**Abstract**

**Purpose:** Cameldid single-domain antibody-fragments (sdAb) have beneficial pharmacokinetic properties, and those targeted to HER2 can be used for imaging of HER2-overexpressing cancer. Labeled with a therapeutic radionuclide, they may be used for HER2-targeted therapy. Here, we describe the generation of a 131I-labeled sdAb as a theranostic drug to treat HER2-overexpressing cancer.

**Experimental Design:** Anti-HER2 sdAb 2Rs15d was labeled with 131I using 131I-SGMIB and evaluated in vitro. Biodistribution was evaluated in two HER2+ murine xenograft models by micro-PET/CT imaging and at necropsy, and under challenge with trastuzumab and pertuzumab. The therapeutic potential of 131I-SGMIB-2Rs15d was investigated in two HER2+ tumor mouse models. A single-dose toxicity study was performed in mice using unlabeled 127I-SGMIB-sdAb at 1.4 mg/kg. The structure of the 2Rs15d–HER2 complex was determined by X-ray crystallography.

**Results:** 131I-SGMIB-2Rs15d bound specifically to HER2+ cells (Kd = 4.74 ± 0.39 nmol/L). High and specific tumor uptake was observed in both BT474/M1 and SKOV-3 tumor xenografted mice and surpassed kidney levels by 3 hours. Extremely low uptake values were observed in other normal tissues at all time points. The crystal structure revealed that 2Rs15d recognizes HER2 Domain 1, consistent with the lack of competition with trastuzumab and pertuzumab observed in vivo. 131I-SGMIB-2Rs15d alone, or in combination with trastuzumab, extended median survival significantly. No toxicity was observed after injecting 127I-SGMIB-2Rs15d.

**Conclusions:** These findings demonstrate the theranostic potential of 131I-SGMIB-2Rs15d. An initial scan using low radioactive 11I-SGMIB-2Rs15d allows patient selection and dosimetry calculations for subsequent therapeutic 131I-SGMIB-2Rs15d and could thereby impact therapy outcome on HER2+ breast cancer patients. Clin Cancer Res; 23(21); 6616–28. ©2017 AACR.

**Introduction**

The HER2 is overexpressed in multiple human cancers including breast, ovarian, colorectal, and urothelial carcinomas (1). Its incidence in breast cancer is about 20%–30% and is often associated with a higher recurrence rate and a shorter time to relapse (2, 3). Upon breast cancer diagnosis, approximately 10% of women have metastatic disease, which is considered incurable. Treatment goals are mainly focused on prolonging overall survival (OS) and progression-free survival (PFS). Therapies targeting HER2 can significantly impact the outcome of HER2+ metastatic breast cancer (4)—since the introduction of anti-HER2 drugs to the standard of care, OS has increased significantly. However, emerging resistance to trastuzumab and the kinase inhibitor lapatinib are frequently observed. Trastuzumab emtansine (TDM1), an antibody–drug conjugate, combines the antitumor effects of trastuzumab with those of the microtubule-inhibitory agent DM1, a cytotoxic agent that is released within target cells. TDM1 has shown therapeutic potential for the treatment of advanced breast cancer patients that progressed after combined treatment with trastuzumab and taxane (5). Unfortunately, most patients eventually progress on TDM1 due to acquired resistance (6). Combining versatile HER2 therapies that can circumvent drug resistance are therefore of high importance (7).

Targeted radionuclide therapy (TRNT) deploys therapeutic radiolabeled molecules like mAbs, mAb fragments, peptides, or synthetic proteins that interact with tumor-associated membrane proteins, and targets both the primary tumor site as well as metastases. The integration of molecular imaging can assist to predict successful TRNT. This theranostic approach aims to include an identical imaging compound (8) to predict targeting and potential toxicity to healthy tissues. Currently, one mAb-based TRNT agent is commercially used, that is, the anti-CD20 mAb 90Y-ibritumomab for treating B-cell non-Hodgkin lymphoma (9–11). Peptide receptor radionuclide therapy (PRRNT) shows efficacy in patients with neuroendocrine tumors (12) and is currently also being investigated in prostate and pancreatic carcinomas (12, 13).

Cameldid single-domain antibody-fragments (sdAb), also referred to as VHHs or nanobodies, may solve some of the...
HER2 is an interesting therapeutic target because it is overexpressed in cancers including breast, ovarian, and gastric. There is a need for strategies to overcome resistance to HER2-targeted therapies for metastatic breast cancer. SdAbs are a promising platform for both imaging and targeted therapy. The 68Ga-labeled HER2-targeting variant was successfully evaluated before in a first clinical study in breast cancer patients to noninvasively detect HER2 expression using PET. We describe here a novel [131I]-labeled sdAb that allows imaging for patient selection and HER2-targeted radionuclide therapy using the same compound. By targeting domain I of HER2, [131I]SGMIB-2Rs15d allows administration to patients who progress on trastuzumab, pertuzumab, or T-DM1. These results indicate that [131I]SGMIB-2Rs15d, with its low toxicity profile and proven therapeutic efficacy, has strong potential as a theranostic drug for clinical translation. A first-in-human study evaluating [131I]SGMIB-2Rs15d in healthy volunteers and HER2+ breast cancer patients is currently ongoing (NCT02683083).

The improved tumor targeting of a [1]SGMIB-labeled anti-HER2 sdAb was first shown with 5F7GGC sdAb (25). Unfortunately, 5F7GGC competes with trastuzumab for binding to domain IV on HER2 (25), thereby compromising its clinical translation and not offering solutions to certain HER2 treatment resistance mechanisms (7). The goal of this study was to generate a potentially more clinically relevant theranostic drug by labeling the anti-HER2 sdAb 2Rs15d with [131I]SGMIB.

Materials and Methods

General

All reagents were purchased from Sigma-Aldrich except when noted. Sodium [131I]iodide in 0.1 N NaOH with a specific activity >185 GBq/mg was purchased from Perkin-Elmer. All reagents used in cell culture experiments were purchased from Gibco BRL except when noted. SdAbs were generated as described previously (18). HER2-targeting 2Rs15d, HER2-targeting but trastuzumab-competing 2Rb17c, and nontargeting R3B23 (control sdAb) were fully characterized previously (18, 20, 26). Trastuzumab (Herceptin) and pertuzumab (Perjeta, Hoffmann-La Roche Ltd) were used as stated in the experiments.

Preparation of [131I]-labeled compounds

[131I]SGMIB was synthesized and purified as reported previously (25), and summarized in Supplementary Materials and Methods. Quality control (QC) was performed by instant thin-layer chromatography (iTLC) using glass microfiber sheets impregnated silica gel strips (Agilent) run with PBS, pH = 7.4. In parallel, radio-size exclusion chromatography (SEC, 0.5 ml/minute, 0.02 mol/L phosphate buffer, and 0.28 mol/L NaCl, pH = 7.4, Superdex 75 5/150 GL, 5 bar) was performed. [131I]-2Rs15d was incubated in PBS at 25°C. Aliquots were obtained up to 144 hours and analyzed with radio-HPLC using a polystyrene divinylbenzene copolymer column (PLRP-S 30 Å, 5 mm, 250/4 mm; Agilent) with the following gradient: (A): 0.1% trifluoroacetic acid in water; (B): acetonitrile): 0–5 minutes, 25% B; 5–7 minutes, 25%–34% B; 7–10 minutes, 75%–100% B; and 10–25 minutes, 100% B, at a flow rate of 1 mL/minute. [131I]-2Rs15d was also incubated in human serum at 37°C for 1 week and analyzed by radio-SEC. [1]SGMIB-sdAbs are further referred to as [1]-sdAbs.

Cell culture conditions

The HER2+ BT474/M1 breast cancer cell line was selected for its increased tumorigenicity (27), while HER2+ JIMT-1 for its resistance toward trastuzumab (28). Both cell lines were cultured in DMEM medium. The HER2+ SKOV-3 and the HER2+/luciferase− SKOV-3.I.P1 (29) ovarian cancer cell lines (ATCC) were cultured using McCoy 5A medium. All media were enriched with 10% FBS, and a mixture of 100 u/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen). Cells were grown in a humidified atmosphere
with 5% CO₂ at 37°C. Prior to use for in vitro and in vivo purposes, cells were detached using trypsin-EDTA. HER2 expression of the different cell lines was confirmed by flow cytometry (Supplementary Materials and Methods). Tumor cell lines all overexpressed HER2, with AMFIs of 7.135, 3.592, and 2430 for BT474/M1, SKOV-3, and JIMT-1, respectively.

Targeting specificity, affinity, and cell-internalizing properties of 131I-2Rs15d.

HER2-binding kinetics of 2Rs15d and unlabeled [127I]-2Rs15d and their noncompeting character with trastuzumab and pertuzumab was assessed via surface plasmon resonance (SPR) as described previously (18). Binding of control sdAbs, 2Rs15d, 2Rb17c, and trastuzumab on BT474/M1 and JIMT-1 cells was evaluated using flow cytometry (Supplementary Materials and Methods). The binding characteristics of [131I]-2Rs15d were evaluated on SKOV-3 and BT474/M1 cells. A total of 8 × 10⁶ cells were adhered overnight and washed two times with PBS prior to addition of radioiodinated sdAbs. Binding specificity was measured by incubating cells with 20 nmol/L of [131I]-2Rs15d and [131I]-control sdAb, and challenged with a 100-fold molar excess of 2Rs15d, trastuzumab, or pertuzumab. Binding of [131I]-2Rs15d, [131I]-2Rb17c, and [131I]-trastuzumab to JIMT-1 cells was assessed in parallel. Binding affinity was determined by incubating the plated cells with serial dilutions of [131I]-2Rs15d, ranging from 0 to 300 nmol/L. A 100-fold molar excess of 2Rs15d was added in parallel to measure the degree of nonspecific binding. Cells were incubated for 1 hour at 4°C, after which unbound activity was washed away. Finally, cells were lysed by addition of 1 mol/L NaOH, and collected. Intracellular retention of [131I]-2Rs15d was evaluated at different time points on BT474/M1 cells. A total of 8 × 10⁶ cells were adhered overnight and washed 2 times with PBS prior to incubation with 25 nmol/L of [131I]-2Rs15d at 4°C for 1 hour. A 100-fold molar excess of unlabeled 2Rs15d was added in parallel to assess nonspecific binding. Next, the unbound fraction was washed away and cells were supplemented with fresh medium and incubated at 37°C for 24 hours. After incubation, supernatants were collected (dissociated fraction) prior to an acid wash of the cells using 0.05 mol/L glycine-HCl pH 2.8, to collect the membrane-bound fraction. Cells were lysed with 1 mol/L NaOH to determine the internalized fraction. All fractions were counted for radioactivity using an automated gamma counter.

Animal models

Normal male C57Bl/6 mice were used to assess blood clearance. Toxicity analysis was performed in normal male and female Swiss albino mice. Female CRL-Nu-FoxN1 nu mice were implanted with a 60-day slow-release estrogen pellet, after which they were inoculated in the right hind leg with 1 × 10⁷ BT474/M1 cells in 1/1 Matrigel/cell culture medium. Tumors were grown to 284 ± 171 mm³ for imaging and dissections, and to 50 ± 30 mm³ for therapy. The same strain was also inoculated with 4 × 10⁶ SKOV-3 cells in cell culture medium and grown until tumors reached 450 ± 100 mm³ for imaging, and intraperitoneally (i.p.) inoculated with 0.5 × 10⁶ SKOV-3 IP1 cells in case of therapy (29). Tumor growth was measured using caliper or bioluminescence imaging after intraperitoneal injection of 150 mg/kg Luciferin (30). All animal protocols were approved by the ethical committee of the Vrije Universiteit Brussel (14-272-5).

Biodistribution of [131I]-2Rs15d via molecular imaging

BT474/M1 and SKOV-3 tumor xenografted mice were intravenously (i.v.) injected in the tail vein with 9.00 ± 0.18 MBq (4.0 µg; 0.32 nmol) [131I]-2Rs15d (n = 3 per model), followed by micro-SPECT/CT imaging after 1, 4, and 24 hours. Mice were anaesthetized using 2% isoflurane and kept warm using a heating pad. Micro-SPECT/CT imaging was performed with a Vector/CIT MILabs system, using a PET-collimator and a spiral scan mode of 94 bed positions (19s per position). For CT, a normal scan mode of only one position was used. The obtained SPECT data were reconstructed with a 0.6 voxel size, 2 subsets, and 7 iterations, after which images were fused and corrected for attenuation based on the CT scan. Images were analyzed using a medical image data analysis tool (AMIDE) and OsiriX. Uptake of [131I]-2Rs15d in organs and tissues was analyzed and expressed as % injected activity per cubic centimeter (%IA/cc).

Blood clearance of [131I]-2Rs15d

Normal C57BL/6 mice were injected intravenously with either 2.55 ± 0.81 MBq (4.0 µg; 0.32 nmol) [131I]-2Rs15d or 1.75 ± 0.04 MBq (4.0 µg; 0.29 nmol) [131I]-control sdAb (n = 6). Blood samples were collected regularly with a microcapillary until 180 min postinjection. Results were expressed as %IA per total blood volume (%IA/TBV), estimated as 7% of the total body weight. The blood half-life was determined through a biphasic nonlinear regression fit.

Biodistribution of [131I]-2Rs15d via serial dissections

Groups of mice with BT474/M1 xenografts (n = 3) were injected intravenously with 0.97 ± 0.34 MBq (2.0 µg; 0.16 nmol) [131I]-2Rs15d or with 1.02 ± 0.05 MBq (2.0 µg; 0.15 nmol) [131I]-control. Mice were euthanized at several time points up to 120 hours, dissected, and major organs and tissues were isolated, weighed, counted, and expressed as %IA per gram of tissue (%IA/g). Urine samples were collected and analyzed using radio-SEC. In parallel, [131I]-2Rs15d was administered to mice that were treated 72 hours prior with a 100-fold molar excess of trastuzumab, pertuzumab (2.4 mg, 16 nmol), or both combined (n = 4). Statistical analyses were performed using one-way ANOVA.

Organ-absorbed doses of [131I]-2Rs15d

The biodistribution data were time-integrated to obtain the residence time per gram tissue (21, 25). Briefly, the area under the curve between 0 and 120 hours was made using the trapezoid integration method. Next, the absorbed doses were calculated using S values for 131I obtained from RADAR phantom units (Unit Density Spheres). The S value for a 1 g sphere (0.0304 Gy/g/MBq-s) was used to calculate all organ doses. In parallel, an estimation of organ absorbed doses was performed by extrapolation to the adult female phantom with OLINDA 1.0 software using a voiding bladder interval of 1 hour. The calculations were based on time-activity curves to determine the number of disintegrations in organs. Organ doses and effective dose were calculated using the appropriate weighing factors.

Therapeutic efficacy of [131I]-2Rs15d

In the first experiment, BT474/M1 tumor xenografted mice (n = 6) received 5 intravenously injections (weekly, for 5 weeks) of either [131I]-2Rs15d (10.83 ± 1.73 MBq; 8.0 µg;...
0.63 nmol/treatment). $^\text{[131I]}$-control sdAb (8.8 ± 2.9 MBq: 8.0 μg: 0.57 nmol/treatment), or vehicle solution. In the second experiment, SKOV-3 JP1 tumor xenografted mice (n = 8) were injected with 5 intravenous doses (weekly, for 5 weeks) of either (i) 8.8 ± 1.4 MBq $^\text{[131I]}$-2Rs15d (8.0 μg: 0.63 nmol/treatment), (ii) trastuzumab at 7.5 mg/kg loading (190 μg: 1.3 nmol): 3.5 mg/kg maintenance (90 μg: 0.6 nmol) + 5.5 ± 2.4 MBq $^\text{[131I]}$-2Rs15d (8.0 μg: 0.63 nmol/treatment), (iii) unlabeled 2Rs15d at 7.5 mg/kg loading (190 μg: 15 nmol): 3.5 mg/kg maintenance (90 μg: 7 nmol), (iv) trastuzumab regimen alone, or (v) an equal volume of vehicle solution. Tumor volume (via caliper or bioluminescence imaging) and animal weight were measured weekly. Dropouts were considered when one of the following endpoints was reached: for subcutaneous tumors (i) tumor size of >1,000 mm³, (ii) >20% weight loss, or (iii) the presence of necrotic tumor tissue; for intraperitoneal tumors (i) exceeding a BIL signal of 5.0 × 10⁷ ph/s/cm²/sr, (ii) severe ascites, or (iii) a sudden >20% weight loss. Survival curves were plotted and analyzed by the log-rank Mantel–Cox test.

Toxicity of $^\text{[127I]}$-2Rs15d

A single dose (1.4 mg/kg) of nonradioactive $^\text{[127I]}$-2Rs15d formulation was administered intravenously to 30 mice. The dose level of 1.4 mg/kg is 1,000 times the expected dose in human as required by the microdosing toxicity guideline of EMEA (CPMP/ICH/286/95), currently used in the first-in-human clinical study in which a single injection of low radioactive $^\text{[131I]}$SGMIB-2Rs15d (<100 μg) is administered. The concurrent control group received vehicle solution only. Parameters evaluated were clinical signs, mortality, changes in body weight and food intake, hematology and clinical chemistry parameters, organ weights, and gross pathology. The mice were sacrificed on day 2 (10 mice/sex/group) and on day 15 (remaining 5 mice/sex/group).

Results

Determination of the HER2–2Rs15d complex crystal structure

The crystal structure of the HER2–2Rs15d complex reveals that the sdAb interacts with an epitope located on HER2 domain 1. This is distinct from the HER2 sites recognized by trastuzumab and pertuzumab (Fig. 1A) and most other reported HER2 binders ( Supplementary Fig. S1A and S1B). As expected, interactions with HER2 are mediated by 2Rs15d residues located in the complementarity determining regions (CDR); however, an almost equal number of amino acids located in the complementarity determining regions

Preparation of $^\text{[131I]}$-labeled compounds

$^\text{[131I]}$SGMIB was synthesized (n = 35) from its tin precursor in 31.6% ± 6.6% radiochemical yield and 98.2% ± 1.2% purity after HPLC purification. The conjugation efficiency of $^\text{[131I]}$SGMIB to biomolecules was 36.5% ± 12.8% (n = 25) for $^\text{[131I]}$-2Rs15d, 36.1% ± 10.0% (n = 7) for $^\text{[131I]}$-control sdAb, 44.2% ± 0.2% (n = 2) for $^\text{[131I]}$-2Rb17c and 57.5% ± 2.1% (n = 2) for $^\text{[131I]}$-trastuzumab with a specific activity ranging from 0.06 to 2.55 MBq/μg. Radiochemical purity was >97% for all prepared compounds.

The stability of $^\text{[131I]}$-2Rs15d was analyzed in PBS at 25°C and in serum at 37°C via radio-HPLC and SEC. $^\text{[131I]}$-2Rs15d was stable in PBS, with >95% intact conjugate up to 72 hours, decreasing to 93% at 144 hours (Fig. 2A). In human serum, 95% of $^\text{[131I]}$-2Rs15d was still intact after 24 hours, gradually decreasing to 87% at 168 hours.

Targeting specificity, affinity, and cellular internalization of $^\text{[1]}$-2Rs15d

Besides binding to recombinant HER2 protein, 2Rs15d and $^\text{[1]}$-2Rs15d were also tested on cancer cells with various levels of functional HER2 expression, namely trastuzumab-sensitive BT474/M1 and SKOV-3 cells, and trastuzumab-resistant JIMT-1 cells (in which HER2 domain IV is obscured by overexpressed MUC4; ref. 28).

Binding affinities to HER2 of 3.99 ± 0.04 nmol/L and 3.62 ± 0.03 nmol/L for 2Rs15d and $^\text{[127I]}$-2Rs15d, were determined by SPR (Fig. 2B and C). $^\text{[131I]}$-2Rs15d bound specifically on BT474/M1 cells, while $^\text{[131I]}$-control sdAb exhibited negligible HER2 binding (Fig. 2D). The noncompeting character of unlabeled 2Rs15d with trastuzumab and pertuzumab was confirmed by SPR measurements (Fig. 1B and C). $^\text{[131I]}$-2Rs15d bound about 1.5 and 4 times better to JIMT-1 cells compared with HER2-domain IV–specific compounds $^\text{[131I]}$-2Rb17c sdAb and $^\text{[131I]}$-trastuzumab respectively, while binding to BT474/M1 was similar for all three (Fig. 1F and C). These observations were confirmed by flow cytometry (Fig. 1H). The binding affinity of $^\text{[131I]}$-2Rs15d, measured by incubating BT474/M1 cells with serial dilutions of $^\text{[131I]}$-2Rs15d, indicated a Kd = 4.74 ± 0.39 nmol/L (Fig. 2E). The cell-associated fraction of $^\text{[131I]}$-2Rs15d remained stable over time, ranging between 20%–30% of initially bound activity (Fig. 2F). At 1 hour, 17.00% ± 0.69% was membrane-bound and 9.13% ± 2.37% was internalized. At 24 hours, 28.79% ± 1.95% of $^\text{[131I]}$-2Rs15d remained cell associated of which about half was internalized and half bound to membrane.

Blood clearance and biodistribution of $^\text{[131I]}$-2Rs15d

Consecutive micro-SPECT/CT images in BT474/M1 (Fig. 3A) and SK-OV-3 (Fig. 3B) subcutaneous tumor xenografted mice were generated and quantified (Fig. 3C; Supplementary Table S3) after intravenous injection of $^\text{[131I]}$-2Rs15d. In the BT474/M1 model, high contrast images were obtained as early as 1 hour postinjection, with most $^\text{[131I]}$-2Rs15d concentrated in kidneys (20.75% ± 4.18% IA/cc) and tumor (6.48% ± 2.58% IA/cc). The accumulation in kidneys dropped significantly to 4.54% ± 0.81% IA/cc after 4 hours, and to a value below 0.5% IA/cc after 24 hours, while the fraction in tumor remained 4.54% ± 0.81% IA/cc after 4 hours and 2.50% ± 1.22% IA/cc after 24 hours. Very low uptake values were measured for thyroid and muscle. Similar results were obtained with the SKOV-3 model, although lower tumor uptake was measured (2.31% ± 0.22% IA/cc after 1 hour and 1.16% ± 0.03% IA/cc after 24 hour), due to the lower HER2 expression compared with BT474/M1.

$^\text{[131I]}$-2Rs15d was cleared from blood in a biphasic manner (Fig. 4A). The calculated half-lives for the initial fast blood pool vanishing phase were about 1.93 ± 0.13 minutes for
Figure 1.
A, Structure of 2R\textsubscript{15}d (cartoon representation) complexed with HER2(1-646)His (surface representation). 2R\textsubscript{15}d (red) binds HER2 domain I (tan; Gin2-Arg196), while pertuzumab and trastuzumab interact with domain II (sky blue; Thr197-Val320) and domain IV (sandy brown; Cys490-Cys566), respectively. HER2 domain III (Cys321-Ala489) is colored in plum. B and C, Competition studies with [\textsuperscript{127}I]-2R\textsubscript{15}d and anti-HER2 mAbs trastuzumab and pertuzumab for binding to HER2. B, Binding of [\textsuperscript{127}I]-2R\textsubscript{15}d and/or trastuzumab and C, [\textsuperscript{127}I]-2R\textsubscript{15}d and/or pertuzumab to immobilized HER2-Fc protein. Competition between two components occurs when the signal obtained by binding of a mixture of the two is lower than the sum of the signals obtained by each component individually. D and E, [\textsuperscript{131}I]-2R\textsubscript{15}d does not compete for HER2 receptor binding with trastuzumab and pertuzumab on BT474/M1 (D) and SKOV-3 cells (E); its binding to HER2 could be blocked only by a 100-fold excess of unlabeled 2R\textsubscript{15}d, but not by a 100-fold excess of unlabeled trastuzumab or pertuzumab. ***, P < 0.0001; ns, not significant, using one-way ANOVA. F and G, Degree of HER2 targeting of [\textsuperscript{131}I]-2R\textsubscript{15}d compared with [\textsuperscript{131}I]-trastuzumab and [\textsuperscript{131}I]-2R\textsubscript{b17}c on trastuzumab-resistant JIMT-1 and trastuzumab-responsive BT474/M1 cells. F, [\textsuperscript{131}I]-2R\textsubscript{15}d binds about 4 times higher to JIMT-1 cells compared with [\textsuperscript{131}I]-trastuzumab, while binding to BT474/M1 was similar for both. G, Binding to JIMT-1 was only 1.5 times higher for [\textsuperscript{131}I]-2R\textsubscript{15}d compared with [\textsuperscript{131}I]-2R\textsubscript{b17}c. ***, P < 0.0001 using Student t test. H, Control sdAb does not bind to HER2 on BT474/M1 and JIMT-1 cells, as determined by flow cytometry (described in Supplementary Materials and Methods). HER2-targeting sdAbs 2R\textsubscript{15}d and 2R\textsubscript{b17}c bind to HER2 on both cell lines, while trastuzumab binds HER2 on BT474/M1 cells but not on JIMT-1 cells.
[131I]-2Rs15d and 1.87/C6 0.13 minutes for [131I]-control sdAb. After 60 minutes, less than 2% IA/TBV was measured in blood for both sdAbs. No significant difference (P = 0.66) was observed.

A summary of the biodistribution data of [131I]-2Rs15d and [131I]-control sdAb in BT474/M1 tumor xenografted mice is presented in Fig. 4B and Supplementary Table S4. The highest tumor uptake for [131I]-2Rs15d was observed after 1 hour, with
a value of 20.22% ± 1.64% IA/g, while only 0.14% ± 0.06% IA/g was observed in tumor after 4 hours for [131I]-control sdAb. Tumor retention of [131I]-2Rs15d decreased to 5.10% ± 1.90% IA/g at 24 hours and to 0.40% ± 0.05% IA/g at 72 hours. In kidneys, 55.63% ± 8.47% IA/g was measured for [131I]-2Rs15d at 1 hour, decreasing rapidly to 0.94% ± 0.52% IA/g at 24 hours and to 0.24% ± 0.14% IA/g at 72 hours. Thyroid uptake was very low at all time points, indicating no substantial dehalogenation occurred. Radioactivity concentration in the other tissues was low. Radio-SEC indicated that about 80% of the radioactivity was present in urine as intact [131I]-2Rs15d at 30 minutes, decreasing rapidly to 15% and 4% at 1 and 3 hours, confirming that [131I]-SGMIB labeling generates rapidly excreted labeled catabolites (21). No significant differences (P = 0.617) in tumor targeting were observed between animals receiving [131I]-2Rs15d alone (11.00% ± 3.94% IA/g) and animals pretreated with trastuzumab (9.31% ± 2.35% IA/g), pertuzumab (8.91% ± 2.06% IA/g), or both (8.59% ± 2.85% IA/g), as presented in Fig. 4C and Supplementary Table S5. Likewise, no differences in normal tissue uptake between the groups was observed.

**[131I]-2Rs15d organ-absorbed doses**

Organ-absorbed doses from 37 MBq of [131I]-2Rs15d are summarized in Supplementary Table S4. The highest absorbed dose was delivered to tumor (11.88 Gy), while kidneys received 8.36 Gy. Doses delivered to other healthy organs and tissues were very low. The absorbed doses calculated for cumulative administration of 46.25 MBq [131I]-2Rs15d and 27.75 MBq of [131I]-2Rs15d + trastuzumab for therapy (see below) are depicted in Fig. 5A. Patient organ-absorbed doses were estimated using OLINDA via extrapolation to the adult female phantom (Table 1). The effective dose for the adult female was 0.0273 mSv/MBq.

**Therapeutic efficacy of [131I]-2Rs15d**

BT474/M1 xenografted mice receiving [131I]-2Rs15d had a significant longer (P < 0.05) median survival of 137.5 days versus...
93.5 and 78 days for mice receiving [\(^{131}\text{I}\)]-control sdAb and vehicle, respectively (Fig. 5B and C). No statistically significant difference in survival was observed between control groups (\(P = 0.98\)). Moreover, half of the animals receiving [\(^{131}\text{I}\)]-2Rs15d showed absence of tumors after 150 days, compared with only 1 of 6 in the vehicle group and 0 of 6 in the [\(^{131}\text{I}\)]-control sdAb group.

In the second experiment performed in an aggressive trastuzumab-responsive metastatic model, SKOV-3.IP1 xenografted mice treated with [\(^{131}\text{I}\)]-2Rs15d had a significantly longer...
Figure 5.
A, Calculated absorbed doses to normal organs and tumor for a cumulative administration of 46.25 MBq \([^{131}I]\)-2Rs15d and trastuzumab + 27.75 MBq of \([^{131}I]\)-2Rs15d, the doses used in the therapy experiments presented in B, C and D, E. B, Tumor volumes (mm\(^3\)) for individual mice in function of time during therapy. Mice were euthanized when tumor size exceeded 1,000 mm\(^3\), a sudden >20% weight loss was measured, or when necrotic tumor tissue presented. C, Survival after treatment with \([^{131}I]\)-2Rs15d or \([^{131}I]\)-control sdAb in mice with BT474/M1 xenografts (n = 6). Mice injected with \([^{131}I]\)-control sdAb reached tumors >1,000 mm\(^3\) between 40 and 140 days, which led to a median survival of 93 days. In the group injected with vehicle solution, three animals reached tumors >1,000 mm\(^3\) between 78 and 113 days. Two animals developed necrotic tumors and were euthanized after 36 and 102 days. After 150 days, one animal in the vehicle group had a small lesion of about 110 mm\(^3\). Three animals treated with \([^{131}I]\)-2Rs15d developed tumors exceeding 1,000 mm\(^3\) between 120 and 127 days after tumor cell inoculation. The other three animals were tumor-free after 150 days. Survival was significantly longer for animals in the treated group compared with those in the control groups (P < 0.05), as determined with log-rank Mantel–Cox test. Moreover, half of the animals receiving \([^{131}I]\)-2Rs15d showed complete absence of tumors after 150 days. D, BLI quantification of tumor tissue in the peritoneum for individual mice in function of time during therapy. Mice were euthanized when the quantified BLI signal exceeded 5.0 \(\times\) 10\(^7\) ph/s/cm\(^2\)/sr, when severe ascites was observed or when a sudden >20% weight loss was measured. E, Therapeutic efficacy of \([^{131}I]\)-2Rs15d, trastuzumab or a combination of both in mice with SKOV-3.IP1 xenografts (n = 8). All animals reached a BLI signal in peritoneum of 5.0 \(\times\) 10\(^7\) ph/s/cm\(^2\)/sr, except for two animals in the trastuzumab group (day 112 and 126) and one in the trastuzumab + \([^{131}I]\)-2Rs15d group (day 88) which were euthanized due to a >20% weight loss. One animal in the PBS (day 39), 2Rs15d (day 39), and trastuzumab (day 88) group, and three animals in the \([^{131}I]\)-2Rs15d group (day 53 and 67) were euthanized due to severe ascites. One animal in the trastuzumab + \([^{131}I]\)-2Rs15d was alive at the end of the study. No significant difference in survival was observed between the groups receiving vehicle and unlabeled 2Rs15d (P = 0.37). Mice treated with \([^{131}I]\)-2Rs15d had a significantly longer median survival of 59 days, versus only 39 days for mice receiving unlabeled 2Rs15d or vehicle solution respectively (P < 0.005, log-rank Mantel–Cox test). Trastuzumab treatment led to a median survival of 89 days, while mice receiving \([^{131}I]\)-2Rs15d + trastuzumab had a median survival of 85 days (difference not significant, P = 0.84, log-rank Mantel–Cox test). Median survival in both groups receiving trastuzumab was significantly longer than that for animals receiving \([^{131}I]\)-2Rs15d alone (P < 0.0001, log-rank Mantel–Cox test).
as if you were reading it naturally.
between MUC4-positive tumors and a shorter DFS in patients receiving adjuvant trastuzumab-based treatment (38). We show here that [131I]-2Rs15d binds four times higher to JIMT-1 compared with [131I]-trastuzumab, while binding to trastuzumab-responsive BT474/M1 was similar for both compounds (Fig. 1F and G). In contrast, the trastuzumab-competing sdAb 2Rs17c outperformed trastuzumab on the same cell line, which implies that a smaller-sized sdAb was less hindered by the presence of MUC4. In addition, as the cytotoxic β-particles of 131I transverse multiple cell diameters, the therapeutic effect of [131I]-2Rs15d might be less influenced by impaired cell-intestinalization or intratumoral HER2 heterogeneity including the presence of truncated HER2 in a subfraction of tumor cells, compared with trastuzumab and T-DM1 (6).

We here showed that [131I]-2Rs15d binds specifically to HER2, and when injected intravenously in mice, it is eliminated from blood rapidly. High contrast micro-SPECT/CT images delineated tumors as early as 1 hour postinjection in two distinct HER2 mouse models. The clearance of [131I]-2Rs15d from kidneys was faster than ever observed for this sdAb (18, 20, 21, 35, 36). For example, dosimetric calculations revealed that 37 MBq of [131I]-2Rs15d achieved an absorbed dose of only 8 Gy to kidneys. This is an important improvement compared with the absorbed doses recalculated for 37 MBq [177Lu]DTPA-2Rs15d, in tandem with 150 mg/kg gelofusin treatment, to kidneys (10 Gy) (21). Analysis of the radioactivity present in urine revealed the increasing presence of radiolabeled metabolites, confirming that [131I]SGMIB indeed goes rise to rapidly clearing catabolites after renal filtration (23). This is an important benefit compared to the previously used radioiodinated chemistry that led to retained radioactivity in kidneys (18, 19, 21).

The tumor uptake for [131I]-2Rs15d was lower than that obtained with [1]I-5F7GCC sdAb. Here, absorbed doses of 45 and 30 Gy were delivered to BT474/M1 tumors from 37 MBq in two independent experiments, and about 18 and 16 Gy to kidneys (25). The difference in tumor uptake between 2Rs15d and 5F7GCC might be attributable to the fact that they target different HER2 epitopes (25). Because [1]I-SGMIB traps radioiodine intracellularly, this effect is more pronounced for highly internalizing sAbs like 5F7GCC than for moderately internalizing sAb like 2Rs15d. The faster washout from tumor in case of 5F7GCC allows higher radioactive levels of [131I]-2Rs15d. This is an important benefit compared to the previously used radioiodinated chemistry that led to retained radioactivity in kidneys (18, 19, 21).

Mice with BT474/M1 xenografts treated with [131I]-2Rs15d had a significantly longer median survival (137.5 days) compared with animals receiving [131I]-control sdAb (93.5 days) and vehicle (78 days) with half of the animals receiving [131I]-2Rs15d exhibiting no visible evidence of tumor after 150 days (Fig. 5B and C). In the SKOV-3-IP1 metastatic model, treatment with [131I]-2Rs15d prolonged median survival with 20 days compared with controls. Animals receiving either trastuzumab alone or in combination with [131I]-2Rs15d lived on average 50 days longer than the controls, and on average 30 days longer than those treated with [131I]-2Rs15d alone. Even more so, 12.5% of mice that received the combination of trastuzumab and [131I]-2Rs15d were alive at the end of the study, confirming that repeated coadministration of [131I]-2Rs15d does not negatively affect therapeutic outcome (Fig. 5D and E). It is important to note that this trastuzumab-responsive SKOV-3-IP1 model is very aggressive and is defined by rapid disease progression (29). Mice were treated with an optimized trastuzumab treatment over 5 weeks and progressed after treatment termination. We anticipated therefore that [131I]-2Rs15d would not outperform the trastuzumab regimen. The limited median survival of mice treated with trastuzumab-alone and the combination might be related to the presence of surviving tumor clones with absent/lower HER2 expression. Whether trastuzumab treatment downregulates HER2 is still unclear (39).

It is encouraging to see that 46.25 MBq of [131I]-2Rs15d shows therapeutic efficacy in two HER2 animal models, with an estimated absorbed dose of 15 Gy to tumor and only 10 Gy to kidneys (Fig. 4A). We did not expect the occurrence of nephrotoxicity, as we are well below the renal toxicity threshold of 23 Gy. A similar radioactive dose to kidneys using [177Lu]-PSMA 61P did not induce any late renal toxicity (40). The therapeutic effect obtained with [131I]-2Rs15d was less pronounced compared with that obtained with [177Lu]DTPA-2Rs15d (21), but in the latter, we administered about 150 MBq resulting in a recalculated absorbed dose to tumor of 40 Gy, compared with 15 Gy [at 46.25 MBq] to tumor in this study. On the basis of dosimetry calculations, we could increase the cumulative activity administered by twofold, without the need for extra kidney-protective measures such as coinjection with gelofusin and/or positively charged amino acids, which could theoretically lead to a tumor absorbed dose of 30 Gy. Future long-term studies of renal toxicity will be required (40).

In addition, therapeutic efficacy of [131I]-2Rs15d will be further assessed in the trastuzumab-resistant JIMT-1 model, which is targetable by 2Rs15d but not by trastuzumab. With these goals in mind, we are currently upscaling the radiochemical process to obtain higher radioactive levels of [131I]-2Rs15d. α-Particle-emitting isotopes might achieve higher therapeutic absorbed doses to tumors compared with β-particles. However, as their path length is shorter, the cell killing efficiency is more influenced by receptor heterogeneity (41). Consequently, α-particle therapy might be more suited in a micro-metastatic setting. In line with antibody-drug conjugates, sAbs have been successfully conjugated with cytotoxic payloads like DM1 or PE38-toxin, showing efficient tumor growth control without systemic toxicity (42–44). However, selecting highly internalizing sAbs will be mandatory to induce significant cytotoxic effects. In addition, receptor heterogeneity might affect therapeutic outcome, which can be effectively addressed with a cytotoxic agent such as 131I with a multicellular range of action.

To our knowledge, this is the first study to describe a theranostic radioiodinated sdAb suitable for clinical translation. [131I]-2Rs15d was successfully applied as an imaging agent using micro-SPECT/CT, and as a therapeutic agent in two distinct HER2 mouse models. Taken together, these data indicate that [131I]-2Rs15d shows promise as a theranostic drug with a low toxicity profile, significant therapeutic efficacy and of potential benefit for patients that progress on trastuzumab, pertuzumab, or T-DM1. We envision the imaging component as a pretreatment scan after the administration of low radioactive dose [1]-2Rs15d. This allows patient selection and exact dosimetry calculations for therapeutic [131I]-2Rs15d, which could impact therapeutic outcome and an understanding of both therapeutic response as well as any normal tissue toxicities that might arise. A first-in-human clinical study is currently ongoing evaluating low radioactive dose [131I]-2Rs15d in healthy volunteers and HER2+ breast cancer patients (NCT02683083).
Disclosure of Potential Conflicts of Interest

M. D’Huyvetter is an employee of Camel-IDS and holds ownership interest (including patents) in Nanobody Therapeutics. G. Raes is an employee of, holds ownership interest (including patents) in, and is a consultant/advisory board member for Camel-IDS. T. Lahoutte is an employee of Camel-IDS, holds ownership interest (including patents) in, and is a consultant/advisory board member for IBA and IRE Belgium. N. Devoogdt is a former employee of Camel-IDS and holds ownership interest (including patents) in Cam Eligid Single Domain Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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References


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