Approvals

The Belgian federal agency for medicines and health products, the regional ethics committee of UZ Brussel, and the radiation protection agency of Belgium approved this study. The study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. Written informed consent was obtained from all participants. The study was registered as a clinical trial with the identifier EudraCT 012-001135-31.

Patient Selection

Twenty adult female breast carcinoma patients with local, locally advanced, or metastatic breast carcinoma that showed a moderate or high expression of HER2 on immunohistochemistry (2+ or 3+) were included in the study. Patients were allowed to enter the study at first diagnosis, at relapse, or under treatment (with the exclusion of any HER2-targeted treatment) if there was at least one documented breast carcinoma lesion. No additional work-up for the detection of potential additional metastasis was required for this study, since tumor-targeting potential was not the primary aim. Exclusion criteria were the male sex, pregnancy, breast feeding, HER2-targeted therapy in the last 30 d before administration, known abnormal liver or kidney function, serious active infection, a recent gastrointestinal disorder with diarrhea, another life-threatening illness, inability to communicate reliably or give informed consent, unwillingness of cooperating with the requirements of the study, or previous participation in the study.

Patients 2 and 11 were withdrawn from the biodistribution and dosimetry study because of decreased renal function and because their liver enzymes were more than 1.5 times above normal at the time of imaging. Their images were, however, evaluated for tumor-targeting potential.

Safety Assessment

For safety evaluation, all patients underwent measurement of vital signs (blood pressure, heart rate, and temperature) and clinical laboratory testing (standard hematologic and comprehensive metabolic panels that included hemoglobin, white blood cells, neutrophils, lymphocytes, platelets, creatinine, blood urea nitrogen, calcium, sodium, potassium, carbon dioxide, lactate dehydrogenase, alanine transaminase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, and albumin) before and 2 h after injection of the compound. Subjective adverse experiences were assessed using open questions before injection, throughout the 2 h that patients were in the nuclear medicine department, and by telephone up to 24 h after injection.

Conjugation of p-SCN-Bn-NOTA to Anti-HER2 Nanobody

The anti-HER2 Nanobody was produced according to good-manufacturing-practice standards. To allow complexation of the $^{68}$Ga radiometal, a p-SCN-Bn-NOTA chelator was conjugated to the Nanobody as described earlier (1). Briefly, Nanobody in 0.05 M sodium carbonate buffer, pH 8.7, was added to p-SCN-Bn-NOTA (10-fold molar excess) and incubated for 2 h at room temperature. The coupling reaction was quenched by adjusting the pH to 7–7.4 using 1N HCl. The conjugate was then purified by size-exclusion chromatography on a Superdex 75 10/300 GL (GE Healthcare) using 0.1 M ammonium acetate, pH 7, as eluent or 0.01 M phosphate-buffered saline.

Synthesis of $^{68}$Ga-HER2-Nanobody

$^{68}$Ga was obtained from a $^{68}$Ge/$^{68}$Ga generator (Eckert and Ziegler), eluted with 0.1N HCl (Merck). The 1.5-mL peak fraction (250–400 MBq) was added to 1 M sodium acetate buffer, pH 5 (1 ml), containing NOTA-Anti-HER2 Nanobody (1.1–3.8 nmol), and the final pH was 4–4.5. The reaction mixture was incubated for 5–7 min at room temperature. Next, the product was purified by gel filtration on a disposable PD10 column (GE Healthcare), equilibrated with 0.01 M...
phosphate-buffered saline, pH 7.4. The compound was finally filtered through a 0.22-µm membrane filter (13 mm; Millipore).

The final solution was analyzed by instant thin-layer chromatography performed on silica gel (Agilent) using 0.1 M sodium citrate, pH 5.0, as eluent to evaluate radiochemical purity ($^{68}$Ga-Nanobody $R_t = 0$, unbound $^{68}$Ga $R_t = 1$). The final product was also analyzed by reverse-phase high-performance liquid chromatography using a polystyrene divinylbenzene copolymer column (PLRP-S 300 Å) to evaluate radiochemical purity and radiochemical identity ($t_R = 12.8$ min).

In addition, quality control of $^{68}$Ga-Nanobody involved analysis of the appearance of the solution, radionuclide identity ($\gamma$-spectrum), presence of $^{68}$Ge, pH, filter integrity (bubble point test), endotoxin (Limulus amebocyte lysate test), and sterility (microbiology).

For the different patient groups, different masses of NOTA-anti-HER2 Nanobody were injected. For the higher masses, the necessary amount of cold NOTA-anti-HER2 Nanobody (in 0.01 M phosphate-buffered saline, pH 7.4) was added to the $^{68}$Ga-Nanobody before final filtration.

$^{68}$Ga-Nanobody PET/CT Imaging

Images were acquired using a Philips Gemini TF PET/CT system (LySO-based PET scanner with time-of-flight capability, an 18-cm axial and 70-cm transaxial field of view, and 64-slice CT). The scanner was accredited by the EANM via the EARL program.

The $^{68}$Ga-Nanobody was injected as an intravenous bolus. Whole-body images were acquired 10, 60, and 90 min after injection. The time per bed position was 1 min (for a total scan time of about 25 min).

Low-dose CT was performed for attenuation correction and localization of hotspots on PET, consisting of slices of 512 by 512 pixels (field of view: 600 mm) at a 5-mm slice thickness, acquired at 120 kV and 50 mAs, resulting in a radiation dose (CT dose index) of 2.9 mGy/scan.

PET images were reconstructed to a 144 × 144 matrix with a 4-mm slice thickness (4-mm isotropic pixels) using the vendor’s standard BLOB-OS-TOF reconstruction with 3 iterations and 33 subsets (at a kernel width of 14.1 cm) with attenuation, scatter, and randoms correction.

Blood and Urine Samples

Blood samples were taken from a peripheral vein before injection of the compound; at 2, 5, 10, and 40 min after injection; and at 1 and 2 h after injection. Urine sample were collected at about 45 min and 2 h after injection. An additional blood sample was collected at least 3 months after tracer injection and used for antidrug antibody (ADA) detection.

Whole-blood and plasma samples were counted against appropriate standards of known dilution in an automatic $\gamma$-well counter and, after correction for decay and background activity, expressed as a percentage of the injected activity (%IA). The blood volume of each volunteer was estimated according to body weight and height, using the Nadler formula and the patient’s hematocrit. The blood activity results of 8 patients were not further used in the analysis because of altered liver or kidney tests (patients 2 and 11), because of injection of the tracer and blood sampling on the same arm (patients 10, 16, 18, 19, and 20), or because of possible interference with $^{99m}$Tc blood activity due to a cardiac blood pool scan 2 d before the study (patient 14). Blood half-lives were calculated with a 2-phase exponential decay model using Prism software (GraphPad Software). Plasma and urine aliquots were analyzed by size-exclusion chromatography or reverse-phase high-performance liquid chromatography to identify possible metabolites.
Detection of ADA

The presence of ADA was analyzed on serum samples of all patients obtained before and at least 3 mo after tracer injection, by sandwich ELISAs of anti-HER2-Nanobody and biotin-anti-HER2-Nanobody and of NOTA-anti-HER2-Nanobody and biotin-NOTA-anti-HER2-Nanobody. Both assay setups were optimized with polyclonal rabbit antibody, obtained after immunization of rabbits with anti-HER2-Nanobody. The plates were coated with 1 µg/mL of Nanobody (anti-HER2-Nanobody or NOTA-anti-HER2-Nanobody) and blocked with Pierce Protein-free blocking buffer (ThermoFisher Scientific), and samples were added in a 1/40 dilution. Detection was made with biotinylated Nanobody (anti-HER2 or NOTA-anti-HER2). Biotinylation was performed with EZ-Link Sulfo-NHS-LC-biotin (Thermo Scientific), according to the manufacturer’s instructions. Streptavidin-HRP (Sigma) was added in 1/2,000 dilution. Staining was performed with tetramethylbenzidine, and the reaction was stopped with 2 M H$_2$SO$_4$. Absorbance was measured at 450 nm.

Serum samples (obtained from ImmunXperts) from 50 healthy donors were analyzed to determine the cutoff value for both setups. Additionally, all samples were analyzed in the presence of 0.5 µg/mL Nanobody to determine the percentage inhibition above which a sample is considered to be confirmed as positive.

Serum samples from all patients were taken before and at least 3 mo after $^{68}$Ga-HER2-Nanobody injection and analyzed in both ELISA sandwiches, in the absence and presence of Nanobody. In every multiwell plate, negative and positive controls (low, medium, and high) were included, according to the recommendations for ADA immunoassays (2).

Region-of-Interest Definition

The $^{68}$Ga-HER2-Nanobody uptake in different organs was determined using regions of interest (ROIs), drawn using MIM contouring software (MIM software Inc., 2014). For biodistribution and dosimetry purposes, organs with tracer uptake were delineated in a semiautomatic (region grow) fashion and manually corrected to ensure that activity on the PET images that was contributing to the organ was included in the ROI (kidneys, intestines, thyroid, whole body, and urinary activity in bladder and ureter). For 5 organs not showing substantial tracer uptake, organ delineation was based on CT data (spleen, heart muscle, lungs, central bone [from top of skull to proximal femora, representing hematopoietic bone marrow] and unaffected breast). Care was taken that activity originating from kidneys, liver, intestines, bladder, and urine was not included in the CT-based ROIs. To reflect normal biodistribution in breast tissue, the activity in the unaffected breast was multiplied by two. For assessment of liver activity, the liver volume was determined using CT-based delineation and multiplied by the average activity in liver parenchyma, as determined by a spheroid ROI of 40-mm diameter, positioned in the right liver lobe. This approach was chosen to overcome the influence of potential motion artifacts in the liver due to breathing, or inhomogeneity in liver activity due to underlying liver metastasis.

Dosimetry

For dosimetric calculations, the OLINDA/EXM software 1.0 (Organ Level Internal Dose Assessment/EXponential Modeling, Vanderbilt University) was used (3). OLINDA/EXM software entails the EXM code, which performs kinetic analysis of biokinetic data for input into the dose calculation algorithms. The organ uptake values per patient were put into the EXM analysis software and a biexponential fit was performed per organ for each patient. OLINDA subsequently calculates the disintegrations per source organ as well as the radiation dose for all target organs from these data. Other input variables were the excretion parameters, put at 100% renal excretion with a biological half-life of 60 min and a voiding bladder interval of 60 min.
Uptake in Tumor Lesions

A sphere-shaped ROI with a diameter of 10 mm was placed within each discernable tumor lesion that measured at least 10 mm on the low-dose CT, obtained for study purposes, or on other available imaging data, using MIM contouring software. For large lesions, the ROI was positioned over the area with the highest uptake. The mean standardized uptake value ($\text{SUV}_{\text{mean}}$), corrected for body weight, within this ROI is reported as the uptake value for a lesion. If available, the uptake in the primary lesion and in the metastasis showing the highest $\text{SUV}_{\text{mean}}$ is reported. Osirix software (Pixmeo) was used for image processing.

Statistical Analysis

Values are reported as mean ± SD. A 1-way ANOVA was conducted to compare the effect of injected Nanobody mass (3 patient subgroups) on tracer uptake in the liver. The statistical analysis used for the assay to ADA was performed as follows. For each set of data of the healthy controls, the data were first analyzed for normality of distribution using the Shapiro–Wilk test. If data were not normally distributed, outliers were identified using the ROUT test ($Q = 5\%$) in GraphPad Prism. After removal of the outliers, data were analyzed again for normality of distribution. If data were normal distributed, the cut-point was calculated as mean + $1.645 \times \text{SD}$. If data were not normally distributed, the cut-point was calculated using the 95th percentile. The percentage inhibition was calculated as $100 \times [1 – (\text{sample with Nanobody}/\text{sample without Nanobody})]$. Each data set was analyzed for normality of distribution. If a nonnormal distribution was found, data were analyzed for outliers. After removal of the outliers, data were again analyzed for normality of distribution. The specificity cut-point was calculated as mean + $3.09 \times \text{SD}$. 
SUPPLEMENTAL FIGURE 1. ADA analysis showed no preexisting or treatment-induced antibodies against the injected Nanobody. Fifty healthy donors were tested in a sandwich ELISA, once in an anti-HER2-Nanobody setup and once in a NOTA-anti-HER2-Nanobody setup, to determine the screening cut-points at 0.128 and 0.118, respectively (A). All patient samples (before and 3 mo after injection with the Nanobody) were analyzed in the absence (screen) and presence (confirm) of anti-HER2-Nanobody (B) or NOTA-anti-HER2-Nanobody (C), in the respective ELISA setup. For B and C, 2 results per patient are shown, representing the measurements on blood samples taken before and 3 mo after injection of the Nanobody. None of the patient samples with a positive screening test showed a signal inhibition above the specificity cut-point for the confirmation assay (defined as 16.16% and 14.98% for anti-HER2-Nanobody or NOTA-anti-HER2-Nanobody, respectively).
## SUPPLEMENTAL TABLE 1. Uptake of $^{68}$Ga-NOTA-Anti-HER2 in Single Organs

<table>
<thead>
<tr>
<th>Organ</th>
<th>Uptake (%IA)</th>
<th>10 min</th>
<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>25.41 ± 8.39</td>
<td>12.47 ± 4.88</td>
<td>8.18 ± 3.07</td>
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<tr>
<td>Kidneys</td>
<td>12.74 ± 2.50</td>
<td>8.14 ± 1.55</td>
<td>6.36 ± 1.23</td>
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<tr>
<td>Intestines</td>
<td>11.76 ± 1.62</td>
<td>6.72 ± 1.11</td>
<td>4.72 ± 0.80</td>
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<tr>
<td>Central bone</td>
<td>3.65 ± 1.02</td>
<td>1.80 ± 0.53</td>
<td>1.20 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>Lungs</td>
<td>2.83 ± 0.56</td>
<td>1.48 ± 0.33</td>
<td>0.98 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>Breasts</td>
<td>1.00 ± 0.54</td>
<td>0.58 ± 0.31</td>
<td>0.39 ± 0.19</td>
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</tr>
<tr>
<td>Spleen</td>
<td>0.48 ± 0.16</td>
<td>0.22 ± 0.09</td>
<td>0.15 ± 0.07</td>
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</tr>
<tr>
<td>Heart muscle</td>
<td>0.40 ± 0.12</td>
<td>0.19 ± 0.06</td>
<td>0.12 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Thyroid</td>
<td>0.12 ± 0.08</td>
<td>0.05 ± 0.03</td>
<td>0.03 ± 0.02</td>
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</tbody>
</table>

## SUPPLEMENTAL FIGURE 2. Total-body activity over time, represented as the average and SD of 18 patients with normal renal and hepatic function. At 1 h after injection, 50% of the tracer had been eliminated from the body.
Supplemental References


**SUPPLEMENTAL FIGURE 3.** Heterogeneous uptake pattern in patient 8, classified as HER2 IHC 2+ but FISH-negative based on a core-needle biopsy. \(^{18}\)F-FDG PET images were obtained 6 d before \(^{68}\)Ga-NOTA-Anti-HER2 PET. (A) Heterogeneous uptake of \(^{68}\)Ga-HER2-Nanobody in the primary tumor, with a pattern that does not match \(^{18}\)F-FDG distribution: area with intense \(^{68}\)Ga-HER2-Nanobody but weak \(^{18}\)F-FDG uptake (arrow); area with faint \(^{68}\)Ga-HER2-Nanobody but intense \(^{18}\)F-FDG uptake (arrowhead). (B) Intense \(^{68}\)Ga-HER2-Nanobody uptake in a bone metastasis located in the coccyx (arrow); absence of uptake in bone metastasis in right iliac bone (arrowhead). (C) Low \(^{68}\)Ga-HER2-Nanobody uptake in muscle (arrow) and lymph node (arrowhead) metastasis.