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Magnetic Resonance Imaging Assays for Dimethyl Sulfoxide Effect on Cancer Vasculature

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Abstract

Objectives—To evaluate the potential of quantitative assays of vascular characteristics based on dynamic contrast-enhanced magnetic resonance imaging (MRI) using a macromolecular contrast medium (MMCM) to search for and measure effects of dimethyl sulfoxide (DMSO) on cancer vasculature with microscopic correlations.

Material and Methods—Saline-treated control (n = 8) and DMSO-treated (n = 7) human breast cancer xenografts (MDA-MB-435) in rats were imaged dynamically by MMCM-enhanced MRI using albumin-(Gd-DTPA)₂₇-(biotin)₁₁ (molecular weight approximately 90 kDa), before and after a 1-week, 3-dose treatment course. After the posttreatment MRI examinations, tumors were perfused with lectin and fixative and subsequently stained with RECA-1 and streptavidin for quantitative fluorescent microscopy. Quantitative MRI estimates of cancer microvessel permeability (K^{PS} ; $\mu\text{L}/\text{min}\cdot 100\text{ cm}^3$) and fractional plasma volume (fPV; %) were based on a 2-compartment kinetic model. Fluorescent microscopy yielded estimates of MMCM extravasation and vascular density that were compared to the MRI results.

Results—DMSO decreased cancer vascular endothelial permeability significantly ($P < 0.05$) from tumor $K^{PS}_{\text{day}0} = 19.3 \pm 8.8\text{ }\mu\text{L}/\text{min}\cdot 100\text{ cm}^3$ to $K^{PS}_{\text{day}7} = 0\text{ }\mu\text{L}/\text{min}\cdot 100\text{ cm}^3$. K^{PS} values in the saline-treated tumors did not change significantly. The amount of extravasated albumin-Gd-(DTPA)₂₇-(biotin)₁₁, as assayed by a fluorescently labeled streptavidin stain that strongly binds to the biotin tag on the MMCM, was significantly ($P < 0.05$) lower in the DMSO-treated cancers than in the control cancers ($57.7\% \pm 5.5\%$ vs. $34.2\% \pm 4.9\%$). Tumor vascular richness as reflected by the MRI-assayed fPV and by the RECA-1 and lectin-stained microscopy did not change significantly with DMSO or saline treatment.

Conclusion—Reductions in cancer microvascular leakiness induced by a 7-day course of DMSO could be detected and measured by dynamic MMCM-enhanced MRI and were confirmed by microscopic measurements of the leaked macromolecular agents in the same cancers. Results support the robustness of an MMCM-enhanced MRI approach to the characterization of cancers and providing first evidence for an in vivo effect of DMSO on cancer blood vessels.

Keywords

dynamic MRI; Biotin-labeled macromolecular contrast media; DMSO; breast cancer; angiogenesis inhibition

Dimethyl sulfoxide (DMSO) is a versatile solvent used widely in synthetic chemistry and has been proposed for a range of possible medicinal applications. Perhaps the first proposed medical application was to use DMSO as a penetrating solvent vector to carry drugs such as tranquilizers through the skin or to deliver heparin or insulin directly through the urinary bladder endothelium.¹ Other reported pharmaceutical actions for DMSO include anti-inflammatory, local analgesic, and bacteriostatic.² DMSO is known to cross the blood–brain barrier and has been effective in the treatment of traumatic brain edema.³ DMSO has also been nominated as a potential antiangiogenic drug by Koizumi et al who reported an inhibition of capillary tube formation in cultured human aortic endothelial cells.⁴ Charlotte Friend made an unanticipated observation that DMSO induced cytodifferentiation and growth arrest in murine erythroleukemia cells.⁵ Although the application of DMSO in cancer therapy has been heavily debated,^{6,7} more recent reports of DMSO effect on vascular network formation and endothelial permeability of the blood–brain barrier^{4,8} encouraged us to test dynamic macromolecular contrast medium (MMCM)-enhanced magnetic resonance imaging (MRI) to detect and quantify possible effects of DMSO on cancer vasculature.

Our interest in DMSO as a possible anticancer agent was initially sparked quite by accident when conducting pilot studies on the effects of thalidomide, a known inhibitor of angiogenesis; 40% DMSO was used to dissolve the thalidomide for intraperitoneal administration. We observed using contrast-enhanced MRI what seemed to be an effect of DMSO on our human breast cancer model, MDA-MB-435, grown in rats. To our surprise, the control tumor bearing rats, given only 40% DMSO as a vehicle control, were observed to have a change in MRI-assayed tumor vascular properties.

Thus, our aim in this study was to evaluate the potential of quantitative assays of vascular characteristics based on dynamic contrast-enhanced MRI using a MMCM to search for and measure effects of DMSO on cancer vasculature with microscopic validations.

MATERIALS AND METHODS

Animal Model

The study was performed with the approval of the Institutional Committee for Animal Research and in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals. Four-week-old female homozygous athymic rats (Hsd: RH-Foxn1^{tmu}, Harlan, Indianapolis, IN, n = 15) were injected with 5×10^6 cells of a poorly differentiated, estrogen-receptor-negative human breast cancer cell line, MDA-MB-435

(ATCC, Manassas, VA). Suspended in a total volume of 0.5 mL as a 1:1 mixture of phosphate buffered saline (PBS) and Matrigel (BD Biosciences, San Jose, CA) the cells were injected subcutaneously into the right mammary fat pad using a 25-gauge needle (Abbott Laboratories, North Chicago, IL). A visual inspection of all animals was conducted every day for general appearance, behavior, and tumor growth. When the tumors reached a volume of approximately 800 mm³, measured in 3 dimensions by caliper, animals were anesthetized by intraperitoneal injection of a combination of 35 mg per kg bodyweight pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) and 0.025 mL per kg bodyweight buprenorphine hydrochloride (Buprenex, Reckitt Benckiser Pharmaceuticals, Richmond, VA). For fast bolus contrast media injection and subsequent administration of lectin (Vector Laboratories, Burlingame, CA), a 25-gauge butterfly catheter (Abbott Laboratories, North Chicago, IL) was inserted in a tail vein. Rats were placed on a heated deuterated (D₂O) water pad to keep the body temperature at physiologic levels during the scan without introducing water (H₂O) flow artifacts into the MR image. All animals were imaged by dynamic contrast enhanced MRI on day 0 and again on day 7. After baseline MRI, the rats were randomly assigned to either the treatment or the control group. The treatment group received ip injections of 40% DMSO (ACS grade, Fisher Scientific Company L.L.C., Fair Lawn, NJ) at a dose of 2.5 mL per kg bodyweight on day 0, day 3, and day 6. The final DMSO formulation was prepared as a saline-DMSO mixture (volume ratio 6:4) and sterile filtered with a nylon filter (0.2 µm, Alltech Inc., Deerfield, IL). The control group received ip injections of prewarmed physiologic saline at a dose of 2.5 mL per kg bodyweight, also on day 0, day 3, and day 6. Tissue preparation and staining procedures are described in the immunohistochemistry section below.

Contrast Medium

The synthesis of biotin-labeled albumin-(Gd-DTPA) conjugate, Figure 1, followed an established literature method⁹ with minor modifications as described below. Briefly, biotin was converted to its 4-sulfono-2-nitrophenyl ester via the dicyclohexyl carbodiimide method in anhydrous dimethylformamide. This active ester thus formed has improved water solubility compared with biotin itself and is also reactive toward amines. Next, the active ester of biotin was added to albumin solution at pH 7.8 in 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid buffer and the reaction was allowed to proceed for 24 hours at room temperature. Subsequently, DTPA bisanhydride, suspended in anhydrous dimethylsulfoxide, was added in portions to the solution adjusted to pH 8.9 with vigorous stirring during 1 hour. The coupling reaction was maintained at pH 8.9 for 8 hours, and then mixed with excess gadolinium nitrilotriacetic acid complex, Gd(NTA)₂, at pH 5.5 to complete Gd³⁺ chelation overnight. After purification by dialysis and size exclusion chromatography, the product solution was lyophilized to give a white, solid material, which was analyzed to be a dual-labeled albumin derivative containing 11 biotin groups (by Avidin-HABA method) and 27 Gd-(DTPA) groups (by ICP-AES), both covalently bound to albumin lysine residues via amide linkages. This macromolecular contrast agent has an actual molecular weight of approximately 90 kDa. Its effective molecular weight is measured to be 180 kDa by size-exclusion HPLC (compared with protein standards). The explanation for what might seem an inconsistency in the fact that true or “actual” molecular weight reflects only the weight of individual atomic components of the molecule while

apparent molecular weight reflects the shape or configuration of the molecule in solution. The latter depends in large part on how much or how little folding of the molecule occurs in solution and on electric charge. At 37°C and clinically relevant 2.0 Tesla, its T_1 -relaxivity was measured to be $10.4 \text{ mM}^{-1}\text{s}^{-1}$ per Gd ion and $280.8 \text{ mM}^{-1}\text{s}^{-1}$ per albumin molecule. The contrast agents were administered at a dose of 0.03 mmol Gd per kg bodyweight. Albumin-(Gd-DTPA)₃₅ was synthesized accordingly following an established literature method¹⁰ as a comparison control in tissue biotin staining.

MRI

MRI was performed using an Omega CSI-II system operating at 2.0 Tesla (Bruker Instruments, Fremont, CA). The system is equipped with Acustar S-150 self-shielded gradient coils. Rats were placed within a birdcage radiofrequency coil (length, 7.6 cm; inner diameter, 4.5 cm) in supine position. A series of 9 precontrast T_1 -weighted inversion recovery centric-ordered fast gradient-recalled echo images (TR/TE = 6.0/1.5 milliseconds, number of acquisitions = 1, flip angle α = 10 degrees, matrix 64×64 , field of view 50×50 , slice thickness = 3 mm), with TI varying between 100 and 2500 milliseconds, were obtained for the calculation by curve fitting of baseline relaxation rates (R1) of tumor tissue in each animal. Because the inversion recovery snapshot FLASH method is not well suited for R1 measurements of flowing blood, the baseline R1 for blood in the inferior vena cava (IVC) was taken to be 0.752 per seconds, which is the mean blood R1 in rats at 37°C and 2.0 Tesla measured previously in more than 200 specimens in our laboratory.¹¹ Subsequently, dynamic contrast-enhanced MRI was performed using a T_1 -weighted 3D-spoiled gradient refocused (SPGR) sequence. After 2 precontrast images, the MMCM is injected as a manual fast bolus via the tail vein followed by the acquisition of 28 postcontrast images with high spatial resolution and TR = 50 milliseconds, TE = 3 milliseconds, NA = 1, flip angle (α) = 90 degree, matrix = $128 \times 128 \times 16$, field of vision = $50 \times 50 \times 48$ mm, partition thickness = 3 mm, acquisition time 1 minute 42 seconds per image.

MRI Data Processing and Kinetic Analysis

A Dell Precision 670 Workstation (Dell Inc., Austin, TX) with a Linux WS v.4 operating system (Red Hat Enterprise, Raleigh, NC) and MR Vision Software (MR Vision Co., Winchester, MA) was used for processing and analysis of the acquired image data. Regions of interest (ROI) were acquired over the blood in the IVC and the tumor periphery for signal intensity measurements at each time point. The tumor periphery was defined as the outer 2 mm rim of the whole tumor, shown in a previous study to be superior to measurements based on the entire tumor area for grading the severity of malignant lesions.¹² Precontrast R1 values were calculated based on the measured signal intensities and the calculated values of precontrast R1 based on the inversion recovery data.¹³ Differences between the precontrast and postcontrast R1 values (R1) in blood and tumor rim were assumed to be directly proportional to the concentration of gadolinium in the tissue.¹³

Estimates of the endothelial transfer coefficient, K^{PS} ($\mu\text{L}/\text{min} \cdot 100 \text{ cm}^3$) and the fractional plasma volume, fPV (%) were calculated based on a 2-compartment kinetic model using the R1 data as described in detail previously.¹⁴ Briefly, a monoexponential function was used to fit the blood R1 data, serving as a forcing function, for the 2-compartment bi-directional

kinetic model (Fig. 2).¹⁵ The fractional rate of reflux from interstitial fluid back to plasma, k (min^{-1}), was not resolvable in the time course of these experiments (1 hour) and was therefore set to zero. The model was used to fit the data for blood and tumor concurrently using the SAAM II software (SAAM Institute, Seattle, WA), which employs a weighted, nonlinear least squares estimation algorithm.¹⁶ Measurement errors in the R1 data were assumed to be independent and Gaussian, with zero mean and fractional standard deviation known within a scale factor determined from the data. Weights were optimally chosen, ie, equal to the inverse of the variance of the measurement error. The precision of the parameter value estimates was determined from the covariance matrix at the least squares fit.

Tumor Fixation by Vascular Perfusion

At the completion of the MR examination on day 7, the rats were perfused intravenously via the tail vein with 1 mL of fluorescein isothiocyanate-labeled *Lycopersicon esculentum* (tomato) lectin (1 mg per mL in 0.9% NaCl; Vector Laboratories, Inc., Burlingame, CA) to provide a means to microscopically assess the perfused population of microvessels¹⁷; lectin adheres to endothelial cells with which it comes into direct contact. After the lectin infusion, the chest cavity was opened rapidly and through a 20-gauge cannula that was inserted into the aorta via an incision in the left ventricle, the entire vasculature was flushed for 2 minutes at a pressure of 140 mm Hg with fixative (1% paraformaldehyde in PBS, pH 7.4; Sigma-Aldrich, St. Louis, MO). Blood and fixative were allowed to exit through an incision placed in the right atrium. Fixed tumors were then excised from the carcass, immersed in 1% paraformaldehyde in PBS for 1 hour at 4°C, rinsed 3 times with PBS, infiltrated with 30% sucrose, frozen in Tissue-Tek Optimal Cutting Temperature (VWR, Brisbane, CA) compound, and processed for immunohistochemistry as described elsewhere in detail.¹⁷ *Lycopersicon esculentum* lectin derived from tomatoes is a stable glycoprotein of about 100,000 Da containing about 50% arabinose and galactose. When injected intravenously this lectin binds *N*-acetylglucosamine oligomers present on the luminal surface of blood vessels. Because the lectin used in this study was labeled with fluorescein isothiocyanate (green fluorescence), the perfused blood vessels appeared green under the fluorescent microscope.

Immunohistochemistry

Cryostat sections (80 μm thickness) of MDA-MB-435 tumors were cut and dried on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). The tissues were permeabilized with PBS containing 0.3% Triton X-100 (Laboratory Chem. Inc., Pittsburgh, PA) and incubated in 5% normal serum (Jackson ImmunoResearch, West Grove, PA) in PBS-plus [PBS containing 0.3% Triton X-100, 0.2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 0.01% thimerosal] for 1 hour at room temperature to block nonspecific antibody binding. Sections were incubated overnight with primary antibodies, listed below, diluted in 5% normal serum and 95% PBS-plus at room temperature. Endothelial cells, whether lectin-perfused or not, were stained with rat monoclonal anti-RECA-1 (1:250, Pharmingen, San Diego, CA) to provide a means to assess the entire population of endothelial cells in the section, regardless of their perfusion status. After incubation in primary antibodies, specimens were rinsed with PBS containing 0.3% Triton X-100 and incubated for 4 to 6 hours at room temperature with secondary antibodies diluted in 5% normal serum in PBS. The secondary antibody for rat anti-RECA-1 was Cy3-labeled goat antimouse IgG (1:400,

Jackson ImmunoResearch, West Grove, PA). Specimens were rinsed with PBS containing 0.3% Triton X-100, postfixed in 4% paraformaldehyde, rinsed again with PBS, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). To detect the extravasated albumin-(Gd-DTPA)₂₇-(biotin)₁₁ in the tumor sections Cy3-labeled streptavidin (1:400, Jackson ImmunoResearch Inc., West Grove, PA) was used for staining. Streptavidin is a tetrameric protein that binds tightly to the small molecule, biotin.

Microscopic Assessments of Tumor Vascularity

The size of the entire microvascular bed composed of endothelial cell-lined vessels stained by RECA-1, defined as the *RECA-1 area-density (%)*, the size of the perfused vascular bed stained by lectin, defined as the *lectin percent areadensity (%)* and the magnitude of the endothelial leak of MMCM stained by streptavidin, defined as the *streptavidin percent area-density (%)* were measured quantitatively in 80 μm -thick sections of each tumor by measuring the proportion of the section's area (2-dimensional area-density) occupied by fluorescence from each of the specific fluorescent markers. Fluorescence was analyzed using ImageJ software (<http://rsb.info.nih.gov/ij>) in digital fluorescence microscopic images, each measuring $960 \times 1280 \mu\text{m}$ (10 $\times$ objective, 1 $\times$ Optovar). On the basis of an analysis of pixel fluorescence intensities, which ranged from 0 to 255, blood vessels were distinguished from background by empirically using only the pixels above a threshold value of 40, thus maximizing the inclusion of only those pixels with specific staining. The area-density was calculated as the percentage of total pixels having a fluorescence intensity value equal to or greater than the threshold.¹⁷ Because the fluorescence threshold was set to provide the greatest signal-to-noise ratio for measuring specific immunoreactivity, some of the faintest immunoreactivity may not have been included. However, as the threshold was applied uniformly, all experimental groups should be similarly affected.

Statistical Analysis

Comparisons of the mean values of either DMSO-treated or saline control tumors over time from baseline to day 7 for K^{PS} and fPV were performed using the paired *t* test. Comparison of baseline values for fPV and K^{PS} in tumors treated either with DMSO or saline was performed using the unpaired *t* test. Statistical testing for fPV and K^{PS} was performed using the GraphPad Prism software (GraphPad Software Inc, San Diego, CA). For the immunohistochemistry data, differences between groups were assessed using analysis of variance followed by the Bonferroni-Dunn test for multiple comparisons. *P* values <0.05 were considered statistically significant.

RESULTS

All procedures were well tolerated in all animal subjects. A series of representative dynamic contrast-enhanced MR images of a subcutaneous MDA-MB-435 tumor xenograft in a rat is provided in Figure 3 and shows strong time-persistent enhancement of blood, seen best in the IVC, and enhancement predominantly of the tumor periphery. The 2-compartment tissue model fit the data well in all experiments. Representative plots of the delta R1 data for tumor rim and blood over time with best fits to the data are shown in Figure 4.

At baseline, tumor volumes did not differ significantly ($P > 0.05$) between the DMSO-treatment group ($n = 7$, $969 \pm 206 \text{ mm}^3$) and the control group ($n = 8$, $1099 \pm 406 \text{ mm}^3$). Tumor volumes increased significantly ($P < 0.05$) over the 1-week course of the study for both the saline-control and DMSO-treatment groups without significant differences between the 2 groups (from $969 \pm 206 \text{ mm}^3$ at baseline to $3108 \pm 758 \text{ mm}^3$ at day 7 for the DMSO group and from $1099 \pm 406 \text{ mm}^3$ at baseline to $2996 \pm 443 \text{ mm}^3$ at day 7 for the control group).

In Table 1, MRI and corresponding microscopic assay results are divided by considerations of vascular endothelial leakiness and vascular richness. Endothelial leakiness to the macromolecular contrast agent, assayed by MRI and expressed as the endothelial transfer coefficient (K^{PS}), declined significantly ($P < 0.05$) in the DMSO-treated tumors

($K_{\text{baseline}}^{\text{PS}} = 19.3 \pm 8.8 \mu\text{L}/\text{min} \cdot 100\text{cm}^3$ to $K_{\text{Day7}}^{\text{PS}} = 0 \mu\text{L}/\text{min} \cdot 100\text{cm}^3$). In the control-saline group tumor K^{PS} values did not change significantly over the 7-day study

($K_{\text{baseline}}^{\text{PS}} = 17.3 \pm 18.0 \mu\text{L}/\text{min} \cdot 100\text{cm}^3$ and $K_{\text{Day7}}^{\text{PS}} = 13.3 \pm 7.7 \mu\text{L}/\text{min} \cdot 100\text{cm}^3$).

Corresponding to the MRI measurements of endothelial permeability, in DMSO-treated tumors the area-density of streptavidin-stained extravasated contrast medium ($34.2\% \pm 4.9\%$) was significantly reduced ($P < 0.05$) compared with the control group ($57.7\% \pm 5.5\%$, Table 1 and Fig. 5 B,C). In saline-treated control tumors albumin-Gd-(DTPA)₂₇-(biotin)₁₁ the streptavidin staining revealed that the contrast medium had extravasated from the microvessels to accumulate widely within the extravascular, interstitial space. Around some vessels the streptavidin staining was quite strong and diffuse whereas around others the staining was less intense and close to the endothelium, suggesting that the vasculature of MDA-MB-435 tumor is heterogeneously leaky. As depicted, DMSO treatment did not only change the quantity of extravasated albumin-Gd-(DTPA)₂₇-(biotin)₁₁ (Fig. 5C), but also the distribution pattern of the contrast agent molecules within extravascular space. In DMSO-treated tumors, the MMCM remained relatively closer to the vascular structures (Fig. 5C) and many tumor vessels showed no surrounding contrast agent extravasation.

No alterations in tumor vascular richness, assayed by MRI as the fPV (%), were observed either for the treatment or for the saline-control group between day 0 and day 7. Immunohistochemically total tumor vascularity and perfused tumor vascularity were assessed by means of area-density measurements of lectin and RECA-1, respectively, on fluorescent sections. No differences were found between the control and the DMSO-treated group either in these tumor vascular richness assays.

DISCUSSION

Quantitative MMCM-enhanced MRI assays of cancer vessel permeability, assayed in vivo as the endothelial transfer coefficient, K^{PS} , demonstrated a significant suppression of microvessel leakiness in human breast cancer xenografts treated with DMSO. To our knowledge this is the first evidence, using any technique, for an in vivo effect of DMSO on cancer or cancer microvessels. Similar MRI assays based on MMCM-enhanced MRI have been used previously to confirm and measure significant reductions in cancer microvascular leakiness induced by a variety of known antiangiogenic drugs including anti-vascular

endothelial growth factor (VEGF) antibodies, VEGF tyrosine kinase inhibitors,¹⁸ cyclooxygenase-2 inhibitors,¹⁹ and a matrix metalloprotease inhibitor.²⁰ DMSO is unlike these above listed antiangiogenic drugs in that its effect on cancer blood vessels has not been previously recognized. One important implication of this recognition is that the choice of DMSO as a solvent or vehicle for any solute in cancer treatment studies should be avoided; observed effects could be from the solvent rather than the studied drug solute. Another important implication of these data is that they support the robustness of our MMCM-enhanced MRI approach to the individual characterization of cancers. A new form of tumor vessel perturbation, induced by DMSO, measured by MRI, and confirmed by quantitative microscopy, can now be added to the list of drug actions detectable and measurable by MMCM-enhanced MRI.

The reduced leak of our macromolecular paramagnetic contrast agent after 7 days of DMSO treatment, measured quantitatively by MRI, was microscopically confirmed in the same tumors by staining directly for the same MRI-detected macromolecules using the biotin tags to which fluorescently-labeled streptavidin is strongly attracted. Corresponding with the MRI-assays of leaked MMCM, the streptavidin percent area-density measurements were significantly lower with DMSO treatment than with saline treatment. The confidence in the conclusion of DMSO-induced suppression of cancer vessel leakiness is heightened by the fact that the MRI measurements and the microscopic measurements were made independently by 2 separate observers (CC and BS) without knowledge of each other's results.

The richness of tumor vascularity did not change with DMSO treatment as reflected either by MRI-derived fPVs or microscopically derived area-density measurements using RECA-1 or lectin staining. In similar studies using other angiogenesis inhibitors we have also observed no or little acute change in fPV while observing significant reductions in K^{PS}.^{19,20} Thus, this study is consistent with these earlier studies in suggesting that assays of the richness of the microvascular network alone may be relatively insensitive to acute effects of angio-active drugs. Although not examined here, it is possible that a DMSO treatment course longer than 7 days or at a higher dose might have resulted in significant changes in vascular richness or in tumor growth, either or both of which effects would support an antiangiogenesis action of DMSO; the observed DMSO effect solely on endothelial permeability should not be equated with a confirmed antiangiogenesis action.

A noteworthy incidental observation is that after DMSO treatment a small leak of the macromolecular probe could still be detected microscopically by streptavidin staining in the same tumors for which no leak was measurable by MRI. This is not surprising in that microscopy would be expected to detect even a few extravasated molecules when the same leaked molecules would produce only a small MRI signal change not easily resolved above background noise. Also as might be predicted, invasive microscopy is more sensitive to the presence and position of molecular probes than is noninvasive MRI.

Our results are consistent with a hypothesis that DMSO is antiangiogenic and possibly could be therapeutic in the clinical management of neoplastic disease. Potential anticancer or antiangiogenic effects of DMSO previously have only been suggested by in vitro tissue

culture observations. Koizumi et al using cultured human aortic endothelial cells were able to demonstrate an inhibitory effect of DMSO on capillary tube formation and a suppression of matrix metalloproteinase-2 production.⁴ Also using tissue cultures, Marks and Breslow reported that DMSO and related polar small-molecular solvent species were seen to induce cytodifferentiation and growth arrest of murine erythroleukemic cells.²¹ Goto et al reported an augmented antiproliferative effect of interferon-alpha in the presence of DMSO on cultured human lung carcinoma cells.²² Adding to this body of cell-culture-derived evidence consistent with anticancer actions of DMSO is our report of significant cancer vessel permeability reduction in a whole breast cancer xenograft model.

The mechanism or mechanisms for the effect of DMSO on cancer vessels, or for the effects observed by others in various cell culture preparations, are unknown. Two possibilities are that DMSO interacts with matrix metalloproteinases⁴ or directly with VEGF in the generally and overall complex process of tumor angiogenesis. In support of and consistent with an effect of DMSO on VEGF, our group has previously applied MRI enhanced with MMCM to demonstrate strong effects on endothelial permeability produced by well-studied antiangiogenic drugs, which directly interfere with VEGF action, specifically, monoclonal anti-VEGF antibodies,²³ and VEGF tyrosine kinase inhibitors.¹⁸

The application of a streptavidin fluorescent marker for binding to a biotin-labeled probe, in this case 11 biotin groups covalently included within albumin-(Gd-DTPA)₂₇-(biotin)₁₁, permitted a detailed tissue microscopic localization of the extravasated macromolecular contrast agent and a direct comparison to the MRI results. van Dijke et al from our laboratory previously reported on the use of a biotin-labeled MMCM for its microscopic tissue localization.⁹ The strength and specificity of the interaction of the biotin-streptavidin system has led to its wide use as an affinity pair in molecular, immunologic, and cellular assays.²⁴ Briefly, biotin is a watersoluble B-complex vitamin (B7) important in the synthesis of fatty acids and in gluconeogenesis. Because biotin is known to be present within all cells of the human body²⁴ one might anticipate the observed subtle background activity from the streptavidin stain present on tissue sections even in animals that received only a nonbiotin-labeled version of the albumin-(Gd-DTPA) macromolecular contrast agent (Fig. 5A). Streptavidin, the binding counterpart of biotin, is a 60 kDa tetrameric protein purified from the bacterium *Streptomyces avidinii*. It is widely used in molecular biology because of its extraordinarily strong affinity for biotin; the dissociation constant (K_d) of the biotin-streptavidin complex is on the order of approximately 10^{-15} mol/L, ranking among one of the strongest noncovalent biologic interactions known.²⁴ The complex forms rapidly and is stable over a wide range of pH and temperature.²⁵

The results of our study are limited. We examined only a single tumor type (MDA-MB-435 breast cancer), grown in an athymic rat model, using only 1 experimental MRI contrast agent not intended for human use, with only a single 1-week, 3-dose course of DMSO. Many variations in these choices could be tested experimentally and might demonstrate enlightening new data. Possible inhibitors/competitors of DMSO effect on cancer vessels were not included in this exploratory study. Future studies of DMSO could provide insights into questions about mechanism of action, dose-dependency, and time profile for DMSO effects.

In conclusion, we were able to demonstrate by 2 independent methods that DMSO notably reduces tumor vessel permeability to a macromolecular diagnostic contrast agent in an in vivo human breast cancer model. The decrease of permeability produced by DMSO and assayed by MRI is confirmed by the immunohistochemical area-density measurements of extravasated albumin-(Gd-DTPA)₂₇-(biotin)₁₁. No significant effects of DMSO on tumor vascular richness were observed either by MRI or fluorescent microscopy. Results support a high sensitivity and robustness of the macromolecular contrast enhanced MRI approach for the characterization of cancer vessels on an individual tumor basis and provide initial evidence for an in vivo effect of DMSO in cancer.

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FIGURE 1.

Schematic structure of albumin-(Gd-DTPA)₂₇-(biotin)₁₁. The belt-like random coil represents the human albumin core element with amino-containing side chains of the lysine residues (small sticks) to which Gd-(DTPA) (gray ovoids) and biotin (white spheres) are covalently attached.

FIGURE 2.

Kinetic model. Shown below is the simple 2-compartment tissue model describing the kinetics of contrast media transport from the plasma space into the interstitial fluid. The endothelial transfer coefficient K^{PS} [$\mu\text{L}/(\text{min} \cdot 100 \text{ cm}^3)$] denotes the clearance of contrast medium from plasma to interstitial water. Denoting the fractional rate of reflux from interstitial fluid back to plasma, the rate constant k (min^{-1}) was not resolvable in the time course of these experiments (1 hour) and is therefore set to zero. The box around the plasma compartment denotes a forcing function representing the mono-exponential disappearance of MMCM from the blood. The kinetics of both compartments, taken together, reflect the dynamic response of the entire tissue/tumor to contrast medium enhancement after bolus intravenous injection.

FIGURE 3.

Representative T_1 -weighted SPGR images, precontrast and at 3 minutes, 15 minutes, 30 minutes, and 60 minutes after injection of albumin-(Gd-DTPA)₂₇-(biotin)₁₁, which were used for the calculation of the endothelial transfer coefficient (K^{PS}) and the fractional plasma volume (fPV). Note the tumor enhancement, most prominent in the rim. The blood enhancement seen in the inferior vena cava (white arrow) and nearby hepatic vein, persisted over the 1-hour course of data acquisition.

FIGURE 4.

Representative fit (solid lines) of model to delta R1 data from blood (Δ) and tumor ($\circ$) using MMCM. The slight deviation of parallel behavior toward convergence of the blood and tumor fits on a semi-logarithmic plot indicates a small leak of the MMCM from the blood into the interstitial space of this tumor. Had the 2 lines been parallel, it would have indicated no measurable leak.

FIGURE 5.

Representative fluorescent-immunohistochemically stained human breast cancer sections depicting leakage of MMCM (streptavidin-biotin reaction) and vascular richness (lectin and RECA-1 antibody). Note that DMSO treatment reduces the extravasation of albumin-(Gd-DTPA)₂₇-(biotin)₁₁ without reducing density of tumor blood vessels. Image (A) shows a negative control 80-micron thick microscopic section of a MDA-MB-435 tumor injected with nonbiotin-labeled albumin-(Gd-DTPA)₃₀ stained with lectin (green) for perfused vessel endothelium and streptavidin (red) for biotin-containing molecules. Seen is a typical distribution of tumor microvessels in green and minimal background red staining possibly because of endogenous biotin, Vitamin B₇. (B). Tumor section from the saline-control group administered albumin-(Gd-DTPA)₂₇-(biotin)₁₁. Note a strong red signal indicating substantial extravascular accumulation of biotin-labeled contrast medium surrounding the

yellow-green tumor microvessels. (C). After 7 days of treatment with DMSO, the density of red-stained extravasated biotin-labeled contrast medium, compared with (B), is strongly reduced indicating a reduction in endothelial permeability and extravascular accumulation of the macromolecules. Confocal microscopic images of tumor vessels in MDA-MB-435 tumors treated with saline (D, E) or DMSO (F, G) for 7 days show that no noticeable change has occurred in the area-density of perfused (green lectin-stained) microvessels nor in the area-density of all microvessels (red, RECA-1 stained), regardless of perfusion status. No difference is observable (D, E, F, G) between saline-control and DMSO-treated groups with regard to vascular area-density. Scale bar: 115 μm in (D, E, F, and G) and 120 μm in (A, B, and C).

Quantitative MRI [K^{PS} , $\mu L / (min \cdot 100\text{ cm}^3)$] and Microscopic (Streptavidin-Biotin Area-Density, %) Assay Results for Endothelial Leakiness and Vascular Richness in our MDA-MB435 Human Breast Cancer Model Before and After a 7-d DMSO-Treatment Protocol

TABLE 1

Endothelial Permeability Assays				Tumor Vascular Richness Assays					
MRI-Assayed Endothelial Transfer Coefficient (K ^{PS})		Streptavidin-Biotin Stained Area-Density (%)		MRI-assayed Fractional Plasma Volume (fPV)		Lectin-stained Area-Density (%) “Perfused Vessels”		RECA-1-stained Area-Density (%) “All Vessels”	
Control	DMSO	Control	DMSO	Control	DMSO	Control	DMSO	Control	DMSO
Day 0	17.3 ± 18.0	19.3 ± 8.8	n/a	n/a	2.7 ± 0.7	3.4 ± 1.3	n/a	n/a	n/a
Day 7	13.3 ± 7.7	0 ^{*†}	57.7 ± 5.5	34.2 ± 4.9 [†]	2.8 ± 0.7	3.5 ± 1.0	17.7 ± 1.6	17.9 ± 0.5	18.8 ± 1.7
									18.8 ± 0.5

MRI indicates magnetic resonance imaging; DMSO, dimethyl sulfoxide; K^{PS} , cancer microvessel permeability; fPV, fractional plasma volume.

* Significant difference ($P < 0.05$) between day 0 (pretreatment) and day 7 (posttreatment).

† Significant difference ($P < 0.05$) compared with the same-day saline control.