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Early Therapy Assessment of Combined Anti-DR5 Antibody and Carboplatin in Triple-Negative Breast Cancer Xenografts in Mice Using Diffusion-Weighted Imaging and ¹H MR Spectroscopy

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Abstract

Purpose—To assess the early response of triple-negative breast-cancer (TNBC) following TRA-8 and carboplatin therapy using DWI and MRS in 2LMP and SUM159 mouse models.

Materials and Methods—Four groups (n = 5/group) of each model were untreated or treated with carboplatin, TRA-8, and combination, respectively. DWI and MRS were applied on 0, 3, and 7 days after therapy initiation, and all tumors were collected thereafter for terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining. The changes in intratumoral apparent diffusion coefficient (ADC) and fat–water ratios (FWRs) were compared with tumor volume changes and apoptotic cell densities.

Results—Mean ADC values of 2LMP and SUM159 tumors significantly increased $4 \pm 4\%$ and $37 \pm 11\%$ during 7 days of combination therapy, respectively, as compared to control groups ($P < 0.05$). Similarly, mean FWRs of 2LMP and SUM159 tumors significantly increased $102 \pm 30\%$ and $126 \pm 52\%$, respectively, for 7 days of combined treatment ($P < 0.05$). The changes of the mean ADC values for 3 days (or FWRs for 7 days) were linearly proportional to either the mean volume changes or apoptotic cell densities in both models.

Conclusion—DWI and MRS assessed the early tumor response to TRA-8 and carboplatin in TNBC mouse models.

Keywords

DWI; MRS; TRA-8; carboplatin; triple-negative breast cancer

TUMOR NECROSIS FACTOR-related apoptosis-inducing ligand (TRAIL) binds several receptors including DR4, DR5, DcR1, DcR2, and osteoprotegerin (1-8), and has presented a high anti-tumor effect in both in vitro (3,5) and in vivo studies (9,10) of several cancer types. However, a concern was raised in its clinical translation due to severe hepatotoxicity revealed during preclinical studies (11), potentially related to its binding to the multiple receptors (1-8). Therefore, a monomeric monoclonal antibody named TRA-8 targeting only DR5 was developed, and provided excellent preclinical efficacy in various cancers without hepatotoxicity (12). Oliver et al recently demonstrated the significant anti-tumor effect of TRA-8 alone or in combination with abraxane (or doxorubicin) in murine models of triple negative (ER/PR/Her2 negative) breast cancer (TNBC) (13), consistent with Rahman et al findings regarding the efficacy of TRAIL for TNBC cell lines (14). TNBC have a higher pathologic complete response (pCR) to chemotherapy (15) and good prognosis (16). However, a higher likelihood of relapse was also observed, when a pCR was not achieved (9,10,16). Therefore, it would be advantageous to achieve a pCR for TNBC with an advanced biological therapeutic agent like TRA-8.

The therapeutic efficacy of TRA-8 could be further improved when combined with carboplatin. Carboplatin, a platinum-based chemotherapeutic agent, induces cell death by means of blocking DNA replication and has been used to treat various cancerous diseases (17). Of interest, TNBC cells have many similar characteristics with BRCA1-mutated cells (18,19) having significant in vitro sensitivity to carboplatin (20). Chang et al reported that the dual combination chemotherapy with docetaxel and carboplatin achieved 26.8% of pCR rate among TNBC patients (21). Furthermore, the improved therapeutic efficacy of TRA-8 combined with carboplatin was validated in ovarian cancer murine models (22).

While the combined use of TRA-8 and carboplatin could be superior to monotherapy, it might induce a range of therapeutic responses among TNBC patients. It would be beneficial to assess early tumor response to therapy for each individual patient to tailor the therapeutic strategy. Diffusion-weighted imaging (DWI) has been used to measure the increased water mobility due to cell death in response to effective therapy, before noticeable change in size or morphology of breast cancer (23-26). Water diffusion is represented with ADC (apparent diffusion coefficient) value.

¹H magnetic resonance spectroscopy (MRS) has been also used to measure early therapeutic response by quantifying lipid concentration; lipid has been observed to increase in apoptotic cells, presumably due to the inhibition of phosphatidylcholine biosynthesis and the activation of phospholipases (27), and fat-water ratio (FWR) has been validated as a surrogate biomarker to evaluate chemotherapy for breast cancer (28,29).

We hypothesized that DWI and MRS would be able to detect the early response of TNBC following the dual combination therapy with TRA-8 and carboplatin. Two TNBC murine models were used to test this hypothesis. The therapeutic efficacy was assessed by means of

monitoring tumor volume and analyzing histologic samples, and correlated with the early changes of ADC and FWR in the tumor region.

MATERIALS AND METHODS

Reagents and Cell Lines

All reagents were from Fisher (Pittsburg, PA) unless otherwise specified. Purified TRA-8 (mouse origin), 2LMP and SUM159 cell lines were provided by Donald Buchsbaum, Ph.D. (University of Alabama at Birmingham, Birmingham, AL). Carboplatin (Hospira, Inc., Lake Forest, IL) was purchased from UAB Hospital pharmacy (Birmingham, AL).

Animal Preparation

Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham. A total of 8 groups of female athymic nude mice (4–5 weeks old, $n = 5$ per group) were used. Groups 1–4 were subcutaneously implanted with 2LMP cells (2 million) in 0.2-mL culture medium into the left flank, while groups 5–8 were implanted with SUM159 cells (4 million). Four weeks after implantation when tumor size was 5–7 mm in diameter, anatomic MR imaging, diffusion-weighted imaging and single voxel MR spectroscopy were performed for all mice on 0 (baseline), 3, and 7 days after therapy initiation. Group 1 was untreated (served as control), and groups 2–4 were treated with carboplatin (30 mg/Kg, i.p., day 1), TRA-8 (0.2 mg, i.p., days 0 and 3), and combination, respectively. Groups 5–8 were treated with the same doses used for groups 1–4, respectively. All mice were killed after imaging on day 7, and tumors were collected for histological analysis. All animals were anesthetized with isoflurane (1–2%) during dosing and imaging.

MR Imaging

Small-animal imaging was performed on a 9.4 Tesla (T) MR imaging system (BioSpec; Bruker BioSpin, Billerica, MA) with a surface coil (Bruker BioSpin) as a receiver. An MR imaging compatible small-animal respiratory gating device (SA instrument, Stony Brook, NY) was used during imaging. Anatomic imaging was acquired with a T_2 -weighted (T_2W) fast spin echo sequence (rapid acquisition with relaxation enhancement). The parameters were as follows: repetition time (TR) = 3000 ms, echo time (TE) = 34 ms, rare factor = 4, field of view (FOV) = 30×30 mm, and matrix size = 128×128 . Continuous 1-mm-thick slices were used to cover the entire tumor region. Diffusion weighted imaging (DWI) was obtained with a diffusion weighted multi-slice 2D spin echo sequence. Four b factors (5, 300, 600, and 1000 s/mm^2) were applied in the x direction in plane with the following parameters; TR=3501 ms, TE = 32 ms, diffusion separation time = 16 ms, diffusion gradient duration = 6 ms, FOV = 30×30 mm, and matrix size = 128×128 . A total of 5–7 slices with 1-mm thickness were used to cover the tumor region. The total acquisition time for T_2W MRI was approximately 2 min, and that for DWI was approximately 30 min. ADC value was calculated by finding the best fitting curve to the equation, $S = S_0 e^{-bD}$, where S is the intensity of the DW image, S_0 is a constant, and D is ADC value. Tumor region was determined in T_2W images, while its necrotic core was determined in ADC maps, using a global thresholding technique (30), where a threshold value was manually determined using

ImageJ (version 1.45i; NIH, Bethesda, MD). ADC values were quantified using software developed with Labview 2010, version 10.0.1 (National Instruments Co., Austin, TX).

MR Spectroscopy

An MR spectroscopic voxel (3–5 mm isotropic voxel) was localized within the ROI. Water was used as the internal reference with nonwater suppressed point resolved spectroscopy (PRESS) sequence. Voxel shimming was performed to increase field homogeneity, and typical line width of the water peak (full-width at half maximum (FWHM)) was 15–22 Hz. The imaging parameters were as follows: TR = 2500 ms, TE = 20 ms, spectral bandwidth = 4006 Hz, 2048 complex data points, and average = 32. Subsequently, the spectrum of metabolites was obtained with the same sequence but with water suppression. Total imaging time for MRS was approximately 30 min. Data analysis was performed in the time domain with the AMARES (Advanced Method for Accurate, Robust and Efficient Spectral fitting) method in jMRUI (v4.0), a Java version of Magnetic Resonance User Interface analysis software (31). The fat–water ratio was the area under the lipid peaks (0.9 ppm and 1.3 ppm) divided by the area under the water peak in this study. The area under lipid peaks was calculated on the spectrum with water suppression; the area under the water peak was calculated on the spectrum without water suppression.

Histological Analysis

Tumor tissue was stained with terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) with the same procedure as Kim et al reported previously (32). Two pictures (X100) were randomly taken in a blinded manner for each tumor slice with a camera (SPOT) on a microscope (Nikon Optiphot-2; Nikon, Melville, NY). The apoptotic (TUNEL) cells were identified by color difference between the target cells (brown) and nontarget cells (blue) or background (pale pink). Color thresholding technique was used to segment the apoptotic cells, while the threshold was manually determined in histogram for blue color of each image. The apoptotic cells were counted in all two pictures per tumor, and then its density was calculated as the number of target cells per unit area (/mm²). Uneven background intensity was corrected using “Rolling Ball” algorithm (33), while the radius was manually determined. The image segmentation and cell counting were implemented using ImageJ (version 1.45i; NIH, Bethesda, MD).

Statistical Analysis

One-way analysis of variance was used to compare mean tumor volumes, ADC values, fat–water ratios, and apoptotic-cell densities between control group and treated groups (34). Pearson correlation coefficients were used to examine the correlation between the changes of tumor volume and ADC values (or FWR) (35). *P* values less than 0.05 were considered significant. Data are presented as mean±standard error. All analyses were performed with SAS, version 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Figure 1 shows representative diffusion weighted images (DWI) of a 2LMP tumor and a SUM159 tumor at four different *b* values (i.e., 5, 300, 600, and 1000 s/mm²) with the same

gray scale and the ADC maps before the therapy initiation. Figure 2a shows the T₂W MR image of a tumor with a voxel drawn for MRS. Figure 2b shows the spectrum without water suppression; signals from all other metabolite peaks were relatively minimal compared with the water signal. Figure 2c shows the signal peaks of several metabolite when water signal was suppressed; the water peak was set at 4.7 ppm, and lipid methyl (–CH₃) and lipid methylene (–(CH₂)_n–) appeared at 0.9 and 1.3 ppm, respectively (27).

Figure 3 presents the percent change of tumor volume, ADC, and fat–water ratio for 3 and 7 days post therapy initiation of the two different TNBC models (2LMP and SUM159). The asterisks or hash marks above bars of the treatment groups represent statistically significant differences compared with the control group (**P* < 0.05; #*P* < 0.01). For 2LMP tumor xenografts, the initial tumor volume, necrotic core size, ADC value, and fat–water ratio were $488 \pm 66 \text{ mm}^3$, $63 \pm 11 \text{ mm}^3$, $10.5 \pm 0.3 \times 10^{-4} \text{ mm}^2/\text{s}$, and 0.102 ± 0.0024 , respectively, without statistical differences among the groups (*P* > 0.05). 2LMP tumor growth was significantly suppressed by either TRA-8 or combination therapy on both days 3 and 7 (*P* < 0.01 on both days). The ADC changes for 2LMP tumors treated with combination therapy were significantly higher than those of the control group on both days 3 and 7 (*P* = 0.04 and 0.02, respectively). Of interest, the mean ADC change of the group treated with either TRA-8 or combination therapy was reduced after day 3, but that of the group treated with carboplatin alone increased during the same time. 2LMP tumor FWR change of the group treated with combination therapy was not different from that of control group on day 3 (*P* = 0.14), but was significantly different on day 7 (*P* = 0.04). For SUM159 tumor xenografts, the initial tumor volume, necrotic core size, ADC value, and fat–water ratio were $192 \pm 26 \text{ mm}^3$, $11 \pm 5 \text{ mm}^3$, $9.2 \pm 0.4 \times 10^{-4} \text{ mm}^2/\text{s}$, and 0.092 ± 0.019 , respectively, with no significant differences among the groups (*P* > 0.05). Combined therapy suppressed SUM159 tumor growth significantly on day 7 (*P* = 0.03), and significantly increased the mean ADC values on both days 3 and 7 (*P* = 0.01 and 0.02, respectively). The mean ADC values of all treated groups with SUM159 tumors increased after day 3. SUM159 tumor FWR changes for the combination-therapy group were significantly higher than that of control group on day 7 (*P* = 0.03), but not on day 3 (*P* = 0.08).

Figures 4a and 4b show the linear correlation between the mean ADC changes at 3 days post therapy initiation and the mean volume changes for 2LMP and SUM159 tumors after 7 days of therapy (*R*² values of 0.81 and 0.97, respectively). However, the correlations of mean ADC changes at 7 days with the mean volume changes at 7 days were much lower (*R*² values of 0.12 and 0.82 for 2LMP and SUM159 tumor groups, respectively). In contrast, the mean FWR changes at 3 days post therapy initiation showed lower correlations with the mean volume changes at 7 days (*R*² = 0.01 and 0.47 for 2LMP and SUM159, respectively), while the mean FWR changes at 7 days were strongly correlated with the mean volume changes at 7 days in both models as shown in Figures 4c and 4d (*R*² = 0.73 and 0.74 for 2LMP and SUM159, respectively).

Figure 5a shows representative microphotographs of TUNEL-stained tumor tissues (either 2LMP or SUM159) of four different groups untreated (control) or treated with carboplatin, TRA-8, and the combination, respectively; the apoptotic cells are indicated with black arrows in each sub-figure. Figures 5b and 5c show the apoptotic cell densities (/mm²) of

2LMP and SUM159 tumors, respectively, while the asterisks (or hash mark) above bars of treated groups represent statistical differences from the control group (* $P < 0.05$; # $P < 0.01$). TRA-8 along and the combination therapy significantly increased the apoptotic cell densities for both TNBC models compared with control, while carboplatin monotherapy was not significantly different ($P > 0.05$). The mean apoptotic cell densities were linearly proportional to the mean ADC changes for 3 days ($R^2 = 0.85$ and 0.95 for 2LMP and SUM159, respectively), and the mean FWR changes for 7 days ($R^2 = 0.80$ and 0.97 for 2LMP and SUM159, respectively), after therapy initiation.

DISCUSSION

DWI and MRS detected TNBC response in 3–7 days after starting therapy with TRA-8 and carboplatin in preclinical models. Early therapy assessment may enable the use of therapeutics for potential responders, maximize the likelihood of a favorable clinical outcome, and save costs by avoiding unnecessary treatment.

The combination therapy with TRA-8 and carboplatin showed an additive efficacy, and significantly suppressed tumor growth in both 2LMP and SUM159 TNBC models during only 7 days after therapy initiation, and the therapeutic efficacy was verified with the significant increase of apoptotic cell density. The combined use of TRA-8 and abraxane did not significantly change the tumor volume in these TNBC models during the same therapeutic period (13). Therefore, a better therapeutic efficacy may be achieved by TRA-8 when used with carboplatin, instead of abraxane.

Both ADC and FWR changes were validated as effective surrogate imaging biomarkers to assess the therapeutic efficacy of TRA-8 in combination with carboplatin. Significant ADC changes were detected only 3 days after the initiation of TRA-8 (or combination) therapy in both TNBC tumor models, but the significant FWR changes required 7 days for detection. Therefore, tumor ADC changes may be used as an earlier imaging biomarker than FWR changes in DR5 targeting therapy with or without chemotherapy.

The mean ADC values for the 2LMP tumors treated with TRA-8 or combination therapy decreased between day 3 and day 7, and this finding is consistent with the results of a previous study with the same animal model (32). However, the ADC values for SUM159 tumors gradually increased over the 7-day period. This discrepancy can be explained by the differences in the size of the central necrotic region. In this study, 2LMP tumors developed significantly larger necrotic cores than SUM159 tumors ($P < 0.01$). The gradient of water concentration in the necrotic core may accelerate the diffusive transport of extracellular water molecules generated by means of apoptosis out of the tumor region, while the gradient of interstitial fluid pressure may accelerate the convective transport.

The present study showed increased fat–water ratio in tumors responding to therapy, consistent with previous studies on patients with locally advanced breast cancer (28,29). MRS-visible lipids have been observed to increase in apoptotic cells in the form of cytoplasmic lipid droplets (27,36). Two resonances at 0.9 ppm and 1.3 ppm were counted as lipids, but not those at 2.1 ppm and 2.8 ppm, because lipid signal at 2.1 ppm was interfered

by underlying protein signal and that at 2.8 ppm was too small. Furthermore, lipid signals at 4.2 ppm and 5.3 ppm could not be included due to water suppression (27,37). The 0.9 ppm and 1.3 ppm peaks were added together under the assumption that T_1 and T_2 values of the two lipid peaks were similar (27). To obtain absolute fat–water ratio, T_1 and T_2 corrections are required (38), but the relative change would be the same in the absence of an absolute correction.

The credibility of this study could have been improved if tumor xenografts directly transferred from the fresh tumor tissues of TNBC patients (i.e., tumorigrafts) had been used (39). Tumorigraft models accurately reflect the genetic diversity and histology of this disease. Furthermore, a larger preclinical studies and/or a phase II clinical trial will need to be followed to validate the results of this study.

In conclusion, the early assessment of therapeutic efficacy is critical to prevent unnecessary treatment and tailor the therapeutic strategy to achieve an improved outcome. ADC and FWR may be used to evaluate TRA-8 and carboplatin for each TNBC patient, and the imaging protocol validated in this study may be translated to the clinical trial of those drugs.

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Figure 1.

Representative DW images and ADC maps. **a:** Representative diffusion weighted images of 2LMP and SUM159 tumor xenografts before therapy initiation with four different b values such as 5, 300, 600, and 1000 s/mm^2 with constant gray scale, while tumors are indicated with white arrows. **b:** ADC maps obtained from the four DW images.

Figure 2.

Representative in vivo ¹H MR spectrum. **a:** A single voxel drawn within the tumor region for MRS. **b,c:** In vivo MR spectrum (b) without water suppression or (c) with water suppression. With the water signal (4.7 ppm) suppression, lipid methyl and lipid methylene peaks are shown at 0.9 ppm, and 1.3 ppm, respectively.

Figure 3.

The changes of tumor volume, ADC, and fat–water ratio. The changes of tumor volume (a,b), ADC (c,d), and fat–water ratio in (a,c,e) 2LMP (e,f) or SUM159 tumor xenografts (b,d,f) for 7 days after therapy initiation. The asterisks or hash marks above bars of the treatment groups represent statistically significant differences compared with the control group (* $P < 0.05$; # $P < 0.01$).

Figure 4.

Relationship between the mean tumor-volume changes and the mean ADC (or fat-water ratio) changes. **a,b:** Linear relationship between the mean tumor-volume changes for 7 days and the mean ADC changes for 3 days post therapy initiation in (a) 2LMP and (b) SUM159 tumor xenografts. **c,d:** Linear relationship the mean tumor-volume changes for 7 days and the mean fat-water ratio changes for 7 days post therapy initiation in (c) 2LMP and (d) SUM159 tumor xenografts.

Figure 5.

Histological analysis of tumor response. **a:** Representative microphotographs of terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining of tumors either untreated (served as control) or treated with carboplatin, TRA-8, and combination. Apoptotic cells are indicated with black arrows in each sub-figure. **b,c:** The apoptotic cell densities in 2LMP (b) or SUM159 (c) tumor xenografts, while the asterisks or hash marks above bars of the treatment groups represent statistically significant differences compared with the control group (* $P < 0.05$; # $P < 0.01$).