Comparative Evaluation of Anti-HER2 Affibody Molecules Labeled with $^{64}$Cu Using NOTA and NODAGA

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Received 4 January 2017; Accepted 8 February 2017; Published 28 February 2017

1. Introduction

Treatment of disseminated breast cancer, which overexpresses human epidermal growth factor receptor type 2 (HER2), with the monoclonal antibody trastuzumab, the antibody-drug conjugate trastuzumab DM-1, or the tyrosine kinase inhibitor lapatinib improves the survival of patients [1]. HER2 overexpression is a predictive biomarker for HER2-targeting therapies [2]. The use of sensitive radionuclide molecular imaging may permit repetitive noninvasive assessment of HER2 expression in breast cancer metastases, addressing the issue of spatial and temporal heterogeneity of HER2 expression. The use of radiolabeled HER2-specific antibodies [3, 4] and their fragments [5] for clinical imaging of HER2 expression has been reported.

Mathematic modeling suggests that for a proteinaceous imaging probe the combination of small size with high affinity provides the highest contrast and consequently the highest sensitivity of radionuclide molecular imaging [6]. Indeed,
excellent contrast was demonstrated in a clinical study that used the smallest possible antibody fragment (VHH) [7]. It is possible to develop even smaller targeting probes by using engineered nonimmunoglobulin scaffold proteins [8], which can be 2- to 4-fold smaller than VHH fragments. Their affinity to selected targets may be in the single-digit nanomolar or subnanomolar range. Other potential advantages of nonimmunoglobulin scaffold proteins include the possibility of their production in prokaryotic hosts or by peptide synthesis, which would dramatically reduce production costs. Moreover, they are structurally stable and can re-fold after denaturation, permitting the use of harsh labeling conditions. Preclinical studies demonstrate the successful application of such scaffold proteins as targeting probes for radionuclide molecular imaging, including DARPinS [9], knottins [10], ADAPTs [11], fibronectin domains [12], and affibody molecules [13]. Clinical studies show that Ga-labeled affibody molecules enable high-contrast imaging of HER2 expression in tumors, can discriminate between breast cancer metastases with high and low HER2 expression, are nontoxic and nonimmunogenic, and inflict a low absorbed dose burden in patients [14, 15].

Clinical PET studies using anti-HER2 affibody molecules labeled with (T1/2 = 67.9 min) demonstrated that discrimination between high and low HER2 expression is better at 4 h postinjection (p.i.) than at 2 h p.i. [14]. This is agreement with preclinical [16] and clinical [17] studies demonstrating that retention of affibody-associated radioactivity is better in tumors with higher HER2 expression compared to tumors with low expression. However, the short half-life of 68Ga complicates imaging later than 4 h after injection. Thus, it would be advantageous to use a positron-emitting label with a longer half-life than the half-life of 68Ga. Candidate labels that could provide imaging several hours after injection include two positron-emitting copper radioisotopes: 61Cu (T1/2 = 3.4 h) and 64Cu (T1/2 = 12.7 h). Both radionuclides can be produced using low-energy cyclotrons available at PET centres [18, 19].

The development of a radiocopper-labeled tracer requires the determination of an appropriate chelator. Although the macrocyclic tetraaza chelator DOTA provides thermodynamically stable and kinetically inert complexes with a variety of radionuclides [20], a DOTA-complex of copper (II) is unstable in vivo. Released radiocopper can bind blood proteins and superoxide dismutase in the liver, deteriorating the imaging contrast [21]. This has been observed also in preclinical studies using affibody molecules and their derivatives. For example, the ZHER2:477 affibody molecule labeled with 64Cu using maleimido-DOTA-conjugated at the C-terminus had a hepatic uptake of 7.1 ± 1.7% ID/g at 4 h p.i. and 10.4 ± 1.2% ID/g at 20 h p.i. in a murine model (Balb/C nu/nu) [22]. When the same clone (designated as PEP08388) was labeled with 111In using the same chelator [16], the hepatic uptake was much lower, 2.1 ± 0.5% ID/g at 4 h p.i. and 1.7 ± 0.3% ID/g at 24 h p.i., in the same murine model. There was no clearance of radionuclide from blood between 4 h (0.52 ± 0.22% ID/g) and 20 h (0.71 ± 0.02% ID/g) in the case of the 64Cu label [22]. In the case of the 111In label, the bloodborne radioactivity was reduced from 0.13 ± 0.02% ID/g at 4 h p.i. to 0.07 ± 0.01% ID/g at 4 h p.i. [16]. In another study, Ren and coworkers [23] compared the biodistribution of an 111In- and 64Cu-labeled DOTA-conjugated 2-helix variant of the anti-HER2 affibody molecule DOTA–MUT-DS. At 1 h after injection, the hepatic uptake of 64Cu-DOTA–MUT-DS was 28±6% ID/g, while the hepatic uptake of 111In-DOTA–MUT-DS was 10 ± 2% ID/g, that is, nearly threefold lower. Clearing of radioactivity from blood was very slow in the case of 64Cu-DOTA–MUT-DS, from 1.5 ± 0.2% ID/g at 1 h p.i. to 1.10 ± 0.02% ID/g at 20 h p.i. In the case of 111In-DOTA–MUT-DS, the blood clearance was much more rapid, from 1.1 ± 0.1% ID/g at 1 h p.i. to 0.18 ± 0.02% ID/g at 20 h p.i. The results of the preclinical studies suggested that the triaza chelators NOTA and NODAGA produce in vivo stable complexes with radiocopper. These chelators and their derivatives have been successfully used to label a number of peptides and antibody fragments with copper-64 [24–27]. Adding to the importance of chelator selection, it has been shown that the charge and geometry of the radiometal-chelator complexes influence the off-target interactions of affibody molecules, modifying biodistribution and imaging contrast [28–31]. To date, 68Ga-NODAGA-ZHER2:Si provides the best contrast among the tested 68Ga-labeled synthetic affibody molecules in preclinical studies [29].

The present study aimed to evaluate the tumor-targeting and imaging properties of the synthetic affibody molecule ZHER2:Si labeled with 64Cu using the NOTA and NODAGA chelators (Figure 1). We further compared their properties with the properties of 68Ga-NODAGA-ZHER2:Si, the best currently available 68Ga-labeled variant.

2. Materials and Methods

2.1. Measurements and Analysis. In the in vitro experiments and ex vivo animal studies, radioactivity uptake was measured using the Wizard2 automated gamma-counter (PerkinElmer). Formulation was accomplished using a VDC-405 dose calibrator (Veenstra Instruments). Radiochemical
yield and purity were determined by radio-instant thin-layer chromatography (radio-ITLC) using ITLC-SG strips (Agilent), with elution in 0.2 M citric acid, pH 2. The ITLC strips were dried and exposed to BAS-TR2025 imaging plates (Fuji Photo Film Co.). To obtain digital images, we scanned the imaging plates with an FLA-5100 scanner (Fuji Photo Film Co). The images were analyzed using AIDA analysis software, version 4 (Raytest). Radio-ITLC results were cross-validated using radio-HPLC with a Jupiter Proteo C12 column (4.6 × 250 mm), at a flow rate of 1.0 mL/min, with a linear CH₂CN/H₂O gradient (10–70% CH₂CN in 12 min) in 0.1% trifluoroacetic acid solution. The radio-HPLC data were concordant with the data from radio-ITLC.

The results are presented as mean ± standard deviation (SD). A P value of <0.05 (based on unpaired two-tailed t-test) was considered to indicate a significant difference between two groups. To evaluate differences between more than two groups, we performed one-way ANOVA analysis with Bonferroni’s multiple comparison test using Prism 5 software (GraphPad Software).

2.2. Production of ⁶⁴Cu. High quality water was deionized (resistance higher than 18 MΩ/cm²) by passing it through the Ultra Clear filtration system (SG Wasseraufbereitung und Regenerierstasion GmbH, Germany). Ultrapure grade aqueous acids (Carl Roth GmbH, Germany) with ppt-levels of metal impurities were used for all solutions during ⁶⁴Ni-electrodeposition, Cu/Ni-separation, and ⁶⁴Cu-formulation. ⁶⁴Cu in the form of [⁶⁴Cu]CuCl₂ was produced via the ⁶⁴Ni(p,n)⁶⁴Cu nuclear reaction, as previously described [18, 32]. In brief, ⁶⁴Cu is produced by proton bombardment of an enriched ⁶⁴Ni target (>98%, 80 mg) with proton energy of 13 MeV using a CC-18/9 cyclotron (D.V. Efremov Institute, St. Petersburg, Russia). Radiochemical isolation of ⁶⁴Cu by anion exchange chromatography and recovery of ⁶⁴Ni were performed as previously described [33, 34]. ⁶⁴Cu was formulated as 16–18 MBq/μL in 0.04 M HCl-solution. The effective specific radioactivity (ESA) was at least 3 TBq/μmol at the end of bombardment (EoB), as determined by titration with 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) (Sigma-Aldrich, Germany).

2.3. Labeling and Stability. We synthetically produced, purified, and characterized affibody molecules containing NOTA or NODAGA chelators coupled via an amide bond to an N-terminal alanine as previously described [28]. Both variants showed a purity of over 97%

Labeling was performed using two different protocols. In Protocol A, affibody molecules (50 μg) were reconstituted in 50 μL 0.55 M ammonium acetate, pH 5.6, and mixed with 150 MBq ⁶⁴CuCl₂ in 10 μL 0.04 M HCl. This mixture was incubated for 45 min at 95°C and then diluted with PBS and analyzed. In Protocol B, following incubation with ⁶⁴CuCl₂, the reaction mixture was combined with a 500-fold excess of Na₂EDTA (10 mg/mL in water, 137 μL). This mixture was then incubated at 95°C for an additional 10 min, and radiolabeled affibody molecules were purified using disposable NAP-5 size-exclusion columns (GE Healthcare).

To test the labeling stability, samples of radiolabeled affibody molecules were incubated for two hours at room temperature with a 500-fold excess of Na₂EDTA. Control samples were diluted with the same volume of PBS. Radio-ITLC was used to measure the percentage of protein-bound radioactivity.

For comparative studies NODAGA-ZHER2:S1 was labeled with ⁶⁸Ga as previously described [29]. For in vitro displacement experiments, the anti-HER2 affibody molecule ZHER2:2395 was labeled with ⁹⁹mTc as previously described [35]. Loading of NODAGA-ZHER2:S1 with ⁶⁴Cu and ⁶⁸Ga and of NOTA-ZHER2:S1 with ⁶⁴Cu was performed following the exact same protocols as for labeling with a fivefold molar excess of metals over proteins.

2.4. Binding to and Processing by HER2-Expressing Cells In Vitro. The HER2-expressing SKOV-3 ovarian carcinoma cell line (American Type Culture Collection, ATCC) was used for binding specificity and cellular processing studies. DMEM medium (Lonza, Belgium) supplemented with 10% fetal bovine serum and penicillin (100 U/mL)–streptomycin (100 μg/mL) (both from Biochrom AG) was used for culturing. For binding and cellular processing experiments, the cells were seeded one day before the experiment in Petri dishes. At the day of the experiment there were approximately 10⁶ cells/dish.

To test the specific binding of ⁶⁴Cu-NODAGA-ZHER2:S1 and ⁶⁴Cu-NOTA-ZHER2:S1 to HER2-expressing SKOV-3 cells, we performed a saturation assay [36] using a 100-fold molar excess of nonlabeled affibody molecules. The binding strengths of Cu-NODAGA-ZHER2:S1, Ga-NODAGA-ZHER2:S1, and Cu-NOTA-ZHER2:S1 were compared via measurement of their concentrations at half-maximum inhibition of ⁹⁹mTc-ZHER2:2395 binding to SKOV-3 cells (IC₅₀), as previously described [30]. Briefly, SKOV-3 cells were incubated for 4 h at 4°C with ⁹⁹mTc-ZHER2:2395 (1 nM) in the presence of gallium- or copper-loaded affibody molecules (concentration range 0–500 nM). After incubation, the cells were washed with 3 mL of medium and treated with 1 mL of trypsin-EDTA solution. The detached cells were collected, and the cell-associated radioactivity was measured. The IC₅₀ values were determined using GraphPad Prism software.

We used a previously validated modified acid wash method for processing of bound conjugates by HER2-expressing SKOV-3 cells [36]. Briefly, the cells were incubated with the labeled compound (1 nM) at 37°C. At predetermined time points, the medium from a set of three dishes was removed. The cells were washed twice with 1 mL of ice-cold medium. To collect the membrane-bound radioactivity, the cells were treated with 0.5 mL of 0.2 M glycine buffer containing 4 M urea, pH 2.0, for 5 min on ice. Dishes were additionally washed with 0.5 mL acidic buffer followed by 1 mL PBS, and the fractions were pooled. To collect radioactivity internalized by the cells, we treated them with 0.5 mL of 1 M NaOH at 37°C for 0.5 h. The dishes were additionally washed with 0.5 mL NaOH solution followed by 1 mL PBS,
and the alkaline fractions were pooled and the percentage of internalized radioactivity was calculated.

2.5. Biodistribution Studies. Animals were cared for in compliance with the guidelines of the International Council of Laboratory Animal Science. All animal procedures were approved by the Animal Ethics Committee of the Provincial Government of Southern Finland and performed following the guidelines of the European Community Council Directives 86/609/EEC.

Female BALB/C nu/nu mice (Scanbur, 8 weeks old at arrival) were used to obtain HER2-positive tumors by subcutaneous inoculation of $1 \times 10^7$ SKOV-3 cells ($n = 28$) or HER2-negative control tumors by inoculation of $1 \times 10^7$ Ramos lymphoma cells ($n = 8$). Experiments were performed 26 days after implantation. The average tumor weight was $0.14 \pm 0.1$ g at the time of the experiment.

For ex vivo measurements, we used a group of four mice for each tracer and data point. Mice bearing SKOV-3 xenografts were injected with $^{64}$Cu-NODAGA-ZHER2:S1 or $^{64}$Cu-NOTA-ZHER2:S1 ($450$ kBq per mouse) via the tail vein. The injected protein dose was adjusted to $5 \mu$g (0.7 nmol). The dose was selected based on our data showing that variation of the injected dose of anti-HER2 affibody molecules between 1 and $10 \mu$g per mouse has no significant effect on the uptake in SKOV-3 xenografts [37]. Biodistribution was measured 2, 6, and 24 h postinjection. Mice bearing Ramos xenografts were injected with the same amounts of protein and radioactivity, and biodistribution was measured 2 h after injection. Similarly, $^{68}$Ga-NODAGA-ZHER2:S1 biodistribution was measured 2 h after injection of $5 \mu$g/500 kBq per mouse. Three mice were injected with $^{64}$Cu-citrate ($450$ kBq per mouse), and radioactivity distribution was evaluated 2 h postinjection. The citrate was used to prevent rapid formation of non-soluble hydroxide. For ex vivo tissue distribution measurements, the animals were anesthetized and exsanguinated, and the organs of interest were excised. Lung, liver, spleen, stomach wall, and kidneys were sampled as whole organs. Besides, radioactivity of gastrointestinal tract (with content) and carcass was measured.

2.6. In Vivo Imaging Studies. SKOV-3 ($n = 2$) or Ramos ($n = 2$) xenograft-bearing mice were injected via the tail vein with $^{64}$Cu-NODAGA-ZHER2:S1 ($9.7 \pm 1.4$ MBq, $5 \mu$g, $120 \mu$L) and $^{64}$Cu-NOTA-ZHER2:S1 ($10.5 \pm 0.9$ MBq, $5 \mu$g, $120 \mu$L). Additionally, SKOV-3 ($n = 2$) xenograft-bearing mice were injected with $^{68}$Ga-NODAGA-ZHER2:S1 ($2.3 \pm 0.1$ MBq, $5 \mu$g, $120 \mu$L). Mice were anesthetized using 2.5% isoflurane/O$_2$ and positioned on a heating pad two at a time in a PET/CT scanner (Siemens Medical Solutions, Inc.) for CT acquisition (10 min) and PET scan in list mode (20 min). Mice bearing SKOV-3 xenografts were scanned at 2, 6, and 24 h postinjection and mice with Ramos xenografts at 2 h postinjection. PET images were reconstructed using an FBP algorithm of two iterations, followed by maximum a posteriori (MAP, 18 iterations) integrative algorithms (Inveon Acquisition Workplace, version 2.0; Siemens Preclinical Solutions). Data were decay-corrected to the time of injection.

### Table 1: Labeling of NOTA-ZHER2:S1 and NODAGA-ZHER2:S1 with $^{64}$Cu and conjugates stability under 2-hour challenge with a 500-fold excess of Na$_2$EDTA.

|                  | Overall yield (%) | Purity (%) | Stability (%) of affibody-associated activity |
|------------------|------------------|------------|----------------------------------------------|
|                  | Protocol A       | Protocol B |
| $^{64}$Cu-NOTA-ZHER2:S1 | 96.2 ± 0.0  | 86.1 ± 0.6 | 96.9 ± 0.0                                    |
| $^{64}$Cu-NODAGA-ZHER2:S1 | 96.9 ± 0.0  | 86.6 ± 1.1 | 96.9 ± 0.0                                    |

*Overall yield is defined as percentage of radionuclide incorporated into affibody molecules at the end of synthesis (Protocol A) or percentage of radionuclide incorporated in affibody molecules at the end of separation (Protocol B) (decay corrected).

3. Results

3.1. Labeling and Stability. Labeling of both NOTA-ZHER2:S1 and NODAGA-ZHER2:S1 with $^{64}$Cu was performed in 0.55 M ammonium acetate, pH 5.6, using two different protocols. Table 1 presents the results of the $^{64}$Cu-labeling experiments and the stability tests. Labeling using Protocol A led to incorporation of $>95\%$ of $^{64}$Cu into the affibody molecules. However, about $6\%$ of the radioactivity was released upon EDTA challenge. We hypothesized that a fraction of the copper was not stably complexed by a macrocyclic chelator but was instead loosely bound to a weak “chelator pocket” formed by amino acids. Protocol B included an EDTA challenging step before purification to strip this weakly bound radiometal. This additional step decreased the overall yield by about $15\%$ but produced conjugates that could withstand the EDTA challenge. The purity was over $98\%$ for both the $^{64}$Cu-labeled NOTA and NODAGA conjugates. Specific activity of 2.5 MBq/µg (17.4 GBq/µmol) was obtained. Due to better stability of the label, Protocol B was used in biological studies.

3.2. Binding to and Processing by HER2-Expressing Cells In Vitro. Figure 2(a) presents the results of the specificity test. Presaturation of receptors with nonlabeled counterparts significantly reduced ($P < 0.00005$) the binding of both $^{64}$Cu-NODAGA-ZHER2:S1 and $^{64}$Cu-NOTA-ZHER2:S1 to HER2-expressing cells, demonstrating the HER2-specificity of both radioligands.
Figure 2: (a) In vitro binding specificity of $^{64}$Cu-NODAGA-ZHER2:S1 and $^{64}$Cu-NOTA-ZHER2:S1 to HER2-expressing SKOV-3 cells. In the blocked group, receptors were presaturated with a 100-fold excess of nonlabeled affibody molecules. Panels (b) and (c) show the cellular processing of $^{64}$Cu-NOTA-ZHER2:S1 (b) and $^{64}$Cu-NODAGA-ZHER2:S1 (c) by SKOV-3 cells. Cells were incubated with the conjugate (1 nM) at 37°C. Data are presented as the mean of three samples ± SD.

Figures 2(b) and 2(c) present the cellular processing of $^{64}$Cu-NODAGA-ZHER2:S1 and $^{64}$Cu-NOTA-ZHER2:S1. Both conjugates showed a low fraction of internalized radioactivity, with less than 10% of cell-associated radioactivity detected at 24 h after incubation. The two conjugates differed somewhat in their overall uptake patterns, with $^{64}$Cu-NODAGA-ZHER2:S1 binding showing an ascending tendency and $^{64}$Cu-NOTA-ZHER2:S1 binding showing a descending tendency after 2 h. Total cell-associated activity at 24 h was significantly higher ($P < 0.05$) for $^{64}$Cu-NODAGA-ZHER2:S1 than for $^{64}$Cu-NOTA-ZHER2:S1.

The relative binding strengths of Cu-NODAGA-ZHER2:S1, Ga-NODAGA-ZHER2:S1, and Cu-NOTA-ZHER2:S1 were compared via measurement of their concentrations at half-maximum inhibition of $^{99m}$Tc-ZHER2:2395 binding to SKOV-3 cells (IC$_{50}$). The IC$_{50}$ values did not significantly differ between natCu-NODAGA-ZHER2:342, natGa-NODAGA-ZHER2:S1, and natCu-NOTA-ZHER2:S1 (Figure 3), suggesting that neither chelators nor metals (in the case of Ga-NODAGA-ZHER2:342) affected the binding strength of ZHER2:S1 to HER2-expressing cells.

3.3. Biodistribution Studies. The tumor-targeting properties of the affibody molecules were compared in BALB/C nu/nu mice bearing implanted human cancer xenografts. To confirm targeting specificity in vivo, we evaluated $^{64}$Cu-NOTA-ZHER2:S1 and $^{64}$Cu-NODAGA-ZHER2:S1 uptake in HER2-positive SKOV-3 xenografts versus HER2-negative Ramos xenografts at 2 h after injection (Figure 4). The highly significant difference ($P < 0.0005$) between uptakes in HER2-positive and HER2-negative xenografts at 2 h postinjection confirmed the in vivo targeting specificity. The uptake of these
Figure 3: Inhibition of $^{99m}$Tc-ZHER2:2395 binding to SKOV-3 cells with natCu-NODAGA-ZHER2:S1, natGa-NODAGA-ZHER2:S1, or natCu-NOTA-ZHER2:S1. The data are presented as mean ± SD of three samples.

Figure 4: Uptake of $^{64}$Cu-NODAGA-ZHER2:S1 (a) or $^{64}$Cu-NOTA-ZHER2:S1 (b) at 2 h after injection in mice bearing either HER2-positive xenografts (SKOV-3) or HER2-negative xenografts (Ramos). The data are presented as mean ± SD for four mice.

Tracers did not differ significantly in any other tissue of mice bearing HER2-positive and HER2-negative xenografts.

Figure 5(a) presents a comparison of the biodistributions of $^{64}$Cu-NOTA-ZHER2:S1, $^{64}$Cu-NODAGA-ZHER2:S1, and $^{68}$Ga-NODAGA-ZHER2:S1 at 2 h postinjection in mice bearing HER2-expressing SKOV-3 xenografts. As is typical for affibody molecules, cleared tracers were reabsorbed in the kidneys. At that time point, radioactivity was localized in the tumors (with no significant difference between the conjugates, $P > 0.017$) and cleared from other normal organs and tissues. Uptake did not significantly differ between $^{64}$Cu-NODAGA-ZHER2:S1 and $^{68}$Ga-NODAGA-ZHER2:S1 in any organ ($P > 0.017$). In contrast, $^{64}$Cu-NOTA-ZHER2:S1 uptake was significantly higher ($P < 0.017$) than that of $^{64}$Cu- and $^{68}$Ga-NODAGA-ZHER2:S1 in all organs, except the kidneys. Renal uptake was significantly lower for $^{64}$Cu-NOTA-ZHER2:S1 ($P < 0.017$). Compared to the other two tracers, $^{64}$Cu-NOTA-ZHER2:S1 showed significantly ($P < 0.017$) lower tumor-to-organ ratios (Figure 5(b)). Compared to both $^{64}$Cu-labeled variants, $^{68}$Ga-NODAGA-ZHER2:S1 provided significantly higher tumor-to-organ ratios ($P < 0.017$), with the exception of the tumor-to-bone and tumor-to-kidney ratios.

Table 2 presents biodistribution data for $^{64}$Cu-NOTA-ZHER2:S1 and $^{64}$Cu-NODAGA-ZHER2:S1 in mice bearing HER2-positive SKOV-3 xenografts at 2, 6, and 24 h after injection. Renal radioactivity levels rapidly decreased, which is unusual for radiometal-labeled affibody molecules. From 2 to
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Uptake (% ID/g)

|                | 64Cu-NOTA | 64Cu-NODAGA | 68Ga-NODAGA |
|----------------|-----------|-------------|-------------|
| Blood          | 1.0 ± 0.2 | 0.32 ± 0.05*| 1.4 ± 0.2   |
| Lung           | 3.0 ± 0.5 | 0.8 ± 0.1*  | 5.9 ± 0.6   |
| Liver          | 10.9 ± 0.6| 2.8 ± 0.5*  | 17 ± 3      |
| Spleen         | 1.6 ± 0.2 | 0.56 ± 0.09*| 2.6 ± 0.7   |
| Stomach        | 3.3 ± 0.3 | 0.8 ± 0.1*  | 5.2 ± 0.3   |
| Kidney         | 207 ± 35  | 290 ± 35*   | 69 ± 7      |
| Tumor          | 17 ± 2    | 12 ± 3*     | 17 ± 4      |
| Muscle         | 0.45 ± 0.06| 0.16 ± 0.05*| 0.55 ± 0.09*|
| Bone           | 1.0 ± 0.1 | 0.35 ± 0.07*| 1.16 ± 0.05 |

Data are presented as mean ± SD for four mice

*Significant difference between 64Cu-NOTA-ZHER2:S1 and 64Cu-NODAGA-ZHER2:S1 at the given time point.

24 h postinjection, renal radioactivity decreased nearly 2.6-fold for 64Cu-NODAGA-ZHER2:S1 and nearly 15-fold for 64Cu-NOTA-ZHER2:S1. Uptake of 64Cu-NOTA-ZHER2:S1 in the blood, lung, liver, spleen, stomach, and gastrointestinal tract increased rapidly, peaking at 6 h postinjection. Uptake of 64Cu-NODAGA-ZHER2:S1 in the blood, lung, liver, spleen, stomach, and blood gradually increased over time. Tumor uptake of 64Cu-NODAGA-ZHER2:S1 did not significantly differ between the 2, 6, and 24 h postinjection time points. On the other hand, tumor uptake of its NOTA-conjugated counterpart was significantly lower at 24 h compared to at 2 and 6 h. Accordingly, the tumor-to-organ ratios for both 64Cu-labeled conjugates decreased over time (Table 3). The tumor-to-organ ratios for 64Cu-NODAGA-ZHER2:S1 were significantly higher than those for 64Cu-NOTA-ZHER2:S1 at all time points.

Table 4 presents the biodistribution of free radiocopper (64Cu-citrate) at 2 h postinjection. Total body retention of radioactivity was 92 ± 5% ID, and blood radioactivity remained at the level of 2.1 ± 0.1% ID/g. Radioactivity accumulated in the lung, liver, spleen, and stomach organs, which all showed prominently increased radioactivity at 6 and 24 h after injection of 64Cu-NOTA-ZHER2:S1 and 64Cu-NODAGA-ZHER2:S1.

3.4. In Vivo PET Studies. The biodistribution data were supported by our results from the imaging experiments (Figures
6 and 7). HER2-positive SKOV-3 xenografts were clearly visualized at all time points using radiocopper-labeled affibody molecules. The uptake of both tracers was much higher in HER2-positive xenografts than in HER2-negative xenografts. At all time points, the hepatic uptake of $^{64}$Cu-NODAGA-ZHER2:S1 was appreciably lower than the uptake of $^{64}$Cu-NOTA-ZHER2:S1. The uptake of $^{64}$Cu-NOTA-ZHER2:S1 over time was clearly decreased in the kidneys and increased in the liver. $^{64}$Cu-NODAGA-ZHER2:S1 and $^{68}$Ga-NODAGA-ZHER2:S1 enabled nearly equal visualization of SKOV-3 xenografts (Figure 7).

### 4. Discussion

The results of this study demonstrated that the macrocyclic chelators NOTA and NODAGA enabled efficient radiocopper labeling of the synthetic anti-HER2 affibody molecule ZHER2:S1 (Table 1). The inclusion of an EDTA challenge before final purification solved the issue of radiocopper being loosely bound to protein. Despite the harsh labeling conditions, radiocopper-labeled affibody molecules specifically bound to HER2-expressing cells in vitro (Figure 2(a)). The chelator-radionuclide combination did not significantly influence the strength of binding to SKOV-3 cells (Figure 3). Internalization of anti-HER2 affibody molecules is typically quite modest [18, 29, 36], but the internalized fractions of $^{64}$Cu-NOTA-ZHER2:S1 and $^{64}$Cu-NODAGA-ZHER2:S1 were unusually small, being two- to threefold lower than the internalized fractions of their $^{68}$Ga- and $^{111}$In-labeled counterparts [18, 29, 36] (Figure 2(b)). This may have been due to a slower internalization rate or, more likely, because of the moderate residualizing properties of the $^{64}$Cu-NOTA and $^{64}$Cu-NODAGA labels and the release of the radiometabolites following intracellular degradation.

Table 4: Biodistribution of $^{64}$Cu-citrate in BALB/C Nu/Nu mice at two hours postinjection.

| Uptake | Per gram (% ID/g) | Per organ (% ID) |
|--------|-------------------|------------------|
| Blood  | 21 ± 0.1          |                  |
| Lung   | 11 ± 1            | 1.6 ± 0.2        |
| Liver  | 36.9 ± 0.5        | 31 ± 1           |
| Spleen | 3.2 ± 0.3         | 0.24 ± 0.04      |
| Stomach| 12.8 ± 0.8        | 1.29 ± 0.06      |
| Kidney | 14.1 ± 0.2        | 3.44 ± 0.07      |
| Muscle | 11.1 ± 0.1        |                  |
| Bone   | 1.8 ± 0.3         |                  |
| GI tract* | 28 ± 1          |                  |
| Carcass| 29 ± 1            |                  |
| Totally| 92 ± 5            |                  |

Data are presented as mean ± SD for three mice.

*The data for the GI tract (with contents) and carcass are presented for the whole sample.

Table 3: Comparison of $^{64}$Cu-NOTA-ZHER2:S1 and $^{64}$Cu-NODAGA-ZHER2:S1 tumor-to-organ ratios in nude mice bearing SKOV-3 xenografts. Data are presented as mean ± SD for four mice.

| Tumor-to-organ ratio | $^{64}$Cu-NOTA-ZHER2:S1 | $^{64}$Cu-NODAGA-ZHER2:S1 | $^{64}$Cu-NOTA-ZHER2:342 | $^{64}$Cu-NODAGA-ZHER2:342 | $^{64}$Cu-NOTA-ZHER2:S1 | $^{64}$Cu-NODAGA-ZHER2:S1 |
|----------------------|-------------------------|---------------------------|--------------------------|---------------------------|-------------------------|---------------------------|
| Blood 17 ± 2 | 38 ± 5 | 12 ± 2 | 36 ± 4 | 8 ± 2 | 21 ± 8 |
| Lung 5.6 ± 0.4 | 15 ± 2 | 2.8 ± 0.5 | 9 ± 2 | 2.1 ± 0.5 | 5 ± 1 |
| Liver 1.6 ± 0.2 | 4.4 ± 0.8 | 1.0 ± 0.1 | 2.6 ± 0.4 | 0.8 ± 0.1 | 21 ± 0.8 |
| Spleen 10.4 ± 0.7 | 22 ± 2 | 6.5 ± 0.6 | 15 ± 3 | 3.2 ± 0.6 | 9 ± 3 |
| Stomach 5.1 ± 0.5 | 15 ± 2 | 3.2 ± 0.8 | 8 ± 1 | 2.8 ± 0.7 | 7 ± 3 |
| Kidney 0.08 ± 0.01 | 0.04 ± 0.01 | 0.24 ± 0.04 | 0.042 ± 0.007 | 0.7 ± 0.1 | 0.10 ± 0.04 |
| Muscle 38 ± 1 | 78 ± 10 | 30 ± 4 | 70 ± 14 | 17 ± 3 | 43 ± 13 |
| Bone 18 ± 2 | 35 ± 3 | 14 ± 3 | 32 ± 9 | 9 ± 3 | 23 ± 7 |

The values for $^{64}$Cu-NOTA-ZHER2:S1 and $^{64}$Cu-NODAGA-ZHER2:S1 significantly differed for each tissue at all time points.
Insight into the nature of this phenomenon might be gained by analyzing the radioactivity distribution at several time points after the injection of radiocopper-labeled affibody molecules (Table 2). The typical biodistribution pattern for radiometal-labeled affibody molecules includes very slow release of radioactivity from tumors, liver, and kidneys, with appreciably more rapid clearance from blood, lung, and intestines, thus, increasing the tumor-to-organ ratios over time [13, 28, 39]. A different pattern was observed with the 64Cu-labeled affibody molecules. Although tumor radioactivity was sufficiently retained, there was a disproportionately rapid decrease of renal radioactivity along with increased uptake in the liver, blood, lung, and intestines (Table 2). These organs that showed elevated uptake also accumulated free copper (Table 4). Radiocopper could not have been directly released from the conjugates in blood/extracellular space, since the conjugation was stable (Table 1), the 64Cu complex with monoamide-NOTA is stable in blood [40], and no free conjugate was available to increase the radioactivity in all tissues except the kidneys. Thus, the unusual biodistribution of 64Cu-NOTA-ZHER2:S1 and 64Cu-NODAGA-ZHER2:S1 was most likely due to radiocatabolite release from the kidneys. This explanation correlates well with the poor intracellular retention of copper (Figure 2).

Importantly, the internalization of affibody molecules by HER2-expressing cells occurs slowly, while such internalization by proximal tubules occurs rapidly [41]. Compared to its complex with monoamide-NODAGA, the complex of 64Cu with monoamide-NOTA is apparently less stable under the conditions of the lysosomal compartment of the renal tubules. Interestingly, earlier publications have not highlighted the phenomenon of 64Cu “leakage” after the processing of complexes with NOTA and NODAGA. However, the prior studies were performed mainly with antagonistic radiopeptides that show slow internalization by cancer cells and low renal uptake. Still, comparison of 64Cu-labeled NOTA- and DOTA-conjugated agonistic bombesin analogs reveals lower tumor and renal radioactivity retention when using NOTA [24], which is in agreement with our present data. Very interesting is an evaluation of 64Cu-labeled A20FMDV2 peptide conjugates for imaging the integrin \( \alpha_v\beta_6 \), since radiometal-labeled A20FMDV2 has high renal uptake [42]. In that study, the distribution of radioactivity after injection of 64Cu-NOTA-A20FMDV2 had the
same features as distribution after injection of $^{64}$Cu-NOTA-ZHER2:SI, that is, rapid release of radioactivity from kidneys, poor retention of radioactivity in tumors, and an increase in hepatic uptake between 1 and 4 hours after injection, with a subsequent decrease at 24 h. Overall, increasing the time between $^{64}$Cu-NOTA-ZHER2:SI or $^{64}$Cu-NODAGA-ZHER2:SI injection and imaging did not improve imaging contrast and did not show any advantage over the use of $^{68}$Ga-NODAGA-ZHER2:SI.

It remains to be evaluated if the same phenomenon is relevant to all affibody molecules and not only to the HER2-targeting ones. However, the high renal reabsorption is typical for all tested radiometal-labeled affibody molecules, including EGFR-, HER3-, PDGFPβ-, and IGFR-IR-specific molecules [30, 43–45]. In all cases, specificity tests based on saturation of receptors in tumors did not decrease renal uptake although the probes were selected to have high affinity to murine counterparts of the human molecular targets. It is likely that this high renal reabsorption is caused by the high affinity of scavenger receptors in the kidneys to the affibody scaffold and is not dependent on target specificity. Thus, the results of this study are, most likely, relevant to all affibody molecules. Moreover, taken into account that all reabsorbed proteins and peptides are directed to the lysosomal compartment of tubuli cells [46], this effect might take place also for other scaffold proteins with high renal reabsorption. This assumption is supported by data concerning biodistribution of $^{64}$Cu-NOTA-A20FMDV2 [42], which has a high renal reabsorption and demonstrates a biodistribution pattern similar to the pattern of $^{64}$Cu-NOTA-ZHER2:SI. A number of alternative chelators for radioiodine have been suggested, such as cross-bridged cyclam derivatives [47, 48] or derivatives of sarcophagine [49, 50]. It should be evaluated if these chelators are better alternatives for labeling of scaffold proteins with $^{64}$Cu or $^{61}$Cu.

5. Conclusion

Our present results suggest that the molecular design of probes based on scaffold proteins with high renal reabsorption should avoid the combination of radiocopper and NOTA/NODAGA amido derivatives. This information is essential to the development of imaging probes based on DARPinS, ADAPTS, and fibronectin domains, which have very high renal reabsorption, similar to that of affibody molecules [9, 11, 12].

Competing Interests

The authors declare that they have no competing interests.

Authors’ Contributions

Vladimir Tolmachev and Cheng-Bin Yim contributed equally to this work.

Acknowledgments

This research was financially supported by grants from the Swedish Cancer Society [Grants CAN 2015/350 and 2014/474], Swedish Research Council [Grants 2015-02353, 2013-5135, and 2015-02509], and Hospital District of Southwest Finland (EVO).

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