Preclinical screening of anti-HER2 nanobodies for molecular imaging of breast cancer

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ABSTRACT Accurate determination of tumor human epidermal growth factor receptor 2 (HER2)-status in breast cancer patients is possible via noninvasive imaging, provided adequate tracers are used. In this study, we describe the generation of a panel of 38 nanobodies, small HER2-binding fragments that are derived from heavy-chain-only antibodies raised in an immunized dromedary. In search of a lead compound, a subset of nanobodies was biochemically characterized in depth and preclinically tested for use as tracers for imaging of xenografted tumors. The selected compound, 2Rs15d, was found to be stable and to interact specifically with HER2 recombinant protein and HER2-expressing cells in ELISA, surface plasmon resonance, flow cytometry, and radioligand binding studies with low nanomolar affinities, and did not compete with anti-HER2 therapeutic antibodies trastuzumab and pertuzumab. Single-photon-emission computed tomography (SPECT) imaging quantification and biodistribution analyses showed that 99mTc-labeled 2Rs15d has a high tumor uptake in 2 HER2+ tumor models, fast blood clearance, low accumulation in nontarget organs except kidneys, and high concomitant tumor-to-blood and tumor-to-muscle ratios at 1 h after intravenous injection. These values were dramatically lower for an irrelevant control 99mTc-nanobody and for 99mTc-2Rs15d targeting a HER2– tumor.—Vaneycken, I., Devoogdt, N., Van Gassen, N., Vincke, C., Xavier, C., Wernery, U., Muyldermans, S., Lahoutte, T., Caveliers, V. Preclinical screening of anti-HER2 nanobodies for molecular imaging of breast cancer. FASEB J. 25, 2433–2446 (2011). www.fasebj.org

Key Words: tracer development • SPECT

Malignant tumors are often characterized by the presence of specific proteins on their cell membrane. These cancer antigens can be expressed in relatively high amounts, and their expression level is often related to a signal transduction cascade that contributes to or promotes the cancer phenotype. Hence, these membrane antigens represent an important target for anticancer treatment. One of the most frequently diagnosed cancers in the European female population is breast cancer (1). Although breast cancer is a very heterogeneous disease (2), 20–30% show overexpression of the human epidermal growth factor receptor 2 (HER2), in most cases caused by the amplification of the HER2 locus in the tumor cells (3). Signaling of this tyrosine kinase receptor causes cell proliferation, enhances cell mobility, and reduces apoptosis. Consequently, HER2 overexpression is associated with tumor aggressiveness and an increased probability for recurrent disease. Targeted therapies for HER2-overexpressing tumors are currently available, such as the specific tyrosine kinase inhibitor lapatinib (GlaxoSmithKline, London, UK), which interacts with the intracellular domain of HER2, and the monoclonal antibodies trastuzumab and pertuzumab (both from Hoffman-La Roche, Basel, Switzerland), which bind to different domains on the extracellular part of HER2 (4, 5). For this reason, determination of the HER2 status of tumors is essential in selecting patients who qualify for anti-HER2 treatment. At present, the HER2 status of breast tumors is determined by 2 clinically validated in vitro tests, immunohistochemistry and/or fluorescence in situ hybridization (4). Both methods require a biopsy that is obtained from the primary tumor, also when metastatic lesions are present (6). However, the HER2 status of the biopsy is not representative for the general HER2 status in the case of intratumoral and intertumoral heterogeneity of HER2 expression (7, 8). A possible strategy that allows evaluation of HER2 expression simultaneously in all present tumor lesions is the use of noninvasive molecular imaging (9). This tech-
unique offers the advantage that the entire tumor lesion, as well as possible metastatic lesions, can be evaluated by a single scanning procedure. In addition, sampling errors with biopsies are avoided, and the procedure can be repeated multiple times so that HER2 expression can be monitored during the disease process. This allows selection of patients that are susceptible to a targeted therapy and to monitor the therapy response. Both antibodies and antibody fragments prove to be suitable for imaging of HER2+ tumors (9, 10, 11). In most cases, it was demonstrated that these diagnostic agents have the ability to bind HER2, both in vitro and in vivo. However, because of their size and interactions with Fc receptors, conventional antibodies are removed too slowly from the blood, resulting in high background activity, and hence good contrast images can only be acquired after hours or days. In addition, the biodistribution often shows, besides high tracer concentrations in the blood, intense accumulation in the liver or excretion through the gastrointestinal tract. Smaller antigen-binding fragments, such as Fabs and scFvs, that are derived from conventional antibodies exhibit a shorter blood half-life but frequently lack sufficient robustness and solubility, resulting in increased nonspecific uptake in nontarget organs (12, 13). Such a background activity is not optimal for the imaging process and limits the clinical use of these tracers. In addition, because of the general inappropriate biophysical and biochemical characteristics of these conventional antibody derivatives, the discovery process for adequate imaging probes can be a tedious process.

Nanobodies offer a solution to these problems. Nanobodies are the antigen-binding fragments from heavy-chain-only antibodies, nonconventional single-chain antibodies occurring naturally in Camelidae. Nanobodies are considered to be the smallest intact antigen-binding fragments derived from a functional antibody. Because of their limited size of ~15 kDa and their general robustness, they possess some important properties that make them very suitable for in vivo radioimmunodetection of tumors and evaluation of tumor antigen expression (14–17).

This report focuses on the identification, production, and purification of a large number of anti-HER2 nanobodies, their subsequent characterization, and the ultimate selection of the lead compound for in vivo tumor imaging with single-photon-emission computed tomography (SPECT). The generation of nanobodies against a new antigen usually provides multiple candidates that are considered for further development. Criteria that we used for the selection process were ease of production and purification, 99mTc labeling efficiency, the affinity of the anti-HER2 nanobodies toward HER2, competition with the monoclonal antibodies trastuzumab and pertuzumab, and in vivo tumor targeting potential and biodistribution in a mouse xenograft model (see Fig. 1). New, preclinically validated 99mTc-labeled HER2 targeting tracers suitable for molecular imaging using SPECT cameras were acquired. Translation to clinical applications is possible.

### MATERIALS AND METHODS

#### Dromedary immunization and evaluation of immune responses

A dromedary was immunized 6 times at weekly intervals with 100 μg HER2-Fc recombinant fusion protein (R&D Systems, Minneapolis, MN, USA) in Gerbu adjuvant. Serum IgG subclasses were separated via protein A and G chromatography, as described previously (18), and tested in flow cytometry on HER2+ and HER2− cells. To this end, CHO cells were made partially HER2+ via 48-h transient transfection using FugeneHD (Roche, Indianapolis, IN, USA) and the plasmid pORF9-hErbB2 (Inviogen, San Diego, CA, USA). Transfected and nontransfected cells (106) were stained sequentially with 5 μg dromedary IgGs, 1 μg rabbit anti-dromedary IgG polyclonal antibody, and 200 ng Alexa Fluor 647-labeled goat anti-rabbit IgG antibody, measured on a FACS Canto II analyzer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

#### Generation of phage-display library and selection of anti-HER2 nanobodies

The anti-HER2 nanobody phage-display library was generated according to published methods (19). In short, lymphocytes were purified from the blood of the immunized animal by density centrifugation, RNA was purified, and total cDNA was generated. This was used as a template for 2-step PCR that amplifies the DNA encoding all variable domains of heavy-chain-only antibodies generated in this immune animal. This pool of amplified nanobody DNA fragments was ligated in the phage-display vector pHEN4 in fusion with phage gene III, and transformed into Escherichia coli TG1 cells to generate a library of 1.5 × 108 transformants.

Next, nanobodies were phage displayed, essentially as described previously (19), and biopannings were performed either on immobilized HER2-Fc protein or on adherent, formalin-fixed HER2+ cells (SKBR3 or BT474). Interacting phages were recovered by alkaline elution and reamplified to use in a second round of biopanning.

After each panning round and for all 3 conditions, colonies were randomly picked, and nanobody-containing periplasmic extracts were made. These extracts were used to screen HER2-specific nanobodies in 2 steps: in ELISA to bind to HER2-Fc protein but not to E-selectin-Fc (a protein unrelated to HER2, but with the same Fc fusion protein); and in flow cytometry to bind to HER2+ SKBR3 or BT474 cells but not to HER2− MDA-MB-435D cells. Sequencing of positive-scoring constructs was determined by an automated DNA sequencer (ABI Prism 3100 genetic analyzer; Applied Biosystems, Foster City, CA, USA), and nanobody protein sequences were aligned.

#### Production and purification of selected nanobodies

All unique anti-HER2 nanobody DNA fragments were cloned in expression vector pHE.N6 to contain a carboxyterminal hexahistidine tail and were produced in 1L Escherichia coli WK6 cultures. Control nanobodies cAbBcII10 and cAbLys3 (20) were produced similarly. Nanobody expression was induced overnight at 28°C with 1 mM isopropyl-β-D-thiogalactoside (IPTG). Periplasmic extracts containing the soluble nanobodies were obtained by osmotic shock. Nanobodies were further purified using immobilized metal affinity chromatography (IMAC) on Ni-NTA resin (Sigma-Aldrich, St. Louis, MO, USA) and gel filtration on Superdex 75 HR 16/60 (Pharmacia, Gaithersburg, MD, USA) in PBS.
Cell lines and culture conditions

CHO cells, the human HER2+ colon carcinoma cell line LS174T, the human HER2+ breast cancer cells SKBR3 and BT474, and the human HER2+ ovarian cancer cell line SKOV3 were obtained from American Type Culture Collection (Manassas, VA, USA). The HER2- breast cancer cell line MDA-MB-435D was kindly provided by Dr. Geert Berx (Department of Molecular and Biomedical Research, University of Ghent, Ghent, Belgium). LS174T cells were cultured in RPMI medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Paisley, UK). SKOV3 and SKBR3 cells were cultured in McCoy’s 5A, MDA-MB-435D, BT474 cells in DMEM, and CHO cells in RPMI medium, all supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin (Invitrogen). All cells were grown in monolayer in Falcon tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at 37°C in a humidified incubator with 5% CO2. Cells were detached with trypsin-EDTA in PBS (Invitrogen).

ELISA

In HER2-binding studies, 100 ng HER2-Fc protein was immobilized on immunosorbent plates (Nunc, Rochester, NY, USA) and blocked with 1% milk. Alternatively, SKOV-3 cells (10⁴/well) were adhered overnight at 37°C in 96-well plates and fixed with formalin for 10 min at room temperature. Periplasmic extracts or purified nanobodies (100 µl) were added for 1 h at room temperature. Nanobody binding was detected by sequential 1-h incubations at room temperature with 1 g/ml anti-HA (pHEN4) or anti-His antibody (pHEN6) (both from AbDSerotec, Oxford, UK), 1 g/ml alkaline-phosphatase-coupled anti-mouse IgG1 antibody, and 2 mg/ml phosphatase substrate (both from Sigma-Aldrich). Wells were washed 3–5 times with PBS-0.1% Tween between each step. OD405 was quantified on an Ultra Microplate reader (Biotek Instruments, Winooski, VT, USA). In saturation-binding experiments, serial dilutions of purified nanobodies from 3.7 × 10⁻¹⁶ M to 0.02 nM were tested in ELISA. OD₄₀₅ signals in function of nanobody concentrations were fit via nonlinear regression and analyzed with GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA) to determine Kₐ values.

Binding of nanobodies in flow cytometry

Cells were collected, washed, and counted. Per 10⁵ cells, either 100 µl periplasmic extract, 1 µg purified nanobody or 20 µl PE-labeled anti-HER2 antibody (BD Pharmingen, Erembodegem, Belgium) was added and incubated for 1 h at
Surface plasmon resonance (SPR)

SPR measurements were performed on a Biacore T100 instrument (GE Healthcare, Little Chalfont, UK). HER2-Fc protein was coupled on a CM5 chip to 740 response units (RU). All analytes were flowed at 30 μl/min in HBS, and the chip was regenerated using 10 mM glycine-HCl pH 2.0. For determination of binding kinetics, sensograms were generated of 1/2-diluted nanobody solutions. Binding was allowed for 180 s and dissociation for 600 s. Curves were fitted using Biacore’s evaluation software using a 1:1 antigen:analyte binding model to retrieve association rate constants (kₐ), dissociation rate constants (kᵩ), and equilibrium dissociation constants (Kᵦ, a measurement of affinity).

For competition experiments, nanobodies and trastuzumab were used at 1 μM and pertuzumab at 10 μM. Trastuzumab and pertuzumab were a kind gift from Gentech (San Francisco, CA, USA). A first 600 s binding phase with either nanobody or antibody was followed by a second 600 s binding phase with a mixture of test compound and competitor. Curves were analyzed using Biacore’s evaluation software and visually interpreted.

Animal models

Female athymic nude mice were injected s.c. in the right hindlimb with either SKOV3 (4*10⁶), MDA-MB-435D (1*10⁶), or LS174T cells (1*10⁶) in PBS under the control of 2.5% isoflurane (Abbott, Ossenigiis-Louvain-la-Neuve, Belgium). Tumors were allowed to grow up to 250–500 mm³. The animal study protocol was approved by the ethical committee for animal research of the Vrije Universiteit Brussel.

Labeling procedure

Nanobodies were labeled with [99mTc(H₂O)₃(CO)₃]⁺ at their His₆-tail, as described previously (17). [99mTc(H₂O)₃(CO)₃]⁺ was added to 1 mg/ml nanobody solution and incubated for 90 min at 50°C. After labeling, the 99mTc-nanobody solution was purified on a NAP-5 column (GE Healthcare) preequilibrated with PBS to remove unbound [99mTc(H₂O)₃(CO)₃]⁺ and passed through a 0.22-μm filter (Millipore, Billerica, MA, USA) to eliminate possible aggregates.

Saturation binding experiments with 99mTc-nanobodies

10⁴ SKOV3 cells were grown for 24 h in 96-well culture plates and formalin fixed. A dilution series of 99mTc-nanobodies (0.15–714 nM in PBS) was added to each well for 1 h at room temperature. Unbound activity was washed away with PBS-0.1% Tween, total bound activity was collected by twice adding 0.1 M glycine-HCl (pH 2.5) for 30 min and counted in a γ counter (Cobra II Inspector 5003; Canberra-Packard, Downers Grove, IL, USA). These experiments were done in triplicate. To determine nonspecific binding, an excess of unlabeled nanobody was added. Specific binding was calculated by subtracting nonspecific binding from total binding. Specific binding values were plotted in function of free 99mTc-nanobody concentration, and resulting saturation curves were fitted using nonlinear regression (GraphPad Prism).

Assessment of 99mTc-nanobody biodistribution via SPECT/microCT imaging and ex vivo dissection analyses

Mice were injected i.v. with 99mTc-nanobodies and imaged at 1 h postinjection (p.i.) using pinhole SPECT and microCT. Animals were dissected at 1.5 h p.i., organ activities were measured against a standard of known activity, and expressed as percentage of injected activity (IA) per gram of tissue. These procedures are described in detail elsewhere (17).

Blood clearance of 99mTc-labeled nanobodies in naive mice

Blood clearance analysis was performed as described previously (17). For determination of the blood clearance, non-tumor-bearing nu/nu mice were injected i.v. with 99mTc-nanobodies. Blood samples were collected at 1, 5, 10, 20, 40, 60, 90, and 120 min p.i. and analyzed with a γ counter (Cobra II Inspector 5003; Canberra-Packard).

Determination of nanobody melting temperature

The melting temperature (Tₘ) of the nanobody was determined by following the circular dichroism signal of the unfolding protein induced by an increase in temperature on a spectropolarimeter (J715; Jasco, Tokyo, Japan). Melting curves were recorded from 35 to 95°C at a temperature gradient of 1°C/min at a fixed wavelength of 205 nm. A protein concentration between 0.1 and 0.2 mg/ml in 50 mM phosphate buffer (pH 7.0) was used. Data analysis was performed as described previously (21).

RESULTS

Dromedary immunization and evaluation of an immune response

In a first step toward the generation of affinity-matured anti-HER2 nanobodies, a dromedary was immunized with a HER2-Fc-recombinant protein, following standard protocols. Dromedaries raise both conventional antibodies (IgG₁ subclass) and heavy-chain-only antibodies (IgG₂ and IgG₃ subclasses) in response to the antigen (18). To evaluate the immune response in the HER2-Fc-immunized dromedary toward cell-surface HER2, the different serum IgG subclasses were purified and tested in flow cytometry. To this end, CHO cells were transiently transfected with a HER2-expressing plasmid, reaching ~50% of cells expressing HER2 (data not shown). All IgG subclasses isolated from the HER2-Fc immunized dromedary equally and specifically recognized cell-surface HER2 (Fig. 2). This demonstrates that cell-surface HER2-targeting heavy-chain-antibodies are generated, and it should be possible to isolate corresponding nanobodies from a nanobody library that is derived from the B lymphocytes of this animal.
Generation and purification of anti-HER2 nanobodies

We next generated a nanobody phagemid library in *E. coli* from the immunized dromedary’s lymphocytes and performed biopannings with phage-displayed nanobodies on either HER2-recombinant protein or HER2/H11001 cells. In total, 240 individual clones were randomly picked, and nanobody-containing periplasmic extracts were used to select HER2-specific nanobodies. Fifty-six clones passed the selection process; of these, 38 were unique. The nanobodies could be further divided into 17 groups based on sequence homology in the CDR3.

In each anti-HER2 nanobody group, at least one representative member was chosen for production as soluble, periplasmic proteins in an *E. coli* expression system. Purification was done by IMAC and gel filtration. Of the 17 nanobody groups, 13 representative nanobodies were purified with a yield > 1 mg/L *E. coli* culture grown in shake flasks.

HER2-recognition by purified nanobodies and binding kinetics

Flow cytometry analysis showed the specific character of HER2-recognition by the purified nanobodies. The anti-HER2 nanobodies recognized HER2+ SKOV3 cells, while no binding was seen with the HER2− cell line MDA-MB-435D or with an irrelevant nanobody (Fig. 3).

Next, the ability of the anti-HER2 nanobodies to recognize HER2 was investigated by ELISA at saturating concentrations of 10 μg/ml. Binding of the anti-HER2 nanobodies to recombinant HER2-Fc protein was significantly greater than the binding observed with an irrelevant nanobody (Fig. 4A). Similarly, anti-HER2 nanobodies bound to formalin-fixed HER2+ SKOV3 cells in ELISA, whereas binding of a control nanobody was negligible (Fig. 4B).

Third, SPR experiments were performed on immobilized HER2-Fc protein to determine binding kinetics parameters (Table 1). 1R94a and 2Rb18a bound their target with relatively poor affinities (high $K_D$), due to both slow association (low $k_a$) and fast dissociation (high $k_d$). All other nanobodies bound to the HER2 target protein with affinities in the low nanomolar range. These good-affinity binders can be grouped into 4 categories (Fig. 5): of all nanobodies tested, 2Rb17c was the fastest to bind, but also to dissociate (Fig. 5A). Inversely, 2Rs15d (Fig. 5B) and 1R135a compensated a slow association phase by a slow release from their target. Nanobodies 2Rb3b (Fig. 5C), 1R133a, and 2Rs23c bound almost equally slowly to the antigen as the previous category but dissociated faster. The final, largest group of nanobodies combined mediocre $k_a$ and $k_d$ values [1R59b (Fig. 5D), 1R119b, 2R5a, 1R136d, and 1R143c].

Finally, we estimated affinities to cellular HER2 by testing dilution-series of purified nanobodies in ELISA on formalin-fixed HER2+ SKOV3 cells. The dose-response curve for 2Rs15d is shown as an example in Fig. 6A. Calculated $K_D$ values are summarized in Table 1. Generally, affinities for cellular HER2 were good and comparable to those for recombinant HER2-Fc. Apart from 1R135a, differences were not >4-fold.

99mTc-labeling and in vitro functionality of anti-HER2 99mTc-nanobodies

Eleven of 13 purified nanobodies could successfully be labeled with 99mTc(I)tricarbonyl. Further purification by gel filtration to eliminate free 99mTc-tricarbonyl resulted in a radiochemical purity > 99%. These 11 99mTc-nanobodies were kept for further characterization. Nanobodies 2Rs23c and 1R94a were discarded because of their insufficient labeling efficiency.

Next, the functionality of 99mTc-nanobodies was studied by saturation binding experiments on formalin-fixed, HER2+ SKOV3 cells. All 99mTc-nanobodies retained their functionality after labeling since their specific binding values showed typical dose-response curves until receptor saturation, as shown in Fig. 6B for...
$^{99m}$Tc-2Rs15d. Calculated $K_D$ values are shown in Table 1 and were consistently higher than those of unlabeled ones on SKOV3 cells. With the exception of 1R59b and 2Rb3b, the relative affinities within each test group were acceptable, and nanobodies 1R119b, 2R5a, 2Rs15d, 1R143c, and 1R135a bound efficiently in both tests.

In vitro competition with trastuzumab and pertuzumab

Competition with trastuzumab and pertuzumab for HER2 binding was monitored for both unlabeled and $^{99m}$Tc-labeled nanobodies.

First, SPR experiments on immobilized HER2-Fc protein were conducted with both nanobodies and therapeutic antibodies at saturating antigen-binding concentrations. Competing nanobodies are defined as those that on saturation of antigen binding sites interfere with HER2-Fc binding of subsequently added antibody, and vice versa. Sensograms of trastuzumab not competing with 2Rs15d and competing with 1R59b are shown as examples in Fig. 7A, B. A summary of these studies is included in Table 1. None of the nanobodies interfered with binding of pertuzumab to its target.

![Figure 3](image-url)

Figure 3. Specific HER2 recognition of purified nanobodies via flow cytometry. A) HER2 mAb and nanobody 2Rs15d bind to HER2+ SKOV3 cells (blue) but not to HER2+ MDAMB435D cells (red). An irrelevant nanobody is negative on both cell types. B) Mean fluorescent intensity of all tested nanobodies on SKOV3 and MDAMB435D cells.

![Figure 4](image-url)

Figure 4. Recognition of immobilized HER2-Fc protein (A) and formalin-fixed, HER2+ SKOV3 cells (B) by nanobodies at 10 µg/ml in ELISA.
all 13 nanobodies tested, only 1R59b and 2Rb17c competed with trastuzumab. Not surprisingly, the latter 2 nanobodies recognized the same epitope since they could dramatically inhibit each others binding to HER2-Fc in SPR experiments (Fig. 7C).

In a second type of experiment, we investigated whether trastuzumab and pertuzumab compete with 99mTc-labeled nanobodies to bind to immobilized HER2-Fc protein or formalin-fixed HER2+ SKOV3 cells. 99mTc-nanobodies were used at sub-Keq concentrations and therapeutic antibodies at 1000-fold molar excess. Unlabeled irrelevant and homologous nano-

![SPR sensograms of representative nanobodies binding to immobilized HER2-Fc protein. Sensograms are from a dilution series of nanobodies from 1.95–125 nM. Note the high $k_a$ and $k_d$ for 2Rb17c (A), low $k_a$ and $k_d$ for 2Rs15d (B; similar to 1R135a), low $k_a$ and medium $k_d$ for 2Rb3b (C; similar to 1R133a and 2Rs23c), medium $k_a$ and $k_d$ for 1R59b (D; similar to 1R119b, 2R5a, 1R136d, and 1R143c).](image-url)
bodies in an equal molar excess were used as negative and positive controls for competition, respectively. Binding of noncompeting $^{99m}$Tc-2Rs15d and $^{99m}$Tc-1R59b competing with trastuzumab for binding to either recombinant HER2-Fc protein or formalin-fixed, HER2$^+$ SKOV3 cells is shown in Fig. 7D. Overall, the conclusions are in agreement to the SPR-competition study results.

In vivo imaging and biodistribution of anti-HER2 $^{99m}$Tc-nanobodies in HER2$^+$ SKOV3 tumor-bearing mice

Tumor-targeting potential of nanobodies was assessed after labeling them with $^{99m}$Tc and monitoring uptake into HER2$^+$ SKOV3 tumors via SPECT and dissection analysis at 1 and 1.5 h, respectively, after intravenous injection. Ex vivo measurements of $^{99m}$Tc-nanobody uptake in tumors and nontarget organs are summarized in Table 2. Quantification of radioactive signals in tumors and various organs based on SPECT images (Supplemental Table S1) was supported by anatomical information obtained by the microCT scans, and results agreed closely with dissection data.

As illustrated in Fig. 8, all $^{99m}$Tc-nanobodies showed a high kidney uptake, an intense activity in the bladder and low blood values, a typical feature of renal-filtered small hydrophilic proteins and peptides. The quantitative analysis of $^{99m}$Tc-nanobody uptake also showed low activities in nontarget organs for most nanobodies. Liver accumulation varied between 0.31 and 8.39% IA/g, depending on the $^{99m}$Tc-nanobody. Notably, all anti-HER2 $^{99m}$Tc-nanobodies demonstrated a higher tumor uptake than the irrelevant $^{99m}$Tc-nanobody (Fig. 8), suggesting specific uptake. Interestingly, a repeated high variation in tumor targeting was observed among the various anti-HER2 $^{99m}$Tc-nanobodies, differences that do not seem to be dictated by an in vitro measured parameter, such as affinity. The highest tumor accumulation was observed with $^{99m}$Tc-nanobodies 2Rs15d, 1R143c, 1R136d, and 2Rb17c, with an uptake of 4.19 ± 0.47, 4.44 ± 0.78, 4.00 ± 1.99 and 3.57 ± 0.13% IA/g, respectively. $^{99m}$Tc-2R5a showed the lowest tumor-targeting potential of 0.78 ± 0.50% IA/g tumor, albeit still 2-fold higher than the uptake of the irrelevant $^{99m}$Tc-nanobody.

A high tumor-to-background ratio is an important criterion to obtain high-contrast images. $^{99m}$Tc-nanobodies 2Rs15d, 1R143c, 1R136d, and 2Rb17c, which showed the highest absolute counts in the tumor, also exhibited the highest tumor-to-blood (T/B) ratios, 16.4 ± 3.6, 14.6 ± 5.9, 9.3 ± 3.8, and 7.8 ± 2.3, respectively, and high tumor-to-muscle (T/M) ratios, 49.6 ± 11.8, 27.4 ± 15.4, 16.2 ± 2.6, and 17.6 ± 1.4, respectively. For comparison, for the nontargeting control $^{99m}$Tc-nanobody, T/B was 1.0 ± 0.1, and T/M was 2.1 ± 0.1 (Table 2).

Additional scrutiny of selected anti-HER2 nanobody lead compounds

On the basis of a combination of good expression yields, affinity properties, amino acid composition, lack of competition with therapeutic antibodies, high SKOV3 tumor uptake, and tumor-to-background ratios, nanobodies 2Rs15d and 1R136d were selected for further studies.

Both lead nanobodies showed equal thermostability, $Tm$ values of 78.5 ± 0.1 and 78.4 ± 0.1°C for 2Rs15d and 1R136d, respectively (Supplemental Fig. S1).

Subsequently, the rate of blood clearance of $^{99m}$Tc-labeled leading nanobodies was compared. $^{99m}$Tc-2Rs15d and $^{99m}$Tc-1R136d are cleared fast from the blood, yielding similar, biphasic blood curves (Supplemental Fig. S2). Calculated blood half-lives of the initial phase were 4.4 and 1.4 min, respectively, and
those of the slow phase were 29.6 and 24.8 min, respectively. At 1 h p.i., percentage IA per total blood volume was <3 and not significantly different ($P > 0.1$) for both tracers.

In addition to the SKOV3 xenograft model, $^{99m}$Tc-labeled lead nanobodies were also evaluated in two other tumor models (HER2$^{+}$ LS174T and HER2$^{-}$ MDA-MB-435D) to test specificity of in vivo tumor targeting, as assessed by SPECT/microCT imaging and biodistribution analysis at 1–1.5 h p.i. Visual interpretation of fused SPECT/microCT images shows that both selected anti-HER2 $^{99m}$Tc-nanobodies accumulate in the HER2$^{+}$ LS174T tumor, but much less in the HER2$^{-}$ MDA-MB-435D tumor (Supplemental Fig. S3). Analyses of imaging quantifications and dissection data are in line with the pictures, demonstrating ~4% IA/g or IA/cm$^3$ uptake in HER2$^{+}$ tumors vs. <0.75% in HER2$^{-}$ xenografts, again indicating specific targeting of these anti-HER2 $^{99m}$Tc-nanobodies (Table 3). Accumulation of $^{99m}$Tc-nanobodies in HER2$^{+}$ tumors and respective tumor-to-background values tended to be higher for $^{99m}$Tc-2Rs15d than for $^{99m}$Tc-1R136d. Uptake in nontarget organs (except kidneys) is similarly low for both $^{99m}$Tc-nanobodies in both models.

**DISCUSSION**

Selected nanobodies (15 kDa) can be compared to other reported HER2-targeting tracers for their applicability in tumor imaging (reviewed by Tolmachev, ref. 9), including full antibodies (150 kDa; refs. 11, 22), Fab's (60 kDa; ref. 23), diabodies (60 kDa; refs. 24, 25), scFvs (30 kDa; refs. 24, 26, 27), DARPins (15 kDa; ref. 26), and affibodies (5 kDa; refs. 11, 28). Ideal tracers are those that combine fast renal clearance and efficient tumor penetration with good apparent affinities, resulting in high tumor uptake and good tumor-to-background ratios.
TABLE 2. Biodistribution of 99mTc-labeled nanobodies in HER2+ SKOV3 xenografted mice based on dissection values (%IA/g) at 1.5 h.p.i.; data are means ± SD.

<table>
<thead>
<tr>
<th>Group</th>
<th>1R135a</th>
<th>1R135b</th>
<th>1R136a</th>
<th>1R136b</th>
<th>1R137a</th>
<th>1R137b</th>
<th>1R138a</th>
<th>1R138b</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>Heart</td>
<td>2.49 ± 0.35</td>
<td>2.80 ± 0.26</td>
<td>2.44 ± 0.35</td>
<td>2.81 ± 0.28</td>
<td>2.50 ± 0.34</td>
<td>2.74 ± 0.29</td>
<td>2.42 ± 0.32</td>
<td>2.78 ± 0.30</td>
<td>2.75 ± 0.33</td>
</tr>
<tr>
<td>Liver</td>
<td>13.6 ± 0.13</td>
<td>15.4 ± 0.12</td>
<td>14.7 ± 0.11</td>
<td>16.2 ± 0.10</td>
<td>15.1 ± 0.12</td>
<td>16.5 ± 0.11</td>
<td>14.5 ± 0.10</td>
<td>16.2 ± 0.10</td>
<td>15.0 ± 0.12</td>
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<td>Kidney</td>
<td>0.38 ± 0.06</td>
<td>0.38 ± 0.06</td>
<td>0.36 ± 0.05</td>
<td>0.37 ± 0.04</td>
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<td>0.37 ± 0.04</td>
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</tr>
<tr>
<td>Spleen</td>
<td>1.24 ± 0.03</td>
<td>1.05 ± 0.03</td>
<td>1.24 ± 0.03</td>
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<td>Lungs</td>
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<td>Muscle</td>
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<td>0.17 ± 0.03</td>
<td>0.16 ± 0.03</td>
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<tr>
<td>Blood</td>
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<td>0.41 ± 0.07</td>
<td>0.57 ± 0.13</td>
<td>0.41 ± 0.07</td>
<td>0.57 ± 0.13</td>
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<tr>
<td>T/B</td>
<td>7.8 ± 2.3</td>
<td>16.4 ± 4.3</td>
<td>7.8 ± 2.3</td>
<td>16.4 ± 4.3</td>
<td>7.8 ± 2.3</td>
<td>16.4 ± 4.3</td>
<td>7.8 ± 2.3</td>
<td>16.4 ± 4.3</td>
<td>7.8 ± 2.3</td>
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</tbody>
</table>

Nanobodies are grouped on the basis of comparable SPR binding characteristics. Biodistribution is based on dissection values (%IA/g) at 1.5 h.p.i.; data are means ± SD.

Nanobodies were assessed for tumor uptake, and tumor-to-background ratios of 11 nanobodies that labeled well with 99mTc were assessed at an early time point (1–1.5 h.p.i.). All tested anti-HER2 99mTc-nanobodies accumulated more in HER2+ SKOV3 tumors than a non-HER2-targeting control 99mTc nanobody, and corresponding T/M and T/B ratios were consistently higher. In addition, specificity of HER2 targeting was confirmed by using at least tumor models for 2 selected lead compounds. Interestingly, important qualitative differences were observed between the various 99mTc-nanobodies in respect not only to tumor uptake, but also to liver accumulation. Breast tumors frequently metastasize to liver. Hence, tracers such as 99mTc-1R135a or 99mTc-1R135b that show elevated liver uptake (both >8% IA/g) should be avoided to visualize HER2 expression in liver lesions.

Nanobodies are easily generated after immunization of Camelidae. From a large pool of anti-HER2 nanobodies, it is necessary to select the most suitable one for in vivo imaging of patients with HER2+ breast cancer. Using our production protocol, we found 38 different nanobodies that recognized HER2, which is, to our knowledge, the largest set ever reported from one immunized animal, after only 2 rounds of biopanning. They were further divided into 17 groups based on sequence similarities in their CDR3. In several groups, variants with 1 to 13 amino acid differences (mostly in framework regions) occur, possibly due to differential in vivo maturation from a common ancestor B-cell clone. Although not investigated, these differences could affect physicochemical properties, such as stability or affinity, but probably not the recognized epitope. Homologous or identical nanobodies were retrieved using different panning conditions (i.e., on recombinant protein or cells). However, several unique nanobodies are only obtained by a particular panning condition. Hence, the use of different sources of antigen for panning results in a higher diversity of binders and thus enlarges the panel of potential applicable nanobodies to choose from.

In search for a lead radiopharmaceutical compound for a phase I clinical trial, we developed a selection strategy based on different criteria. One of these criteria, the production efficiency, is important in view of industrial applications. The production yield of the nanobodies varied, depending on the clone, from 0.1 to 17.9 mg/L E. coli culture. If desired, yields can be upscaled further by optimizing expression conditions or by using other production hosts (29).

We chose 13 well-producing nanobodies for a comparative study. To select the anti-HER2 nanobody with the highest potential as molecular imaging tracer, tumor uptake, and tumor-to-background ratios of 11 nanobodies that labeled well with 99mTc were assessed at an early time point (1–1.5 h.p.i.). All tested anti-HER2 99mTc-nanobodies accumulated more in HER2+ SKOV3 tumors than a non-HER2-targeting control 99mTc nanobody, and corresponding T/M and T/B ratios were consistently higher. In addition, specificity of HER2 targeting was confirmed by using at least 2 other tumor models for 2 selected lead compounds. Interestingly, important qualitative differences were observed between the various 99mTc-nanobodies in respect not only to tumor uptake, but also to liver accumulation. Breast tumors frequently metastasize to liver. Hence, tracers such as 99mTc-1R135a or 99mTc-1R135b that show elevated liver uptake (both >8% IA/g) should be avoided to visualize HER2 expression in liver lesions.

Efficient blood clearance and tumor penetration rates are inversely proportional to tracer molecular weights, whereas efficiency of tumor accumulation is related to tracer affinities and specificity of targeting. Fast, specific tumor targeting is an important criterion for good clinical tracers since, mainly for comfort of the patient and logistics, fast diagnosis is preferred (9).
Differences in liver accumulation could be attributed to differences in lipophilicity or potential cross-reactivity with the murine HER2 ortholog, which is expressed in the liver. All nanobodies were found to be equally stable in serum, as determined by gel filtration (data not shown), similarly to what we have shown before with a $^{99m}$Tc-anti-EGFR nanobody (30). Nonetheless, the qualitative differences in tumor targeting and extratumoral tissue uptake among a panel of different nanobodies argue for the necessity of a full biodistribution assessment of all tracers in relevant in vivo models, since their performance is difficult to predict by mere in vitro measured parameters of unlabeled compounds, such as affinity. Indeed, all unlabeled nanobodies were shown to specifically bind to HER2 in vitro, with affinities in the nanomolar range, while no affinity parameter correlated with tumor-targeting capacities.

On the basis of ex vivo analyses, $^{99m}$Tc-nanobodies 2Rs15d, 1R136d, 1R143c, and 2Rb17c showed the highest accumulation in the tumor of 3.6 to 4.4% IA/g. Also, the SPECT quantifications presented these $^{99m}$Tc-nanobodies as the best tumor targeting tracers. T/B and T/M ratios ranged from 7.8 to 16.4 and from 16.2 to 49.6, respectively, at 1.5 h p.i. Also, activities in liver and blood were low for these tracers. The choice of these nanobodies as optimal $^{99m}$Tc-labeled compounds for evaluation of HER2 tumor positivity via SPECT imaging is further supported by their good affinities for HER2 in both the unlabeled and $^{99m}$Tc-labeled format. Given their noncompetitive nature, based on in vitro experiments, the first 3 $^{99m}$Tc-nanobodies could potentially be used for evaluation of HER2 status in breast cancer patients undergoing anti-HER2-antibody therapy, whereas 2Rb17c could be suited to determine the presence of the trastuzumab-epitope prior to therapy initiation.

Besides nanobodies, DARPins and affibodies seem to meet the requirements of excellent anti-HER2 imaging tracers as well, as they are small and robust, have good affinity, have highly specific tumor uptake, and have good tumor-to-background ratios shortly after tracer administration (15, 16, 17, 30, 31). Both DARPins and

![Figure 8. Representative transverse, coronal, and sagittal views of fused SPECT/CT images of HER2$^+$ SKOV3 tumor-bearing mice 1 h after i.v. injection of $^{99m}$Tc-labeled nanobodies. Note good tumor targeting by anti-HER2 $^{99m}$Tc-2Rs15d and low tumor accumulation by an irrelevant control $^{99m}$Tc-nanobody.](image)
TABLE 3. In vivo targeting of HER2+ and HER2− tumors by lead anti-HER299mTc-nanobodies in xenografted mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>99mTc-2Rs15d</th>
<th>99mTc-1R136d</th>
<th>SPECT (%IA/cm³)</th>
<th>Dissection (%IA/g)</th>
<th>SPECT (%IA/cm³)</th>
<th>Dissection (%IA/g)</th>
<th>SPECT (%IA/cm³)</th>
<th>Dissection (%IA/g)</th>
<th>SPECT (%IA/cm³)</th>
<th>Dissection (%IA/g)</th>
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<tbody>
<tr>
<td>Heart</td>
<td>0.93 ± 0.10</td>
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<td>0.37 ± 0.06</td>
<td>0.37 ± 0.06</td>
<td>0.37 ± 0.06</td>
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<tr>
<td>Liver</td>
<td>3.24 ± 0.33</td>
<td>0.23 ± 0.05</td>
<td>1.66 ± 0.28</td>
<td>1.66 ± 0.28</td>
<td>1.66 ± 0.28</td>
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</tr>
<tr>
<td>Spleen</td>
<td>0.39 ± 0.20</td>
<td>0.12 ± 0.02</td>
<td>0.39 ± 0.15</td>
<td>0.39 ± 0.15</td>
<td>0.39 ± 0.15</td>
<td>0.39 ± 0.15</td>
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<td>0.39 ± 0.15</td>
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<tr>
<td>Muscle</td>
<td>3.00 ± 0.12</td>
<td>0.10 ± 0.02</td>
<td>3.00 ± 0.12</td>
<td>3.00 ± 0.12</td>
<td>3.00 ± 0.12</td>
<td>3.00 ± 0.12</td>
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<tr>
<td>Lungs</td>
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<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
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<tr>
<td>Blood</td>
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<td>0.30 ± 0.10</td>
<td>0.30 ± 0.10</td>
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<tr>
<td>T/M</td>
<td>2.17 ± 0.12</td>
<td>0.49 ± 0.08</td>
<td>2.17 ± 0.12</td>
<td>2.17 ± 0.12</td>
<td>2.17 ± 0.12</td>
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<td>2.17 ± 0.12</td>
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<tr>
<td>T/B</td>
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<td>0.62 ± 0.08</td>
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<td>2.33 ± 0.14</td>
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</tbody>
</table>

Biodistribution of 99mTc-labeled anti-HER2 nanobodies 2Rs15d and 1R136d injected i.v. in mice bearing either HER2 LS174T or HER2 MDA-MB-435D tumors. Biodistribution is assessed either by quantification of activities in tumors and tissues based on SPECT images at 1 h p.i. or dissection values at 1.5 h p.i.; data are means of 6 mice/nanobody. T/M, tumor-to-muscle ratio; T/B, tumor-to-blood ratio.

**In vivo targeting of HER2+ and HER2− tumors by lead anti-HER299mTc-nanobodies in xenografted mice.** The researchers concluded that the small size of affibodies is nonimmunoglobulin, artificial protein scaffolds, in which particular surface-exposed residues are randomized and selected for binding to target proteins. Hereby, HER2-binders are generated with SPR affinities similar to those of nanobodies, in the low- to high-nanomolar range, however, with suboptimal imaging properties (32, 33). The advantage of nanobodies in comparison to these artificial scaffolds is that nanobodies are easily generated, whereas DARPin and affibodies need further complex and time-consuming in vitro mutagenesis to reach affinities in the subnanomolar range and to obtain optimal imaging features. Tumor-targeting properties of these in vivo matured binders are excellent, with tumor uptake values at 1 h p.i. in the SKOV3 xenograft model of 9% IA/g for the 90 pM H10-2-G3 DARPin and 17% IA/g for the 22 pM ZHER2:342 affibody, with corresponding T/B ratios, similar to those of our lead nanobody, of 13 and 15, respectively (26, 28). Hence, our lead nanobody shows a lower absolute tumor uptake than the best affibodies and DARPin, but a comparable tumor-to-background ratio early after administration. For diagnosis, good contrast images due to high tumor-to-background ratios are of more importance than high tumor uptake. For comparison, SKOV3 tumor uptake of a 9 nM trastuzumab-derived scFv was 3% IA/g, but only a T/B ratio of 1.0 was obtained with additional high liver uptake (26). Also, because of its slow clearance, imaging with labeled trastuzumab antibody is only possible several hours after administration, and even then T/B ratios reach only 1.3 at 3 d p.i (11).

The influence of tracer affinity on tumor targeting potential remains a controversial issue. Two studies using either anti-HER2 scFvs (27) or DARPin (26) investigated the relationship between these two parameters in a comparative study using stepwise lower affinity mutants (as determined by SPR) of the same binder. Adams et al. (27) concluded that scFv C6.5 derivatives (30 kDa) with affinities between 1 and 100 nM are optimal for a significant tumor uptake. scFv tracer affinity > 100 nM resulted in a lower tumor uptake, but affinities < 1 nM also failed to result in higher tumor penetration and uptake, mainly due to perivascular localization. In contrast, Zahnd et al. (26) obtained a different relationship with DARPin: tumor uptake increased with enhanced affinity, achieving a maximum with the binder showing the best affinity (90 pM) while retaining a homogeneous tumor distribution. The researchers concluded that the small size of DARPin (15 kDa, similar to nanobodies) and good affinity (and in particular the slow off-rate) are key determinants for efficient tumor targeting (26). In our study, using a variety of nanobodies with unrelated sequences, the best tumor-targeting nanobodies, 2Rs15d, 1R136d, 1R143c, and 2Rb17c, had affinities (as determined by SPR) between 2.7 and 8.1 nM. However, binders with equally good affinity, such as 2R5a and 1R119b, targeted tumors poorly. Similarly, nanobody off-rate also did not correlate well with tumor uptake, as
with 99mTc and remained functional, as determined by ligand-binding studies. Calculated affinities for 
99mTc-nanobodies were consistently lower than those of unlabeled ones, but to different degrees. It remains to be investigated whether this is due to either partial denaturation of less heat-stable ones, or because of the effects of structural modifications introduced by the tricarbonyl core. It must be borne in mind that radio-labeling procedures involve chemical modifications of the protein structure that might affect size, charge distribution, or folding of the protein to some extent. Nevertheless, affinities of 
99mTc-nanobodies tend to correlate better with tumor uptake than affinities of unlabeled ones. Indeed, 3 of 5 
99mTc-nanobodies < 20 nM \(K_D\) (except 1R119b and 1R135a) targeted well and all 4 with >99 nM \(K_D\) targeted HER2 \(^+\) tumors less. Hence, the affinity of labeled tracers may be a better indicator of tumor-targeting potential than that of their unlabeled counterparts. Other parameters, such as stability, solubility, and the targeted epitope, might be equally important.

All injected \(99mTc\)-labeled nanobodies used in this study accumulate intensely in the kidneys. This is typical for small hydrophilic proteins and is caused by efficient renal clearance and nonspecific reabsorption by proximal tubuli (34). Because of the sufficiently large anatomical distances, high kidney signals should not interfere with imaging of primary breast tumors. However, noninvasive HER2 status determination in the proximity of the kidneys might be more problematic. In addition, high kidney radioactivities might cause nephrotoxicity if using nanobodies labeled with radionuclides with therapeutic properties (34). Therefore, reduced kidney uptake is always desired. We have recently shown that it is feasible to lower nanobody retention by the kidneys without affecting tumor uptake or tumor-to-background ratios (30).

In clinical reality, PET tumor imaging is gaining interest over SPECT. The radiolabeling of PET tracers with, for instance, \(^{68}Ga\) or \(^{18}F\) usually occurs through complexation with chelating groups or using prosthetic groups that are covalently attached to the tracer backbone. Most commonly, free amine groups are the acceptors of such reactive groups. For such applications, it is, therefore, important to select nanobodies that are amenable to coupling at lysine residues without disturbing antigen binding. On the basis of sequence analyses, nanobodies 2R15d and 1R136d are preferred and are selected for additional studies, since they have 6 and 5 lysine residues, respectively, only in framework regions. Nanobodies 1R143c and 2Rb17c are less ideal for amine couplings since they contain a lysine residue in CDR3, the largest antigen-binding-loop.

**CONCLUSIONS**

A panel of anti-HER2 nanobodies from a phage-display library derived from \(in vivo\)-matured dromedary heavy-chain antibodies was screened by various techniques in an effort to identify a new tracer for molecular imaging of tumor HER2 expression. Taking together all \(in vitro\) and \(in vivo\) studies and on the basis of its superior tumor uptake and tumor-to-background ratios, we ultimately selected 2R15d as a lead compound for further development as a clinical radiopharmaceutical.

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