High-resolution single-photon emission computed tomography and X-ray computed tomography imaging of Tc-99m–labeled anti-DR5 antibody in breast tumor xenografts

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Abstract

A murine, apoptosis-inducing monoclonal antibody (mTRA-8) targeting human DR5 was radiolabeled with Tc-99m. The binding affinity ($K_d$) and the number of DR5 receptors were measured in MD MBA-231–derived 2LMP cell lines that were “sensitive” or “resistant” to mTRA-8 killing. Single-photon emission computed tomography and X-ray computed tomography (SPECT/CT) evaluated the Tc-99m–mTRA-8 retention and distribution within xenograft tumors; biodistribution analyses confirmed the levels. Scatchard assays showed specific and high binding affinity of Tc-99m–mTRA-8 to DR5; the killing efficacy of mTRA-8 was unchanged by Tc-99m labeling. There was no significant difference between sensitive and resistant 2LMP cells for $K_d$ values (1.5 ± 0.3 nmol/L = acid labile), or DR5 receptors (mean/cell = 11,000). SPECT/CT imaging analyses at 6 h after injection of Tc-99m–mTRA-8 revealed the second 1.5 mm shell from the surface of the mammary fat pad tumors ($n = 5$; 5,627 mm³) retained 12.7 ± 1.4%ID/g, higher than the other shells, with no difference between the sensitive and resistant 2LMP tumors. Binding of Tc-99m–labeled mTRA-8 in tumor was specific; excess unlabeled mTRA-8 blocked Tc-99m–mTRA-8 retention in tumor by 45%. Retention of Tc-99m–labeled isotype antibody in tumor was consistent with the blocking study, and 30% lower. These studies show that SPECT/CT imaging provided detailed distribution information of Tc-99m–labeled mTRA-8 within breast tumor xenografts. Imaging could provide a mechanism to assess DR5 modulation when DR5 therapy is combined with chemotherapy and radiation, and thereby aid in optimizing the dosing schedule. [Mol Cancer Ther 2007;6(3):866–75]

Introduction

The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is considered to have potential for cancer treatment because it induces apoptosis in most tumor cells (1–4). Of concern, the significant cytotoxicity of TRAIL in human hepatocytes may limit the clinical application of TRAIL (5). To date, five receptors for TRAIL have been identified (6–13); two of them, DR4 (TRAIL-R1) and DR5 (TRAIL-R2; refs. 7–10), can transduce the death signal, whereas the others, DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (6, 11–13), function as decoy receptors by blocking or modulating the TRAIL-mediated signaling pathway. The hepatotoxicity of TRAIL may be related to its binding to multiple receptors, a limitation that would be overcome by using an agonist antibody that targeted only one death receptor. In fact, a novel murine, monomeric monoclonal antibody (mTRA-8) was developed specifically to target DR5, and was previously reported to be highly efficacious for cancer therapy in animal models (14). DR5 is predominantly expressed in most cancer cells, but not in normal cells including hepatocytes (14). As reported, mTRA-8–induced apoptosis in nine breast cancer cell lines in vitro and the 2LMP cell line in vivo (15) without toxicity for normal human hepatocytes (14). Each cell line had a unique degree of DR5 expression and sensitivity to mTRA-8–induced cytotoxicity (14, 15). To date, imaging DR5 in vivo has not been reported, a shortcoming considering the advantage that imaging would provide to validate the delivery of agonist antibodies to tumors, and to understand mechanisms of natural or acquired resistance to DR5-mediated apoptosis (16). Furthermore, imaging would provide a method to evaluate the modulation of DR5 receptor expression that may result from either chemotherapy (15, 17) or radiation (4, 18).

The primary aim of this study was to characterize Tc-99m–labeled mTRA-8 by detailed in vitro analyses, and to
subsequently image in vivo distribution within xenograft breast tumors at high spatial resolution by single-photon emission computed tomography (SPECT) imaging. Tc-99m is cost-efficient and used for preclinical and clinical purposes extensively, and was therefore chosen for the initial studies. A secondary aim was to characterize a related breast cancer subclone that was resistant to mTRA-8 killing. Both breast cancer cell lines expressed luciferase and were subclones of MD MBA231. The two lines are referred to as “2LMP-sensitive” or “2LMP-resistant.”

The Tc-99m-mTRA-8 was evaluated using Scatchard assays to measure DR5 binding affinity and the number of DR5 receptors. The functionality of Tc-99m-mTRA-8 was evaluated by cytotoxicity assay. The in vivo distribution of Tc-99m-mTRA-8 was tested in athymic nude mice bearing either s.c. or mammary fat pad 2LMP tumors. A high-resolution SPECT/X-ray computed tomography (CT) instrument was used for imaging the Tc-99m-mTRA-8 that was injected in the animals, whereas bioluminescence imaging was applied to monitor tumor growth. The in vivo distribution of Tc-99m-mTRA-8 in the xenografted tumors imaged by SPECT was compared with the region of viable cells in tumor slices assessed by bioluminescence imaging and histology, and statistically evaluated in 1.5 mm shells of the tumors. Finally, the retention of Tc-99m-mTRA-8 (percentage of injected dose per gram) in tumor and normal tissues was confirmed by biodistribution. These studies show an in vivo SPECT imaging method to evaluate DR5 expression at high resolution within tumors, providing a mechanism to image the modulation of DR5 expression during therapy.

Materials and Methods
Reagents and Cell Lines
All reagents were from Fisher (Pittsburgh, PA) unless otherwise specified. Purified mTRA-8 was provided by Sankyo Co., Ltd. (Tokyo, Japan). Purified mouse IgG1 K isotype control antibody was purchased from eBioscience, Inc. (San Diego, CA). Fresh Tc-99m was obtained from Birmingham Nuclear Pharmacy (Birmingham, AL). The 2LMP subclone of the human breast cancer cell line MDA-MB-231 was obtained from Dr. Marc Lippman (Georgetown University, Washington, DC) and maintained in MEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). An adeno-associated virus vector encoding firefly luciferase was obtained from Dr. Ponnazhagan (University of Alabama at Birmingham, Birmingham, AL) and was used to make a stable luciferase-positive clone. This clone was determined to have identical sensitivity to mTRA-8 killing, as compared with the parent 2LMP cell line (data not shown). Luciferin was purchased from Xenogen, Inc. (Alameda, CA). In order to establish 2LMP cells that were resistant to mTRA-8, a nude mouse bearing a luciferase-positive 2LMP tumor in the mammary fat pad was i.v. injected six times with mTRA-8 (200 μg, twice a week for 3 weeks), and the surviving tumor was collected after 9 weeks and cultured. The cells remained luciferase-positive, but compared with the original luciferase-positive 2LMP cell line, differed in their sensitivity to mTRA-8. These luciferase-positive cells were referred to as “resistant” 2LMP cells, whereas the original luciferase-positive cells from which they originated were referred to as “sensitive” 2LMP cells. All 2LMP cells reported in this publication are luciferase-positive, but are denoted as only 2LMP.

HYNIC Conjugation and Radiolabeling
A fresh 1.8 mmol/L solution of succinimidyl 6-hydrazinonicotinate (HYNIC; courtesy of Dr. Gary Bridger, AnorMED, Inc., Langley, British Columbia) in dimethylformamide was prepared. Forty picomoles was transferred to glass vials, followed by freezing at −90°C, then the solutions were vacuum-dried using Advantage Benchtop Freeze Dryer (Virtis Co. Inc., Gardiner, NY) with the shelf temperature at −75°C and trap at −90°C. The vials were sealed under vacuum, and kept frozen at −80°C until use. Each vial was reconstituted with 1.0 mL of sodium phosphate buffer [0.15 mol/L (pH 7.8)] containing 1 mg of mTRA-8 (HYNIC/mTRA-8 molar ratio = 6; ref. 19). After a 3-h incubation at room temperature, the mixture was transferred to Slide-A-Lyzer dialysis cassette having molecular weight cutoff of 10,000 (Pierce, Rockford, IL) and then immersed into 1,000 mL of PBS (pH 7.4) overnight at 4°C. The HYNIC-modified mTRA-8 was labeled with Tc-99m using SnCl2/tricine as the transfer ligand (20), and unbound Tc-99m was removed by G-25 Sephadex size exclusion chromatography. The radiolabeling yield was ~60%. The protein concentrations of the collected fractions were measured by Lowry assay (21). The level of Tc-99m binding to mTRA-8 was always >96%, as measured by TLC using separate strips eluted with saturated saline and methyl ethyl ketone.

Cell Viability Assay
Two assays were conducted with mTRA-8, whereas three assays were done with Tc-99m-labeled mTRA-8 following decay. A total of 1,000 cells were added to each well of 96-well plates (1,000 cells/well), and the cells were incubated overnight at 37°C in culture medium. The DR5 agonist antibodies were diluted to eight different concentrations (range, 10–10,000 ng/mL) and added to four to six wells per concentration. A final addition of secondary cross-linking antibody (goat anti-mouse antibody for mTRA-8 and goat anti-human for humanized mTRA-8; Southern Biotechnology, Birmingham, AL) was made to yield a final concentration of 5 μg/mL. After 24 h, the ATP level was determined using the ATP lite assay (Perkin-Elmer, Boston, MA). The light emission from the wells of the plates was measured using an IVIS-100 imaging system (Xenogen) and quantified using the vendor software. The luminescent exposure time was 120 s, whereas binning was 8. Axial field of view was 15 cm, and f-stop was 1. The region of interest was drawn manually around the area of each individual well in the culture plate, and the intensity of light emitted from the region of interest was measured. Data were normalized to light emission of untreated cells with secondary cross-linking antibody and reported as means ± SE.
**In vitro Binding Assay**

The two 2LMP cell lines were plated in separate 24-well plates, respectively (500,000 cells per well), and incubated overnight at 37°C in culture medium [DMEM, with 2 mmol/L L-glutamine, 1 mmol/L Na pyruvate, 10% fetal bovine serum (pH 7.4); 0.2 mL/well]. The Tc-99m-mTRA-8 was diluted in binding buffer [DMEM, with 30 mmol/L Hepes, 2 mmol/L L-glutamine, 1 mmol/L Na pyruvate, 1% bovine serum albumin (pH 7.4)] to eight concentrations; the highest incubation concentration was ~10 nmol/L, with 2-fold subsequent dilutions. Each concentration was made in duplicate; a third well at each concentration included excess unlabeled mTRA-8 (41.6 µg per well) for blocking. This control was needed to measure nonspecific binding. The mTRA-8 was incubated with the live, attached 2LMP cells for 1 hour at 37°C. Previous experience indicated that the Tc-99m-mTRA-8 did not bind at 0°C (data not shown). The free Tc-99m-mTRA-8 (not bound to cells) was washed off with 0.2 mL of ice-cold HBSS (pH 7.2), and 0.2 mL of NaC2H3O2-HBSS (pH 4.0) was added to each well and incubated for 5 min at room temperature to isolate the acid-labile component, presumably the surface-bound component. After the acid-labile fraction was collected, 0.2 mL of NaOH (1 mol/L) was added to each well and incubated for 10 min at room temperature to isolate the binding component that was not removed with the original acid wash. This binding component was referred to as the acid-stable component, and was obtained by dissolving the cells. This component would represent internalized antibody, or surface-bound antibody that was resistant to the acid washing. All acid-stable fractions were assayed for protein concentration, as were known numbers of cells to determine the protein content. The Tc-99m activity in the samples was measured using a gamma-ray counter (MINAXI Auto-gamma 5000 series Gamma Counter; Packard Instrument Company, Downers Grove, IL). The binding assays were repeated thrice independently. The data were analyzed using GraphPad Prism (ver. 4.0, San Diego, CA) after correction of Tc-99m for decay, and after correction for nonspecific binding. Data were fitted with nonlinear regression analyses assuming one site of binding, enabling the determination of the binding affinity (Kd) and the number of DR5 receptors (assuming one antibody per DR5; refs. 22, 23). Because the number of total cells were known, the receptor number per cell could be determined.

**Animal Tumor Model**

Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee. Four experiments were done with female athymic nude mice (8 weeks old). Injection vehicle was the culture medium (0.2 mL/site). Experiment 1 evaluated Tc-99m-mTRA-8 and included four groups of nude mice that were implanted with 1 million cells per site. Mice in group 1 (n = 10) were implanted in the right mammary fat pad with the 2LMP-sensitive cells. Mice in group 2 (n = 9) were implanted in the right mammary fat pad with the 2LMP-resistant cells. Mice in group 3 (n = 5) were implanted s.c. in the right and left flanks with 2LMP-sensitive cells. Mice in group 4 (n = 5) were implanted s.c. in the right and left flank with 2LMP-resistant cells. Experiment 2 evaluated Tc-99m-isotype control antibody and included five nude mice implanted s.c. with 2LMP-sensitive cells (20 million cells). Experiment 3 included two groups of nude mice (n = 4 per group) implanted in the mammary fat pad with 2LMP-sensitive cells (1 million cells); groups 1 and 2 were used to evaluate Tc-99m-mTRA-8 and Tc-99m-isotype control antibody, respectively. Experiment 4 evaluated in vivo-specific binding of mTRA-8 to DR5 receptors and included...
two groups of nude mice (n = 3 per group) implanted s.c. in the right and left flanks with 2LMP-sensitive cells (1 million cells per site). In experiment 1, half of the mice implanted with the mammary fat pad tumors (groups 1 and 2) and all mice with s.c. tumors (groups 3 and 4) received an i.v. dose of 22.2 MBq of Tc-99m-mTRA-8 (12 μg). The remaining half of the mice with mammary fat pad tumors (groups 1 and 2) received an i.v. dose of 7.4 MBq (26 μg). The i.v. dose of Tc-99m-isotype antibody for mice in experiment 2 was 1.5 MBq (13 μg). In experiment 3, the i.v. doses for group 1 (Tc-99m-mTRA-8) and group 2 (Tc-99m-Isotype antibody) were 10.8 MBq (9.4 μg) and 4.4 MBq (9.6 μg), respectively. In experiment 4, group 1 received i.v. Tc-99m-mTRA-8 at 3.7 MBq (9.0 μg), whereas group 2 received i.v. Tc-99m-mTRA-8 at 3.8 MBq (9.3 μg) mixed together in the same syringe with unlabeled mTRA-8 (1 mg). Imaging (experiments 1, 3, and 4) and biodistribution analyses (all experiments) were conducted at 4 to 5 weeks after the tumor cell implantation.

In vivo Imaging Studies

Mice were induced and maintained with isoflurane gas anesthesia for all studies, and monitored continuously to allow the lowest dose (typically 1–1.5%) to prevent movement. Imaging studies were conducted using X-SPECT, a SPECT/CT dual-modality imaging instrument manufactured by Gamma Medica, Inc., (Northridge, CA) to monitor detailed Tc-99m-mTRA-8 distributions in the tumors in vivo. For SPECT imaging, a total of 64 projections (data matrix size, 56 × 56 per projection) were acquired with a 30 s acquisition time per projection, using a pinhole collimator with a 1 mm tungsten pinhole insert. The field of view for mammary fat pad tumors was 45 to 50 mm, whereas the radius of rotations was 3.3 to 3.7 cm. Images were reconstructed using an ordered subsets expectation maximization algorithm (8 subsets and 20 iterations). In SPECT imaging, the data matrix size of each individual projection was 56 × 56, and that of the final reconstructed image was 56 × 56 × 56. The expected spatial resolution of the current study was ~1.3 mm, based on the acquisition variables, and using published data from previous calibration measurements with the same detector and pinhole collimator (24). Quantification accuracy provided by the SPECT imager was measured to within 10% error, using known calibration sources (25). The total photon count of 64 projections was ~180,000 at 6 h after 22.2 MBq dose injection in experiment 1. The fourth-order Butterworth digital filtrations (fc = 0.25, fm = 0.15) provided by the vendor software were applied for all SPECT images to enhance the image quality. For the CT system, the X-ray tube was operated at a voltage of 50 kVp, and an anode current of 0.6 mA. 256 projections were acquired to obtain the CT images, and acquisition time per projection was 0.5 s. In CT imaging, the data matrix size of each individual projection was 256 × 256, and that of the final reconstructed image was 512 × 512 × 512. The coregistration of SPECT and CT images was done.
using computer software, IDL Virtual Machine (Research System Inc., Boulder, CO). After coregistration, the data matrix size of each image was transformed to $256 \times 256 \times 256$. The SPECT/CT images were acquired at 6 h following injection of Tc-99m-mTRA-8 or Tc-99m-isotype control antibody. Studies attempting to collect SPECT/CT data for five times over 20 h indicated changes in distribution of the labeled antibodies due to prolonged anesthesia and recovery (data not shown). Therefore, the current study used only 6-h time points as this was shown not to alter biodistribution kinetics. A 60 W heat lamp warmed the animal bodies while they were under anesthesia. A consistent color scale was applied to all SPECT images after correction for radioactive decay and dose.

To analyze tumor growth according to tumor location and cell line, all mice in experiment 1 were imaged repeatedly (total number = 7, over 4 weeks) after implantation using the IVIS-100 imaging system. Each mouse was i.p. injected with luciferin at a dose of 2.5 mg (0.1 mL) and imaged under isoflurane anesthesia after 15 min. Luminescent exposure time was 60 s, photographic binning was 4, axial field of view was 25 cm, and f-stop was 1. The region of interest was drawn manually around the tumor area, and the light emitted from the region of interest was measured using the vendor software. For each mouse, light emission from the tumor region of interest was normalized to the initial light emission of the same region at the time of tumor cell implantation.

**Ex vivo Imaging Study and Histology**

Mammary fat pad tumors ($n = 4$ per group) were dissected at 24 h after Tc-99m-mTRA-8 or Tc-99m-isotype control antibody injection and split into two halves of $\sim 5$ mm thickness. The tumor dissection was done 10 min after the mice were injected i.p. with luciferin (2.5 mg within 0.1 mL). The planar bioluminescence images of the tumor halves were acquired to visualize the viable cell distributions (exposure time, 5 s; axial field of view, 10 cm; binning, 8; f-stop, 1), and for comparison with in vivo integrated SPECT image obtained by integrating a series of tomographic images of the same tumors. A consistent pseudocolor scale was applied to all bioluminescent images as well as SPECT images. The tumor halves were fixed initially in 10% neutral-buffered formalin prior to paraffin processing and embedding. The tumor halves were subsequently sectioned (10 $\mu$m thickness) and subjected to H&E staining to determine viable tumor tissue.

**Tumor Image Analysis**

For the distribution analysis of Tc-99m-mTRA-8 within tumors, SPECT images from six mice bearing 2LMP mammary fat pad tumors (groups 1 and 2; $n = 3$ per tumor type) were selected. The images were collected at $\sim 6$ h after Tc-99m-mTRA-8 injection. Tumor boundary was determined based on the intensity difference between the tumor and animal body in SPECT images, whereas threshold was manually set by visual inspection. From the segmented tumor images, the averaged volumes of the sensitive and resistant tumors were $4,846 \pm 932$ and $5,559 \pm 642$ mm$^3$ (mean $\pm$ SE), respectively, whereas the linear dimension of a unit voxel was 0.82 mm. The isodistance shells with 1.5 mm thickness beginning from the tumor surface were established for each individual tumor in the following steps: first, a binary three-dimensional tumor image was created based on the segmented SPECT image. Second, a three-dimensional distance image was created for the binary three-dimensional tumor image generated in the first step. The Euclidean distance was used for distance measurement from

![Figure 3](mct.aacrjournals.org)
the surface of the tumor. Third, the isodistance shells were created based on the three-dimensional distance image with a shell thickness of 1.5 mm. The random topological structure of the tumor was maintained when isodistance shells were constructed. The intensity of each shell was calculated by summing all photon counts within the shell and was converted into the percentage of injected dose per gram (%ID/g) using tumor weight measured after dissection and photon collection efficiency of the SPECT imager. The photon collection efficiency was measured using a cylindrical phantom (25 mm diameter and 25 mm height) containing aqueous Tc-99m pertechnetate solution (15 MBq). Cumulative intensities relative to the distance from the tumor surface were acquired in both the tumor types, and statistical comparison was conducted. The image segmentation was done using ImageJ, version 1.33u (NIH, Bethesda, MD), and isodistance shells were generated using computer software written with MATLAB, version 7.0.4 (The MathWorks, Inc., Natick, MA).

**Biodistribution**

All mice in experiment 1 were sacrificed at 24 h and the tumors, livers, and blood were collected for analyses. The mice in experiments 2 to 4 were terminated after 24 h and the dose retentions in tumor and the other normal tissues ($n = 15$) were measured. All samples were weighted and the Tc-99m activity was measured using a calibrated gamma ray counter (MINAXIγ Auto-gamma 5000 series Gamma Counter; Packard Instrument Company), decay corrected to dosing time, and converted to absolute radioactivity. The exact dose of radioactivity and antibody protein (via Tc-99m-mTRA-8—specific activity) was known for each mouse by measuring the dosing syringe before and after injection with a Atomlab 100 dose calibrator (Biodex Medical Systems, Shirley, NY), and therefore, the percentage of the injected dose (%ID) per gram of tissue (%ID/g) was determined. The ex vivo analyses were combined with the shell analyses as described in the previous section.

**Statistical Analyses**

One-way ANOVAs (26) was carried out using SAS, version 8.2 (SAS Institute Inc., Cary, NC) to compare sensitive versus resistant tumor cells for binding affinity of Tc-99m-mTRA-8 ($K_d$) and DR5 receptor number. ANOVA was also used to compare sensitive versus resistant tumors for retention (%ID/g) of Tc-99m-mTRA-8 in whole tumor or tumor shells. Bonferroni correction (26) was applied to adjust $P$ values for multiple comparisons. The Tc-99m-mTRA-8 distributions within the tumors for the sensitive and resistant groups were compared using the Kolmogorov-Smirnov test (27). ANOVA was used to compare the sensitive 2LMP tumor retention of Tc-99m-mTRA-8 versus that of Tc-99m-isotype control antibody. Finally, ANOVA was used to compare the sensitive 2LMP tumor retention of Tc-99m-mTRA-8 as compared with the tumor retention of Tc-99m-mTRA-8 with excess unlabeled mTRA-8 (1 mg).

**Results**

**In vitro Assays: Cell Viability and Scatchard Analyses**

Figure 1A presents the viability of the 2LMP cells following overnight treatment with several concentrations of mTRA-8. Treatment with mTRA-8 at 100 ng/mL killed approximately 70% of the sensitive 2LMP cells, whereas only 10% of resistant 2LMP cells were killed. No significant differences were detected between the mTRA-8 that was Tc-99m–labeled and the stock unlabeled mTRA-8 (Fig. 1B). Figure 1C and D presents representative Scatchard plots...
for Tc-99m-mTRA-8 binding to the two 2LMP cell lines, after correction for nonspecific binding (insets are saturation binding curves). For the sensitive 2LMP cells, the $K_d$ for the acid-labile binding component was 1.26 ± 0.07 nmol/L (mean ± SE), and that for the acid-stable binding component was 2.70 ± 0.32 nmol/L. For the resistant 2LMP cells, the $K_d$ for the acid-labile binding component was 1.82 ± 0.41 nmol/L, whereas that for the acid-stable binding component was 3.78 ± 0.85 nmol/L. The binding affinity for the acid-labile binding component in the sensitive 2LMP cell line was not significantly different from that in the resistant 2LMP cell line ($P = 0.2511$). Also, for acid-stable binding components, the difference between binding affinities of the sensitive and resistant cell lines was not significant ($P = 0.2990$). The average number of DR5 receptors per cell was 9,392 ± 1,005 for the sensitive 2LMP cells and 12,108 ± 1,933 for the resistant 2LMP cells, which were not significantly different ($P = 0.2806$).

**In vivo Tumor Imaging**

Bioluminescence imaging data analyses for the four groups of mice in experiment 1 revealed that they grew at a similar rate (data not shown). The mean light emission from the tumor regions increased ~150-fold from days 6 to 36 after implantation. At the SPECT imaging time, the average weight of s.c. tumors was 0.6 g, whereas that of mammary fat pad tumors was 3.0 g for all experiments.

Figure 2 shows SPECT/CT volume renderings and SPECT images (coronal view) showing distribution of Tc-99m-mTRA-8 (A, B) or Tc-99m-isotype control antibody (C) in mammary fat pad 2LMP tumors sensitive (A, C) or resistant (B) to mTRA-8 killing at 6 h after dose injection. The spacing between the neighboring SPECT images in Fig. 2A and B is 0.8 mm with the axial field of view of 45 mm, whereas that in Fig. 2C is 0.9 mm with the axial field of view of 50 mm. The white-dotted circle in each subfigure indicates the tumor region. Based on visual inspection, the increased retention of Tc-99m-mTRA-8 at the periphery of the tumor region was noted, whereas Tc-99m-isotype control antibody was distributed more uniformly within the tumor. No significant difference of Tc-99m-mTRA-8 distribution was noted between the 2LMP tumors that were sensitive or resistant to mTRA-8. The same dose distributions were observed in SPECT images at 24 h after injection (data not shown).

**Ex vivo Tumor Imaging**

Figures 3 and 4 show an *in vivo* integrated SPECT image having ~5 mm thickness of a representative 2LMP tumor with ~10 mm diameter at 6 h after Tc-99m-mTRA-8 (Fig. 3A) or Tc-99m-isotype control antibody (Fig. 4A) injection, the bioluminescence image of the tumor half (B), and photograph (C) and histology (D) of the cut surface. The purple region of the histology indicates the area of viable cells. The tumor in Fig. 4 is the one shown in Fig. 2C. The Tc-99m-mTRA-8 retention was highest within the viable cell region assessed by the bioluminescence imaging or histology (Fig. 3), whereas the Tc-99m-isotype control antibody was more uniformly distributed in the tumor at a much lower level (Fig. 4).

**Tc-99m-mTRA-8 Distribution Analysis**

The distances from the surfaces of the sensitive 2LMP tumors ($n = 3$) in which the normalized cumulative intensity, considered as the normalized cumulative uptake of Tc-99m-mTRA-8 as well, reached 80% and 90%, were 3.2 ± 0.1 and 3.8 ± 0.1 mm (mean ± SE), respectively, whereas those of the resistant 2LMP tumors ($n = 3$) were 3.4 ± 0.2 and 4.3 ± 0.3 mm, respectively (Fig. 5A). Based on the Kolmogorov-Smirnov test, no statistically significant difference for the dose distributions between these two tumor types was detected (asymptotical, $P = 0.9684$). Figure 5B shows the dose retention (%ID/g, $n = 5$; mean and SE) at each 1.5 mm shell averaging the five tumors with matching size. The retention at the second shell was highest and significantly different ($P < 0.0125$) from those at the others except the third shell after Bonferroni adjustment of $P$ values.

**Biodistribution**

The liver and blood retentions of Tc-99m-mTRA-8 at 24 h after injection did not differ ($P > 0.05$) between any of the groups in experiment 1, averaging 6.7 ± 0.5 and 13.2 ± 0.7%ID/g ($n = 29$), respectively. The means and SEs of the retentions (%ID/g) in tumor and normal tissues of the mice in experiments 1 and 3 with Tc-99m-mTRA-8 injection are
presented in Table 1, together with those in experiments 2 and 3, in which the Tc-99m-isotype control antibody was administered. In Table 1, the tumor uptake of Tc-99m-mTRA-8 (9.5 ± 0.6%ID/g) was found to be significantly higher than that of control antibody (6.7 ± 0.4%ID/g; P = 0.0004). In experiment 4, the tumor retention of Tc-99m-mTRA-8 alone (14.4 ± 1.8%ID/g) was significantly higher than that of Tc-99m-mTRA-8 with excess unlabeled mTRA-8 (8.0 ± 0.7%ID/g; n = 6 per group; P = 0.0079), confirming in vivo-specific binding of mTRA-8 to DR5 in the tumor.

There was no significant difference (P > 0.05) in Tc-99m-labeled mTRA-8 retention for s.c. tumors versus mammary fat pad tumors. Similarly, the retention of Tc-99m-mTRA-8 in sensitive 2LMP tumors was not statistically different (P > 0.05) when compared with resistant 2LMP tumors. Table 2 summarizes the %ID/g in tumors for the two 2LMP cell lines and different implant locations. Although not statistically different, the resistant s.c. 2LMP tumors had 15% less retention of Tc-99m-mTRA-8, as compared with sensitive s.c. tumors. Similarly, the resistant mammary fat pad tumors had 17% less retention of Tc-99m-mTRA-8, as compared with sensitive mammary fat pad tumors.

### Table 1. Retentions of Tc-99m-labeled mTRA-8 and isotype control antibody (%ID/g) in each organ at 24 h after i.v. injection, together with tumor-to-blood ratios

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percentage of injected dose per gram (mean ± SE)</th>
<th>Difference (P)</th>
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<tr>
<td></td>
<td>mTRA-8</td>
<td>Isotype antibody</td>
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<tr>
<td>Heart</td>
<td>4.42 ± 0.46 (n = 9)</td>
<td>5.56 ± 0.29 (n = 8)</td>
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<td>Stomach</td>
<td>0.58 ± 0.09 (n = 9)</td>
<td>1.27 ± 0.18 (n = 9)</td>
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<td>Large intestine</td>
<td>1.23 ± 0.32 (n = 9)</td>
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<tr>
<td>Small intestine</td>
<td>0.89 ± 0.08 (n = 9)</td>
<td>1.46 ± 0.14 (n = 9)</td>
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<td>Cecum</td>
<td>1.25 ± 0.38 (n = 9)</td>
<td>1.40 ± 0.07 (n = 9)</td>
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<td>Spleen</td>
<td>3.04 ± 0.26 (n = 9)</td>
<td>4.87 ± 0.49 (n = 9)</td>
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<td>7.16 ± 2.19 (n = 9)</td>
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<td>3.44 ± 0.40 (n = 9)</td>
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<td>Right kidney</td>
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<td>4.77 ± 0.44 (n = 9)</td>
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<tr>
<td>Leg muscle</td>
<td>0.55 ± 0.07 (n = 9)</td>
<td>2.57 ± 0.53 (n = 9)</td>
</tr>
<tr>
<td>Reproductive organs</td>
<td>3.19 ± 0.37 (n = 9)</td>
<td>5.55 ± 0.59 (n = 9)</td>
</tr>
<tr>
<td>Brain</td>
<td>0.29 ± 0.03 (n = 9)</td>
<td>0.34 ± 0.02 (n = 9)</td>
</tr>
<tr>
<td>Femur</td>
<td>1.17 ± 0.12 (n = 9)</td>
<td>2.68 ± 0.62 (n = 9)</td>
</tr>
<tr>
<td>Liver</td>
<td>5.84 ± 0.79 (n = 14)</td>
<td>7.30 ± 0.28 (n = 9)</td>
</tr>
<tr>
<td>Blood</td>
<td>11.26 ± 0.79 (n = 14)</td>
<td>13.58 ± 0.88 (n = 9)</td>
</tr>
<tr>
<td>Tumor</td>
<td>9.45 ± 0.60 (n = 14)</td>
<td>6.72 ± 0.41 (n = 9)</td>
</tr>
<tr>
<td>Tumor/blood</td>
<td>0.87 ± 0.06 (n = 14)</td>
<td>0.51 ± 0.03 (n = 9)</td>
</tr>
</tbody>
</table>

NOTE: P values were provided for statistical comparisons.

### Discussion

The present studies used SPECT/CT imaging to evaluate Tc-99m-labeled mTRA-8 retention in breast tumors at high spatial resolution. One necessary aspect of SPECT probe evaluation is to measure binding parameters to the target cells. These studies confirmed the specific and high binding affinity of the Tc-99m-mTRA-8 for DR5-expressing breast cancer cells (Kd = 1.5 and 3.2 nmol/L, for the acid-labile and acid-stable binding components, respectively). Furthermore, the studies confirmed that the total DR5 receptors available for binding were relatively low in number (mean, 11,000). Of importance, the binding affinity of mTRA-8 for DR5 and the number of DR5 receptors did not differ between the sensitive and resistant 2LMP breast cell lines. Finally, the Tc-99m labeling procedure did not alter the functionality of the mTRA-8 for killing the cells (Fig. 1B).

In spite of the low number of DR5 receptors expressed in breast cancer cells, in vivo imaging by SPECT showed sufficient sensitivity to detect nonuniform distribution of Tc-99m-mTRA-8 in the tumor xenografts. A higher retention of Tc-99m-mTRA-8 at the periphery of tumors was detected without any distinguishable difference between

### Table 2. Retentions of Tc-99m-mTRA-8 (%ID/g) in sensitive and resistant 2LMP tumors in two different implant sites (s.c. flank and mammary fat pad) with P values for statistical comparison at 24 h after i.v. injection

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Percentage of injected dose per gram (mean ± SE)</th>
<th>Difference (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitive</td>
<td>Resistant</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>10.37 ± 0.52 (n = 9)</td>
<td>8.79 ± 0.61 (n = 10)</td>
</tr>
<tr>
<td>Mammary fat</td>
<td>9.45 ± 0.60 (n = 14)</td>
<td>7.87 ± 0.94 (n = 9)</td>
</tr>
</tbody>
</table>
the sensitive and resistant 2LMP tumors (Fig. 2), and was consistent with the area of live cells assessed by ex vivo bioluminescence imaging or histology (Fig. 3). On the contrary, relatively low tumor uptake and uniform distribution of Tc-99m-isotype control antibody were observed (Figs. 2 and 4). Therefore, the increased uptake of Tc-99m-mTRA-8 at the tumor periphery resulted from the in vivo – specific binding of Tc-99m-mTRA-8 to DR5 receptors on viable 2LMP cells. An additional in vivo SPECT imaging was done in another group of mice (n = 4) at five different time points (0, 3, 6, 9, and 20 h) after Tc-99m-mTRA-8 injection, to monitor the kinetics of the antibody more dynamically (data not shown). In this experiment, the tumor uptake was decreased by ~30% at 24 h after dosing, probably due to prolonged anesthesia. Therefore, the frequency of image acquisition must be considered as changes induced in the animal due to data collection could alter the antibody delivery to the tumor.

The retention of Tc-99m-mTRA8 in tumor as determined by biodistribution analyses (%ID/g) represents an average for the entire tumor. As SPECT revealed the retention within the tumor was not uniform, it was desirable to subdivide the tumor volume into several isodistance shells and estimate %ID/g in those shells, and thereby improve the accuracy of determining delivery. The SPECT imaging data was comprehensively analyzed, allowing the determination of %ID/g within 1.5 mm shells of the tumors. The second shell from the outside of the tumor surface always showed the highest retention of the Tc-99m-mTRA-8 at 6 h postinjection. Based on the specific activity of the Tc-99m-mTRA-8, and assuming that 1 g of tumor would equal 10^9 cells, the retention of 12.7%ID/g in the second shell would represent a total of 6,700 mTRA-8 molecules per tumor cell. This number compares favorably with 11,000, the average DR5 receptors per cell that was determined by Scatchard analyses. This method might be easily applied to analyze the distribution of radioactive tracer in any target organs. The accuracy of this approach is highly dependent on the definition of the target boundary and the spatial resolution provided by the imaging system. In current study, only SPECT images were used to segment the tumor area because CT imaging did not provide sufficient contrast in the inner boundary of the tumor adjacent to the body. However, higher soft tissue contrasts can be achievable in CT imaging with the use of pertinent imaging contrast agents. Alternately, a magnetic resonance imaging technique may be employed for better segmentation, after accurate coregistration of both the SPECT and magnetic resonance images. The in vivo results showed a similar retention of Tc-99m-mTRA-8 for sensitive and resistant 2LMP, whereas in vitro Scatchard analyses confirmed similar binding properties and receptor numbers. In contrast, by cytotoxicity assay, the resistant 2LMP cells did not have the same capacity to transduce the death signal as the sensitive 2LMP cells. Death receptors often undergo oligomerization after binding of agonist antibodies, a process that is also referred to as “capping” (28–33). This process is likely to be independent of binding of the mTRA-8 antibody to DR5, and may explain the difference between the two cell lines that differ in their sensitivity to mTRA-8 killing. For example, the resistant cells bind mTRA-8, but may lack the efficacy to form the oligomer that is required for the death signal. Two lines of evidence support this concept. First, in vitro killing with monomeric mTRA-8 requires a secondary cross-linking antibody. Second, human tumors growing in certain FcR knockout nude mice are not sensitive to mTRA-8 treatment. Fc receptors may thus assist in the formation of the oligomer. In the current studies, the mTRA-8/DR5 receptor oligomer complex may stabilize retention of the Tc-99m-mTRA8 in the sensitive tumors, and thereby explain the slightly higher retention values as compared with the resistant tumors.

Recent reports suggest that the number and membrane location of tumor-specific apoptosis receptors can be increased by exposure to chemotherapy drugs or radiation to favor apoptosis (31, 34–39), a process that may also be linked to increased signaling due to oligomer formation. The SPECT imaging methods reported in the current study have sufficient sensitivity and spatial resolution to measure changes in DR5 expression within tumors. Noninvasive imaging during chemotherapy and/or radiation therapy could provide a mechanism to assess DR5 modulation, and could aid in optimizing the dosing schedule.

Acknowledgments

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Molecular Cancer Therapeutics

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