human MDA-MB-231 breast carcinoma cells stably expressing mCherry fluorescent protein (abbreviated MDA-MB-231-mCherry) were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5% FBS, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 2x nonessential amino acids (Invitrogen) and 1 µg/ml blasticidin (Sigma-Aldrich) (33). Human GILM2 breast carcinoma cells (kindly provided by Dr. Janet Price, M.D. Anderson Cancer Center) were grown in DMEM/F12 media supplemented with 10% FBS, 100 IU/ml penicillin/streptomycin, and 1x insulin/transferrin/sodium selenite mix (Invitrogen) (34). Human MDA-MB-435-Lung2 cells (abbreviated 435-Lung2 cells, kindly provided by Dr. Janet Price, M.D. Anderson Cancer Center) were maintained in MEM supplemented with 5% FBS, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1x nonessential amino acids, and 1x MEM vitamins (Invitrogen). 435-Lung2 cells are a highly metastatic variant of MDA-MB-435 cancer cells (35). Although the primary tumor origin of MDA-MB-435 cells is unclear, recent studies indicate these cells have a basal-like gene expression profile and may be breast cancer cells with lineage infidelity (36-38). Human MCF-10A breast epithelial cells stably expressing H-RasV12 or empty vector (39) were grown in DMEM/F12 media supplemented with 5% horse serum
(Invitrogen), 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin (Sigma-Aldrich), and 100 IU/ml penicillin-streptomycin. Human BxPC3 pancreatic adenocarcinoma cells (kindly provided by Dr. Hidayatullah Munshi, Northwestern University) were cultured in DMEM supplemented with 10% FBS and 100 IU/ml penicillin/streptomycin. Mapatumumab and lexatumumab were kindly supplied by Dr. Robin Humphreys (Human Genome Sciences) (15, 16). Doxorubicin (Sigma-Aldrich) was dissolved in DMSO.

**Immunoblotting.** Proteins were immunoblotted from whole cell lysates as described (39). Primary antibodies against TRAIL-R1, TRAIL-R2 (Stressgen), β-actin (Sigma-Aldrich), PARP and caspase-8 (BD Biosciences) were used.

**TRAIL receptor cell surface expression.** Cells were washed once with PBS and incubated with TRAIL-R1, TRAIL-R2 or control IgG1 mAbs conjugated with allophycocyanin (BioLegend) in PBS containing 2.5% BSA for 45 min at 4°C in the dark. Cells were then washed three times with PBS containing 2.5% BSA, resuspended in PBS containing DAPI (1 μg/ml), and analyzed by flow cytometry.

**Cell viability assay.** A MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was used to analyze the effect of mapatumumab (0-1 μg/mL), lexatumumab (0-2 μg/mL) or doxorubicin (0-0.5 μM) on cell viability. Cells were cultured in 96-well plates (2.5 x 10^3 cells/well). Cell viability was assessed after 24, 48 or 72 h. The number of viable cells was determined in triplicate wells by measuring A_{490 nm} 1 h after adding the MTS reagent (Promega) as described by the manufacturer. Cell viability was expressed as the percentage of viable cells: A_{exp group}/A_{control} x 100.
**Crystal violet cell survival assay.** Cells were grown on six-well plates (3 x 10^5 cells/well) and treated with PBS vehicle, mapatumumab (0.5 µg/mL), lexatumumab (0.5 µg/mL) or doxorubicin (0.2 µM for MDA-MB-231 and 435-Lung2 cells or 0.5 µM for GILM2 cells) for 48 h. After treatment, the cells were washed and cultured in fresh media, which was changed every 2 days for 5-7 days. Surviving cells were stained with crystal violet as described (17).

**Annexin V-labeling assay.** Cells were treated with mapatumumab (0.5 µg/mL), lexatumumab (0.5 µg/mL) or doxorubicin (0.2 µM for MDA-MB-231 and 435-Lung2 cells or 0.5 µM for GILM2 cells) for 16 h. Apoptotic cells were identified by flow cytometry using the Annexin-PE Apoptosis Detection Kit I (BD Bioscience) as described by the manufacturer.

**Orthotopic model of metastatic breast cancer.** Triple-negative MDA-MB-231-mCherry breast cancer cells (1 x 10^6) in 100% Matrigel (BD Bioscience) were injected into the ducts of both 4th mammary glands of 4- to 5-week old female athymic nu/nu mice (Harlan Sprague-Dawley) as described (33). To characterize the metastatic phenotype of this orthotopic model, mice were euthanized at 2-week intervals from 2-10 weeks after tumor inoculation (6-8 mice/time point). The lungs and up to twelve lymph nodes (2 inguinal, 2 axillary, 2 brachial, 4 superficial and 2 deep cervical) were harvested from each euthanized mouse. Metastases were identified by mCherry fluorescence using a SteroDiscovery.V12 fluorescence microscope with an AxioCam MRm digital camera (Zeiss). Fluorescent pseudocolored images representing light emitted from metastatic lesions superimposed over a grayscale reference image were created using ImageJ software. A subset of metastases was confirmed by H&E staining as described in “Tumor histology and immunohistochemistry”.

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To determine the effect of each therapeutic agent on tumor burden in this model, mice (10-11 per group) were randomized to one of the following treatment groups: vehicle (10% DMSO \textit{i.p.}), mapatumumab (20 mg/kg \textit{i.p.}), lexatumumab (20 mg/kg \textit{i.p.}) or doxorubicin (5 mg/kg \textit{i.p.}). Treatment was initiated 17 days after tumor inoculation. Vehicle and doxorubicin were administered weekly (3 total doses), while mapatumumab and lexatumumab were administered twice weekly (6 total doses). Mammary tumors were measured with calipers, and tumor volume was calculated using the formula: volume=length x width$^2$ x $\pi/6$. Mice were euthanized 45 days after tumor inoculation. Lymph nodes and lungs were dissected from each mouse and mCherry-fluorescent metastases were identified as described in the preceding paragraph. Lung metastatic tumor burden was quantitated using NIH ImageJ analysis to determine the number of mCherry-fluorescent metastases per lung and the percentage of the lung surface area of the metastases. All procedures involving animals were approved by the Northwestern University Institutional Animal Care and Use Committee.

\textit{Tumor histology and immunohistochemistry.} Formalin-fixed tissue samples were embedded in paraffin blocks, sectioned and H&E stained by the Northwestern University Pathology Core Facility. Cleaved caspase-3 was detected by immunohistochemistry with a polyclonal antibody that recognizes the cleaved, active subunit of caspase-3 (Cell Signaling) using the DakoEnVision+System-HRP (DAB) kit according to the manufacturer’s instructions. The percentage of tumor cells that were positive for cleaved caspase-3 was scored in five randomly selected fields from three tumors per treatment group using an Axiovert 2000 microscope (Zeiss).
Statistics. ANOVAs with Bonferroni posttests were performed using Prism 4 GraphPad Software to assess statistical significance.

Results

Human metastatic, triple-negative cancer cells are more sensitive to cell death induction by lexatumumab than mapatumumab. We chose three human triple-negative cancer cell lines, MDA-MB-231, GILM2 and MDA-MB-435-Lung2 (abbreviated 435-Lung2), which metastasize to the lungs for these studies (34-36). Before evaluating the sensitivity of these cell lines to human agonistic mAbs targeting TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab), we examined the expression of these proapoptotic TRAIL receptors in the cell lines by immunoblotting. Both TRAIL-R1 and TRAIL-R2 were highly expressed in each of these metastatic cancer cell lines (Fig. 1A). Moreover, flow cytometry revealed that both TRAIL receptors were expressed on the cell surface of each cancer cell line (Fig. 1B). Despite expression of both proapoptotic death receptors, all three cancer cell lines were more sensitive to lexatumumab (Lexa) than mapatumumab (Mapa) as determined by an MTS cell viability assay in dose-response and time course studies (Fig. 1C). The IC$_{50}$ values for lexatumumab were 0.13 µg/mL for MDA-MB-231 cells, 0.23 µg/mL for 435-Lung2 cells, and 0.19 µg/mL for GILM2 cells. The cytotoxic agent doxorubicin (Dox) was used as a positive control in these studies. In contrast, human BxPC3 pancreatic adenocarcinoma cells were more sensitive to mapatumumab than lexatumumab at the highest concentration tested (Fig. 1D), consistent with a prior report (31). To determine whether mapatumumab or lexatumumab suppressed long-term cell survival, cancer cell lines were treated with vehicle, mapatumumab (0.5 µg/mL), lexatumumab (0.5 µg/mL) or Dox (0.2-0.5 µM) for 48 h, washed, and then grown for 5-7 days in the absence of
these agents. Under these conditions, cancer cells treated with vehicle or mapatumumab remained viable and continued to grow following the removal of these agents (Fig. 1E). In contrast, treatment with lexatumumab or doxorubicin led to a dramatic decrease in the number of viable cancer cells. Collectively, these results indicate that lexatumumab is more effective than mapatumumab at inducing cell death and inhibiting long-term cell survival in these human metastatic, triple-negative cancer cell lines.

Lexatumumab activates caspase-8 and induces apoptosis in human metastatic, triple-negative cancer cells more robustly than mapatumumab. TRAIL receptor agonists initiate apoptosis by activating the apical caspase-8 in the extrinsic pathway (5, 6). Treatment of metastatic, triple-negative cancer cell lines with lexatumumab resulted in proteolytic cleavage of procaspase-8 and its p43/p41 processing intermediate (labeled “cleaved”) as detected by reduced intensity of one or both of these isoforms on immunoblots (Fig. 2A). Mapatumumab was less potent at inducing caspase-8 processing, and doxorubicin, an activator of the intrinsic apoptotic pathway, produced little caspase-8 activation under the conditions tested. In addition, lexatumumab treatment resulted in more robust proteolysis of the caspase-3 substrate PARP (40) than mapatumumab, consistent with enhanced activation of downstream caspases by caspase-8. To measure cell death, MDA-MB-231, 435-Lung2, and GILM2 cells were treated with vehicle, mapatumumab (0.5 µg/mL), lexatumumab (0.5 µg/mL) or Dox (0.2-0.5 μM) for 16 h and annexin V positive cells were scored by flow cytometry. Consistent with our MTS data and long-term survival data (Figs. 1C and 1E), all three metastatic cancer cell lines were more sensitive to lexatumumab than mapatumumab (Fig. 2B). To determine whether transformed breast epithelial cells were more sensitive to these agents than non-transformed cells, we treated MCF-10A breast epithelial cells stably expressing oncogenic H-RasV12 or empty vector with 1 µg/mL lexatumumab or
mapatumumab for 16 h and scored annexin V positive cells (Fig. 2C, *left panel*). Transformed MCF-10A-H-RasV12 cells were more sensitive to lexatumumab than non-transformed MCF-10A-Vector cells, confirming the preferential proapoptotic activity of this agent against cancer cells. Interestingly, neither of these cell lines was sensitive to mapatumumab, although they expressed TRAIL-R1 (data not shown). The enhanced sensitivity of transformed MCF-10A-H-RasV12 cells to lexatumumab was also observed in dose-response experiments using an MTS assay (Fig. 2C, *right panel*). Taken together, these results indicate that lexatumumab potently induces caspase-8 activation and apoptosis via the extrinsic pathway *in vitro* in three different metastatic, triple-negative cancer cell lines and underscore the enhanced sensitivity of transformed cells to this proapoptotic agent.

**Characterization of metastases in an MDA-MB-231-mCherry orthotopic model of breast cancer.** Human MDA-MB-231 breast cancer cells are triple-negative with a basal-like gene expression profile (36). Although MDA-MB-231 breast cancer cells readily metastasize to the lung after tail vein injection in nude mice, they only rarely metastasize to the lung after standard mammary fat pad inoculation (35). Because direct intraductal injection into the lactiferous duct of the fourth mammary gland has recently been demonstrated to promote lymph node and visceral metastases of ER-positive breast cancer cells (41), we postulated that intraductal injection of MDA-MB-231 cells would augment their metastatic potential. In these experiments, we used MDA-MB-231 cells stably expressing a mCherry fluorescent protein (MDA-MB-231-mCherry cells) to facilitate tracking metastases and quantifying tumor burden in mice. Specifically, $1 \times 10^6$ MDA-MB-231-mCherry cells resuspended in Matrigel were injected bilaterally into the lactiferous duct of the 4th mammary gland ($1 \times 10^6$ cells per injection) of female athymic nude mice, and mice were autopsied at various time points to determine
metastatic tumor burden (Table 1 and Fig. 3). The first lymph node metastases were detected in two of six mice as early as two weeks after intraductal inoculation. The number of metastatic lymph nodes per mouse increased with time, and larger lymph nodes metastases were more prevalent at later stages. Lung metastases were detected as early as the fourth week; a few micrometastases were found in only one mouse out of six at this time point. The prevalence of lung metastases and metastatic tumor burden progressed with time. Hence, we have developed a bona fide orthotopic model that recapitulates the aggressive nature of triple-negative breast cancer, including its propensity to metastasize to lungs, making it an ideal model for evaluating novel antimetastatic therapies.

**Lexatumumab suppresses metastases more robustly than mapatumumab in an orthotopic model of triple-negative breast cancer.** To examine the antitumor activity of TRAIL receptor agonists in vivo, we treated female athymic nude mice with established MDA-MB-231-mCherry mammary tumors (10-11 mice per group) with vehicle, mapatumumamb (20 mg/kg twice weekly) lexatumumab (20 mg/kg twice weekly) for three weeks beginning 17 days after intraductal injection of tumor cells, a time when a subset of mice have disseminated disease as determined by lymph node metastases (Table 1). Doxorubicin (5 mg/kg weekly x 3), a commonly used cytotoxic agent in breast cancer, was used as a control. Both lexatumumab and mapatumumab inhibited mammary tumor growth and there was a trend toward smaller tumors in the lexatumumab group, but this difference was not statistically significant (Fig. 4A). Doxorubicin was more effective at inhibiting mammary tumor growth than either TRAIL receptor agonist (Fig. 4A). However, doxorubicin treatment resulted in weight loss, and two doxorubicin treated mice died during the study (data not shown). Both TRAIL receptor agonists and doxorubicin induced apoptosis in mammary tumors as determined by cleaved caspase-3

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immunostaining (Fig. 4B). Consistent with our *in vitro* findings, lexatumumab induced apoptosis more potently than mapatumumab in mammary tumors *in vivo*.

To evaluate the impact of each therapeutic agent on metastases *in vivo*, mice were euthanized 45 days after intraductal tumor inoculation, and metastases (lymph nodes and lung) were scored at autopsy. Both lexatumumab and doxorubicin reduced the number of lymph node metastases, while mapatumumab did not (Table 2). All three agents reduced lung metastases compared to vehicle control (Table 2 and Fig. 4C). Importantly, only one of ten mice in the lexatumumab group developed lung metastases, whereas 5 of 11 mice in the mapatumumab and doxorubicin groups developed lung metastases. To more precisely quantitate the effects of each treatment on metastatic tumor burden in the lungs, the number of metastases per lung (Fig. 4C, *left panel*) and the surface area of lung occupied by metastases (Fig. 4C, *right panel*) was calculated by fluorescence imaging. Although all 3 agents reduced the number of lung metastases and surface area occupied by lung metastases, lexatumumab was more effective than mapatumumab or doxorubicin at reducing lung metastatic burden. Collectively, our findings point to TRAIL-R2 as a promising therapeutic target in metastatic triple-negative breast cancer.

**Discussion**

Although recent studies point to a critical role of the endogenous TRAIL proapoptotic pathway in metastasis suppression, the specific contribution of TRAIL-R1 versus TRAIL-R2 in mediating these actions *in vivo* has not been delineated (7-10). Hence, it remains to be determined whether TRAIL-R1 or TRAIL-R2 is a better therapeutic target in metastatic solid tumors such as breast cancer. To directly address this question, we examined the sensitivity of human metastatic, triple-negative cancer cells to fully human agonistic mAbs targeting TRAIL-
R1 (mapatumumab) or TRAIL-R2 (lexatumumab) *in vitro* and *in vivo*. We have demonstrated that lexatumumab is more effective than mapatumumab in activating caspase-8, inducing apoptosis and inhibiting long-term survival of several metastatic, triple-negative cancer cell lines *in vitro*. The mechanism(s) underlying the enhanced sensitivity of these metastatic cancer cell lines to lexatumumab is unclear: both TRAIL-R1 and TRAIL-R2 are expressed on the cell surface of these cancer cell lines. We also observed that transformed human mammary epithelial cells are more sensitive to lexatumumab than non-transformed cells, consistent with the well-documented tumor-selectivity of proapoptotic receptor agonists (5, 6). Additionally, we have demonstrated that lexatumumab is more effective than mapatumumab at suppressing lung and lymph node metastases in an orthotopic model of triple-negative breast cancer. Interestingly, we observed that lexatumumab is also more effective than doxorubicin at suppressing metastases *in vivo* at doses of doxorubicin that were associated with systemic toxicity and some mortality, even though doxorubicin treatment reduced primary tumor burden more robustly than lexatumumab. Hence, our results point to a promising therapeutic role for specifically targeting TRAIL-R2 in metastatic breast cancer that is consistent with the documented physiologic function of the TRAIL pathway in metastasis suppression.

Although TRAIL and an agonistic TRAIL-R2 mAb (Apomab) were previously reported to inhibit bone metastases induced by intratibial injection of breast cancer cells (42, 43), our findings are the first to demonstrate the antimetastatic activity of TRAIL receptor agonists in an orthotopic model of breast cancer that more faithfully recapitulates the entire metastatic cascade *in vivo*, including stromal invasion, intravasation, extravasation and lung colonization. Moreover, our studies are the first direct comparison of the therapeutic efficacy of TRAIL-R1 and TRAIL-R2 agonists in a murine model of breast cancer. Although human breast cancer cell lines and
tumors express both TRAIL-R1 and TRAIL-R2, the enhanced antimetastatic activity of lexatumumab in vivo may reflect the greater sensitivity of breast cancer cells to TRAIL-R2-mediated apoptosis and/or the requirement of TRAIL-R2 for detachment-induced anoikis (11, 26-28). Collectively, our findings support the clinical translation TRAIL-R2 agonists, such as lexatumumab, in metastatic breast cancer.

Our results are consistent with a recent report demonstrating that TRAIL-induced apoptosis is mediated specifically by TRAIL-R2 in a subset of triple-negative breast cancer cell lines in vitro (32). Triple-negative breast tumors, which express basal epithelial genes, such as cytokeratin 5 and vimentin, are associated with poor survival due to frequent metastases to the lung and brain within the first five years of diagnosis (44). Although many breast cancer cell lines are resistant to TRAIL alone, triple-negative breast carcinoma cell lines with a mesenchymal phenotype, defined by vimentin expression, are selectively sensitive to TRAIL-induced apoptosis in vitro, while triple-negative breast cancer cells which express epithelial markers such as E-cadherin are largely resistant to TRAIL (26, 32). These latter findings suggest that the epithelial mesenchymal transition (EMT), a process implicated in tumor progression to an invasive, metastatic phenotype (45), may be accompanied by the acquisition of TRAIL-sensitivity. Intriguingly, the expression of TRAIL-R2, but not TRAIL-R1, is higher in malignant breast tumors than normal breast tissue and is associated with estrogen receptor-negative status, lymph node metastases and poor survival, suggesting that TRAIL-R2 levels may increase with disease progression (27, 28). Taken together, these results suggest that TRAIL-R2 may be a promising drug target in poor prognosis triple-negative breast cancer, which currently lack targeted therapies.
Consistent with this idea, agonistic mAbs targeting TRAIL-R2, including the murine TRA-8 mAb and the human Apomab, have been shown to inhibit mammary tumor growth in murine models, including triple-negative tumors (14, 43). Our work provides the first in vivo evidence for the efficacy of lexatumumab in reducing primary and metastatic tumor burden in a murine model of breast cancer. As noted, humanized agonistic mAbs targeting death receptors have several potential advantages over recombinant TRAIL in the clinic, including improved pharmacokinetics, lack of decoy receptor binding, and activation of antibody-mediated cellular cytotoxicity (18). Indeed, multiple TRAIL-R2 mAb agonists (lexatumumab, PRO95780, conatumumab and CS-1008) have been tested in phase I dose-escalation studies in patients with advanced solid tumors (18, 19, 21, 23, 25). Specifically, lexatumumab was well tolerated at doses up to 10 mg/kg with a terminal t\(_{1/2}\) of 13.7-16.4 days and no evidence of immunogenic reactions (23, 25). Moreover, lexatumumab treatment resulted in stable disease in 9 of 31 (29%) or 12 of 37 (35%) patients. The safety and tolerability of TRAIL-R2 agonists as single agents in these phase I studies provide a strong foundation for combining them with cytotoxic agents in future clinical trials. Multiple preclinical studies have demonstrated that TRAIL-R2 agonists and chemotherapy or radiation act synergistically to reduce tumor burden in murine models of several solid tumors (14, 15, 46, 47). Chemotherapy and radiation enhance the sensitivity of cancer cells to TRAIL receptor agonists by increasing the expression of TRAIL-R2 and/or Bax, thereby providing additional rationale for therapeutically targeting TRAIL-R2 (15, 26, 48).

Overall, we have demonstrated that TRAIL-R2 is the principal proapoptotic death receptor in metastatic, triple-negative cancer cells and shown that targeting TRAIL-R2 results in greater antimetastatic activity in vivo than targeting TRAIL-R1 in an orthotopic model of triple-negative breast cancer. Although TRAIL-induced apoptosis in lymphoid malignancies and other
solid tumors, such as pancreatic adenocarcinomas, appears to be mediated predominantly by TRAIL-R1 (30, 31), our results strongly underscore the rationale for specifically targeting TRAIL-R2 in poor prognosis triple-negative breast cancer, which frequently metastasizes to the lungs and brain despite adjuvant chemotherapy and radiation (44). Of note, the differential sensitivity of various tumor types to TRAIL-R1 or TRAIL-R2 agonists is poorly understood and will require further study to delineate the underlying molecular mechanisms. Nevertheless, given the lack of targeted agents for triple-negative breast cancer, the clinical translation of TRAIL-R2 agonists, alone and/or in combination with chemotherapy, has the potential to improve the clinical outcome for these patients who are at high risk for disease progression in the first few years after diagnosis.

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References


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Table 1. Lymph node and lung metastases in MDA-MB-231-mCherry Orthotopic Model

<table>
<thead>
<tr>
<th>Week</th>
<th>Mice with metastases, n (%)</th>
<th>Lymph node metastases per mouse*</th>
<th>Mice with lung metastases, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2/6 (33%)</td>
<td>0.7 ± 0.5</td>
<td>0/6 (0%)</td>
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<td>4</td>
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<td>1/6 (17%)</td>
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<td>6.3 ± 0.6</td>
<td>3/8 (37%)</td>
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<td>8</td>
<td>7/7 (100%)</td>
<td>8.3 ± 0.5</td>
<td>6/7 (86%)</td>
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<tr>
<td>10</td>
<td>8/8 (100%)</td>
<td>10.3 ± 0.5</td>
<td>7/8 (88%)</td>
</tr>
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</table>

*Data are the mean ± SEM.

Table 2. Effect of Therapeutic Agents on Metastases in the MDA-MB-231-mCherry Orthotopic Model

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Dox</th>
<th>Mapa</th>
<th>Lexa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>9.2 ± 0.7</td>
<td>5.9 ± 0.8*</td>
<td>7.0 ± 1.0</td>
<td>3.7 ± 0.9***</td>
</tr>
<tr>
<td>metastases per</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse*</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mice with lung</td>
<td>10/11 (91%)</td>
<td>5/11 (45%)</td>
<td>5/11 (45%)</td>
<td>1/10 (10%)</td>
</tr>
<tr>
<td>metastases, n (%)</td>
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*Data are the mean ± SEM. *P < 0.05, ***P < 0.001 versus vehicle control
Figure Legends

Fig. 1 Human metastatic, triple-negative cancer cells are more sensitive to lexatumumab-induced cell death. A, Immunoblot of TRAIL-R1 and TRAIL-R2 expression in human metastatic, triple-negative cancer cells. B, Cell surface expression of TRAIL-R1 and TRAIL-R2 by flow cytometry. Cells were incubated with allophycocyanin conjugated control IgG1 (black), TRAIL-R1 (red, upper panels) or TRAIL-R2 (red, lower panels) mAb and analyzed by flow cytometry. C, MTS cell viability assay of metastatic cancer cells treated with different doses of mapatumumab (Mapa) or lexatumumab (Lexa) for 48 h (upper panels, mean ± SEM, n=3). **P < 0.01 or ***P < 0.001 lexatumumab vs. mapatumumab-treated cells. MTS assay of cancer cells incubated with vehicle, mapatumumab (0.5 µg/ml), lexatumumab (0.5 µg/ml) or doxorubicin (Dox, 0.2 µM for MDA-MB-231 and 435-Lung2 cells or 0.5 µM for GILM2 cells) for 24, 48 or 72 h (lower panels, mean ± SEM, n=3). *P < 0.05, **P < 0.01 or ***P < 0.001 versus vehicle-treated cells. D, MTS cell viability assay of human BxPC3 pancreatic adenocarcinoma cells treated with different doses of mapatumumab (Mapa) or lexatumumab (Lexa) for 48 h (mean ± SEM, n=3). **P < 0.01 mapatumumab vs. lexatumumab-treated cells. E, Long-term cell survival assay of metastatic cancer cells treated with vehicle, mapatumumab (0.5 µg/ml), lexatumumab (0.5 µg/ml) or doxorubicin (0.2-0.5 µM) for 48 h. Cells were washed and cultured for 5-7 additional days in the absence of drugs. Viable cells were stained with crystal violet. Data shown is representative of three independent experiments.

Fig. 2 Lexatumumab activates caspase-8 and initiates apoptosis in metastatic, triple-negative cancer cells more robustly than mapatumumab. A, Metastatic cancer cells were treated with vehicle, mapatumumab (0.5 µg/ml), lexatumumab (0.5 µg/ml) or doxorubicin (0.2-
0.5 µM) for 24 h. Procaspe-8, cleaved caspase-8, PARP and cleaved PARP were detected by immunoblotting. B, Cancer cells were treated with vehicle, mapatumumab (0.5 µg/ml), lexatumumab (0.5 µg/ml) or doxorubicin (0.2 µM for MDA-MB-231 and 435-Lung2 cells or 0.5 µM for GILM2 cells) for 16 h. Apoptosis was measured by Annexin V labeling using flow cytometry. C, MCF-10A breast epithelial cells stably expressing oncogenic H-RasV12 or empty vector were treated with mapatumumab (1.0 µg/ml) or lexatumumab (1.0 µg/ml) for 16 h and cell death was measured by Annexin V labeling (left panel). MCF-10A-H-RasV12 or MCF-10A-Vector cells were treated with varying concentrations of lexatumumab for 48 h and cell viability was measured by MTS assay (right panel, mean ± SEM, n=3).

**Fig. 3. Characterization of metastases in the MDA-MB-231-mCherry orthotopic model of breast cancer.** MDA-MB-231-mCherry breast cancer cells (1 x 10⁶ cells in Matrigel) were injected bilaterally into the ducts of the 4th mammary gland in female athymic nude mice. Fluorescent pseudocolored images of whole lungs and lymph nodes showing metastatic nodules at 4 and 10 weeks after tumor cell injection. H&E staining confirms the presence of tumor cells in lungs and lymph nodes at 4 and 10 weeks (low and high magnification fields in middle and lower panels, respectively). Arrowheads indicate metastatic nodules containing tumor cells.

**Fig. 4. Enhanced metastasis suppression by lexatumumab in vivo in an orthotopic model of triple-negative breast cancer.** MDA-MB-231-mCherry cells were injected intraductally into both 4th mammary glands of female nude mice. Seventeen days later, mice were randomized into four groups (10-11 mice per group) and treated with vehicle, doxorubicin (5 mg/kg once weekly), mapatumumab (20 mg/kg twice weekly) or lexatumumab (20 mg/kg twice weekly) for 21 days. Mice were assessed for survival, weight changes, and tumor burden. Representative whole lungs and lymph nodes are shown in Figure 4B. H&E staining confirms the presence of tumor cells in lungs and lymph nodes at 10 and 14 days (low and high magnification fields, respectively). Arrowheads indicate metastatic nodules containing tumor cells.
three weeks. A, Percent original mammary tumor volumes at various time points in each treatment group (mean ± SEM, n=10-11 mice per group). **P < 0.01 or ***P < 0.001 versus vehicle-treated mice. B, Percent active caspase-3-positive tumor cells in mammary tumors one week after treatment with vehicle, doxorubicin, mapatumumab or lexatumumab (mean ± SEM, n=3 tumors per group). **P < 0.01 or ***P < 0.001 versus vehicle. C, Number of metastases per lung surface (left panel) or percent surface area of the lung occupied by metastases (right panel) as determined by ex vivo fluorescence imaging of isolated whole lungs (mean ± SEM, n=10-11 mice per group). *P < 0.05, **P < 0.01 or ***P < 0.001 for the indicated comparisons.
Figure 1
Figure 2
Figure 3

4 Weeks

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10 Weeks

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Figure 4
Clinical Cancer Research

Enhanced Metastasis Suppression by Targeting TRAIL Receptor 2 in a Murine Model of Triple-Negative Breast Cancer

Dmitry Malin, Feng Chen, Carol Schiller, et al.

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