Original Article
Tumor-specific targeting by Bavituximab, a phosphatidylserine-targeting monoclonal antibody with vascular targeting and immune modulating properties, in lung cancer xenografts

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Abstract: Bavituximab is a chimeric monoclonal antibody with immune modulating and tumor-associated vascular disrupting properties demonstrated in models of non-small cell lung cancer (NSCLC). The molecular target of Bavituximab, phosphatidylserine (PS), is exposed on the outer leaflet of the membrane bi-layer of malignant vascular endothelial cells and tumor cells to a greater extent than on normal tissues. We evaluated the tumor-targeting properties of Bavituximab for imaging of NSCLC xenografts when radiolabeled with ^{111}In through conjugation with a bifunctional chelating agent, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). In vitro binding of ^{111}In-DOTA-Bavituximab to PS was determined by enzyme-linked immunosorbent assay (ELISA). Biodistribution of ^{111}In-DOTA-Bavituximab was conducted in normal rats, which provided data for dosimetry calculation. Single-photon emission computed tomography/computed tomography (SPECT/CT) imaging was performed in athymic nude rats bearing A549 NSCLC xenografts. At the molar conjugation ratio of 0.54 DOTA per Bavituximab, the PS binding affinity of ^{111}In-DOTA-Bavituximab was comparable to that of unmodified Bavituximab. Based on the quantitative SPECT/CT imaging data analysis, ^{111}In-DOTA-Bavituximab demonstrated tumor-specific uptake as measured by the tumor-to-muscle ratio, which peaked at 5.2 at 72 hr post-injection. In contrast, the control antibody only presented a contrast of 1.2 at the same time point.These findings may underlie the diagnostic efficacy and relative low rates of systemic vascular and immune-related toxicities of this immunoconjugate. Future applications of ^{111}In-DOTA-bavituximab may include prediction of efficacy, indication of tumor immunologic status, or characterization of radiographic findings.

Keywords: Bavituximab, phosphatidylserine, lung cancer, immunotherapy, vascular targeting, [111]In

Introduction

Under normal cellular homeostasis, phosphatidylserine (PS) is an anionic membrane phospholipid preferentially located on the inner leaflet of the plasma membrane. This asymmetry is maintained by ATP-dependent aminophospholipid translocases that catalyze the transfer of aminophospholipids from the external to the internal plasma membrane [1, 2]. In certain physiologic states, such as apoptosis and cell activation, PS asymmetry is disrupted, resulting in exposure of PS on the external membrane leaflet [3-6]. This process occurs due to

inactivation of aminophospholipid translocases or the activation of scramblases and ABC floppases [7]. Stresses within tumors, such as hypoxia and acidity, also trigger these events through the generation of reactive oxygen species [6]. Exposed PS in the cellular microenvironment restricts the production of pro-inflammatory cytokines and increases the production of anti-inflammatory cytokines. This role is consistent with PS exposure during apoptosis, a class of cell death that does not induce inflammatory responses [8]. On apoptotic cells, PS exposure results in down-regulated macro-

phage secretion of TNF-α and IL-1β, and upregulated production of TGF-β and IL-10 [8]. In addition, PS exposure suppresses immune responses by reducing dendritic cell maturation and antigen presentation [9]. Therefore, PS can be applied for targeted cancer immunotherapy. The molecular distribution of PS appears to result in favorable pharmacodynamic properties. In normal cells, including vascular endothelium, PS is segregated to the inner membrane leaflet and is therefore not available for antibody binding. In contrast, the exposure of PS on cancer cells and vascular endothelial cells in the tumor microenvironment permits this antibody to target the tumor microenvironment.

Bavituximab is a human-mouse chimeric IgG1 monoclonal antibody that targets PS by recognizing the PS-binding protein β2 glycoprotein-1 (β2GP1). Specifically, by cross-linking two β2GP1 molecules at surface of cells with exposed PS, Bavituximab localizes to tumors [10]. Once bound, Bavituximab activates host effector (immune) functions, such as antibody dependent cellular cytotoxicity (ADCC), resulting in tumor vessel destruction. Additionally, Bavituximab conveys anticancer effects by reversing the immune and inflammatory suppression normally exerted by tumors. Specifically, PS-targeting antibodies such as Bavituximab have been shown to result in promotion of pro-inflammatory pathways, recruitment of tumoricidal M1 macrophages, dendritic cell maturation and antigen presentation leading to adaptive T lymphocyte responses [11, 12].

The PS-targeting antibody, Bavituximab, has completed phase 1 [13] and phase 2 testing in lung cancer [14] and is currently under investigation in combination with chemotherapy and other agents for a number of malignancies, including lung, breast, rectal, liver, and prostate cancer. A phase 3 trial in previously treated advanced nonsquamous NSCLC is underway [15]. Clinical experience with Bavituximab to date shows relatively low rates of systemic vascular and immune-related toxicities. These observations also suggest tumor-specific targeting by Bavituximab. In this study, we presented a proof-of-concept method for the imaging evaluation of an ^{111}In-labeled Bavituximab immunoconjugate by single-photon emission

computed tomography (SPECT) with the goal to understand the low rates of systemic vascular and immune-related toxicities of Bavituximab seen clinically along with its anti-tumor effects.

Materials and methods

All chemicals were of reagent grade and used as received. All chemicals, solvents, and reagents were purchased from Sigma-Aldrich and Fisher Chemical unless otherwise noted. The [111]InCl$_3$ solution in 0.1 M HCl was purchased from Perkin-Elmer. The [64]CuCl$_2$ solution was purchased from Washington University, St. Louis. Rituximab (human IgG1 chimeric mAb) was obtained from the University of Texas Southwestern pharmacy. Radio-TLC analysis was performed on a Rita Star Radioisotope TLC Analyzer (Straubenhardt, Germany) to monitor the radiolabeling reaction. Instant Thin Layer Chromatography Medium (ITLC) acted as the supporter, and 1 × PBS buffer was the developing eluent.

Preparation of DOTA-Bavituximab conjugate

The DOTA-Bavituximab conjugate formation was via the coupling reaction between the activated 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) bifunctional chelating agent and free lysine amino groups in the antibody. The conjugation was undertaken in aqueous conditions. Briefly, a solution of 9.4 mg (19.9 μmol) of DOTA·4H$_2$O in 300 μL of H$_2$O was adjusted to pH 5-5.5 with 1 M NaOH and set in an ice-water bath. To the DOTA solution was added 1.9 mg (9.94 μmol) of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 76 μL of H$_2$O. Then, 4.3 mg (19.9 μmol) of N-Hydroxysulfosuccinimide (s-NHS) in 86 μL of H$_2$O freshly prepared was added, and the reaction mixture was stirred in the ice-water bath for 30 min and then used without separation for the protein conjugation. An aliquot of 5.8 mg of Bavituximab (provided by *Peregrine Pharmaceuticals, Tustin, CA*) in 0.6 mL of PBS, pH 7.4, was pre-conditioned by 4 times of washing with 0.1 M Na$_2$HPO$_4$, pH 7.5, using an Amicon® Ultra 4 mL filter (50,000 Da) at 4°C. Subsequently, the solution of N-Hydroxysulfosuccinimidyl DOTA (DOTA-OSSu) (0.66 μ mol) was added to the preconditioned Bavituximab solution. After the pH was adjusted to 8.0, the reaction mixture was incubated at 4°C over-
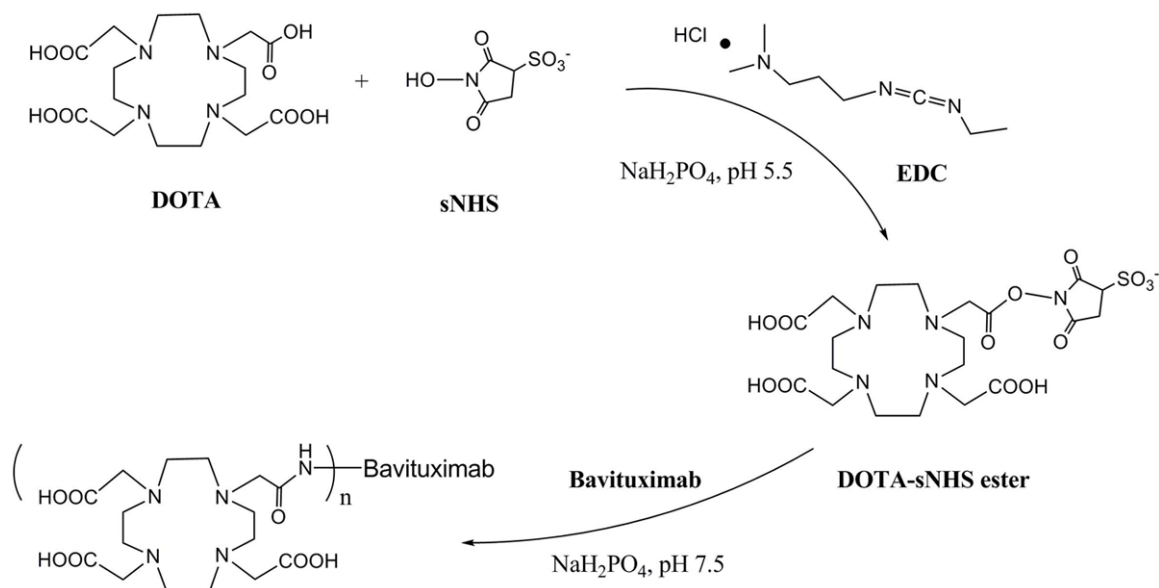
Figure 1. Schematic conjugation procedure of Bavituximab with a bifunctional chelator, 1,4,7,10-tetraazacyclodo-decane-1,4,7,10-tetraacetic acid (DOTA). EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; s-NHS: N-Hydroxy-sulfosuccinimide.

night with continuous mixing. The DOTA-Bavituximab conjugate was separated and purified by centrifuging the reaction mixture 5 times in an Amicon® Ultra 4 mL filter (50,000 Da) at 4°C using 0.1 M NaOAc, pH 5.0 as the washing solution. The same procedure was also applied to the control antibody (Rituximab).

Determination of average number of chelates per antibody molecule

The average number of DOTA chelator per Bavituximab molecule was determined by the isotope dilution method. Briefly, a series of standardized $CuCl_2$ dilutions was prepared to react with the DOTA-Bavituximab conjugates. For each Cu(II) concentration, 3 µL of DOTA-Bavituximab containing approximately 150 pmol of the conjugate was added to 56 µL of 0.1 M NH_4OAc, pH 5.0, followed by adding 40 µL of the standardized $CuCl_2$ solution and 1 µL of $^{64}CuCl_2$. The reaction mixture was incubated at 37°C for 1 h, after which 10 µL of 5 mM diethylenetriaminepentaacetic acid (DTPA) was added to remove non-specifically bound $^{64}Cu(II)/Cu(II)$. Radio-TLC was performed to analyze the labeling reaction yields. The number of chelator per antibody molecule was calculated from the ratio of counts remaining at the origin to the total number of counts, using the method of Meares et al. [16].

Indium-111 (^{111}In) radiolabeling of DOTA-Bavituximab

The isotope of ^{111}In was selected for this work to match the biological half-life of Bavituximab. To label DOTA-Bavituximab with ^{111}In, 230 MBq of $^{111}InCl_3$ in 10 µL of 0.1 M HCl was added to 1.6 mg of DOTA-Bavituximabin 300 µL of 0.1 M sodium acetate, pH 5.0. The reaction mixture was incubated at 37°C for 0.5 h, and then the reaction mixture was mixed with 1 µL of 5 mM DTPA solution. The reaction mixture was allowed to stand at room temperature for 5 min, and the radiolabeling yield was ~90% as accessed by radio-ITLC. The ^{111}In-DOTA-Bavituximabwas purified by a Bio-Spin P6 columnto reach > 95% of radiochemical purity. The same procedure was also applied to the control antibody (Rituximab).

Characterization of ^{111}In-DOTA-Bavituximab target binding

For phospholipid ELISAs, 50 µl of PS in *n*-hexane (10 µg/mL) was added to wells of Immulon 1B 96-well plates. The solvent was evaporated at room temperature and the plates were blocked for 1 h with 1% BSA in PBS and then washed with PBS. Unlabeled Bavituximab, Rituximab, and ^{111}In-DOTA-Bavituximab were pre-diluted in 10% fetal bovine serum to pro-
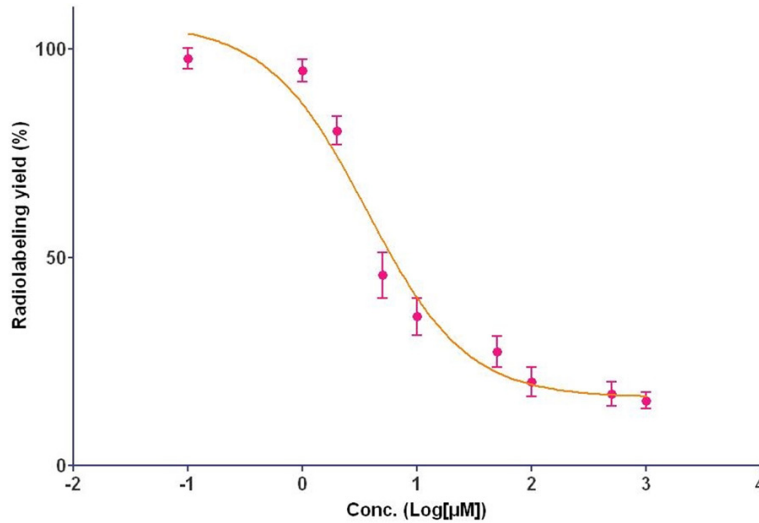
Figure 2. Determination of the average number of DOTA per Bavituximab using an isotopic dilution method.
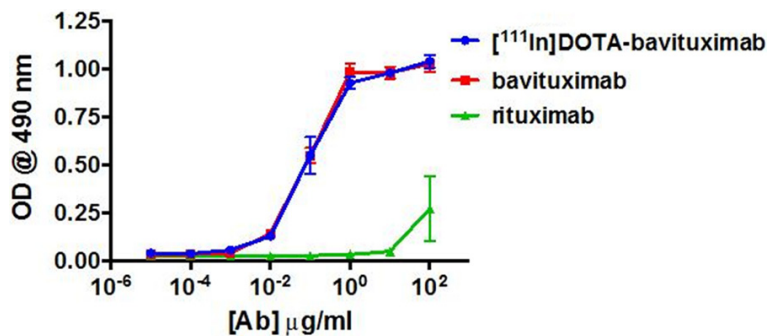
Figure 3. Phosphatidylserine (PS) binding by ^{111}In-DOTA-Bavituximab, native Bavituximab, and control antibody (Rituximab).

Use Committee (IACUC). A549 human lung carcinoma cells-were maintained at 37°C in a mixture of 5% CO_2 and 95% air in Gibco® DMEM supplement-ed with 10% fetal bovine serum (Sigma Aldrich). The cells were allowed to grow until 90% confluent. Cells were washed with PBS and mixed with Basement Membrane Matrix BD Matrigel™ (BD Biosciences) in a 1:1 ratio such that there would be 5 million cells/300 μl of suspension. The cell/Matrigel suspension was stored on ice until implantation into animals. Athymic nude rats were chosen because the use of the xenograft model calls for the use of immune-suppressed animals and because Bavituximab interacts with rat, but not mouse, β2GP1. Animals were housed in a pathogen-free facility. Fifteen 5-week old female athymic nude rats were purchased from the National Cancer Institute. 5 million cells were injected subcutaneously into the right flank of each rat. Tumor growth was monitored by caliper measurement three times per week. Tumors were allowed to grow until reaching a diameter of 1.5 cm for SPECT/CT imaging.
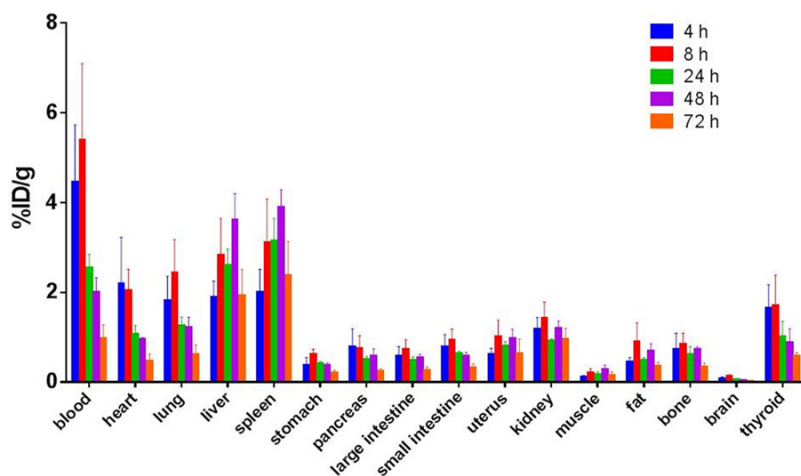
Dosimetry studies

Distributions of radioactivity were determined in normal rats with tail vein injection of ^{111}In-DOTA-Bavituximab. The organs of interest [blood, heart, lung, liver, spleen, stomach, pancreas, large intestine, small intestine, uterus, kidney, muscle, fat, bone, brain, and thyroid] were excised after whole-body perfusion at 4, 8, 24, 48, and 72 hr post-injection (p.i.) (n = 4), and then the radioactivity was quantitated by automatic gamma counter. The concentration in each sample was expressed as percentage injected dose per gram (%ID/g) [17].

Radiation dose estimates for human organs were calculated based on mass-based extrapo-

vide β2GP1 and further diluted in blocking buffer at an initial concentration of 200 μg/mL and 2-fold dilutions were performed in a separate 96-well plates (100 μl per well). The dilutions (100 μl/well) were then transferred from the dilution plates to PS-coated plates. The plates were incubated for 1 h at room temperature. The plates were washed and bound antibody was detected with goat anti-human IgG conjugated to HRP (1:2000 in blocking buffer) and developed with the chromogenic substrate ODP. The plates were read at 490 nm with a microplate reader (BioTek Instruments, Winooski, VT).

Generation of A549 xenograft model

All animal studies were approved by the UT Southwestern Institutional Animal Care and

Figure 4. Biodistribution of ^{111}In-DOTA-Bavituximab in normal rats (4, 8, 24, 48, 72 hr; n = 4) as percent injected dose per gram of tissue (%ID/g).

tumors was used to determine the optimum exposure time, achieving at least 20,000 counts per projection. The CT imaging was performed using 360 projections per rotation with 45 kVp, 1500 ms exposure, and the binning factor of 1:4. The SPECT image reconstruction was carried out using HiSPECT NG (Sciviswissenschaftliche Bildverarbeitung GmbH, Germany) with 35% smoothing, 100% resolution, and 3 × 3 iterations (Standard mode). The quantification of the organ activity was performed using Bioscan's In Vivo Scope 1.43 software package (Bioscan, Washington, DC). After co-registration of the CT and SPECT images, a cylindrical region of interest (ROI) was drawn, encompassing the organs in all planes containing the organs. The total activity in the tumor was quantified as percentage injected dose per gram (%ID/g) by normalizing the signal imaged at a given location for the amount of tracer injected into the subject.

lation of animal data. Specifically, the concentration in the animal organs was converted to a concentration in human organs by multiplying the animal concentration by a ratio of the total body weight of the animals and humans [18]. Then the percentage in the human organs was derived using organ masses taken from a standard model of the human body for either adult males [19]. Data were fit using the SAAM II software [20]. Time integrals of activity [21] were entered into the OLINDA/EXM software [22] using the adult male model. Time-activity data in the bladder was fit with a single, in-growing exponential, and no bladder voiding was assumed. From the observed activity in the intestines, 5% of the administered activity was assumed to enter the gastrointestinal tract (at the small intestine) and follow normal transit kinetics thereafter.

Results

Antibody modification and radiochemistry

The conjugation of DOTA to Bavituximab (**Figure 1**) was performed by combining the antibody with 15 molequiv of DOTA-OSSu intermediate at pH 8 and the reaction was allowed to proceed overnight at 4°C. The molar conjugation ratio of DOTA to Bavituximab was determined by the ^{64}Cu/[nat]Cu isotope dilution method (**Figure 2**). Under the reaction conditions, the desired low chelator per antibody ratio (0.54) was achieved. The radiolabeling yield with ^{111}In varied from 50% to over 95% depending on the relative amounts between ^{111}In and the DOTA-Bavituximab conjugate. After purification, the radiochemical purity of ^{111}In-DOTA-Bavituximab was determined to be > 95% by radio-TLC analysis. The average specific activity of ^{111}In-DOTA-Bavituximab in this study was about 140 MBq/mg. Rituximab without PS-specific binding [10, 23] was chosen as the negative control. Its DOTA conjugation and radiochemistry followed the same procedure as that for Bavituximab.

SPECT/CT imaging

SPECT/CT Imaging was performed using a NanoSPECT/CT [Plus]System (Bioscan, Washington, DC, USA) on A549 tumor bearing rats. After the intravenous injection of around 14-16 MBq of ^{111}In-DOTA-Bavituximab or control antibody (^{111}In-DOTA-Rituximab) (n = 1), SPECT images were acquired at 48, 72, and 96 hr p.i. The SPECT data were collected with 4 detector arrays collimated with multi-pinhole apertures giving a post-reconstruction resolution of 1.23 mm. Tumor focused images were acquired with 20 projections per 360° rotation, with a movement of 48 mm per 360°. The count rate of the

Table 1. Estimates of the human radiation dosimetry (adult male model) obtained from the biodistribution data summarized in **Figure 4**. Results were obtained by employing a mass-based extrapolation of the biodistribution data to estimate the human organ concentrations used as input for the OLINDA/EXM dosimetry software

Target Organ	Estimated Dose	
	mSv/MBq	rem/mCi
Adrenals	1.59E-01	5.89E-01
Brain	9.44E-02	3.49E-01
Breasts	8.46E-02	3.13E-01
Gallbladder Wall	1.89E-01	6.98E-01
LLI Wall	2.26E-01	8.36E-01
Small Intestine	1.59E-01	5.87E-01
Stomach Wall	1.41E-01	5.21E-01
ULI Wall	1.81E-01	6.71E-01
Heart Wall	1.46E-01	5.42E-01
Kidneys	1.61E-01	5.95E-01
Liver	2.79E-01	1.03E+00
Lungs	1.34E-01	4.95E-01
Muscle	1.10E-01	4.08E-01
Ovaries	1.58E-01	5.84E-01
Pancreas	1.70E-01	6.27E-01
Red Marrow	1.16E-01	4.31E-01
Osteogenic Cells	3.14E-01	1.16E+00
Skin	7.32E-02	2.71E-01
Spleen	2.29E-01	8.49E-01
Testes	1.06E-01	3.93E-01
Thymus	1.20E-01	4.42E-01
Thyroid	1.21E-01	4.46E-01
Urinary Bladder Wall	1.70E-01	6.28E-01
Uterus	1.54E-01	5.69E-01
Total Body	1.20E-01	4.46E-01
Effective Dose Equivalent	1.61E-01	5.97E-01
Effective Dose	1.50E-01	5.54E-01

^{111}In-DOTA-Bavituximab target binding

Immunoreactivity of ^{111}In-DOTA-Bavituximab was determined by ELISA. As shown in **Figure 3**, the PS binding curve of ^{111}In-DOTA-Bavituximab was almost identical to that of the intact Bavituximab, which correlated with their calculated EC_{50} values (^{111}In-DOTA-Bavituximab: 0.23 µg/mL; Bavituximab: 0.18 µg/mL). The radiolabeled control antibody Rituximab did not demonstrate significant PS binding at sub-µg/mL concentrations. These data indicated that the immunoreactivity of Bavituximab was not significantly altered by DOTA conjugation or radiolabeling, showing the plausibility of using

^{111}In-DOTA-Bavituximab for in vivo assessment of the PS status.

Dosimetry

In order to investigate the dosimetry of ^{111}In-DOTA-Bavituximab for potential clinical application, biodistribution was performed in normal/healthy (non-tumor-bearing) rats at multiple time points (4, 8, 24, 48, 72 hrp.i.) (**Figure 4**, **Table 1**). As expected, liver and spleen demonstrated the most activity. Human radiation dose estimates based on an adult male model revealed thatthe skin received the least radiation exposure (7.32×10^{-2} mSv/MBq or 2.71×10^{-1} rem/mCi), while most organs received around 0.08-0.15 mSv/MBq. Not surprisingly, the bone and liver received the highest doses, which were around 0.31 and 0.28 mSv/MBq, respectively. The effective dose was approximately 0.15 mSv/MBq.
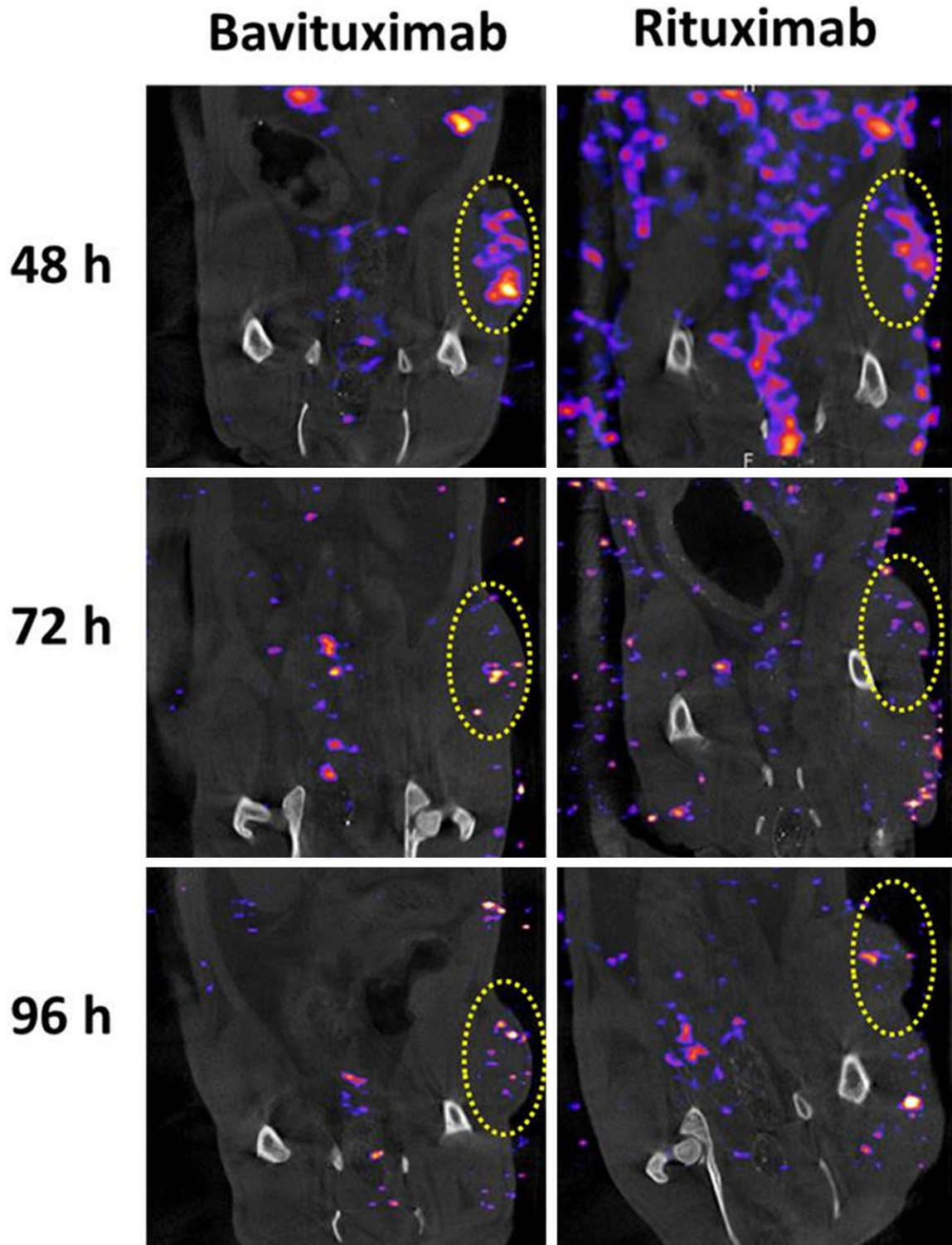
SPECT/CT imaging

Small animal SPECT/CT imaging studies were conducted in athymic nude rats bearing A549 NSCLC xenografts. Representative coronal SPECT/CT images are presented in **Figure 5**. A549 tumors were clearly visualized by ^{111}In-DOTA-Bavituximab at 48 hrp.i. when the images provided the greatest tumor contrast. At 72 and 96 hrp.i., A549 tumors could still be identified by ^{111}In-DOTA-Bavituximab. Besides A549 tumors, other surrounding tissues were observed with minimal uptake within the SPECT imaging window. In contrast, ^{111}In-DOTA-Rituximab could not show significant contrast of tumor to the surrounding tissues, indicating only non-specific distributionwas associated with this control antibody.

The quantitative imaging analysis was performed using the manufacturer's software. Tumor-to-muscle ratio, the metric applicable in clinical practice, was calculated based on the imaging quantification data (**Figure 6**). At all time-points, ^{111}In-DOTA-Bavituximab demonstrated a substantially increased tumor-to-muscle ratio (3.7, 5.2, and 3.8 at 48, 72, and 96 hrp.i., respectively) compared to ^{111}In-DOTA-Rituximab (2.4, 1.2, and 0.2 at 48, 72, and 96 hrp.i., respectively).

Discussion

Phosphatidylserine (PS) is a membrane phospholipid normally segregated to the inner mem-

Figure 5. Representative SPECT/CT images of ^{111}In-DOTA-Bavituximab and radiolabeled control antibody (^{111}In-DO-TA-Rituximab) in rats bearing A549 NSCLC tumors (coronal view) at 48, 72, and 96 hr p.i. Yellow circles denote uptake in tumors.

brane leaflet. In apoptotic cells or activated cells such as tumor vascular endothelial cells, PS is exposed on the outer membrane leaflet.

In addition, PS in the tumor microenvironment generates an immunosuppressive signal allowing the tumor to escape immune surveillance.
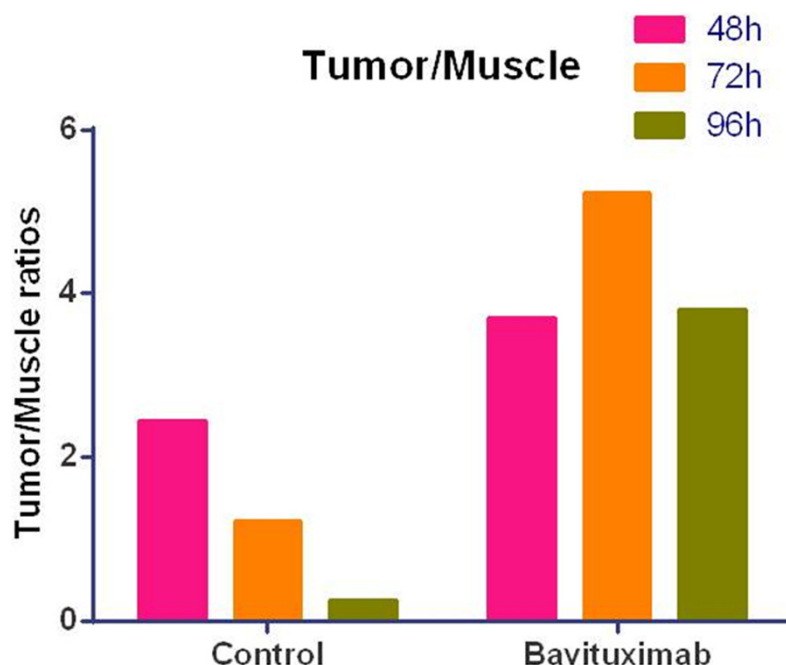
Figure 6. Tumor-to-muscle uptake ratios in A549 NSCLC tumor-bearing rats treated with ^{111}In-DOTA-Bavituximab and radiolabeled control antibody (^{111}In-DOTA-Rituximab) derived from SPECT/CT imaging quantitative analysis.

PS-targeting antibodies, such as Bavituximab, reawaken antitumor immunity, exert a vascular targeting effect, and have demonstrated anti-tumor effects in a number of cancer models [11, 24-27]. In clinical studies, Bavituximab has been investigated in numerous malignancies. In lung cancer, encouraging initial efficacy and safety results have led to phase 3 development. In this study, the ^{111}In-labeled Bavituximab was prepared to explain its low rates of systemic vascular and immune-related toxicities seen clinically and help understand its anti-tumor effects. The tumor-specific activity shown from the in vivo imaging of ^{111}In-DOTA-Bavituximab can help distinguish Bavituximab from other immune modulating and vascular targeting agents.

The isotope of [111]In ($t_{1/2}$: 67 hr) was selected for labeling to best match the biological half-life of Bavituximab (approximately 48 hr) [13]. Our DOTA modification and radiolabeling did not appear to alter immunoreactivity of Bavituximab to PS, as ^{111}In-DOTA-Bavituximab exhibited almost identical PS binding in the ELISA assay as unconjugated Bavituximab antibody did. The biodistribution data showed minimal uptake across the whole rat body within a 72-hr period.

Meanwhile, the SPECT/CT imaging data revealed PS-specific tumor targeting from ^{111}In-DOTA-Bavituximab, not the control antibody of Rituximab. These results correlate with the low rates of systemic vascular and immune-related toxicities of Bavituximab seen clinically, due to the unique feature of PS between normal and cancerous environments.

Dosimetric analysis suggested that ^{111}In-DOTA-Bavituximab could be administered at radiographically useful doses to human patients without imposing significant amount of radiation doses. As with any dose estimates based on data obtained from an animal model, uncertainties always exist in the actual human dose estimates, particularly in the extrapolation models used to produce the human dose calculations and the assumptions applied about excretion of activity. Nevertheless, the acquired dosimetry data still provides a reasonable basis for predicting the approximate human dosimetry.

In this work, we achieved a ratio of 0.54 DOTA per Bavituximab molecule by controlling the conjugation conditions. We intentionally aimed for such a low ratio as it would not alter antibody immunoreactivity after DOTA modification and ^{111}In-labeling. Another consideration was that the resulted ^{111}In-DOTA-Bavituximab might have a significant amount of non-specific binding during the circulation, which would result in a high level of systemic radiation exposure, so high specific activity was not pursued. Although the radiolabeled antibody appeared to have good tumor targeting, absolute tumor uptake may have been compromised by this low ratio due to the resulted low specific activity. The low specific activity might also lead to the lower tumor-to-muscle ratios than those observed with the previously reported [74]As-Bavituximab [23], in addition to other determinants of in vivo tumor targeting. Further studies on ^{111}In-DOTA-

Bavituximab at higher specific activity are warranted if this radioimmunoconjugateis intended for PS-targeted imaging.

Although the A549 NSCLC tumors could be visualized on SPECT/CT images at 48 hrp.i., we noticed the relatively weak tumor accumulation shown at 72 and 96 hr p.i. Low specific activity of ^{111}In-DOTA-Bavituximab aside, the suboptimal sensitivity of SPECT imaging with ^{111}In might play a role as the NanoSPECT/CT system is optimized for the detection of [99m]Tc's gamma emission at 141 keV. Therefore, positron emission tomography (PET) will offer advantages to the development of Bavituximab-based immuno-diagnostic techniques for noninvasive assessment of PS status before and after therapeutic interventions of cancer. Actually, immunoPET with Bavituximab would be straightforward when labeled with [89]Zr or [124]I [28, 29]. Arsenic-74 is another choice but perhaps not ideal due to limited availability and a not-so-well-established radiochemistry procedure [30].

In addition to this proof of principle study, there are numerous potential clinical applications for PS-targeting radiolabeled Bavituximab. Such a radiolabeled antibody could yield insights into Bavituximabtherapeutic efficacy, indicate a tumor's immunologic status, or distinguish between malignant and benign molecularimaging findings. Because standard tissue immunohistochemistry cannot determine whether PS is internalized or exposed on the outer membrane leaflet, only with an *in vivo* diagnostic can tumors be characterized as PS-positive for the purpose of predicting response to PS-directed therapy. Furthermore, because PS is segregated to the inner cell membrane leaflet in most normal tissues, a PS-targeting imaging agent might assist with the determination of whether radiographic abnormalities represent malignancy. Preclinical studies have shown that cancer treatments such as cytotoxic chemotherapy, ionizing radiation, and certain kinase inhibitors enhance PS flipping [25]. The extent to which such effects occur in patients, with which agents they occur most, and whether these effects predict outcomes might be evaluated with a radiolabeled PS-targeting antibody. Separately, *in vivo* characterization of tumor PS exposure might provide insight into a tumor's immunomodulating properties and the potential role for immunotherapies such as vaccines and checkpoint inhibitors. Other possibilities include conjugation of Bavituximab to a therapeutic radioisotope, toxin, or drug to capitalize on the antibody's apparent tumor specificity.

In conclusion, we demonstrated that ^{111}In-DOTA-Bavituximab preserved the in vivo PS targeting of Bavituximab, an acceptable dosimetry profile, and specific accumulation in NSCLC xenografts. These findings may underlie the efficacy and low rates of systemic vascular and immune-related toxicities of Bavituximab seen clinically. In the future, potential clinical applications of ^{111}In-DOTA-Bavituximab may include prediction of Bavituximab efficacy, indication of tumor immunologic status, or distinguishing between malignant and benign radiographic findings. In the near term, modifications of the current radiolabeled compound may improve its future performance, such as altering the DOTA: Bavituximab ratio and employing PET radioisotopes.

Disclosure of conflict of interest

Dr. Gerber reports grants from the American Society of Clinical Oncology, grants from Peregrine Pharmaceuticals, during the conduct of the study. Dr. Hao has nothing to disclose. Dr. Watkins has nothing to disclose. Dr. Barbero has nothing to disclose. Dr. Stafford has nothing to disclose. Dr. Anderson has nothing to disclose. Dr. Holbein has nothing to disclose. Dr. Oz reports grants from NIH NCRR grant (1S10RR029674-01) during the conduct of the study. Dr. Mathews has nothing to disclose. Dr. Thorpe reports grants from Peregrine Pharmaceuticals during the conduct of the study; grants from Peregrine Pharmaceuticals outside the submitted work. Dr. Brekken

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