Methionine Deprivation Induces a Targetable Vulnerability in Triple-negative Breast Cancer Cells by Enhancing TRAIL Receptor-2 Expression

Elena Strekalova, Dmitry Malin, David M. Good* and Vincent L. Cryns

Department of Medicine, University of Wisconsin Carbone Cancer Center, University of Wisconsin School of Medicine and Public Health, Madison, WI.

*Current address: Covance, Inc., 3301 Kinsman Blvd., Madison, WI.

Grant support: Breast Cancer Research Foundation (VLC), Susan G. Komen for the Cure Postdoctoral Fellowship Award (DM), and P30CA14520 University of Wisconsin Comprehensive Cancer Center core facility support.

Correspondence to: Vincent L. Cryns, Department of Medicine, MFCB 4144, 1685 Highland Avenue, Madison, WI 53705 USA. Phone: 608-262-4786; Fax: 608-263-9983. E-mail: vlcryns@medicine.wisc.edu.

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.

Manuscript word count: 5000; Total number of figures and tables: 6

Running Title: Methionine Depletion Enhances Sensitivity to TRAIL Receptor-2 Agonists

Key words: methionine depletion; breast cancer; TRAIL; nutrition; therapeutics
Translational Relevance

Despite their potential tumor-selectivity and safety, proapoptotic TNF-related apoptosis-inducing ligand (TRAIL) receptor agonists, including recombinant TRAIL and humanized agonistic monoclonal antibodies, have been largely disappointing in clinical trials in patients with advanced malignancies. Here we report that methionine depletion enhances the sensitivity of triple (estrogen receptor, progesterone receptor and HER2)-negative breast cancer cells to lexatumumab, a proapoptotic humanized agonistic monoclonal antibody targeting TRAIL receptor-2 (TRAIL-R2). Methionine depletion augments lexatumumab-induced caspase activation and apoptosis by increasing TRAIL-R2 mRNA and cell surface expression. Notably, these effects were not observed in untransformed breast epithelial cells, suggesting that methionine stress selectively unmasks a targetable vulnerability in transformed cells. In addition, dietary methionine deprivation enhances the antitumor effects of lexatumumab in an orthotopic model of metastatic triple-negative breast cancer. Taken together, our results provide proof-of-principle preclinical evidence to support a clinical trial of dietary methionine restriction in combination with proapoptotic TRAIL-R2 agonists.

Abstract

Purpose: Many neoplasms are vulnerable to methionine deficiency by mechanisms that are poorly understood. Because gene profiling studies have revealed that methionine depletion increases TNF-related apoptosis-inducing ligand receptor-2 (TRAIL-R2) mRNA, we postulated that methionine stress sensitizes breast cancer cells to proapoptotic TRAIL-R2 agonists.

Experimental Design: Human triple (ER/PR/HER2)-negative breast carcinoma cell lines were cultured in control or methionine-free media. The effects of methionine depletion on TRAIL

2, Strekalova et al.
receptor expression and sensitivity to chemotherapy or a humanized agonistic TRAIL-R2 monoclonal antibody (lexatumumab) were determined. The melanoma-associated antigen MAGED2 was silenced to delineate its functional role in sensitizing TNBC cells to methionine stress. An orthotopic TNBC model was utilized to evaluate the effects of dietary methionine deficiency, lexatumumab or the combination.

**Results:** Methionine depletion sensitized TNBC cells to lexatumumab-induced caspase activation and apoptosis by increasing TRAIL-R2 mRNA and cell surface expression. MCF-10A cells transformed by oncogenic H-Ras, but not untransformed cells, and matrix-detached TNBC cells were highly sensitive to the combination of lexatumumab and methionine depletion. Proteomics analyses revealed that MAGED2, which has been reported to reduce TRAIL-R2 expression, was suppressed by methionine stress. Silencing MAGED2 recapitulated features of methionine deprivation, including enhanced mRNA and cell surface expression of TRAIL receptors and increased sensitivity to TRAIL receptor agonists. Dietary methionine deprivation enhanced the antitumor effects of lexatumumab in an orthotopic metastatic TNBC model.

**Conclusion:** Methionine depletion exposes a targetable defect in TNBC cells by increasing TRAIL-R2 expression. Our findings provide the foundation for a clinical trial combining dietary methionine restriction and TRAIL-R2 agonists.
Introduction

Transformed cells are selectively vulnerable to specific amino acid deficiencies, including methionine, arginine, leucine, serine and others (1-4). Depletion of the essential amino acid methionine inhibits cell cycle progression and induces apoptosis in cancer cells \textit{in vitro} and suppresses tumor growth \textit{in vivo} in preclinical models of diverse tumor types (5-9). Strikingly, supplementation with homocysteine renders normal cells largely resistant to methionine depletion, while transformed cells remain sensitive to methionine deprivation in the presence of homocysteine (10, 11). In addition, administration of the methionine-degrading enzyme methioninase mimics many of the antitumor actions of methionine depletion \textit{in vitro} and \textit{in vivo} (1, 12, 13). Both methionine deprivation and methioninase have been reported to enhance the cytotoxicity of chemotherapy drugs in some but not all studies; these chemosensitizing effects have been attributed to methionine stress-induced cell cycle blockade (14-17). Methionine depletion reduces the free concentration of intracellular methionine despite normal rates of methionine synthesis from homocysteine in tumor cells (18, 19). Although methionine plays an integral role in many biochemical pathways including protein and polyamine synthesis and methylation of nucleic acids and proteins, the molecular mechanisms underlying the “methionine dependence” of many neoplasms remain poorly understood (20). Clearly, a more detailed understanding of the cellular response to methionine deprivation would greatly facilitate the development of more effective combination therapies that act synergistically with methionine stress.

Gene expression analyses have revealed that both tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its proapoptotic receptor TRAIL-R2 mRNA are upregulated in
methionine-dependent CNS tumor cell lines in response to methionine depletion (21). Although the functional relevance of the observed increase in TRAIL/TRAIL-R2 mRNA was not explored, these findings suggest that methionine stress may sensitize cancer cells to proapoptotic TRAIL receptor agonists. TRAIL/Apo2L is a promising cancer therapy that preferentially induces apoptosis in transformed cells by binding to its proapoptotic death receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5, and activating procapases-8/-10 by a FADD-dependent mechanism in the extrinsic apoptotic pathway (22). Moreover, TRAIL and agonistic monoclonal antibodies (mAbs) targeting TRAIL-R1 or TRAIL-R2 inhibit primary tumor growth and metastatic tumor burden in preclinical models of diverse tumor types including breast cancer (23-28). We have recently reported that a human mAb targeting TRAIL-R2 (lexatumumab) is more effective than an agonistic TRAIL-R1 mAb (mapatumumab) in inducing apoptosis and suppressing lung metastases in an orthotopic model of clinically aggressive triple (ER/PR/HER2)-negative breast cancer (28). Recently, recombinant TRAIL (dulanermin) and agonistic mAbs targeting TRAIL-R1 or TRAIL-R2 have been evaluated in clinical trials in patients with advanced malignancies (29-34). Although these early stage clinical trials have demonstrated the safety and tolerability of TRAIL receptor agonists, they have been largely disappointing from a therapeutic standpoint (35). We postulated that methionine deprivation would enhance the sensitivity of triple-negative breast cancer (TNBC) cells to TRAIL-R2 targeted therapies such as lexatumumab and augment its antitumor activity in vivo.

Here we report that methionine depletion robustly sensitizes a panel of human TNBC cells to lexatumumab-induced caspase activation and apoptosis by increasing TRAIL-R2 mRNA and cell surface expression. Strikingly, MCF-10A cells transformed by oncogenic H-Ras, but not
untransformed cells, were highly sensitive to the combination of lexatumumab and methionine depletion. Proteomics analyses revealed that the melanoma-associated antigen MAGED2, which has been reported to inhibit TRAIL-induced apoptosis by reducing TRAIL-R2 expression (36), was suppressed by methionine depletion. Silencing MAGED2 recapitulated features of methionine deprivation, including enhanced TRAIL receptor expression and increased sensitivity to TRAIL receptor agonists. In addition, dietary methionine deprivation enhanced the antitumor effects of lexatumumab in an orthotopic model of metastatic TNBC. Collectively, our findings indicate that methionine stress selectively induces a targetable vulnerability in transformed cells by increasing TRAIL-R2 expression.

Material and Methods

Cell culture and reagents

Human MDA-MB-231 TNBC cells stably expressing mCherry fluorescent protein and GILM2 TNBC cells were cultured as described previously (28). MDA-MB-468 TNBC cells stably expressing mCherry fluorescent protein were generated by lentiviral transduction as described (37) and were maintained in DMEM supplemented with 10% FBS and 100 IU/mL penicillin/streptomycin (Invitrogen). Human MCF-10A breast epithelial cells stably expressing H-RasV12 or empty vector were cultured as described previously (38). For methionine deprivation, complete medium was formulated by supplementing RPMI 1640 with additional nutrients to closely match the original media for each cell line. Methionine-free medium (0% Met) was formulated in the same way as complete medium, without addition of 15 mg/L L-methionine. Lexatumumab was generously supplied by Dr. Robin Humphreys (Human Genome Research. on April 15, 2017. © 2015 American Association for Cancer Research.
Sciences). Doxorubicin, Vinorelbine, 5-Fluorouracil, Gemcitabine and Docetaxel were purchased from Sigma-Aldrich.

**Immunoblotting**

Proteins were immunoblotted from whole-cell lysates as described (38). Primary Abs against MAGED2, β-actin (Sigma-Aldrich), PARP (BD Biosciences) and caspase-3 (Cell Signaling Technology) were used.

**Cell viability assay**

An MTS assay (Promega) was used to measure cell viability as described (28). Cells were plated in 96-well plates (2.5 x 10^3 cells/well) overnight. The next day, cells were washed with PBS, and complete or 0% Met medium was added. After 72 hours, MDA-MB-468 and GILM2 cells were treated with doxorubicin (3.4 µM), lexatumumab (4 µg/ml), gemcitabine (4 µM), vinorelbine (4 µM), docetaxel (4 nM) or 5-Fluorouracil (4 µg/ml), while MDA-MB-231 cells were treated with doxorubicin (2 µM), lexatumumab (2 µg/ml), gemcitabine (2 µM), vinorelbine (2 µM), docetaxel (2 nM) or 5-Fluorouracil (2 µg/ml). Cell viability was assessed after 48 hours of drug treatment. Cell viability was expressed as the percentage of viable cells compared to control vehicle-treated cells in complete medium.

**Crystal violet cell survival assay**

Cells were seeded on 6-well plates (3 x 10^5 cells/well). The next day, cells were washed with PBS, and complete or 0% Met medium was added. After 24 hours, cells were treated with vehicle, doxorubicin (3.4 µM) or lexatumumab (4 µg/ml) for MDA-MB-468 and GILM2 cells or
with vehicle, doxorubicin (2 µM) or lexatumumab (2 µg/ml) for MDA-MB-231 cells for 48 hours. MCF-10A cells were treated with vehicle, doxorubicin (3.4 µM) or lexatumumab (2 µg/ml) for 24 hours. Surviving cells were stained with crystal violet as described (26).

**Flow cytometry**

For the Annexin V apoptosis assay, cells were cultured in complete or 0% Met medium for 48 hours, treated with vehicle, doxorubicin (3.4 µM) or lexatumumab (2 µg/ml) for 6 hours, and then analyzed by flow cytometry using the Annexin-PE Apoptosis Detection Kit (BD Bioscience). To measure cell surface expression of TRAIL receptors, cells were washed once with PBS and incubated with TRAIL-R1, TRAIL-R2 or control IgG1 mAb conjugated with allophycocyanin (BioLegend) in PBS containing 2.5% BSA for 45 minutes at 4°C in the dark. Cells were then washed 3 times with PBS containing 2.5% BSA, resuspended in PBS containing 4′,6-diamidino-2-phenylindole (DAPI, 1 µg/mL), and analyzed by flow cytometry.

**Anoikis assay**

mCherry-labeled MDA-MB-231 cells were grown in complete growth medium or 0% Met medium containing 1% methylcellulose on Corning Costar Ultra-Low attachment plates (Fisher Scientific) at a density of $1.0 \times 10^4$ cells/well (96-well plates) for 48 hours prior to drug treatment (MTS assay) or $2.0 \times 10^5$ cells/well (6-well plates) for 72 hours (flow cytometry).

**MAGED2 siRNA experiments**

siRNAs targeting the sequences GGGAUACAUCAUUCACUCU (MAGED2-1) or GGGCAAUGAUUUUGGUGAA (MAGED2-2) of human MAGED2 and non-silencing control

8, Strekalova et al.
siRNA were purchased from Sigma-Aldrich. Cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen).

**Real-time PCR**

cDNA was synthesized from total RNA using iScript™ cDNA Synthesis Kit (Bio-Rad). Primers for MAGE-D2 (forward 5-TCAGCACCTTAGAGCCCCTGGA-3, reverse 5-CAGATGACAGCCTCAGGGTCAAGGTCAAGG-3), TRAIL-R1 (5-CAGAGGGATGGTCAAGGTCAAGG-3, reverse 5-CCACAACCTGAGCCCCTGGA-3), TRAIL-R2 (forward 5-CGCTGCACCAGGATGGTCAAGGTCAAGG-3, reverse 5-GTGCCTTCTTCGACTGACA-3) and gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (5-GAAGGTGAAGGTCGGAGTC-3, reverse 5-GAAGATGGTGATGGGATTTC-3) were purchased from Integrated DNA Technologies. PCR amplification was performed using iQ™ SYBR Green supermix (Bio-Rad) and a CFX96 Real Time PCR sequence detection system (Bio-Rad). Experiments were performed twice, each in triplicate. A comparative Ct method was used to compare the RNA expression in samples to that of the control in each experiment.

**Orthotopic model of metastatic TNBC**

All animal experiments were approved by the IACUC at the University of Wisconsin-Madison. MDA-MB-468-mCherry TNBC cells (2 × 10⁶) in 100% Matrigel (BD Bioscience) were injected into the ducts of the 4th mammary glands of 4- to 5-week-old female NOD scid IL2 receptor γ chain knockout (NSG) mice (Jackson Laboratory) as described (37). Mice were randomized into four treatment groups (10 mice per group): (1) a control 15% protein diet (Teklad TD.01084) plus vehicle (PBS i.p. twice weekly, 6 doses), (2) control diet plus lexatumumab (10 mg/kg i.p. 9, Strekalova et al.)
twice weekly, 6 doses), (3) an isocaloric 15% protein methionine-free (0% methionine, Teklad TD.140119) diet plus vehicle, or (4) a methionine-free diet plus lexatumumab (10 mg/kg i.p. twice weekly, 6 doses). The composition of each diet is listed (Supplementary Table S1). Mice were placed on their respective diets 8 weeks after tumor cell inoculation, and treatment (vehicle or lexatumumab) was initiated 0.5 weeks later. The diets were continued throughout the treatment period. Mammary tumors were measured with calipers and tumor volume was calculated as described (39). mCherry-fluorescent metastases were visualized in isolated whole lungs using a Leica MZ10F fluorescent stereomicroscope and tumor burden was scored using NIH ImageJ analysis as described (28). For quantitating the number of lung macrometastases, only metastases (≥0.1 mm) were scored.

Statistics

ANOVAs with Bonferroni posttests were done using Prism 4 GraphPad Software to assess statistical significance.

Results

Methionine depletion sensitizes TNBC cells to lexatumumab

To determine whether methionine depletion enhances the cytotoxicity of lexatumumab and diverse chemotherapy agents, three human TNBC cell lines (MDA-MB-231-mCherry, GILM2 and MDA-MB-468) were grown in control or methionine-free media for 72 hours and then treated with vehicle, lexatumumab, doxorubicin, gemcitabine, vinorelbine, docetaxel or 5-fluorouracil. Methionine deprivation alone was moderately cytotoxic against two of the three TNBC cell lines as determined by an MTS cell viability assay (Fig. 1A) and inhibited cell
proliferation in all three TNBC cell lines as determined by a reduction in Ki67-positive cells (Supplementary Fig. S1). Of all the cancer therapeutics evaluated, only the combination of methionine depletion and lexatumumab resulted in enhanced cytotoxicity in all three TNBC cells compared to treatment in control media. To confirm these findings using a second assay, TNBC cells were grown in control or methionine-free media for 72 hours, then treated with vehicle, doxorubicin or lexatumumab for 48 hours, and viable cells scored by crystal violet staining. Under these conditions, methionine depletion dramatically sensitized TNBC cells to lexatumumab, but had no or little effect on the cytotoxicity of doxorubicin (Fig. 1B). Pretreatment of TNBC cells with methioninase, an enzyme that degrades methionine, also sensitized TNBC cells to lexatumumab (Supplementary Fig. S2A). In addition, MCF-10A breast epithelial cells transformed by oncogenic H-RasV12 were more sensitive to methionine depletion alone or the combination of methionine depletion and lexatumumab than untransformed MCF-10A cells expressing empty vector (Fig. 1C). Collectively, these results indicate that methionine depletion robustly sensitizes TNBC cells to the proapoptotic death receptor agonist lexatumumab and point to the potential tumor-selectivity of this combination.

**Methionine deprivation augments lexatumumab-induced caspase activation and apoptosis in TNBC cells**

To determine whether methionine deprivation enhances caspase activation by lexatumumab, TNBC cells were grown in control or methionine-free media, treated with vehicle, doxorubicin or lexatumumab, and then analyzed by immunoblotting. Methionine deprivation resulted in more robust proteolysis of the caspase substrate PARP as detected by decreased full-length PARP and/or increased cleaved product compared to TNBCs grown in control media (Fig. 2A). In
addition, methionine deprivation augmented lexatumumab-induced proteolytic processing of procaspase-3 as detected by diminished procaspase-3 expression. In contrast, methionine restriction had little or no effect on doxorubicin-induced PARP cleavage or procaspase-3 processing under the conditions tested. Moreover, pretreatment of TNBC cells with methioninase augmented lexatumumab-induced PARP cleavage (Supplementary Fig. S2B). These findings indicate that methionine restriction enhances caspase activation by lexatumumab.

To quantitate the effect of methionine restriction on apoptosis, TNBC cells were grown in methionine-free media for 48 hours, treated with vehicle, doxorubicin or lexatumumab for 6 hours, and Annexin V-positive cells were scored by flow cytometry. Consistent with our MTS and crystal violet data, methionine depletion profoundly sensitized all three TNBC cell lines to lexatumumab-induced apoptosis (Fig. 2B). In contrast, methionine deprivation had little effect on doxorubicin-induced cell death. Furthermore, transformed MCF-10A-H-RasV12 cells were more sensitive to methionine depletion and the combination of methionine depletion and lexatumumab than untransformed MCF-10A-Vector cells, confirming the preferential proapoptotic activity of this treatment against cancer cells (Fig. 2C). However, methionine deprivation had little effect on doxorubicin-induced cell death in either transformed or untransformed MCF-10A cells. Taken together, these findings indicate that methionine depletion potently augments caspase activation and apoptosis by lexatumumab and underscores the enhanced sensitivity of transformed cells to this combination.

**Methionine deprivation preferentially increases the cell surface expression of TRAIL-R2 in TNBC cells and sensitizes matrix-detached cells to lexatumumab**
To delineate the mechanisms by which methionine depletion enhances the sensitivity of TNBC cells to lexatumumab, we first examined its effects on the expression of TRAIL-R1 and TRAIL-R2. Methionine deprivation for 72 hours resulted in robust induction of TRAIL-R2 and TRAIL-R1 mRNA levels in all three TNBC cell lines as determined by real-time PCR (Fig. 3A). Similarly, treatment of TNBC cells with methioninase for 48 hours increased TRAIL-R1 and TRAIL-R2 mRNA levels (Supplementary Fig. S2C). Moreover, methionine depletion increased the cell surface expression of TRAIL-R2 in all TNBC cell lines as determined by flow cytometry but had modest or no effect on TRAIL-R1 cell surface expression (Fig. 3B). Intriguingly, methionine deprivation increased the cell surface expression of both TRAIL-R1 and TRAIL-R2 in transformed MCF-10A-H-RasV12 cells but not in untransformed MCF-10A-Vector cells (Fig. 3C). These results suggest that methionine restriction sensitizes TNBC cells to lexatumumab by increasing the cell surface expression of TRAIL-R2.

Because resistance to extracellular matrix detachment-induced apoptosis (“anoikis”) is a hallmark of metastatic carcinoma cells (40), we examined whether methionine deprivation enhanced cell surface expression of TRAIL-R2 and sensitized matrix-detached cells to lexatumumab. Similar to the results obtained in adherent cells, methionine depletion increased cell surface expression of TRAIL-R2 to a greater extent than TRAIL-R1 in mCherry-labeled MDA-MB-231 cells grown in suspension on Ultra-low attachment plates (Fig. 3D). Moreover, methionine depletion sensitized matrix-detached mCherry-labeled-MSA-MB-231 TNBC cells to lexatumumab as determined by MTS assay (Fig. 3E). Collectively, these results indicate that methionine depletion sensitizes matrix-detached TNBC cells to lexatumumab, supporting an antimetastatic activity for this novel combination.
Methionine deprivation reduces MAGED2 protein levels but does not affect MAGED2 mRNA levels

To identify proteins that might mediate the effects of methionine depletion on TRAIL receptor expression, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) of MDA-MB-231-mCherry and GILM2 TNBC cells grown in methionine-free or control media for 72 hours. Proteomics analysis revealed a number of proteins that were differentially expressed in TNBC cells in response to methionine depletion (Supplementary Table S2). One particularly intriguing candidate was MAGED2/MAGD2, which was downregulated by methionine depletion in both TNBC cell lines and has been previously reported to inhibit TRAIL-induced apoptosis by reducing TRAIL-R2 expression (36). Immunoblotting confirmed that MAGED2 protein levels were decreased in MDA-MB-231-mCherry, GILM2 and MDA-MB-468 TNBC cells grown in methionine-free media compared to cells cultured in control media (Fig. 4A). In contrast, methionine depletion did not affect MAGED2 mRNA levels (Fig. 4B), indicating that methionine deprivation regulates MAGED2 via a post-transcriptional mechanism.

Silencing MAGED2 increases cell surface expression of TRAIL receptors and sensitizes TNBC cells to TRAIL receptor agonists

To investigate the functional role of MAGED2 downregulation in the lexatumumab-sensitizing effects of methionine depletion, we transiently silenced MAGED2 in MDA-MB-231-mCherry cells using two different siRNAs (si-MAGED2-1 and si-MAGED2-2) targeting MAGED2. Both MAGED2 siRNAs robustly reduced MAGED2 mRNA and protein levels compared to a control scrambled siRNA (Fig. 5A). Silencing MAGED2 increased TRAIL-R1 and TRAIL-R2 mRNA levels (Fig. 5B) and cell surface expression of both death receptors (Fig. 5C). Consistent with
these effects, silencing MAGED2 sensitized MDA-MB-231 TNBC cells to lexatumumab and the TRAIL-R1 agonist mapatumumab as determined by crystal violet staining (Fig. 5D) and MTS assay (Fig. 5E). Collectively, these results indicate that silencing MAGED2 mimics many of the effects of methionine depletion, including enhanced cell surface expression of TRAIL receptor and increased sensitivity to TRAIL receptor agonists.

Dietary methionine deprivation enhances the antitumor effects of lexatumumab in an orthotopic model of metastatic TNBC

To examine the antitumor activity of methionine deprivation in combination with lexatumumab in vivo, female NSG mice with established MDA-MB-468-mCherry mammary tumors were fed a control or methionine-free diet (Supplementary Table S1) and treated with vehicle or lexatumumab (10 mg/kg twice weekly) for 3 weeks. All three interventions (methionine-free diet, lexatumumab alone, and methionine-free diet plus lexatumumab) inhibited tumor growth compared to vehicle-treated mice on a control diet, although the combination of methionine-free diet plus lexatumumab was more effective than diet alone or lexatumumab alone at the completion of the study (Fig. 6A). Both the methionine-free diet and the methionine-free diet plus lexatumumab resulted in modest weight loss (Fig. 6B). To evaluate the impact of treatment on lung metastases in vivo, mice were euthanized 13 weeks after intraductal tumor inoculation, and mCherry-fluorescent metastatic lesions in the lungs were identified at autopsy. Notably, only mice receiving the methionine-free diet in combination with lexatumumab had a significant reduction in tumor burden as defined as the percentage surface area occupied by lung metastases or number of macrometastases ≥ 0.1 mm compared to vehicle-treated mice on control diets (Fig. 6C). TRAIL-R2 mRNA levels were increased in mammary tumors from mice treated with the
methionine-free diet compared to mice on a control diet (Fig. 6D), while MAGED2 protein levels were reduced in mammary tumors from mice on the methionine-free diet (Fig. 6E). These findings indicate that dietary methionine deprivation inhibits mammary tumor expression of MAGED2 and enhances TRAIL-R2 expression and the antitumor effects of lexatumumab in an orthotopic model of metastatic TNBC.

Discussion

We have demonstrated that methionine deprivation unmasks a targetable vulnerability of transformed cells by augmenting TRAIL-R2 mRNA levels and cell surface expression, resulting in enhanced sensitivity to proapoptotic TRAIL-R2 agonists such as lexatumumab. Similar results were obtained when we used methioninase to degrade methionine, a therapeutic strategy that has shown promise in preclinical studies (1, 12, 13, 15). Strikingly, the effects of methionine depletion on increasing cell surface expression of TRAIL-R2 and enhancing sensitivity to lexatumumab are largely confined to transformed cells, as untransformed MCF-10A-Vector cells were resistant to these effects. These findings are consistent with the reported tumor-selectivity of methionine deprivation and TRAIL receptor agonists when used individually (10, 11, 22, 24, 26, 41). Methionine depletion also sensitized matrix-detached TNBC cells to lexatumumab, supporting an antimetastatic activity for this combination. In addition, we have shown that a brief exposure to a methionine-free diet is well tolerated by mice and enhances the antitumor activity of lexatumumab against mammary tumors and lung metastases in vivo in an orthotopic MDA-MB-468 TNBC model that is resistant to lexatumumab alone. Intriguingly, this combination therapy suppressed lung metastases more robustly than it inhibited mammary tumor growth, consistent with the reported function of TRAIL as a metastasis suppressor and our prior
preclinical observations with lexatumumab monotherapy in a different orthotopic model of metastatic TNBC (MDA-MB-231) that was sensitive to this agent (28, 42, 43). Our current findings point to methionine deprivation as a novel nutritional intervention to augment the antitumor efficacy of proapoptotic TRAIL receptor agonists, which have been largely disappointing in clinical trials (29-34). Moreover, phase I and phase II trials of a methionine-free diet alone or in combination with chemotherapy in patients with metastatic or recurrent solid tumors have confirmed the safety and tolerability of intermittent or continuous short-term methionine deprivation (44-46), suggesting that our findings could be readily translated into a clinical trial. Collectively, our results indicate that methionine depletion induces a selective vulnerability to lexatumumab by increasing expression of its molecular target and strongly support combining these interventions to enhance the therapeutic impact of TRAIL-R2 agonists. In support of this idea, we have recently demonstrated that metastatic TNBC cells are more sensitive to lexatumumab than the TRAIL-R1 agonist mapatumumab in vitro and in vivo (28), pointing to TRAIL-R2 as a promising therapeutic target in metastatic TNBC.

From a mechanism standpoint, we have shown that methionine depletion suppresses the protein levels of MAGED2, which provides a molecular link between methionine stress and enhanced TRAIL receptor sensitivity. Specifically, stably silencing MAGED2 mimics many of the effects of methionine depletion, including enhanced mRNA and cell surface expression of TRAIL receptors and increased sensitivity to TRAIL receptor agonists. However, methionine stress augments the cell surface expression of TRAIL-R2 to a greater extent than TRAIL-R1, while MAGED2 silencing enhances cell surface expression of both receptors, suggesting that additional proteins that are differentially regulated by methionine depletion contribute to its

17, Strekalova et al.
effects on TRAIL receptors. MAGED2 is a ubiquitously expressed member of the melanoma-associated antigen (MAGE)-II family that has been implicated in apoptosis-resistance by inhibiting TRAIL-R2 expression and/or antagonizing p53 function (36, 47, 48). Indeed, MAGED2 was previously identified as a negative regulator of TRAIL-R2 expression and TRAIL-induced apoptosis in melanoma cells (36). However, the mechanisms by which MAGED2 suppressed mRNA levels and cell surface expression of TRAIL-R2 were not delineated. Although we have demonstrated that methionine stress downregulates MAGED2 expression by a post-transcriptional mechanism, the details of this mechanism have yet to be elucidated. Furthermore, it remains to be determined how MAGED2 regulates TRAIL receptor expression. In contrast to the previous report (36), p53 is not required for the MAGED2-dependent effects on TRAIL-R2 expression in our experiments, as silencing MAGED2 in p53-mutant MDA-MB-231 TNBC cells suppressed TRAIL-R2 expression. Clearly, further studies are warranted to illuminate the molecular pathways linking methionine stress, reduced MAGED2 expression and enhanced TRAIL receptor levels. Nevertheless, our findings unequivocally point to the importance of MAGED2 and TRAIL-R2 in linking methionine stress to enhanced sensitivity to TRAIL receptor agonists.

In addition, our mechanistic observations may also be relevant to other nutritional interventions that have been reported to inhibit tumor growth, such as protein or caloric restriction, as these interventions result in decreased methionine intake (49). Indeed, protein restriction was recently reported to reduced cancer incidence in individuals aged 50-65 and inhibit tumor burden in murine models of cancer (50). Although serum IGF-1 levels were reduced by protein restriction in this study, it will be interesting to examine whether protein restriction sensitizes transformed
cells to TRAIL agonists by increasing TRAIL-R2 expression. Such studies could lead to additional strategies to combine nutritional interventions and targeted therapies to improve clinical outcomes.

In summary, our results demonstrate that metabolic stress exposes distinctive vulnerabilities in transformed cells that can be targeted with rationally selected therapies. Specifically, methionine depletion exposes a targetable defect in TNBC cells by robustly increasing cell surface expression of TRAIL-R2 and rendering them more sensitive to TRAIL-R2 agonists \textit{in vitro} and \textit{in vivo}. Our findings provide proof-of-principle preclinical evidence to support a clinical trial combining dietary methionine restriction and proapoptotic TRAIL-R2 receptor agonists.

**Acknowledgements**

We are indebted to Mark Burkard and members of the Cryns lab for critical reading of the manuscript, Joshua Coon for use of the mass spectrometer, and Robin Humphreys for providing lexatumumab.
References


20, Strekalova et al.


Figure Legends

Figure 1. Methionine deprivation sensitizes TNBC cells to lexatumumab. **A**, MTS cell viability assay of TNBC cells cultured in normal growth media (control) or methionine-free media (0% Met) and treated for 48 hours with vehicle, doxorubicin (3.4 µM), lexatumumab (4 µg/ml), gemcitabine (4 µM), vinorelbine (4 µM), docetaxel (4 nM) or 5-Fluorouracil (4 µg/ml) for MDA-MB-468 and GILM2 cells or doxorubicin (2 µM), lexatumumab (2 µg/ml), gemcitabine (2 µM), vinorelbine (2 µM), docetaxel (2 nM) or 5-Fluorouracil (2 µg/ml) for MDA-MB-231 cells (mean ± SEM, n = 3). **B**, Crystal violet cell survival assay of TNBC cells cultured in control or 0% Met media and treated for 48 hours with vehicle, doxorubicin (3.4 µM) or lexatumumab (4 µg/ml) for MDA-MB-468 and GILM2 cells or doxorubicin (2 µM) or lexatumumab (2 µg/ml) for MDA-MB-231 cells. Left panel: representative images. Right panel: quantification performed by counting cells in 3 fields of each well (mean ± SEM, n = 3). **C**, Crystal violet cell survival assay of MCF-10A breast epithelial cells stably expressing oncogenic H-RasV12 or empty vector grown in 0% Met media and treated with vehicle, doxorubicin (3.4 µM) or lexatumumab (2 µg/ml) for 24 hours. Left panel: representative images. Right panel: quantification performed by counting cells in 3 fields of each well (mean ± SEM, n = 3). In all panels, *, P < 0.05, **, P < 0.01, ***, P < 0.001.

Figure 2. Methionine deprivation augments lexatumumab-induced caspase activation and apoptosis in TNBC cells. **A**, TNBC cells were grown in control or 0% Met media for 48 hours and then treated for 24 hours with vehicle, doxorubicin (3.4 µM) or lexatumumab (2 µg/ml) for MDA-MB-468 and GILM2 cells or doxorubicin (2 µM) and lexatumumab (1 µg/ml) for MDA-MB-231 cells. Procaspase-3, PARP, and cleaved PARP were detected by immunoblotting. **B**, 24, Strekalova et al.
TNBC cells were cultured in control or 0% Met media and treated with vehicle, doxorubicin (3.4 µM) or lexatumumab (2 µg/ml) for 6 hours. 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 grown in control or 0% Met media and were treated with vehicle, doxorubicin (3.4 µM) or lexatumumab (2 µg/ml) for 6 hours. Apoptosis was quantitated by Annexin V labeling.

**Figure 3. Methionine deprivation preferentially increases the cell surface expression of TRAIL-R2 in TNBC cells and sensitizes matrix-detached cells to lexatumumab.** A, TNBC cells were cultured in control or 0% Met media for 72 hours followed by isolation of total RNA. TRAIL-R1 (left panel) and TRAIL-R2 (right panel) mRNA levels were measured by Real-time PCR and normalized to expression in TNBC cells grown in control media. B and C, Cell surface expression of TRAIL-R1 and TRAIL-R2 in TNBC cells (B) or MCF-10A breast epithelial cells stably expressing oncogenic H-RasV12 or empty vector (C). Cells were grown in control or 0% Met media for 72 hours, incubated with control IgG, TRAIL-R1 or TRAIL-R2 mAb, and analyzed by flow cytometry. Grey bar: negative control cells incubated with IgG. Black line: cells cultured in control media and incubated with TRAIL-R1 (bottom panels) or TRAIL-R2 (top panels) Ab. Red line: cells grown in 0% Met media and incubated with TRAIL-R1 (bottom panels) or TRAIL-R2 (top panels) Ab. D, Cell surface expression of TRAIL-R1 and TRAIL-R2 in mCherry-labeled MDA-MB-231 cells grown in suspension in control or 0% Met media. Cells were incubated with control IgG, TRAIL-R1 or TRAIL-R2 mAb and analyzed by flow cytometry. Grey bar: negative control adherent cells incubated with IgG. Black line: cells cultured in suspension in control media and incubated with TRAIL-R1 (bottom panel) or...
TRAIL-R2 (top panel) Ab. Red line: cells grown in suspension in 0% Met media and incubated with TRAIL-R1 (bottom panel) or TRAIL-R2 (top panel) Ab. E, MTS cell viability assay of mCherry-labeled MDA-MB-231 cells grown in suspension in control or methionine-free media and treated with vehicle of lexatumumab. Cells were grown in complete growth medium (control) or methionine-free media (0% Met) containing 1% methylcellulose on Ultra-Low attachment plates for 48 hours and then treated with vehicle or lexatumumab (1 µg/ml) for 24 hours (mean ± SEM, n = 3). **, P < 0.01

Figure 4. Methionine deprivation reduces MAGED2 protein levels but does not affect MAGED2 mRNA levels. A, Immunoblot of MAGED2 protein expression in TNBC cells grown in control (Met +) or 0% Met (- Met) media for 72 hours. B, Real-time PCR analysis of MAGED2 mRNA levels in TNBC cells grown in control or 0% Met media for 72 hours. MAGED2 mRNA levels were normalized to expression in TNBC cells cultured in control media.

Figure 5. Silencing MAGED2 increases cell surface expression of TRAIL receptors and sensitizes TNBC cells to TRAIL receptor agonists. A, MDA-MB-231 cells were transfected with a scrambled control siRNA or one of two different siRNAs targeting MAGED2 (MAGED2-1 and MAGED2-2). Left panel: Real-time PCR analysis of MAGED2 mRNA levels 24 hours after siRNA transfection. MAGED2 mRNA levels were normalized to control siRNA-transfected cells. Right panel: Immunoblot analysis of MAGED2 48 hours after siRNA transfection. B, Real-time PCR analysis of TRAIL-R1 and TRAIL-R2 mRNA levels in MDA-MB-231 cells transfected with control or MAGED2 siRNAs. C, Cell surface expression of TRAIL-R1 and TRAIL-R2 in MDA-MB-231 cells transfected with control or MAGED2
siRNAs. Grey bar: negative control. Black line: control siRNA. Red line: MAGED2 siRNA. D, Crystal violet cell survival assay of MDA-MB-231 cells transfected with control or MAGED2 siRNA and treated with vehicle, lexatumumab (0.1 µg/ml) or mapatumumab (0.1 µg/ml) for 72 hours. Left panel: representative images. Right panel: quantification performed by counting cells in 3 fields of each well (mean ± SEM, n = 3). E, MTS cell viability assay of MDA-MB-231 cells transfected with control or MAGED2 siRNA and treated with vehicle, lexatumumab (1 µg/ml) or mapatumumab (1 µg/ml) for 24 hours (mean ± SEM, n=3). In (D) and (E), *, P < 0.05, **, P < 0.01, ***, P < 0.001.

**Figure 6. Dietary methionine deprivation enhances the antitumor effects of lexatumumab in an orthotopic model of metastatic triple-negative breast cancer.** Female NSG mice with orthotopic MDA-MB-468-mCherry mammary tumors were randomized into four groups (10 mice per group): control diet plus vehicle, control diet plus lexatumumab (10 mg/kg twice weekly for 6 doses, indicated by arrows), methionine-free diet plus vehicle, or methionine-free diet plus lexatumumab (10 mg/kg twice weekly for 6 doses). Mice were started on their respective diets 8 weeks after tumor cell inoculation, and the diets were continued throughout the treatment period. A, percentage original mammary tumor volume (at 8 weeks) in each treatment group (mean ± SEM, n = 10 mice per group). B, Body weight of mice. C, Representative whole lung images by fluorescence microscopy. The percentage of the surface area occupied by lung metastases and the number of fluorescent macrometastases per lung (mean ± SEM, n = 10 mice per group). D, Real-time PCR analysis of TRAIL-R2 mRNA levels in mammary tumors isolated from mice receiving control diet or methionine-free diet. mRNA levels were normalized to expression in mice receiving the control diet. E, Immunoblot of MAGED2 expression in
mammary tumors from mice receiving control diet or methionine-free diet. In (A) through (C), *, P < 0.05, **, P < 0.01, ***, P < 0.001 versus vehicle-treated mice.
Figure 1

(A) Viability (%) of MDA-MB-231, GILM2, MDA-MB-468 cell lines treated with Vehicle, Dox, Lexa, Gem, Vin, Docetaxel, 5-Fu.

(B) Number of cells of MDA-MB-231, GILM2, MDA-MB-468 cell lines treated with Vehicle, Dox, Lexa.

(C) Number of cells of MCF-10A with Vector and RAS.
<table>
<thead>
<tr>
<th></th>
<th>MDA-MB-231</th>
<th>GILM2</th>
<th>MDA-MB-468</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Dox</td>
<td>-</td>
<td>Dox</td>
</tr>
<tr>
<td></td>
<td>Lexa</td>
<td>Lexa</td>
<td>Vehicle</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARP</td>
<td>Cleaved</td>
<td>Cleaved</td>
<td>Cleaved</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procasp-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2**

![Graphs showing the effect of Met, Dox, and Lexa on various cell lines](image-url)
Figure 3

Panel A: Graphs showing TRAIL-R1 and TRAIL-R2 mRNA expression in MDA-MB-231, GILM2, and MDA-MB-468 cells under control and 0% Met conditions.

Panel B: Flow cytometry histograms for MDA-MB-231, GILM2, and MDA-MB-468 cells showing TRAIL-R1 and TRAIL-R2 expression.

Panel C: Flow cytometry histograms for MCF-10A and MCF-10A-RAS cells showing TRAIL-R1 and TRAIL-R2 expression.

Panel D: Flow cytometry histograms for MCF-10A and MCF-10A-RAS cells showing TRAIL-R1 and TRAIL-R2 expression.

Panel E: Bar graph showing the number of cells under control and 0% Met conditions, with Vehicle and Lexa treatments.

Note: Figures A, B, C, D, and E are from a study on the expression of TRAIL receptors in different cell lines under metabolic stress conditions.
A

MDA-MB-231  GILM2  MDA-MB-468

Met  +  -  +  -  +  -

MAGED2 →

β-Actin →

B

![Bar graph showing MAGED2 mRNA expression](image)

MAGED2 mRNA (fold expression)

<table>
<thead>
<tr>
<th></th>
<th>231</th>
<th>GILM2</th>
<th>468</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0% Met</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Figure 4
Figure 5

A

B

C

D

E

Downloaded from clincancerres.aacrjournals.org on April 15, 2017. © 2015 American Association for Cancer Research.
Figure 6

A. % original tumor volume vs Time (wk)

B. Body weights (g) vs Time (wk)

C. Imaging of metastases in different treatment groups.

D. TRAIL-R2 mRNA expression.

E. Western blot analysis of MAGED2 and β-Actin.
Methionine Deprivation Induces a Targetable Vulnerability in Triple-negative Breast Cancer Cells by Enhancing TRAIL Receptor-2 Expression

Elena Strekalova, Dmitry Malin, David M. Good, et al.

Clin Cancer Res Published OnlineFirst February 27, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-2792

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/03/24/1078-0432.CCR-14-2792.DC2

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.