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Preclinical Breast Cancer Models to Investigate Metabolic Priming by Methionine Restriction

Elena Strekalova, Dmitry Malin, Harisha Rajanala, and Vincent L. Cryns

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

Abstract

We have developed a novel therapeutic paradigm (“metabolic priming”) for cancer whereby restriction of the essential amino acid methionine activates a number cell stress response pathways that can be selectively targeted to enhance the therapeutic impact of methionine restriction. One example of metabolic priming is the combination of methionine restriction with proapoptotic TRAIL receptor-2 (TRAIL-R2) agonists. Methionine restriction enhances the cell surface expression of TRAIL-R2 selectively in transformed breast epithelial cells and renders them more susceptible to cell death induction by TRAIL-R2 agonists in cellular and murine models of breast cancer. This methods review will focus on preclinical models of breast cancer to investigate metabolic priming by methionine restriction. Multiple cell-based methods are detailed to measure cell viability, cell survival, caspase activity, apoptosis and matrix detachment-induced cell death (anoikis). In addition, we describe an orthotopic model of metastatic breast cancer that utilizes mCherry-fluorescently labeled human breast cancer cells. This model captures the entire metastatic cascade from the mammary gland to the lung and mimics key features of the human disease. These breast cancer models can be readily adapted to other tumor types. Overall, we provide a step-wise, translationally relevant approach to study metabolic priming in the context of cancer.

Keywords

methionine; metabolism; TRAIL; metastasis; apoptosis; therapeutics; murine models

1. Introduction

Depletion of the essential amino acid methionine has been demonstrated to initiate apoptosis and inhibit cell cycle progression in broad range of tumor cell lines and primary tumor cells (1–4). These cellular effects of methionine restriction lead to suppression of tumor growth in many murine models of cancer (5, 6). Although clinical trials of dietary MR in patients with advanced solid tumors have demonstrated the safety of prolonged dietary MR for up to 39 months, these studies have failed to demonstrate significant clinical activity of MR alone or in combination with cytotoxic agents (7–9). We postulated that a more detailed

understanding of the cellular stress response to MR would enable us to utilize MR to expose targetable vulnerabilities in tumor cells and enhance the therapeutic efficacy of MR by specifically targeting these pathways. Viewed from this context, we envision MR as novel paradigm to metabolically prime tumor cells to respond to cancer drugs that target stress response pathways induced by MR.

As proof of principle, we observed that MR robustly enhances the cell surface expression of the proapoptotic TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2/DR5) in clinically aggressive triple-negative breast cancer cells (TNBC), which lack expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (10). The proapoptotic TRAIL receptor pathway has been extensively studied as a potential cancer therapy because soluble recombinant TRAIL ligand and agonistic antibodies targeting proapoptotic TRAIL receptors (TRAIL-R1 and TRAIL-R2) have demonstrated robust tumor-selective activity in preclinical models with little toxicity (11). However, these agents have not demonstrated efficacy in clinical trials in patients with advanced malignancies (12–15).

We postulated that MR would prime TNBC cells to respond to TRAIL-R2 agonists by enhancing the cell surface expression of its molecular target, TRAIL-R2. To this end, we observed that preincubating a panel of human TNBC cells in methionine-free media selectively sensitized them to the agonistic TRAIL-R2 monoclonal antibody lexatumumab but had little effect on their sensitivity to diverse cytotoxic agents (10). Intriguingly, untransformed human breast epithelial cells failed to upregulate cell surface expression of TRAIL-R2 in response to MR and were largely resistant to the combination of MR and lexatumumab, underscoring the potential tumor-selectivity of our metabolic priming approach. We also tested this approach in an orthotopic model of TNBC that recapitulates many features of the human disease, including triple-negative receptor expression and extensive visceral metastases including the lungs (10, 16). In this metastatic murine model, a three-week administration of a methionine-free diet alone or lexatumumab alone modestly inhibited primary tumor growth, but neither agent suppressed lung metastases (10). However, the combination of MR and lexatumumab was more effective at inhibiting primary tumor growth than either intervention alone and resulted in significant suppression of lung metastases. These antitumor effects of MR were accompanied by enhanced expression of TRAIL-R2 expression in mammary tumors and modest weight loss (10). Overall, these findings underscore the potential utility of MR to metabolically prime tumors cells to proapoptotic TRAIL-R2 agonists by augmenting cell surface expression of their molecular target.

In this methods review, we will focus on cellular models of breast cancer to investigate the effects of MR on cell viability, apoptosis, and caspase activation as well as murine breast cancer models to examine the antitumor effects of MR *in vivo*. Although the focus is clinically aggressive TNBC models and the TRAIL receptor pathway, these methods could be readily adapted to incorporate diverse tumor-types and other molecular vulnerabilities induced by MR. As noted previously, our strategy utilizes MR to metabolically prime tumors to respond to rationally selected cancer drugs based on exposed molecular liabilities rather

than envisioning MR as a stand-alone therapy. Our goal is to provide a detailed description of relevant preclinical breast cancer models to facilitate investigation in this exciting field.

2. Materials

2.1 Cell lines

1. Human TNBC cell lines: MDA-MB-231 and MDA-MB-468 TNBC cells are available from ATCC, (see Note 1) while GILM2 TNBC cells are described elsewhere (17).
2. Human breast epithelial cell lines: Human MCF-10A breast epithelial cells stably expressing oncogenic RasV12 or empty vector (see Note 2) are described previously (18).
3. For *in vivo* experiments, MDA-MB-468 cells stably expressing mCherry fluorescent protein (468-mCherry) are utilized to enable fluorescence imaging of tumors (10).

2.2 Cell culture reagents

1. MDA-MB-231 TNBC cells are maintained in DMEM/F12 medium supplemented with 10% FBS and 100 units/mL penicillin/streptomycin (Thermo Fisher Scientific). MDA-MB-468 and MDA-MB-468-mCherry cells are cultured in DMEM with 10% FBS and 100 units/mL penicillin/streptomycin (Thermo Fisher Scientific). GILM2 cells are grown in DMEM/F12 supplemented with 10% FBS, Insulin/Transferrin/Sodium Selenite mix and 100 units/mL penicillin/streptomycin (Thermo Fisher Scientific). MCF-10A stably expressing RasV12 or empty vector are cultured in DMEM/F12 medium supplemented with 5% horse serum (Thermo Fisher Scientific), 20 ng/ml EGF (Sigma-Aldrich), 10 µg/ml insulin (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 0.5 mg/ml hydrocortisone (Sigma-Aldrich), and 100 units/mL penicillin/streptomycin.
2. For MR experiments, RPMI-1640 medium without methionine (Thermo Fisher Scientific) is supplemented with other nutrients and FBS (MR media, see Note 3) to closely match the original media for each cell line (complete media).

2.3 Cell viability reagents

1. Cell viability is measured using a colorimetric MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega).

¹-Early passage cell lines should be used for experiments and tested periodically for Mycoplasma contamination using commercially available kits (*e.g.*, Universal Mycoplasma Detection Kit, ATCC). Cell lines obtained from other investigators and from ATCC more than one year ago should be authenticated.

²-MCF-10A cells stably expressing oncogenic Ras or empty vector serve as isogenic models of transformed or untransformed breast epithelial cells (18), respectively to evaluate the tumor-selectivity of therapies.

³-Serum contains modest concentrations of methionine (approximately 3 µM or 0.3 µM for 10% FBS). For dose response experiments at lower methionine concentrations, dialyzed FBS and methionine-free media should be used. Recent findings suggest that fasting methionine levels in healthy human subjects range from 3–30 µM (20).

2. Surviving cells can also be stained with crystal violet (Sigma-Aldrich, see Note 4).

2.4 Apoptosis reagents

1. Apoptotic cells are scored by flow cytometry-based annexin V labeling (Annexin-PE Apoptosis Detection Kit, BD Bioscience).
2. Caspase-3/–7 activity is measured in cell lysates using a luminescent assay (Caspase-Glo 3/7, Promega).
3. HPLC-purified doxorubicin is purchased from Sigma-Aldrich.
4. Agonistic monoclonal antibodies against TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab) were obtained from Human Genome Sciences.

2.5 Anoikis reagents

1. TNBC cells are grown in adherent culture as in Section 2.2 or in suspension on Corning Costar Ultra-Low Attachment Plates (Thermo Fisher Scientific) in media supplemented with 1% methylcellulose (Sigma-Aldrich) to prevent cell aggregation.

2.6 TRAIL receptor expression reagents

1. Total RNA is used to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad).
2. Oligonucleotide primers are purchased from Integrated DNA Technologies.
3. Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) against TRAIL-R1 (Abcam, ab59047), TRAIL-R2 (Abcam, ab53319) and control IgG (Abcam, ab37356) are used for cell surface detection of TRAIL receptor expression.

2.7 Mice and murine model reagents

We have established a fluorescent orthotopic model of metastatic TNBC using mCherry-labeled human MDA-MB-468 cells in female NOD scid IL2 receptor γ chain knockout (NSG) mice (Jackson Laboratory) (10) (see Notes 5 and 6). Whenever possible, pharmacologic grade drugs and reagents are used.

1. Female 4–5 week old NSG mice (see Note 7) are purchased from Jackson Laboratories.

⁴. Multiple assays are used to assess cell viability (MTS and crystal violet) and apoptosis (annexin V and caspase activation) to validate findings.

⁵. The use of fluorescently labeled human TNBC cells facilitates visualization of primary and metastatic tumors (10, 19, 21). In addition, fluorescently labeled circulating tumor cells can be directly isolated from blood in tumor-bearing mice (22). Alternative approaches include the use of bioluminescently labeled cancer cells.

⁶. The orthotopic model of metastatic TNBC in this chapter captures the entire metastatic cascade from primary tumor to lung metastases and faithfully recapitulates the human disease with regard to triple-negative status and lung metastases. This is a major advantage over many other metastatic models, such as tail vein injection, which only model the later stages of metastasis (extravasation and organ colonization) (23, 24).

2. Control diet: 15% protein diet (Envigo/Teklad TD.01084).
3. MR diet: isocaloric 15% protein methionine-free (0% methionine, Envigo/Teklad TD.140119).
4. mCherry-flourescent metastases are identified at autopsy using a Leica MZ10F fluorescent stereomicroscope.

3. Methods

3.1 MTS cell viability assay

1. Trypsinize actively growing TNBC cells and count viable cells by trypan blue exclusion using a hemocytometer. Plate $3\text{--}5 \times 10^3$ cells in each well of a 96-well plate (triplicate wells). Allow the cells to adhere overnight (see Notes 8 and 9).
2. Wash cells with PBS and add control or MR media for 72 h.
3. Add doxorubicin (0–3.4 μM) or agonistic TRAIL receptor mAbs (0–4 $\mu\text{g/ml}$) for an additional 48 h.
4. Cell viability is measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Add 20 μl MTS reagent to each well of the 96-well plate. Measure absorbance at 490 nm in triplicate wells 1 h later using a microplate reader. Cell viability is expressed as the percentage of viable cells relative to control cells (see Notes 10 and 11).

3.2 Crystal violet cell survival assay

1. Plate cells (3×10^5 cells/well) on 6-well plates. Allow cells to adhere overnight.
2. Wash cells with PBS and add complete or MR media for 48 h.
3. Doxorubicin (0–3.4 μM) or TRAIL receptor mAbs (0–4 $\mu\text{g/ml}$) are added for an additional 24–48 h.
4. Surviving cells are fixed and stained with crystal violet solution (40% ethanol, 60% PBS, and 0.5% crystal violet) and counted (see Note 12).
5. Representative images and quantitation of multiple experiments are presented (Fig. 1).

⁷The use of NSG mice, which lack B cells, T cells and NK cells, rather than athymic nude mice, which lack only T cells, enhances the metastatic potential of orthotopic mammary tumors (25). However, they may also be less hardy than athymic nude mice and require reductions in dosing of toxic agents such as chemotherapy drugs.

⁸MTS assay experiments are performed in triplicate wells to minimize variability due to plating differences. The use of multi-channel pipettes (e.g., 8 channels) enhances speed and fidelity of cell plating, particularly for large experiments.

⁹We typically use 100 μl of media/well in 96-well plates to provide adequate “cushion” to prevent dislodging cells during media manipulations.

¹⁰Statistical significance of intergroup differences is assessed by ANOVA with appropriate post-tests using GraphPad Prism software. Cell model experiments are performed at least three times.

¹¹MTS cell viability results can be normalized to control cells and expressed as fold-differences if desired.

¹²Crystal violet cell survival assays can also be performed with a drug/media “washout” period. Treated cells are returned to control media for several days to assess the long-term impact of transient MR and/or drug treatment.

3.3 Annexin V labeling

1. Grow TNBC cells (3×10^5 cells/well) in 6-well plates in complete or MR media for 48 h.
2. Add doxorubicin (0–3.4 mM) or TRAIL receptor mAb (0–2 µg/ml) for an additional 6 h.
3. Collect supernatant. Trypsinize cells and collect by gentle centrifugation for 5 min at 1200 rpm in a table top centrifuge.
4. Annexin V labeling is determined using the Annexin-PE Apoptosis Detection Kit (BD Bioscience). Wash cells in PBS and resuspend at 1×10^6 cells/ml in 1X Annexin V Binding Buffer (10 mM Hepes/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM CaCl_2).
5. Place 100 µl of resuspended cells in a 5 ml conical tube wrapped in aluminum foil to exclude light. Add 5 µl of PE Annexin V and DAPI (1 µg/ml) and incubate at RT for 15 min.
6. Analyze cells in a flow cytometer (LSRII, BD Biosciences). Annexin V-PE positive cells are detected using a blue laser (Ex 496 nm/Em 578 nm), while DAPI positive cells are counted using a violet laser (Ex 358 nm/Em 460 nm).
7. Flow cytometry data is presented using annexin V labeling on the x axis and DAPI staining on the y axis (Fig. 2, see Note 13).

3.4 Caspase-3/7 activity assay

1. Seed TNBC cells (2.5×10^3 cells/well) on black-welled 96-well plates overnight. Allow to adhere overnight.
2. Wash cells in PBS and add 100 µl complete or MR media for 48 h.
3. Add doxorubicin (0–3.4 µM) or TRAIL receptor mAb (0–2 µg/ml) for an additional 16–24 h.
4. Caspase-3/–7 activity is measured using the Caspase-Glo 3/7 kit (Promega). Add 100 µl of Caspase GLO reagent with added Hoechst dye (10 µM) and incubate for 1 h at room temperature. Caspase activity is detected by measuring luminiscense using a multi-mode microplate reader (Synergy H1, BioTek).
5. In order to normalize the signal to cell number, media is removed from the plate, and cells are washed once with PBS. Nuclei are visualized by Hoescht staining and measuring fluorescence (Ex 361 nm/Em 497 nm) using the same microplate reader.

¹³For annexin V labeling experiments, treated cells are scored in each quadrant of the flow cytometry analysis (Fig. 2). Viable cells are double negative for Annexin V and PE (left lower quadrant). Early apoptotic cells are positive for Annexin V because they have externalized phosphatidylserine and negative for DAPI because they have intact plasma membranes preventing DAPI staining of nuclei (right lower quadrant). Late apoptotic/necrotic cells are double positive for Annexin V and DAPI (right upper quadrant). Cellular/nuclear debris are Annexin V negative and DAPI positive (left upper quadrant). We prefer to show flow cytometry results directly because they contain all this data.

3.5 Anoikis assay

1. Seed 1.0×10^4 cells/well in 96-well Corning Costar Ultra-Low attachment plates in complete or MR media supplemented with 1% methylcellulose (Sigma-Aldrich) for 48 h.
2. Add vehicle, doxorubicin (0–3.4 μM) or agonistic TRAIL receptor mAb (0–2 $\mu\text{g/ml}$) for an additional 24 h.
3. Measure cell viability by MTS assay in 96-well plates as described in Section 3.1.
4. The annexin V (Section 3.3) and caspase-3/7 (Section 3.4) can be modified using TNBC cells grown on Ultra-Low attachment plates to assess the impact of matrix detachment on apoptosis and caspase activation, respectively.

3.6 TRAIL receptor expression

RT-PCR

1. Grow TNBC cells (3×10^5 cells/well) in 6-well plates in complete or MR media for 48 h.
2. Isolate total RNA using SpinSmart™ Total RNA Mini Purification Kit (Denville Scientific) and then synthesize cDNA from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturers' protocols.
3. cDNAs are PCR amplified using the iQ SYBR Green Supermix (Bio-Rad) and detected with CFX96 Real-Time PCR Detection System (Bio-Rad). The following primer pairs are used: TRAIL-R1 (forward 5'-CAGAGGGATGGTCAAGGTCAAGG-3, reverse 5'-CCACAACCTGAGCCGATGC-3), TRAIL-R2 (forward 5'-CGCTGCACCAGGTGTGATT-3, reverse 5'-GTGCCTTCTTCGCACTGACA-3), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used to normalize gene expression. Primers for GAPDH are (forward 5'-GAAGGTGAAGGTCGGAGTC-3, reverse 5'-GAAGATGGTGATGGGATTTTC-3). 50 – 400 ng cDNA is amplified in a 25 μl reaction volume containing a SYBR Green Supermix (Bio-Rad). Cycling conditions are 95°C for 5 min, followed by a 40-cycle amplification at 95°C for 15 sec, and 59°C for 45 sec. Experiments are performed two times and each sample is analyzed in triplicate.
4. Real-time PCR results are represented as Ct values (the threshold PCR cycle for detecting the PCR product). To compare the different RNA samples, we used a comparative Ct method whereby the RNA expression in each sample was normalized to GAPDH expression in each experiment.

Flow cytometry

1. Culture TNBC cells (3×10^5 cells/well) in 6-well plates in complete or MR media for 72 h.

2. Trypsinize and collect cells by gentle centrifugation for 5 min at 1200 rpm in a tabletop centrifuge.
3. Wash cells with PBS and incubate with FITC-conjugated mAbs against TRAIL-R1, TRAIL-R2 or control IgG in ice cold PBS containing 2.5% BSA for 45 min in the dark.
4. Wash cells three times with cold PBS containing 2.5% BSA and resuspend in PBS containing DAPI (1 µg/ml).
5. Analyze cells using a LSRII flow cytometer. FITC positive cells are detected using a blue laser (Ex 495 nm/Em 519 nm). To exclude non-viable cells from the analysis, DAPI positive cells are detected with a violet laser (Ex 358 nm/Em 460 nm) and excluded.

3.7 Fluorescent orthotopic models of metastatic breast cancer

1. Allow female 4–5 week old NSG mice to recover from the stress of transportation for up to one week in the vivarium. NSG mice are maintained in appropriate barrier facilities as warranted by their immunocompromised nature.
2. Resuspend 2×10^6 MDA-MB-468-mCherry TNBC cells in 100% Matrigel (BD Bioscience).
3. Anesthetize mice using 2.5–5% vaporized isoflurane via nose cone and then inject TNBC cells into the lactiferous ducts of the 4th mammary gland bilaterally using a 27-gauge needle (see Note 14).
4. Measure tumors weekly with Vernier calipers and calculate tumor volume using the formula: tumor volume (mm^3) = (length x width²) x $\pi/6$ (19). Weigh mice weekly.
5. Randomize mice to treatment groups when mean tumor volume is 100–150 mm^3 (typically 2–3 weeks depending on the tumor model). A typical experimental design would include 4 treatment groups: control diet + vehicle, MR diet + vehicle, control diet + TRAIL receptor agonist, and MR diet + TRAIL receptor agonist (see Note 15). We typically give agonistic TRAIL receptor mAbs 10 mg/kg twice weekly by intraperitoneal injection using a 27-gauge needle.
6. Monitor mice daily on therapy (see Note 16). Weigh mice and measure tumor volumes weekly.
7. Euthanize mice in three weeks or sooner if mice develop tumors > 1.5 cm in any dimension or show signs of distress. Perform terminal intracardiac puncture

¹⁴. Intraductal injection of breast cancer cells, in comparison to the standard mammary fat pad injection, facilitates metastasis in murine models (26).

¹⁵. We typically use 10 mice per group (each with two primary mammary tumors) to evaluate the therapeutic impact of treatments on primary and metastatic tumor burden.

¹⁶. Our metabolic priming approach combines short-term (days to few weeks) complete MR (methionine-free diet) in combination with TRAIL receptor agonists. Mice lose weight on a methionine-free diet (approximately 2 gm over three weeks (10)) but can tolerate this diet for at least 5 weeks.

under deep isoflurane anesthesia to obtain blood for complete blood count, electrolytes, liver function and kidney function tests.

8. Dissect euthanized mice and remove lungs as a single intact organ.
9. Image lungs *ex vivo* by fluorescence microscopy and capture images with digital camera.
10. Quantitate metastatic tumor burden using NIH Image J software to analyze digital images for number of fluorescent metastases per lung and % surface area of lung occupied by metastases (Fig. 3).
11. The number of lung metastases can also be scored by routine hematoxylin and eosin (H&E) staining of formalin-fixed paraffin embedded (FFPE) sections of lung tissue. H&E staining of FFPE sections of liver, brain, and kidney are performed to assess for systemic toxicity. Briefly, deparaffinized and dehydrated sections (10 µm) are incubated with hematoxylin for 3 min, washed with tap water and then with deionized water, incubated with eosin for 1 min and then washed with 100% ethanol.

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Fig. 1.

Crystal violet survival assay of MDA-MB-468 TNBC cells grown in control media or methionine-free media for 24 h and then treated with vehicle, doxorubin (3.4 μ M) or the TRAIL-R2 agonist lexatumumab (4 μ g/ml) for an additional 48 h. Representative images (left panel) and quantitative analysis of three independent experiments (right panel). **, $P < 0.01$, ***, $P < 0.001$. Reproduced from (10) with permission from the American Association for Cancer Research.

Fig. 2.

Annexin V flow cytometry analysis of MDA-MB-468 TNBC cells grown in control media or methionine-free media for 48 h and then treated with vehicle, doxorubin (3.4 μ M) or the TRAIL-R2 agonist lexatumumab (2 μ g/ml) for an additional 6 h. The percentage of TNBC cells in each quadrant is indicated. Reproduced from (10) with permission from the American Association for Cancer Research.

Fig. 3. MDA-MB-468-mCherry lung metastases in female NSG mice treated with vehicle, lexatumumab (10 mg/kg *i.p.* twice weekly for 3 weeks), methionine-free diet for 3 weeks or methionine-free diet plus lexatumumab. Representative fluorescent *ex vivo* images of lungs (left) and quantitation of percentage of lung surface area occupied by metastases and number of macrometastases (at least 0.1 mm). *, $P < 0.05$, **, $P < 0.01$. Reproduced from (10) with permission from the American Association for Cancer Research.