
Site-Specific Radiometal Labeling and Improved Biodistribution Using ABY-027, A Novel HER2-Targeting Affibody Molecule–Albumin-Binding Domain Fusion Protein

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Because of their better penetration, smaller targeting proteins may be superior to antibodies for radioimmunotherapy of solid tumors. Therefore, Affibody molecules (6.5 kDa) have a potential for being suitable as targeted moiety for radiolabeled therapeutic proteins. Previous studies have demonstrated that a fusion of an Affibody molecule with an albumin-binding domain (ABD) provides a strong noncovalent binding to albumin *in vivo*. This strong noncovalent binding can be used for reduction of the renal uptake of the Affibody molecule while maintaining a size smaller than that of an antibody, which is important when using residualizing radionuclide labels conjugated to Affibody molecules. The goal of this study was to design and evaluate a new targeting Affibody–ABD fusion protein with improved biodistribution properties for radionuclide therapy. **Methods:** A novel Affibody-based construct, Z_{HER2:2891}-ABD₀₃₅-DOTA (ABY-027), was created by fusion of the reengineered HER2-binding Affibody molecule Z_{HER2:2891} to the N terminus of the high-affinity ABD₀₃₅, and a maleimido-derivative of DOTA was conjugated at the C terminus of the construct. Binding and processing of ¹⁷⁷Lu-ABY-027 by HER2-expressing cells were evaluated *in vitro*. Targeting of HER2-expressing SKOV-3 xenografts was evaluated in BALB/C *nu/nu* mice and compared with targeting of previously reported ABD-(Z_{HER2:342})₂. **Results:** The binding affinity (dissociation constant) of ABY-027 to HER2 (74 pM) was the same as for the parental Z_{HER2:2891} (76 pM). ABY-027 was stably labeled with ¹⁷⁷Lu and ¹¹¹In with preserved specific binding to HER2-expressing cells *in vitro*. *In vivo* receptor saturation experiments demonstrated that targeting of SKOV-3 xenografts in BALB/C *nu/nu* mice was HER2-specific. ¹⁷⁷Lu-ABY-027 demonstrated substantially (2- to 3-fold) lower renal and hepatic uptake than previously assessed HER2-specific Affibody-based albumin-binding agents. Tumor uptake of radiolabeled ABY-027 at 48 h after injection was 2-fold higher than that for previously reported ABD-(Z_{HER2:342})₂. **Conclusion:** An optimized molecular design of an ABD fusion protein resulted in an Affibody molecule construct with better properties for therapy. Fully preserved *in vivo* targeting of the fusion protein was shown

in xenografted mice. Site-specific coupling of DOTA provides a uniform conjugate and creates the potential for labeling with a broad range of therapeutic radionuclides. The biodistribution of ¹⁷⁷Lu-ABY-027 in a murine model suggests it is more suitable for therapy than alternative approaches.

Key Words: HER2; affibody molecule; ¹⁷⁷Lu; targeting therapy; albumin

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During the last decades, impressive progress in the treatment of disseminated cancer has been achieved. Conventional chemotherapy has been supplemented with new therapeutic modalities that exploit the recognition of unique molecular features of cancer cells or the microenvironment in tumor tissue, resulting in increased selectivity of several therapies and reduced toxicity to healthy tissues. One cancer-associated molecular target for therapy is the human epidermal growth factor receptor type 2 (HER2, Neu, ErbB2). HER2 is a member of the epidermal growth factor tyrosine kinase receptor family involved in the regulation of cell proliferation, motility, and apoptosis suppression. HER2 is overexpressed in many types of carcinomas and is considered to be part of the malignant phenotype (1). A recent review reports a HER2 overexpression frequency of 25% for breast cancer, 20%–70% for hormone-refractory prostate cancers, and 35%–55% for colorectal cancer (1). In contrast, HER2 expression in healthy adult tissues is low or nondetectable. Treatment of HER2-expressing breast cancer with trastuzumab, a HER2-specific antibody, significantly prolongs survival of breast cancer patients (2). Unfortunately, many HER2-expressing breast cancers have primary resistance, and others develop treatment-induced resistance to the HER2-binding monoclonal antibody trastuzumab within 1 y (3). Resistance often develops despite sustained HER2 expression (3).

The persistence of HER2 expression may be used for targeting a payload to the tumor cells, including cytotoxic

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agents and radionuclides (4). Radionuclide therapy offers the advantage of a cross-fire effect occurring when nuclides delivered to cancer cells irradiate neighboring malignant cells, which can overcome issues associated with heterogeneous expression of a particular malignant target or nonuniform penetration of targeting agents into the tumor tissue (5). Radioimmunotherapy of non-Hodgkin lymphoma using the radiolabeled antibodies ^{131}I -tositumomab and ^{90}Y -ibritumomab tiuxetan has demonstrated high response rates and constitutes an encouraging example of targeted radionuclide therapy (6). However, targeted radionuclide therapy of solid tumors using immunoglobulins remains a challenge (7). The major reasons are the long residence time of the large (150 kDa) immunoglobulins in circulation, resulting in irradiation of healthy tissues, such as bone marrow, and slow extravasation and penetration into the tumor, limiting the therapeutic effect (8). The slow diffusion and poor penetration of antibodies into solid tumors has raised interest in smaller targeting proteins as carriers for radionuclides and other payloads. Proteins with a mass below the cutoff for renal clearance (~60 kDa) are rapidly lost from the bloodstream (9). Despite the short circulatory half-life, high-affinity proteins may yield high levels of solid tumor targeting, probably because of greatly superior tumor penetration, as exemplified with a 6.5-kDa HER2-binding Affibody (Affibody AB) molecule (10,11).

Our approach is to make targeted therapeutic agents based on Affibody molecules. Affibody molecules constitute a new class of affinity proteins based on a 58-amino-acid residue protein domain derived from staphylococcal protein A (12,13). Affibody molecules combine small size (~6.5 kDa) with high affinity and specificity for the target. The most studied is the $Z_{\text{HER2:342}}$ Affibody molecule, binding to HER2 with high affinity (dissociation constant [K_D], 22 pM) (11). The clinical utility for imaging of HER2 expression in patients with metastasized breast cancer was demonstrated using a chemically manufactured variant of $Z_{\text{HER2:342}}$ labeled with ^{111}In or ^{68}Ga (14). The main obstacle for using Affibody molecules with residualizing radiometal labels for radionuclide therapy is their predominant renal excretion associated with nearly quantitative tubular reabsorption (15–17). The renal concentration of radioactivity in a murine model at 4 h after injection was as high as 250 percentage injected dose per gram (%ID/g) for Affibody variants labeled with ^{111}In or ^{177}Lu using a DOTA chelator (15,16).

One of the approaches for the generation of the new targeting agents is to assemble the targeting molecule from smaller parts, providing the desired biologic and pharmacologic properties. In our previous studies (17,18), we demonstrated that fusion of the anti-HER2 Affibody molecule dimer ($Z_{\text{HER2:342}})_2$ to a 5-kDa, non-cysteine-containing, 3-helix-bundle albumin-binding domain (ABD)₀₃₅ modified the pharmacokinetics. The residence time in blood was appreciably extended, and the renal uptake was reduced 20- to 30-fold in comparison with non-ABD-fused Affibody molecules. The ^{177}Lu -CHX-A''-DTPA-ABD-($Z_{\text{HER2:342}})_2$ conjugate

demonstrated a curative effect in murine microxenografts with high HER2 expression (17). In a parallel study, Dennis et al. showed that a fusion protein of a Fab fragment and an albumin-binding peptide was superior to the Fab fragment in terms of tumor penetration (19). Serum albumin has previously been shown to preferentially locate in tumors, compared with normal tissue (20), and the favorable tumor penetration of an albumin-associated fusion protein might be a result of combining favorable localization with high-affinity binding to a protein on the tumor cell surface.

In the present study, the improved HER2-targeted Affibody molecule $Z_{\text{HER2:2891}}$ has been combined with an engineered ABD fusion protein ABD₀₃₅, which has a high affinity (K_D , 50–500 fM) for human serum albumin (21). ABD₀₃₅ also displays higher affinity for murine serum albumin than the parent ABD, found in the HER2-binding fusion protein used in our previous study. The Affibody molecule $Z_{\text{HER2:2891}}$ is an engineered derivative of $Z_{\text{HER2:342}}$ used in that study. The target-binding surface has been retained, but the nonbinding surface has been optimized and is distinctly different from the parental molecule (22). $Z_{\text{HER2:2891}}$ was recently validated in a clinical trial and was shown to target HER2-expressing metastatic lesions in breast cancer patients (23). In the fusion protein, denoted ABY-027, the Affibody moiety was inserted N-terminally of the ABD, to separate the HER2 and the albumin-binding surfaces as far as possible. In the Affibody molecule, the HER2-binding surface is located on helices 1 and 2, whereas in the ABD the albumin-binding surface is located on helices 2 and 3 of the triple helical protein domain. Last, the chelating group used for labeling the fusion protein was site-specifically introduced, using a unique cysteine residue specifically introduced for this purpose.

The goal of this study was to evaluate the biodistribution and targeting properties of the new potential anti-HER2 therapeutic agent $Z_{\text{HER2:2891}}$ -ABD₀₃₅-C-DOTA (further denoted ABY-027).

MATERIALS AND METHODS

General

^{177}Lu -lutetium and ^{111}In -chloride were from Covidien. Buffers used for conjugation and labeling were purified from metal contaminations using a Chelex 100 resin (Bio-Rad Laboratories). The NAP-5 size-exclusion columns were from GE Healthcare. The radioactivity was measured using an automated γ -counter with a 7.62-cm (3-in) NaI(Tl) detector (1480 WIZARD; Wallac Oy). The radioactivity distribution on the instant thin-layer chromatography strips was measured by a Cyclone Storage Phosphor System and analyzed using the OptiQuant image analysis software (PerkinElmer). CHX-A''-DTPA-ABD-($Z_{\text{HER2:342}})_2$ was produced and labeled with ^{111}In as described earlier (17,18).

Production of $Z_{\text{HER2:2891}}$ -ABD₀₃₅-DOTA (ABY-027)

A DNA fragment encoding $Z_{\text{HER2:2891}}$ (22) was subcloned into a pET (Novagen)-derived expression vector containing a gene encoding ABD₀₃₅ (21) with a C-terminal cysteine, resulting in the expression vector pAY02107. Soluble protein was produced in *Escherichia coli* and purified using human serum albumin

(HSA)-Sepharose (CNBr-activated Sepharose 4FF; Amersham Biosciences AB) as described earlier (17). The $Z_{\text{HER2:2891-ABD}_{035}}$ was conjugated to maleimide-DOTA (Macrocyclics) as described by Ahlgren et al. (24). The conjugate was analyzed by high-performance liquid chromatography and online mass spectrometry (HPLC-MS) using an Agilent 1100 HPLC/MSD equipped with Zorbax 300SB-C18 (4.6×150 , $3.5 \mu\text{m}$) column. The binding kinetics and affinity of ABY-027 for HER2-Fc chimera and for HSA were measured using a Biacore 2000 instrument (GE Healthcare) according to methods described earlier (21,22).

Labeling Chemistry

For labeling, an aliquot of 50 μg of ABY-027 in 0.5 M sodium acetate containing ascorbic acid (10 mg/mL), pH 5.5, was mixed with a predetermined amount (65–76 MBq) of ^{177}Lu or ^{111}In and incubated at 60°C or 80°C for 30 min. A metal-to-ligand ratio of 1:5 was used for labeling. For routine quality control of the labeling, silica gel-impregnated glass fiber sheets for instant thin-layer chromatography (Gelman Sciences Inc.), eluted with 0.2 M citric acid, were used.

For a stability test, 2 aliquots of ^{177}Lu -ABY-027 were mixed with a 500-fold molar excess of ethylenediaminetetraacetic acid, incubated for 60 min at ambient temperature and analyzed as described above. An amount of non-protein-bound ^{177}Lu was compared with a standard not exposed to ethylenediaminetetraacetic acid.

In Vitro Studies

The HER2-expressing ovarian carcinoma cell line SKOV-3 (American Type Culture Collection, LGC Standards AB), displaying approximately 1.6×10^6 HER2 receptors per cell (25), was used in this study. All experiments were performed in triplicate.

The binding specificity for HER2-expressing cells of the ^{177}Lu -ABY-027 conjugates labeled at 60°C or 80°C was tested using methods described earlier (17). For HER2 blocking, a 1,000-fold excess of nonlabeled Affibody molecule was used. Cells were incubated with labeled conjugate (0.5 nM) for 1 h at 37°C, and the percentage of cell-bound radioactivity was determined in blocked and nonblocked samples.

The antigen-binding capacity of ^{177}Lu -ABY-027 labeled at 60°C or 80°C was determined using methods described earlier (17). A solution of ^{177}Lu -ABY-027 in cell culture medium (1 mL, 0.06 pmol) was added to cell pellets to provide an approximately 100-fold molar excess of receptor over conjugate. The cells were incubated for 5 h at 4°C, and the percentage of cell-bound radioactivity was determined.

The cellular retention and processing of ^{177}Lu -ABY-027 by HER2-expressing cells were studied according to the method described earlier (26). Cells were incubated with radiolabeled Affibody conjugate (5 nM) for 1 h at 4°C. The medium was then exchanged, and incubation was continued at 37°C. The membrane-associated radioactivity was determined at preselected time points. The percentage of small (<5 kDa) radiocatabolites in the incubation medium was determined by analysis using NAP-5 size-exclusion columns.

In Vivo Studies

The animal studies were approved by the Local Ethics Committee for Animal Research. The tumor was grafted by subcutaneous injection of approximately 10^7 SKOV-3 cells in the hind legs of female BALB/c *nu/nu* mice. Tumors were allowed to

grow for 4–5 wk before experiments. In all biodistribution studies, groups of 4–6 animals were used. At predetermined time points, the animals were euthanized by heart puncture under anesthesia (ketamine [Ketalar, 10 mg/mL; Pfizer] and xylazine [Rompun, 1 mg/mL; Bayer], 20 μL of solution per gram of body weight). Blood and organ samples were excised and weighed, and their radioactivity was measured. The tissue uptake values were calculated as %ID/g.

For comparison, 2 groups of mice were injected with 50 kBq of ^{111}In -ABY-027 (10 μg) and ^{111}In -CHX-A"-DTPA-ABD- $(Z_{\text{HER2:342}})_2$ (16 μg) in 100 μL of phosphate-buffered saline (PBS), and biodistribution was measured at 48 h after injection. The average tumor weight was 0.2 ± 0.1 g.

To evaluate the influence of the injected ABY-027 protein dose on tumor uptake and biodistribution of ^{177}Lu -ABY-027, 3 groups of mice were injected with 20 kBq in 100 μL of PBS per mouse. The protein dose was adjusted to 1, 10, or 50 μg per mouse by mixing with nonlabeled ABY-027. The biodistribution was compared at 48 h after injection. The average tumor weight was 0.14 ± 0.07 g.

To evaluate the biodistribution and tumor-targeting capacity of the labeled compound, and to obtain input data for dosimetry calculations, 24 mice were injected with 120 kBq of ^{177}Lu -ABY-027 (protein dose, 10 μg in 100 μL of PBS) each. The average tumor weight was 0.10 ± 0.05 g. At predetermined time points (4, 24, 48, 72, 168, and 336 h after injection), a group of 4 mice was euthanized, and the concentration of the radioactivity in tumors and tissues was determined. To confirm HER2-specific tumor targeting of ^{177}Lu -ABY-027, 4 mice were injected with 1 mg each of nonlabeled ABY-027. One hour later, each mouse was injected with 120 kBq of ^{177}Lu -ABY-027 (protein dose, 10 μg in 100 μL of PBS). The animals were euthanized at 48 h after injection, and the biodistribution of radioactivity was determined.

Dosimetry Calculations

The dosimetry calculations were performed according to methods described earlier (17). Briefly, the organ uptake values from the biodistribution study, noncorrected for physical half-life, were time-integrated by the trapezoid method to obtain the residence time per gram of tissue. The extrapolated area was less than a few percent in all organs. In the absorbed dose calculations, S values for ^{177}Lu were obtained from RADAR (Radiation Dose Assessment Resource) phantoms (unit density spheres) (<http://www.doseinfo-radar.com/>). The S value for a 1-g sphere (0.0233 mGy/MBq·s) was used generally to calculate all organ doses. This simplified dosimetry calculation is motivated by the fact that the low-energy β -particles in the ^{177}Lu decay are locally absorbed, and photons and other penetrating radiations are contributing to a low extent, meaning that the crosstalk between different organs in the mouse is negligible.

Statistics

Statistical analyses were performed by unpaired, 2-tailed *t* tests using Prism (version 4.00; GraphPad Software) for Windows (Microsoft). A *P* value below 0.05 was considered significant.

RESULTS

Characterization of $Z_{\text{HER2:2891-ABD}_{035}}$ -DOTA (ABY-027)

The identity and purity of $Z_{\text{HER2:2891-ABD}_{035}}$ -cys and $Z_{\text{HER2:2891-ABD}_{035}}$ -DOTA were characterized by HPLC-MS and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Both analyses confirmed the expected mass of the

constructs (12,122 and 12,646 Da, respectively). The purity of Z_{HER2:2891}-ABD₀₃₅-DOTA was above 98%, as determined by HPLC-MS. No variants other than the 1:1 chelator-to-protein ratio were detected. The binding kinetics of ABY-027 to the extracellular domain of HER2 were measured using surface plasmon resonance. The association rate constant (k_a) was $(8.78 \pm 0.06) \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, the dissociation rate constant (k_d) was $(6.45 \pm 0.03) \times 10^{-4} \text{ s}^{-1}$, and the dissociation constant ($K_D = k_d/k_a$) was 74 pM. A retained high-albumin-binding affinity was confirmed and shown to be similar to that of the single-domain ABD₀₃₅ used as control. Because of high affinity, it was not possible to determine the k_d using surface plasmon resonance

Labeling Chemistry

The labeling of ABY-027 was rapid and efficient under the selected conditions. Labeling with ¹⁷⁷Lu at 60°C provided yields of $94\% \pm 7\%$ after 15 min and $98\% \pm 2\%$ after 30 min ($n = 6$). At 80°C, the yields were $96\% \pm 3\%$ and $97\% \pm 1\%$ after 15 and 30 min, respectively. The specific radioactivity was 1.3–1.5 MBq/μg (16.4–19.3 GBq/μmol), depending on the specific radioactivity of ¹⁷⁷Lu-lutetium chloride. Challenge with a 500-fold molar excess of ethylenediaminetetraacetic acid during 1 h did not reveal any measurable release of radioactivity. Labeling yields were 98.8% for ¹¹¹In-ABY-027 and 99.1% for ¹¹¹In-CHX-A''-DTPA-ABD-(Z_{HER2:342})₂.

Cellular Binding and Retention

The binding of ¹⁷⁷Lu-ABY-027, labeled at 60°C or 80°C, was significantly decreased by preincubation with an excess of nonlabeled compound that demonstrated receptor-mediated binding (Fig. 1). The binding-competent fraction of ¹⁷⁷Lu-ABY-027 labeled at 60°C during 30 min was $88\% \pm 1\%$. Increasing the labeling temperature up to 80°C significantly decreased the binding-competent fraction to $81\% \pm 2\%$ ($P = 0.02$). Because labeling at 80°C did not provide higher yield or more rapid labeling, further biologic experiments were performed with conjugates labeled at 60°C.

Cellular retention and processing experiments demonstrated good retention of ¹⁷⁷Lu radioactivity after interrupted incubation of SKOV-3 cells with ¹⁷⁷Lu-ABY-027 (Fig.

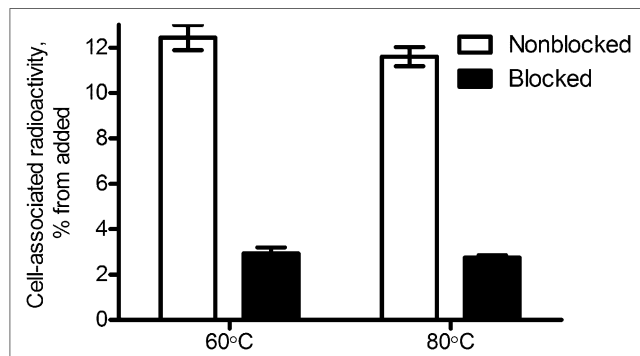


FIGURE 1. In vitro specificity test for ¹⁷⁷Lu-ABY-027 labeled at 60°C and 80°C on SKOV-3 cells (average of 3 ± SD).

2A): half of the cell-associated radioactivity was retained at 24 h after interrupted incubation. Most of the radioactivity release occurred during the first 4 h. Only half of the cell-associated radioactivity had been internalized after 24 h. Size-exclusion chromatography of incubation medium showed that after 24 h of incubation approximately 40% of the radioactivity in the medium was associated with low-molecular-weight (<5 kDa) radiocatabolites, indicating that some of the conjugate was processed intracellularly and radiocatabolites were excreted (Fig. 2B).

Tumor Uptake and Biodistribution

The direct comparison of the biodistribution pattern of ¹¹¹In-ABY-027 and ¹¹¹In-CHX-A''-DTPA-ABD-(Z_{HER2:342})₂ in tumor-bearing mice (Table 1) demonstrated a 2-fold-higher tumor uptake and 3-fold-lower radioactivity retention in kidneys for ¹¹¹In-ABY-027. The radioactivity concentration in other studied tissues was slightly higher for ¹¹¹In-ABY-027. Data for ¹¹¹In-CHX-A''-DTPA-ABD-(Z_{HER2:342})₂ were in good agreement with published results (17,18).

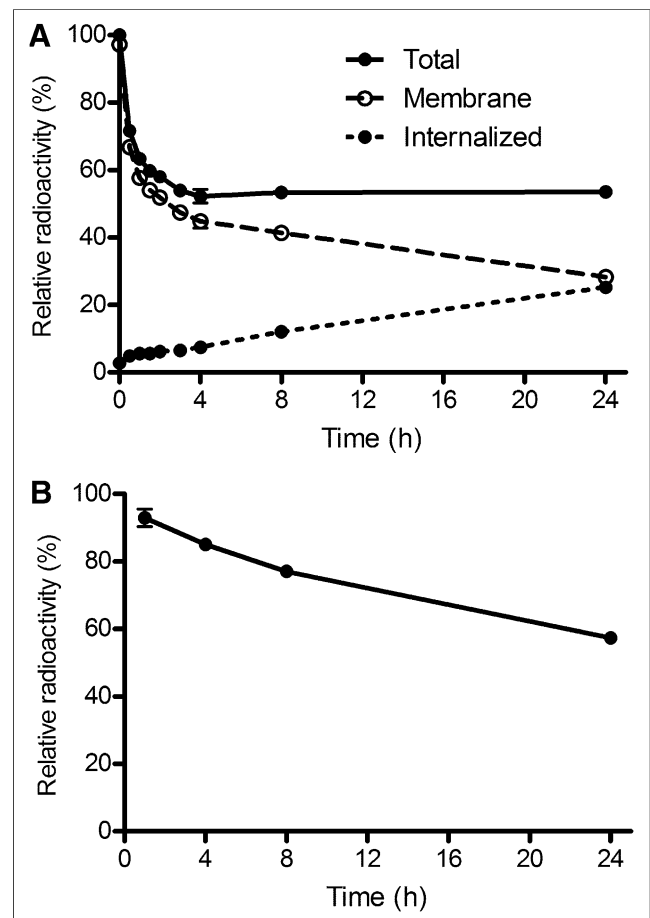


FIGURE 2. (A) Cellular processing of ¹⁷⁷Lu-ABY-027 in SKOV-3 cells after interrupted incubation (average of 3 samples ± SD). In each sample, radioactivity was normalized by cell-associated radioactivity at time of interruption. (B) Size-exclusion analysis of incubation medium after interrupted incubation of ¹⁷⁷Lu-ABY-027 with SKOV-3 cells (average of 2 ± SD).

TABLE 1

Comparison of Biodistribution of Radiolabeled Potential Affibody-Based Therapeutics in Tumor-Bearing Mice

Organ	Concentration of radioactivity, %ID/g at 48 h after injection					
	¹¹¹ In-ABY-027*	¹¹¹ In-CHX-A''-DTPA-ABD-(Z _{HER2:342}) ₂ ^a	¹⁷⁷ Lu-ABY-027*	¹⁷⁷ Lu-CHX-A''-DTPA-ABD-(Z _{HER2:342}) ₂ * (17)	¹¹¹ In-DOTA-ABD-(Z _{HER2:342}) ₂ [†] (18)	¹¹¹ In-DOTA-HSA-(Z _{HER2:342}) _n * (33)
Tumor	43 ± 3	22 ± 7	17 ± 7	26 ± 4	12 ± 3	17 ± 1
Blood	5.9 ± 0.8	4.7 ± 1.0	5.3 ± 0.4	5.5 ± 0.8	4.6 ± 1.0	3.0 ± 0.9
Lung	4.5 ± 0.4	3.4 ± 0.7	3.5 ± 0.5	3.5 ± 0.5	3.3 ± 0.6	2.6 ± 0.6
Liver	7.0 ± 0.9	6 ± 2	3.0 ± 0.7	5.7 ± 0.6	5.2 ± 0.8	14 ± 3
Spleen	5.4 ± 0.8	3.2 ± 0.9	2.9 ± 0.5	3.8 ± 0.4	5 ± 2	9 ± 1
Kidney	6.0 ± 0.6	17 ± 3	3.9 ± 0.6	15.9 ± 0.8	18 ± 2	5.7 ± 0.9
Muscle	1.0 ± 0.2	0.7 ± 0.2	1.4 ± 0.4	1.0 ± 0.3	0.7 ± 0.2	1.0 ± 0.3
Bone	1.54 ± 0.08	1.0 ± 0.3	1.2 ± 0.4	1.8 ± 0.6	1.7 ± 0.6	3.33 ± 0.05

*SKOV-3 ovarian carcinoma xenografts, high HER2 expression.
[†]LS174T colorectal carcinoma xenografts, low HER2 expression.

Data on the influence of ABY-027 protein dose on tumor uptake and biodistribution of ¹⁷⁷Lu-ABY-027 in mice bearing SKOV-3 xenografts are presented in Figure 3. No dose-dependent significant difference in organ accumulation of radioactivity at 48 h after injection of 1, 10, or 50 µg of ABY-027 can be seen, except from a slightly (but significantly) higher retention of radioactivity in the carcass in mice injected with 1 µg of conjugate. The apparent lower tumor concentration of ¹⁷⁷Lu in mice injected with 50 µg of conjugate was not significantly separate from the value obtained with 10 µg (*P* > 0.05). In further experiments, a dose of 10 µg per animal was used.

Data on the biodistribution of ¹⁷⁷Lu-ABY-027 in BALB/C *nu/nu* mice bearing SKOV-3 xenografts at different time points after injection are presented in Figure 4 and Table 2. The mean tumor weight was 98 ± 42 mg in these experiments. Figure 4 presents the results of the targeting specificity test. The tumor radioactivity concentration at 48 h after injection was decreased from 17 ± 7 to 5 ± 1 %ID/g (*P* < 0.015) by preinjection of 1 mg of nonlabeled ABY-027. There was no significant difference in the radioactivity concentrations in all other organs and tissue samples. The decrease of ¹⁷⁷Lu-ABY-027 uptake in the tumor by presaturation of HER2 confirms that the tumor-targeting is receptor-mediated.

¹⁷⁷Lu-ABY-027 demonstrated efficient targeting of HER2-expressing SKOV-3 xenografts in nude mice (Table 2). Already at 24 h after injection, the tumor radioactivity concentration was equal to the radioactivity concentration in blood and higher than in all other studied organs and tissue samples. The concentration of ¹⁷⁷Lu in tumors peaked at 48 h after injection, followed by slow washout. Up to 14 d after injection, the concentration of the radioactivity in the tumor exceeded the concentrations in any other organs. The blood clearance was slow, with an elimination half-life of 41 h. The concentration of radioactivity in excretory organs (kidneys and liver) was lower than that in tumors and blood, peaking between 24 and 48 h after injection.

Dosimetry Calculations

The fraction of decays per gram of the administered activity (the residence time) was calculated by integrating the kinetic data (area under the curve) obtained in BALB/c *nu/nu* mice bearing SKOV-3 tumor xenografts and is presented in Table 3. The contribution of extrapolated data was small, less than 10%. The residence time was multiplied with a conversion factor to obtain the absorbed dose given in Table 3. The data on a previous ABD-fused Affibody molecule variant, ¹⁷⁷Lu-CHX-A''-DTPA-ABD-(Z_{HER2:342})₂ (17), are provided for comparison. The data were obtained in the same xenograft model and calculated using the same method. The comparison suggests that the ratio of doses to tumor and bone is equal for both conjugates. At the same time, tumor-to-liver (4.9 for ¹⁷⁷Lu-ABY-027 vs. 2.8 for ¹⁷⁷Lu-CHX-A''-DTPA-ABD-(Z_{HER2:342})₂) and tumor-to-kidney (3.9 for ¹⁷⁷Lu-ABY-027 vs. 1.4 for ¹⁷⁷Lu-CHX-A''-DTPA-ABD-(Z_{HER2:342})₂) dose ratios were appreciably higher for ¹⁷⁷Lu-ABY-027.

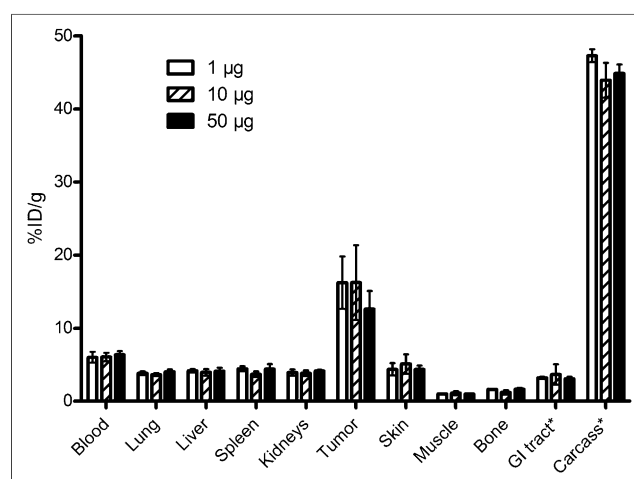


FIGURE 3. Uptake of radioactivity at 48 h after injection of 1, 10, or 50 µg of ¹⁷⁷Lu-ABY-027 in SKOV-3-xenografted mice (average of 4–6, %ID/g ± SD). *For gastrointestinal (GI) tract (with content) and carcass, data are presented as %ID per whole sample.

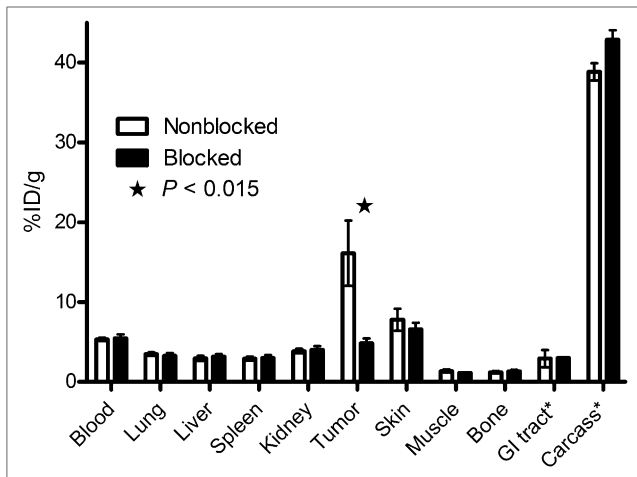


FIGURE 4. In vivo binding specificity of ^{177}Lu -ABY-027, 48 h after injection (average of 4, %ID/g \pm SD). Blocked group was preinjected with 1 mg of nonlabeled ABY-027. *For gastrointestinal (GI) tract (with content) and carcass, data are presented as %ID per whole sample.

DISCUSSION

Targeted delivery of cytotoxic radionuclides is one potential option for treatment of HER2-expressing malignant tumors that are resistant to receptor-blocking therapies. Numerous attempts have been made, with smaller proteins having better extravasation and penetration in tumors than antibodies. However, several studies have demonstrated that the use of small HER2-targeting proteins such as Fab'₂ (27), diabodies (28), DARPin (29), nanobodies (30), and Affibody molecules (16,24) labeled with radiometals all resulted in a high concentration of radioactivity in the kidneys. In each case, the renal concentration

of radioactivity exceeded the tumor concentration several-fold, suggesting a high risk of severe nephrotoxicity as a side effect of targeted radionuclide therapy.

We have previously shown that fusion of a dimeric anti-HER2 Affibody molecule with an ABD provided a protein capable of strong binding to albumin in vivo (17,18). This binding prevented rapid glomerular filtration and tubular reabsorption and endowed a conjugate with favorable targeting properties for radiotherapy. Dosimetry calculations demonstrated that the dose to the tumor exceeded the dose to the kidney 1.4-fold, and curative treatment in a microxenograft model was possible (17). However, a further reduction of renal uptake would be desirable, and the present study was based on the hypothesis that a stronger binding to albumin might reduce the renal uptake of the radiolabeled conjugate. Furthermore, a strong binding to albumin is also expected to minimize losses due to lysosomal catabolism after pinocytosis of albumin carrying the conjugate, by enabling maximal use of neonatal Fc receptor-mediated salvage of albumin (31). Thus, ABD₀₃₅ with femtomolar affinity for albumin may offer a pharmacokinetic advantage as a fusion partner to new targeting agents.

The surface plasmon resonance experiment showed that ABY-027 binds to HER2 with an affinity (K_D , 74 pM) identical to the affinity of parental Z_{HER2:2891} (K_D , 76 pM). This finding supported our assumption that the HER2-targeting moiety should preferably be situated at the N terminus of the fusion protein to preserve the affinity by minimizing steric interference from ABD. The construct was efficiently and stably labeled with ^{177}Lu at 60°C with preserved HER2-binding capacity (Fig. 1). The cellular processing experiment suggested a moderate internalization rate (Fig. 2), although more rapid than that for the parent anti-HER2 Affibody molecule (24).

TABLE 2
Biodistribution of ^{177}Lu -ABY-027 (Injected Dose, 10 μg) in BALB/C *nu/nu* Mice Bearing HER2-Expressing SKOV-3 Xenografts

Organ	Concentration of radioactivity (%ID/g)					
	4 h	1 d	2 d	3 d	7 d	14 d
Tumor	1.1 \pm 0.5	8 \pm 1	17 \pm 7	15 \pm 3	7 \pm 2	1.3 \pm 0.2
Blood	5 \pm 1	8 \pm 2	5.3 \pm 0.4	3.4 \pm 0.4	0.39 \pm 0.05	0.020 \pm 0.003
Heart	1.3 \pm 0.3	2.7 \pm 0.5	2.4 \pm 0.3	1.9 \pm 0.2	0.7 \pm 0.2	0.21 \pm 0.04
Lung	2.1 \pm 0.4	4.1 \pm 0.7	3.5 \pm 0.5	2.4 \pm 0.2	0.6 \pm 0.1	0.13 \pm 0.03
Liver	1.0 \pm 0.2	2.8 \pm 0.8	3.0 \pm 0.7	2.5 \pm 0.3	1.2 \pm 0.2	0.52 \pm 0.03
Spleen	0.6 \pm 0.1	2.3 \pm 0.5	2.9 \pm 0.5	2.5 \pm 0.6	1.9 \pm 0.6	1.0 \pm 0.6
Pancreas	0.45 \pm 0.06	1.3 \pm 0.2	1.0 \pm 0.2	0.8 \pm 0.2	0.27 \pm 0.05	0.10 \pm 0.03
Stomach	0.41 \pm 0.05	1.5 \pm 0.2	1.5 \pm 0.6	0.9 \pm 0.1	0.25 \pm 0.04	0.08 \pm 0.01
Kidney	2.6 \pm 0.4	4.3 \pm 0.8	3.9 \pm 0.6	2.98 \pm 0.09	1.3 \pm 0.2	0.39 \pm 0.06
Muscle	1.4 \pm 0.4	1.3 \pm 0.2	1.4 \pm 0.4	0.9 \pm 0.2	0.36 \pm 0.08	0.09 \pm 0.03
Bone	0.7 \pm 0.2	1.3 \pm 0.2	1.2 \pm 0.4	1.1 \pm 0.2	0.5 \pm 0.3	0.5 \pm 0.2
Intestines*	0.9 \pm 0.3	1.9 \pm 0.5	3 \pm 2	1.3 \pm 0.4	0.40 \pm 0.07	0.10 \pm 0.02
Carcass*	58 \pm 15	46 \pm 2	39 \pm 2	32 \pm 2	12 \pm 1	6.1 \pm 0.4

*Data for intestines with content and carcass are presented as %ID per whole sample. Data are presented as average of %ID/g \pm SD, $n = 4$.

TABLE 3
 Dosimetry of ^{177}Lu -ABY-027 in Mice in Comparison with ^{177}Lu -CHX-A''-DTPA-ABD-(Z_{HER2:342})₂ (17)

Organ	^{177}Lu -ABY-027 (Gy/MBq)	^{177}Lu -CHX-A''-DTPA-ABD-(Z _{HER2:342}) ₂ (Gy/MBq)
Blood	0.42 ± 0.04	0.51 ± 0.3
Heart	0.21 ± 0.01	0.25 ± 0.02
Lung	0.27 ± 0.02	0.33 ± 0.01
Liver	0.28 ± 0.01	0.76 ± 0.03
Spleen	0.32 ± 0.04	0.53 ± 0.4
Pancreas	0.091 ± 0.008	0.12 ± 0.01
Stomach	0.10 ± 0.01	0.097 ± 0.003
Intestine	0.10 ± 0.01	0.11 ± 0.005
Kidney	0.35 ± 0.02	1.49 ± 0.07
Tumor	1.37 ± 0.22	2.1 ± 0.2
Muscle	0.11 ± 0.01	0.09 ± 0.001
Bone	0.13 ± 0.02	0.21 ± 0.05

Data are presented as average Gy/MBq ± SD, *n* = 4.

As expected, a clear effect on the half-life of ABD binding to the serum albumin was demonstrated. The elimination half-life of the parental Z_{HER2:2891} Affibody molecule from blood was increased 80-fold, from 0.5 h (24) to 41 h. Furthermore, in SKOV-3-xenografted mice biodistribution experiments with ABY-027 labeled with ^{177}Lu showed more than a 3-fold reduced renal accumulation of radioactivity, compared with ^{177}Lu -CHX-A''-DTPA-ABD-(Z_{HER2:342})₂ (17), with retained tumor uptake. Targeting of HER2-expressing xenografts was specific, because saturation of HER2 by preinjection of an excess of nonlabeled ABY-027 caused a significant decrease of tumor uptake (Fig. 4). At the same time, targeting was equally efficient in a broad range of injected protein doses (Fig. 3). Thus, ^{111}In -ABY-027 provides an efficient expansion of the plasma half-life and a reduction of renal uptake of Affibody molecules as well as specific and improved targeting of HER2-expressing xenografts in comparison with previously reported ABD-(Z_{HER2:342})₂ (Table 1). Importantly, the circulatory half-life of ^{177}Lu -ABY-027 in mice was 2- to 2.5-fold shorter than the half-life of radiolabeled anti-HER2 antibodies (25,32), and the molecular weight of ^{177}Lu -ABY-027-albumin adduct (~80 kDa) is approximately half the weight of an antibody. These together should reduce problems seen when using antibodies for radioimmunotherapy, such as an excessively long residence time in blood and poor tumor penetration. It has been previously shown that the penetration of a Fab-albumin-binding peptide-albumin complex (size, ~120 kDa) was much better than what would be assumed from its size and at least equal to that of the noncomplexed Fab (19). We have previously shown a curative effect of targeted radionuclide therapy in mice carrying SKOV-3 xenografts using an anti-HER2 Affibody molecule fused with the first-generation ABD (17), in contrast to the mAb pertuzumab yielding only prolonged survival in the same tumor model (25).

As discussed above, the current Affibody ABD fusion protein is a development from the first-generation HER2-binding ABD fusion protein (17,18). Recently, Hoppman

et al. (33) evaluated the alternative approach of direct chemical coupling of Z_{HER2:342} to HSA. We have made a comparison of the biodistribution of these different constructs, using the data from 48 h after injection because this was the only common time point (Table 1). The study of the construct ^{111}In -DOTA-ABD-(Z_{HER2:342})₂ was performed in mice xenografted with the low-HER2-expressing cell line LS174T, compared with the other 3 constructs studied, possibly explaining the lower tumor values in xenografts of the high-HER2-expressing cell line SKOV-3. Furthermore, in addition xenografts based on the same cell line may show batch-to-batch variability and variation in biodistribution pattern of the same conjugate labeled with different radionuclides (34). Therefore, comparisons are preferably done head-to-head in the same batch of xenografts, as with ^{111}In -ABY-027 and ^{111}In -CHX-A''-DTPA-ABD-(Z_{HER2:342})₂ in the present study. However, data from normal tissues should be directly comparable. Despite multiple HER2 bindings, the HSA conjugate did not provide the highest tumor accumulation, compared with the fusion proteins analyzed in the high-HER2-expressing xenograft model. Furthermore, the high levels in the liver and spleen, as well as the faster clearance, suggest negative effects of multiple random conjugations on the properties of albumin. On the contrary, the novel fusion protein based on affinity-matured ABD provided the lowest kidney values and also the lowest values in the liver and spleen. A comparison of dosimetry of ^{177}Lu -ABY-027 and ^{177}Lu -CHX-A''-DTPA-ABD-(Z_{HER2:342})₂ (17) are presented in Table 3. Both calculations were based on the same methodology, and the biodistribution input data were obtained in the same animal model, BALB/C *nu/nu* mice bearing SKOV-3 xenografts. A curative effect was obtained after injection of 21.6 MBq of ^{177}Lu -CHX-A''-DTPA-ABD-(Z_{HER2:342})₂ (17). With ^{177}Lu -ABY-027, the same dose to tumor would be achieved with 33.1 MBq. With the specific activity used in the present study, this administration of such activity would require injection of 22–25 μg of ^{177}Lu -ABY-027 per mouse. As shown in the

present study, injection of such a protein dose is in a range where small differences in the injected protein dose insignificantly affect tumor uptake. A comparison at equal dose to tumor shows that the dose to blood and bone would be similar for the 2 constructs and that ¹⁷⁷Lu-ABY-027 would give a 2.8- and 1.8-fold-lower dose to kidney and liver, respectively.

CONCLUSION

Z_{HER2:2891}-ABD₀₃₅-DOTA (ABY-027) is a fusion protein having a biodistribution suitable for therapy and showing retained HER2-specific binding. The capacity of specific targeting of HER2-expressing xenografts was shown in vivo. In a murine model, ¹⁷⁷Lu-ABY-027 provided an appreciable reduction of renal and hepatic uptake of radioactivity in comparison with alternative approaches. Site-specific conjugation of DOTA provides a uniform conjugate and creates the potential for labeling with a broad range of therapeutic radionuclides. The unique cysteine can also be used for site specific coupling of other effector functions.

DISCLOSURE

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