Pretargeted radioimmunoimaging with a biotinylated D-D\textsubscript{3} construct and 99mTc-DTPA-biotin: strategies for early diagnosis of small cell lung cancer

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Abstract

Objective: Pro-gastrin releasing peptide (ProGRP) plays an oncogenic role in small cell lung cancer (SCLC). The anti-ProGRP\textsubscript{(31-98)} monoclonal antibody D-D\textsubscript{3} can selectively accumulate in SCLC xenografts in nude mice. This study evaluated the effectiveness of a new pretargeting procedure for the early diagnosis of SCLC.

Methods: D-D\textsubscript{3} was radiolabeled with technetium-99m (99mTc) using a three-step pretargeting method. Mice with SCLC xenografts were treated with different labeling regimens, and the biodistribution and radioimmunoimaging were explored. The percentage injected dose per gram (%ID/g) in various organs, tumor/non-tumor (T/NT) ratio, and tumor/background (T/B) ratio were also calculated.

Results: In vivo distribution experiments revealed that 99mTc-DTPA-biotin was metabolized in the liver and kidney, with rapid elimination in the blood. The T/B ratio was highest in mice treated with biotinylated antibody D-D\textsubscript{3} + avidin + 99mTc-DTPA-biotin. Single-photon emission...
computerized tomography imaging further confirmed that the T/B ratio was highest in this group at all time points.

**Conclusions:** In contrast to directly labeled D-D3, pretargeting technology displayed specific enhancement and signal amplification in tumors, which could increase the target tumor uptake of $^{99m}$Tc and provide a new approach for the early diagnosis of SCLC.

**Keywords**
Biotin-avidin, ProGRP$_{(31-98)}$, monoclonal antibody D-D3, biodistribution, radioimmunoimaging, small cell lung cancer

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**Introduction**

Lung carcinoma is the leading cause of cancer-related deaths worldwide, resulting in 1.6 million deaths every year.¹ As a pathological subtype of lung cancer, small cell lung cancer (SCLC) is characterized by high aggressiveness and early metastasis, and accounts for approximately 13% to 20% of all cases.²,³ There is an urgent need to develop a novel antibody with low toxicity, high specificity, and good targeting capability for radioimmunoimaging (RII), to facilitate the prompt diagnosis and effective treatment of SCLC.

In 1978, McDonald et al.⁴ discovered that SCLC stimulated the secretion of gastrin-releasing-peptide (GRP), thus identifying GRP as a potential tumor marker in SCLC. GRP is also expressed in the central and enteric nervous systems and in prostate and breast cancers.⁵,⁶ However, although the clinical application of the neuropeptide GRP is restricted by its instability in serum and short half-life, its precursor, pro-gastrin-releasing peptide (ProGRP), can effectively discriminate between non-SCLC and SCLC in patients, and has been used as a sensitive and reliable tumor marker for SCLC.⁷ More recently, the Chinese Institute for Radiation Protection selected amino acids 31 to 98 of ProGRP (which are common to all three translation products produced by differential mRNA splicing) and produced recombinant ProGRP$_{(31-98)}$. This recombinant peptide was then used as an antigen to immunize BALB/c mice, and the monoclonal antibody D-D3 was successfully prepared.⁸

In the present study, we aimed to investigate the potential of a three-step pretargeting biotin-avidin system (BAS) in combination with anti-ProGRP$_{(31-98)}$ D-D3 for diagnosing SCLC, by evaluating the biodistribution and RII performance in nude mice with SCLC xenografts. We synthesized and characterized biotinylated D-D3, $^{99m}$Tc-diethylene-triamine-pentaacetate (DTPA)-biotin, and compared pretargeting and direct radiolabeling methods in terms of biodistribution and RII.

**Materials and methods**

**Preparation and identification of biotinylated monoclonal D-D3 antibody**

Anti-ProGRP$_{(31-98)}$ monoclonal antibody D-D3 (5 mg/mL; Chinese Institute for Radiation Protection, Taiyuan, China) was dissolved in sodium bicarbonate solution (0.1 mol/L, pH 9.2), and
biotin-activated ester (Sigma Co., MO, USA) was dissolved in dimethyl sulfoxide. The biotin-activated ester and antibody were mixed at a ratio of 8:1 at room temperature for 3 to 4 hours, and the mixture was then purified on a Sephadex G50 chromatography column (Amersham Pharmacia Biotech, GE Healthcare Life Sciences, Shanghai, China). The degree of biotinylation, titer, and activity of the biotinylated antibody were determined using a HABA/Avidin kit and by enzyme-linked immunosorbent assay, respectively (Sigma Co.), according to the manufacturer’s instructions. ProGRP protein expression levels in target tissues were detected by immunohistochemistry using the above biotinylated antibody as the primary antibody.

**99mTc radiolabeling**

**Radiolabeling of D-D3 antibody.** D-D3 was labeled with $^{99m}$TcO$_4^-$ (Xinke Pharmaceutical Co., Shanghai, China) using 2-mercaptoethanol. D-D3 antibody (500 μg, 5 mg/mL) was reduced by 1 μL 2-mercaptoethanol at 30°C for 30 minutes, before radiolabeling. The reduced antibody was then purified on a 2 cm × 6 cm Sephadex G50 column (Soochow University, Suzhou, China) and eluted with the phosphate-buffered saline (PBS; 0.05 mol/L, pH 7.0) at a flow rate of 1 mL/minute. The effluent was collected in 20 centrifuge tubes, and each sample was diluted to 0.5 mL. The mixture was incubated at room temperature for 15 minutes and coloration was measured with a UV/vis spectrophotometer (UNICO Co., WI, USA) at an absorbance of 280 nm. SnCl$_2$ (5 μg; Sigma) was then added to the reduced D-D3 antibody with agitation, followed by 50 μL $^{99m}$TcO$_4^-$ (370 MBq/100 μL), and the mixture was gently shaken and incubated at room temperature for 15 minutes. $^{99m}$Tc-D-D$_3$ was purified by gel column separation, as above. The radiochemical purity was estimated by instant thin-layer chromatography using Xinhua #1 filter paper (Radiation Injury Centre, Soochow University, Suzhou, China) as support and saline as the solute. The stability was determined by adding 50 μL $^{99m}$Tc-D-D$_3$ to 100 μL human serum or normal saline and incubation at 37°C for 1, 2, 4, 6, 8, 10, and 12 hours. The immunocompetence of the labeled product was determined by cell conjugation assay. Briefly, 5 × 10$^6$ NCI-H446 cells were collected, centrifuged twice with PBS, permeabilized with 0.01% Triton X-100 for 1 hour at room temperature, and then incubated with $^{99m}$Tc-D-D$_3$ at 4°C for 4 hours. At the end of the incubation, cells were harvested by centrifugation (111.8 × g twice for 5 minutes). Each supernatant was collected in separate vials, and the cells and supernatants were counted separately for radioactivity using a single-well gamma counter (Nuclear Annular Optical Instrument Co., Ltd., Shanghai, China). The immunobinding rate was $B/T \times 100\%$, where $T$ was the total radioactivity count (including supernatant and cells) and $B$ was the radioactivity count of the precipitated cells.

**Radiolabeling of DTPA-biotin.** Briefly, 50 μL (2 mg/mL) DTPA-biotin (Sigma) was added to 5 μg SnCl$_2$ solution (1 mg/mL) and 45 μL $^{99m}$TcO$_4^-$ (370 MBq/100 μL), followed by mixing at 37°C for 10 minutes. The labeling ratios were determined at different volumes of $^{99m}$TcO$_4^-$ (10, 25, 45, 100, and 200 μL), amounts of SnCl$_2$ (1, 2, 5, 10, and 20 μg), and pH (1, 4, 6.5, 7.4, and 9), using paper chromatography. $^{99m}$Tc-DTPA-biotin was then incubated with normal saline (NS) and healthy serum at 37°C, respectively, and the radiochemical purity was determined at different time points (1, 2, 4, 6, 8, 10, and 12 hours) to assess the stability of the labeled product.
Cell culture and animal models

The human small cell lung cancer cell line NCI-H446 was obtained from the Chinese Academy of Sciences Cell Centre (Shanghai, China) and maintained in Dulbecco’s Modified Eagle Medium (Zhejiang Tian Hang Biological Technology Co., Ltd., Hangzhou, China) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. Female athymic nude mice (3 weeks old) were purchased from Shanghai Experimental Animal Center (China). NCI-H446 cells (2 × 10⁷) in 200 μL Matrigel were injected subcutaneously into the right armpit in each mouse. All animal-related procedures were conducted in accordance with the University of Soochow University Guidelines (Guide for the Care and Use of Laboratory Animals in Experimental Animal Center of Soochow University), and were approved by the Ethics Committee of Soochow University (approval no. ECSU-2016083).

Biodistribution study

Pretargeting biodistribution in nude mice. Forty-five female nude mice (body weight about 30 g) were divided randomly into nine groups (n = 5). Each mouse was administered a three-step pretargeting procedure. Briefly, the mice were injected sequentially with 100 μL (200 μg) of biotinylated antibody D-D₃ (at time [t] = −48 hours), 100 μL (200 μg) of clearing agent (avidin; Sigma) (t = −24 hours), and finally 50 μL (0.148 MBq) of ⁹⁹ᵐTc-DTPA-biotin (t = 0); group b: injected via the tail vein with 100 μL (200 μg) of D-D₃ (t = −48 hours), 100 μL (200 μg) avidin (t = −24 hours), and 50 μL (0.148 MBq) of ⁹⁹ᵐTc-DTPA-biotin (t = 0); group c: injected via the tail vein with 100 μL (200 μg) NS (t = −48 hours), 100 μL (200 μg) avidin (t = −24 hours), and 50 μL (0.148 MBq) of ⁹⁹ᵐTc-DTPA-biotin (t = 0); and group D: directly injected via the tail vein with 50 μL ⁹⁹ᵐTc-D-D₃ (0.148 MBq). Similar to the biodistribution study, all mice were sacrificed at the designated time points (1, 2, 4, 8, 12, and 24 hours) by cervical dislocation after injection of the radiotracer. Blood and selected organs were collected, and tissues and organs were blotted, weighed, and counted in a gamma scintillation counter (Shanghai Nuclear Institute Rihuan Photoelectric Instrument Co.) with standards of the injected dose. The %ID/g and tumor/non-tumor (T/NT) ratio were calculated.

RII study

The mice were divided into four groups, a, b, c, and d, when the tumor diameter reached 10 mm, similar to the biodistribution study. Five mice from each group were selected for gamma imaging studies.
Before conducting the imaging studies, thyroid uptake of $^{99m}$Tc was blocked by pretreatment with 0.1% potassium iodide in 5% glucose solution for 5 days. Tumor-bearing mice in groups a, b, and c were injected intraperitoneally with 500 μL (7.4 MBq) $^{99m}$Tc-DTPA-biotin, and mice in group D were injected with 500 μL (7.4 MBq) $^{99m}$Tc-D-D3. SPECT/CT scans (Siemens, Munich, Germany) were conducted at 1, 2, 4, 8, 12, and 24 hours after injection. The mice were placed on a single-photon emission computerized tomography (SPECT)/computed tomography bed, ventral side down. Images with an acquisition matrix of 128 × 128 at a preset count of 500 kilocounts were taken using a planar scintigraphic camera. Regions of interest were drawn over the tumors, and the tumor/background (T/B) ratios were determined.

Statistical analysis

Quantitative data were expressed as mean± standard deviation. Results were compared among more than two groups by one-way ANOVA. A P value < 0.05 was considered statistically significant.

Results

Preparation and identification of biotinylated monoclonal D-D3 antibody

The biotinylated monoclonal antibody D-D3 was successfully constructed. The average molar biotin:antibody ratio was 2.5, with a quality ratio of active biotin ester and antibody of 8:1. The titer of biotinylated D-D3 was 1:3,200, and the immunocompetent fraction was 90.92%. Immunohistochemistry demonstrated positive granules in the cytoplasm of NCI-H446 SCLC cells when using biotinylated D-D3 (group b) and D-D3 (group c) as primary antibodies, with similar intense expression of ProGRP (Figure 1). However, no obvious staining was found in the negative control group, in which PBS was used instead of primary antibody (group d).

$^{99m}$Tc radiolabeling

Radiolabeling of D-D3 antibody. The labeling rate and radiochemical purity of $^{99m}$Tc-D-D3 were 78.69±3.11% and 92.14±3.55%, respectively. The elution curve of the Sephadex G50 chromatographic column is shown in Figure 2a. After incubation with NS at 37°C for 8 hours, the radiochemical purity was 64.73±0.34%, and this fell to 54.45±0.95% at 12 hours (Figure 2b). The radiochemical purity after incubation with healthy human serum at 37°C for 8 hours was 65.49±0.45%, falling to 52.92±0.84% at 12 hours. The immunobinding rate of $^{99m}$Tc-D-D3 to NCI-H446 cells was 82.1±2.45%.

Radiolabeling of DTPA-biotin. The radiochemical purity of $^{99m}$Tc-DTPA-biotin was 89.45±0.24%. The elution curve of the Sephadex G50 chromatographic column is shown in Figure 2c. The radiochemical purity after incubation with NS at 37°C for 8 hours was 65.46±1.86%, and this fell to 58.54±0.25% at 12 hours (Figure 2d). The radiochemical purity after incubation with healthy human serum at 37°C for 8 hours was 70.36±1.46%, falling to 60.81±1.01% at 12 hours. The $^{99m}$Tc radio-labeling rate of DTPA-biotin was affected by labeling volume, pH, and the amount of SnCl2 solution, and the reaction conditions were optimized by changing the above factors. The labeling ratio increased to 88.50% after the addition of SnCl2 (5 μg) to the 100 μL labeling volume (pH 7.4) (Figure 3).

Biodistribution studies

Pharmacokinetics of biotinylated D-D3 and $^{99m}$Tc-DTPA-biotin in nude mice. Immediately before sacrifice, nude mice underwent the
three-step pretargeting protocol with biotinylated D-D3, followed by clearing agent (avidin) and 99mTc-DTPA-biotin. The % ID/g data for various organs at different time points are summarized in Table 1. At 5 minutes post-injection of 99mTc-DTPA-biotin, the radiotracer was mainly distributed in the blood, liver, and kidney, but was also detected in the intestine, bone, and brain. The %ID/g values in all organs were reduced after 1 hour, especially in blood ($P = 0.009$).

Biodistribution in nude mice with SCLC xenografts. 99mTc-DTPA-biotin accumulated in tumor xenografts in nude mice (Figure 4a-1). As early as 4 hours post-injection, tumor uptake reached a maximum of 1.816 ± 0.001%ID/g. Despite a subsequent decline to 0.598 ± 0.069%ID/g, the level remained higher than in the other observed organs. The T/NT ratios for other organs and tissues increased gradually with time, and peaked at 24 h post-injection (Figure 4a-2). Owing to the low blood level and rapid blood clearance, the tumor-to-blood ratio increased significantly over time (1.016 ± 0.186, 5.533 ± 0.004 and 8.891 ± 0.704 at 1, 4, and 24 hours after injection, respectively; all $P < 0.05$). The %ID/g between blood and tumor was similar at each time point in the negative

Figure 1. Expression of pro-gastrin releasing peptide (ProGRP) protein in small cell lung cancer using different primary antibodies. Sections stained with hematoxylin (original magnification ×400). Hematoxylin–eosin staining showed that the tumor cells were large and round, with nucleoli and nuclear separation (a). Intense expression of ProGRP in tissues stained with (b) biotynlated D-D3 and (c) D-D3 primary antibodies, respectively, and no obvious staining in negative control using PBS as primary antibody (d).
Figure 2. Elution curves and radiochemical purity. (a) Elution curve of $^{99m}$Tc-D-D$_3$ and (b) radiochemical purity of $^{99m}$Tc-D-D$_3$ in vitro and in vivo at different time points. (c) Elution curve of $^{99m}$Tc-DTPA-biotin and (d) radiochemical purity of $^{99m}$Tc-DTPA-biotin in vitro and in vivo at different time points.

Figure 3. Labeling rates of $^{99m}$Tc-DTPA-biotin. (a) Labeling rate of $^{99m}$Tc-DTPA-biotin as a function of labeling volume. Reaction conditions: 50 μL DTPA-biotin (2 mg/mL), 5 μg SnCl$_2$ (1 mg/mL), and $x$ μL $^{99m}$TcO$_4^-$ (370 MBq/100 μL) at pH 7.4, with mixing at 37°C for 10 minutes. (b) Labeling rate of $^{99m}$Tc-DTPA-biotin as a function of amount of SnCl$_2$. Reaction conditions: 50 μL DTPA-biotin, $x$ μg SnCl$_2$ (1 mg/mL), and 45 μL $^{99m}$TcO$_4^-$ (370 MBq/100 μL) at pH 7.4, with mixing at 37°C for 10 minutes. (c) Labeling rate of $^{99m}$Tc-DTPA-biotin as a function of pH. Reaction conditions: 50 μL DTPA-biotin, 5 μg SnCl$_2$ (1 mg/mL), and 45 μL $^{99m}$TcO$_4^-$ (370 MBq/100 μL) at pH x, with mixing at 37°C for 10 minutes.
Table 1. Biodistribution of biotinylated D-D3, avidin, and $^{99m}$Tc-DTPA-biotin in nude mice.

| Organ (%ID/g) | Time after injection of $^{99m}$Tc-DTPA-biotin |
|--------------|-----------------------------------------------|
|              | 5 minutes | 15 minutes | 30 minutes | 1 hour | 2 hours | 4 hours | 8 hours | 12 hours | 24 hours |
| Heart        | 1.828 ± 0.08 | 1.791 ± 0.040 | 1.169 ± 0.019 | 1.037 ± 0.105 | 0.831 ± 0.033 | 0.622 ± 0.018 | 0.204 ± 0.006 | 0.054 ± 0.000 | 0.032 ± 0.001 |
| Liver        | 2.40 ± 0.110 | 1.527 ± 0.024 | 1.314 ± 0.225 | 1.028 ± 0.309 | 0.826 ± 0.107 | 0.698 ± 0.147 | 0.643 ± 0.035 | 0.459 ± 0.041 | 0.214 ± 0.011 |
| Spleen       | 1.852 ± 0.671 | 1.568 ± 0.039 | 1.093 ± 0.022 | 0.927 ± 0.194 | 0.753 ± 0.121 | 0.656 ± 0.197 | 0.639 ± 0.190 | 0.503 ± 0.035 | 0.188 ± 0.025 |
| Lung         | 1.308 ± 0.016 | 1.057 ± 0.484 | 1.016 ± 0.172 | 0.813 ± 0.270 | 0.619 ± 0.095 | 0.416 ± 0.017 | 0.174 ± 0.012 | 0.105 ± 0.003 | 0.083 ± 0.006 |
| Kidney       | 3.691 ± 0.465 | 4.155 ± 0.017 | 4.748 ± 0.490 | 3.179 ± 0.247 | 2.317 ± 0.923 | 1.542 ± 0.163 | 0.713 ± 0.092 | 0.524 ± 0.114 | 0.369 ± 0.043 |
| Stomach      | 1.641 ± 0.005 | 1.463 ± 0.331 | 1.080 ± 0.598 | 0.957 ± 0.081 | 0.914 ± 0.044 | 0.873 ± 0.053 | 0.699 ± 0.088 | 0.314 ± 0.011 | 0.115 ± 0.001 |
| Intestine    | 0.667 ± 0.247 | 0.564 ± 0.163 | 0.479 ± 0.034 | 0.391 ± 0.114 | 0.346 ± 0.026 | 0.205 ± 0.061 | 0.091 ± 0.015 | 0.075 ± 0.002 | 0.054 ± 0.004 |
| Muscle       | 0.875 ± 0.130 | 0.811 ± 0.051 | 0.775 ± 0.128 | 0.504 ± 0.056 | 0.367 ± 0.029 | 0.279 ± 0.018 | 0.172 ± 0.036 | 0.109 ± 0.003 | 0.068 ± 0.001 |
| Bone         | 0.549 ± 0.027 | 0.504 ± 0.090 | 0.370 ± 0.091 | 0.368 ± 0.0712 | 0.337 ± 0.012 | 0.219 ± 0.059 | 0.071 ± 0.001 | 0.045 ± 0.012 | 0.029 ± 0.011 |
| Brain        | 0.495 ± 0.209 | 0.383 ± 0.119 | 0.348 ± 0.078 | 0.238 ± 0.042 | 0.214 ± 0.051 | 0.115 ± 0.022 | 0.049 ± 0.007 | 0.026 ± 0.006 | 0.013 ± 0.000 |
| Blood        | 3.13 ± 0.251 | 2.190 ± 0.090 | 1.350 ± 0.062 | 1.319 ± 0.165 | 1.165 ± 0.090 | 0.682 ± 0.014 | 0.212 ± 0.002 | 0.089 ± 0.013 | 0.075 ± 0.003 |

Values given as mean ± standard deviation. N = 5 per group.
control group (b), with no antibody D-D3 (Figure 4b-1), and the T/NT ratio was significantly lower in group B compared with group a (Figure 4b-2). $^{99m}$Tc-DTPA-biotin was mainly metabolized through the kidney in group c (blank control), with little uptake in blood and muscle (Figure 4c-1), and the tumor/kidney ratio was $<$0.2 at all time points (Figure 4c-2). $^{99m}$Tc was used to directly label D-D3 in the positive control (group d), and $^{99m}$Tc-D-D3 was mainly distributed in the liver and kidney, with lower intensities in the muscle, bone, and brain. Tumor uptake reached a maximum at 8 hours post-injection ($0.785 \pm 0.086\%$ID/g) (Figure 4d-1). The T/NT ratios for blood, muscle, and brain at 24 hours post-injection were $2.018 \pm 0.155$, $4.817 \pm 0.589$, and $7.449 \pm 1.102$, respectively (Figure 4d-2). Compared with group a, the direct labeling method resulted in higher blood uptake and a lower T/NT ratio. The tumor/blood and tumor/muscle ratios at 12 and 24 hours post-injection were significantly higher in the experimental (group a) compared with the other three control groups ($P < 0.05$ or $< 0.01$) (Table 2).

**RII study**

The tumor mass in SCLC xenografted nude mice was observed at 2 hours after injection of $^{99m}$Tc-DTPA-biotin in group a (Figure 5a). The radioactivity uptake of the tumor mass increased gradually with time, and the tumor contours gradually

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**Figure 4.** Biodistribution of $^{99m}$Tc-DTPA-biotin and $^{99m}$Tc-D-D3 at different time points in nude mice with small cell lung cancer xenografts. (a) Pretargeted with biotinylated D-D3, clearing agent avidin, and $^{99m}$Tc-DTPA-biotin; (b) pretargeted with biotin, clearing agent avidin, and $^{99m}$Tc-DTPA-biotin; (c) pretargeted with normal saline and $^{99m}$Tc-DTPA-biotin; and (d) $^{99m}$Tc-D-D3 alone. (1) Columns indicate mean %ID/g and error bars represent standard deviation (SD); (2) lines represent tumor/non-tumor (T/NT) and error bars represent SD.
became clear. The tumor mass was apparent on imaging at 4 hours, and was still visible at 24 hours. There was no apparent accumulation of radioactivity in the tumor masses in groups b and c from 1 to 24 hours, and the radiotracer was mainly metabolized through the urinary system (Figure 5b and c). Radioactivity in the tumor accumulated gradually over time in group d, and a visible tumor image was

Table 2. Tumor/blood and tumor/muscle ratios between experimental and control groups.

| Group | 12 hour tumor/blood | 12 hour tumor/muscle | 24 hour tumor/blood | 24 hour tumor/muscle |
|-------|---------------------|----------------------|---------------------|---------------------|
| a     | 8.347 ± 0.21        | 7.003 ± 2.187        | 8.891 ± 0.704       | 6.913 ± 1.398       |
| b     | 1.237 ± 0.257       | 0.840 ± 0.094        | 1.549 ± 0.331       | 1.711 ± 0.122       |
| c     | 1.194 ± 0.109       | 0.774 ± 0.241        | 1.541 ± 0.490       | 1.092 ± 0.429       |
| d     | 1.920 ± 0.350       | 3.628 ± 0.817        | 2.018 ± 0.155       | 4.817 ± 0.589       |

Values given as mean ± standard deviation. N = 5 per group. Regarding 12 hour tumor/blood, 12 hour tumor/muscle, 24 hour tumor/blood, and 24 hour tumor/muscle: group a vs group b: P < 0.01, 0.008, < 0.01, and 0.003; group a vs group c, P < 0.01, 0.008, < 0.01, 0.002; and group a vs group d, P < 0.01, 0.036, < 0.01, 0.075, respectively.

Figure 5. Single-photon emission computerized tomography radioimmunoimaging of nude mice with transplanted small cell lung cancer xenografted tumors at 1, 2, 4, 8, 12, and 24 hours after injection of 7.4 MBq $^{99m}$Tc-DTPA-biotin or $^{99m}$Tc-D-D3. (a) Pretargeted with biotinylated D-D3, avidin, and $^{99m}$Tc-DTPA-biotin; (b) pretargeted with biotin, avidin, and $^{99m}$Tc-DTPA-biotin; (c) pretargeted with normal saline and $^{99m}$Tc-DTPA-biotin; and (d) $^{99m}$Tc-D-D3 alone. Tumors indicated by arrows.
acquired at 8 hours (Figure 5d). The T/B ratios in the corresponding contralateral sites at various time points are summarized in Table 3. The T/B ratios in group a increased progressively with time and were significantly higher compared with the other three groups ($P < 0.05$ or $< 0.01$). The T/B ratio in group a peaked at 5.22 ± 1.013 at 24 hours post-injection, while the ratios in groups b and c remained relatively steady, and were <2 at 24 hours post-injection. Although the T/B ratios in group d were higher than in groups b and c, peaking at 4.159 ± 0.285 at 24 hours post-injection, they were still lower than in group a.

**Discussion**

We previously labeled anti-ProGRP(31-98) monoclonal D-D3 antibody with $^{131}$I and showed that $^{131}$I-D-D3 was specifically and highly concentrated in ProGRP-expressing malignant sites. However, it was cleared slowly in the blood, with a long tumor imaging time (approximately 72 hours). The long physical half-life of $^{131}$I (8.04 days) and high gamma ray energy thus limit its use for imaging. We therefore selected $^{99m}$Tc, which has a shorter half-life (6.02 hours) and lower energy (140 keV), to label anti-ProGRP(31-98) monoclonal D-D3 antibody in subsequent experiments, with some satisfactory results. However, the large molecular weight of the complete monoclonal antibody means that the metabolism of $^{99m}$Tc-D-D3 in the blood is also slow, and the optimal RII time is still long. In addition, considerable effort has been made to develop novel methods for detecting SCLC at an early stage of tumor growth, before it becomes invasive, with the aim of improving patient survival by allowing earlier and more effective treatments.

The pretargeting concept was proposed by Reardan et al. in 1985, as an alternative to direct coupling of a chelate and antibody. Unlike direct targeting strategies which use concurrent administration of targeting agents in one system, pretargeting involves separating the accumulation of targeting agents at the tumor lesion from the subsequent delivery of the imaging drug molecules, which can shorten the chelation time of radioactive markers in vivo, thus reducing the dose absorbed by normal tissues and avoiding damage caused by directly radiolabeled antibodies. Hnatowich et al. introduced the BAS for RII in 1987, with high sensitivity, specificity, and stability. Paganelli et al. subsequently explored the potential of BAS by devising a three-step procedure based on the administration of a cold biotinylated antibody, followed by an excess of cold avidin, and finally a radiolabeled biotin derivative. Rossi and Mcbride et al. found that

| Group | Time after injection of radiotracer |
|-------|------------------------------------|
|       | 1 hour | 2 hours | 4 hours | 8 hours | 12 hours | 24 hours |
| a     | 1.819 ± 0.312 | 2.370 ± 0.435 | 3.053 ± 0.704 | 3.607 ± 0.233 | 4.641 ± 1.118 | 5.22 ± 1.013 |
| b     | 1.108 ± 0.123* | 1.205 ± 0.153* | 1.390 ± 0.648* | 1.421 ± 0.075* | 1.501 ± 0.0212* | 1.580 ± 0.247* |
| c     | 1.093 ± 0.214# | 1.212 ± 0.166# | 1.412 ± 0.024# | 1.465 ± 0.027# | 1.509 ± 0.031# | 1.639 ± 0.068# |
| d     | 1.467 ± 0.263 | 1.780 ± 0.094† | 2.015 ± 0.158† | 2.841 ± 0.122† | 4.34 ± 0.103† | 4.159 ± 0.285† |

Values given as mean ± standard deviation. $N=5$ per group. *$P < 0.01$ compared with group a; # $P < 0.05$ compared with group c; † $P > 0.05$ compared with group d.
pretargeting procedures could be used in combination with $^{99m}$Tc or $^{124}$I for superior imaging capability compared with directly radiolabeled antibody fragments. Recent studies also showed that pretargeted scintigraphy may improve imaging for early tumor detection, diagnosis, and treatment.$^{16-21}$ In addition, pretargeting technology has the advantage of allowing the application of short half-life nuclides, such as $^{99m}$Tc.

The metabolism of $^{99m}$Tc-DTPA-biotin in nude mice was consistent with a two-compartment model with first-order absorption. The %ID/g of blood and major organs was quickly reduced, to <0.1%ID/g in blood at 24 hours. This rapid clearance was favorable compared with direct labeling, and these properties are beneficial for radioimmunological imaging and related applications in the diagnosis and treatment of diseases. The in vivo biodistribution of $^{99m}$Tc-DTPA-biotin in nude mice bearing SCLC xenografts showed that radiotracer levels in the blood were still low in group a, and the tumor/blood ratio increased significantly over time. The blood/tumor %ID/g in group b was similar at each time point, and the T/NT ratio was significantly lower than in group a. The results for group c showed that $^{99m}$Tc-DTPA-biotin was mainly metabolized through the kidney, with little uptake in the blood and muscle, and the T/NT ratio was significantly lower than in group a. The tumor/blood ratio in group d was higher than in groups b and c but lower than in group a, demonstrating that the three-step procedure of BAS had high binding affinity and specificity.

SPECT images in group a were consistent with the biodistribution results, showing high concentrations of the tracer in liver and kidney. There are at least two possible explanations for this finding: first, because of the administration volume, we injected intraperitoneally, which might have affected tumor accumulation, and second, the biotinylated D-D$_3$, avidin, and $^{99m}$Tc-DTPA-biotin might have been deposited and excreted through the liver and kidney. Additionally, the tumor mass was apparent on imaging at 4 hours and the tumor/blood ratio peaked at 24 hours. The radioactivity in groups b and c was mainly metabolized by the urinary system. Tumor radioactivity in group d accumulated gradually over time, and a visible tumor image was acquired at 8 hours, consistent with the time for conventional RII of 12 to 14 hours.

This study had some limitations. First, the study required several injections of milligrams of protein within a specific time frame, which may have caused an immune response and thus complicated the outcomes. Further studies are therefore needed to explore the optimal reaction conditions, such as dose, interval, and time of administration. Second, because of the administration volume, we used intraperitoneal rather than tail vein injection, which might have affected the tumor accumulation, led to higher radioactivity accumulation in the liver and kidney, and might have resulted in complex formation between the biotinylated antibody and radiolabeled biotin in the circulation. Finally, unlike Fab fragments, D-D$_3$ is a full antibody with a long circulating half-life, which may lead to poor pharmacokinetic characteristics of the radiotracer. In addition, the potential toxicity of antibodies, such as human anti-mouse antibody (HAMA), means that they should be used with caution in future study. Indeed Aarts et al.$^{22}$ found that HAMA developed in 61% of patients treated with a murine antibody, while Liu et al.$^{23}$ showed that antibodies derived from human sequences could effectively locate novel therapeutic targets and may be less immunogenic than those derived from rodent sequences.
In conclusion, our results suggest that pretargeting technology using D-D$_3$ and $^{99m}$Tc was effective in nude mice with SCLC xenografts, and the superior signal amplification could increase the radiopharmaceutical concentration in the tumor. Further studies are planned to determine how to reduce the accumulation of radioactivity in other organs, while increasing the tumor concentration and avoiding an immunogenic response to the avidin–biotin interaction, such as by adopting intravenous injection, using smaller antibody fragments, and bioorthogonal click chemistry reactions.

**Declaration of conflicting interest**

The authors declare that there is no conflict of interest.

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