Site-Specific Radiiodination of HER2-Targeting Affibody Molecules using 4-Iodophenethylmaleimide Decreases Renal Uptake of Radioactivity

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Affibody molecules are small scaffold-based affinity proteins with promising properties as probes for radionuclide-based molecular imaging. However, a high reabsorption of radiolabeled Affibody molecules in kidneys is an issue. We have shown that the use of 125I-3-iodo-((4-hydroxyphenyl)ethyl)maleimide (IPEM) for site-specific labeling of cysteine-containing Affibody molecules provides high tumor uptake but low radioactivity retention in kidneys. We hypothesized that the use of 4-iodophenethylmaleimide (IPEM) would further reduce renal retention of radioactivity because of higher lipophilicity of radiometabolites. An anti-human epidermal growth factor receptor type 2 (HER2) Affibody molecule (ZHER2:2395) was labeled using 125I-IPEM with an overall yield of 45 ± 3%. 125I-IPEM-ZHER2:2395 bound specifically to HER2-expressing human ovarian carcinoma cells (SKOV-3 cell line). In NMRI mice, the renal uptake of 125I-IPEM-ZHER2:2395 (24 ± 2 and 5.7 ± 0.3% IAg−1 at 1 and 4 h after injection, respectively) was significantly lower than uptake of 125I-IHPEM-ZHER2:2395 (50 ± 8 and 12 ± 2% IAg−1 at 1 and 4 h after injection, respectively). In conclusion, the use of a more lipophilic linker for the radiiodination of Affibody molecules reduces renal radioactivity.

Introduction

Malignant transformation is often associated with an aberrant expression of certain types of cell-surface proteins, for example, receptors, cell adhesion molecules, or proteins active in embryonic development.[1] Molecular recognition of these proteins can be used for specific treatment of malignant cells, for example, targeted therapy. Monoclonal antibodies (Mabs) are the most used kind of targeting agents, which may act by preventing mitogenic signaling[2] or by eliciting antibody-dependent or complement-dependent cytotoxicity.[3] Antitumor action of Mabs might be further enhanced by conjugation of cytotoxic drugs or radionuclides.[4] However, there is an appreciable inter- and intrapatient heterogeneity in expression of molecular targets. Apparently, tumors that do not express particular targets would not respond to a particular targeting therapy. Therefore, the targeted treatment should be personalized, that is, adjusted to the tumor molecular abnormality profile of each particular cancer case.[5] In vivo visualization of cell-surface target proteins using radionuclide molecular imaging can personalize anticancer treatment by the selection of patients who would most likely benefit from a particular targeted therapy.[6]

A possible approach to the development of imaging agents is the radiolabeling of therapeutic Mabs using nuclides emitting gamma quanta that can be detected outside the patient’s body.[7] The use of Mabs as imaging agents has, however, certain downsides. Antibodies are relatively bulky proteins (150 kDa); this limits their rates of extravasation, tumor penetration, and blood clearance of unbound tracers.[7] Therefore, imaging is possible only several days after injection. In addition, antibodies have a tendency to accumulate in tumors nonspecifically due to an “enhanced permeability and retention” (EPR) effect, which might cause false positive diagnoses.[7] Small engineered scaffold affinity proteins, for example, Affibody molecules, are strong alternatives to antibodies in the development of imaging agents.[8] Affibody molecules are small (7 kDa) three-helical cysteine-free scaffold proteins derived from the immunoglobulin-binding domain of staphylococcal receptor protein A.[9] Randomization of surface amino acids on helices 1 and 2 of Affibody molecules creates large combinatorial libraries enabling the selection of high-affinity binders to different proteins, including cancer-associated ones.[10] The small size and high affinity (in low nanomolar and subnanomolar range) makes them good candidates for development of imaging probes.[11] Affibody-based agents have been generated for the imaging of several cancer-associated molecular targets, for example human epidural growth factor receptor type 2 (HER2),[12] insulin-like growth factor-1 receptor

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IGF-1R, epidermal growth factor receptor (EGFR), and carbonic anhydrase IX (CAIX). Preclinical studies have demonstrated that Affibody molecules provide a much higher contrast than radio-labeled Mabs and enable imaging only a few hours after injection. Clinical studies have demonstrated the capacity of Affibody molecules to image HER2 expression in breast cancer metastases.

The high renal retention is a major dosimetry problem in the case of radionuclide therapy and might complicate imaging of metastases in the lumbar region. On the other hand, radiomolecules are cleared rapidly from kidneys when Affibody molecules are labeled using nonresidualizing halogens, such as Br, I, or different iodine radioisotopes. Importantly, internalization of receptor-bound Affibody molecules by cancer cells is slow, which makes residualizing properties of a label not critical for successful targeting. Thus, the use and further development of nonresidualizing is a promising way to reduce renal retention of radioactivity of radio-labeled Affibody molecules.

Among radiomolecules, iodine radioisotopes offer a variety of properties as labels for targeting proteins and peptides; for example, (131)I (τ/2 = 13.3 h) is suitable for imaging using single-photon emission computed tomography (SPECT), (124)I (τ/2 = 4.18 d) for positron emission tomography (PET), and (131)I (τ/2 = 8 d) for therapy. The long-lived (125)I (τ/2 = 59.4 d) is a convenient surrogate nuclide, which is commonly used in the development of radiodiodination techniques. Direct radiodiodination of Affibody molecules destroys binding, presumably due to the presence of tyrosine in the binding site. To overcome this problem, Affibody molecules were indirectly labeled using N-succinimidyl para-iodobenzoate (SPIB) as a precursor. The resulting (125)I-para-iodobenzoate (125I-PtIB)-ZHER2.342 has demonstrated high-contrast imaging of HER2-expressing xenografts in mice and low renal retention of radioactivity. A disadvantage of that method is that it is not site-specific. There are seven amino groups in ZHER2.342 (N-terminus and six lysines), which result in a mixture of proteins with a different number of labeled prosthetic groups per protein at different positions. A meta-analysis of biodistribution studies suggested that the biodistribution profile of (125I-PtIB)-ZHER2.342 was not quite reproducible, possibly because of some deviation in labeling conditions resulting in different compositions of such a mixture.

The introduction of a unique thiol group by engineering a cysteine into the Affibody molecule allows a site-specific coupling of a linker molecule by thiol-directed chemistry. Both indirect radiohalogenation of cysteine-containing Affibody molecules using 3-bromo-(4-hydroxyphenethyl)maleimide (BrHPEM) or 3-iodo-(4-hydroxyphenethyl)maleimide (IHPEM) (1) as an intermediate precursor resulted in a homogenous radio-labeled product with preserved binding specificity. Surprisingly, the use of BrHPEM and IHPEM resulted in four to seven-fold decrease of renal retention of radioactivity at 4 h after injection, in comparison with the use of para-halobenzoate-based labels. This demonstrated that positioning of the linker molecule influences the biodistribution profile. However, the exact mechanism of the decrease is not clear. We speculate that exoproteases are predominantly featured in the renal proteolysis of Affibody molecules, and a terminal placement results in a more rapid formation of “leaky” lipophilic radiometabolites. As lipophilicity of radiometabolites is an important factor in their decreased intracellular retention, the use of a more lipophilic pendant group for C-terminal radioiodination of cysteine-containing Affibody molecules should further reduce renal retention of radioactivity. N-(4-(125)I-iodophenethyl)maleimide (125I-IPEM) (2), which has been earlier developed for site-specific labeling of antigen-binding fragments (Fab fragments), would be a suitable linker, as it both permits site-specific labeling of cysteine-containing Affibody molecules and is more lipophilic than IHPEM.

The goal of this study was to test the hypothesis that the use of 125I-IPEM for indirect radioiodination of Affibody molecules provides a conjugate that is capable of specific binding to its molecular target cells and provides lower renal retention of radioactivity than its 125I-IPEM-labeled counterpart. The anti-HER2 Affibody molecule ZHER2.2395, with a C-terminal cysteine was used as a model in this study. Labeling of ZHER2.2395 using 125I-IPEM was established, and properties of 125I-IPEM-ZHER2.2395 and 125I-IPEM-ZHER2.2395 were directly compared in vitro and in vivo.

### Results and Discussion

**Synthesis of N-(4-(tri-n-butylstannylphenethyl)amine)phenethylmaleimide (7)**

Synthesis of a precursor for iododestannylation, N-(4-(tri-n-butylnyl)phenethyl)-maleimide (7), was performed according to Scheme 1. 4-Bromophenethylamine (3) was protected with phthalimide using phthalic anhydride and provided 4 in 81%. A palladium-catalyzed metal–halogen exchange using bis(tri-butyltin) yielded 5 in 83%. Deprotection of the phthalimide group using methylamine (94% yield) and a subsequent reaction with maleimide provided 7 in 30%. Synthesis of nonradioactive iodophenethylmaleimide (8) for the use as a chroma-
4-Iodophenethylamine was reacted with maleimide to yield N-[4-iodophenethyl]-maleimide (9) in 38% yield.

Radioiodination of 7

Precursor 7 was radioiodinated using Chloramine-T as an oxidant (Scheme 3). As our goal was “one-pot” labeling, that is, performing radioiodination of a precursor and conjugation of radio-IPEMt to reducted Affibody molecules without intermediate purification, we tried to determine a minimum amount of 7 that provides a stable high yield. In the first series of labeling experiments, the amount of 7 dissolved in ethanol was varied, while the amount of oxidant was constant (40 μg, ~140 nmol) and reaction time was 10 min. The influence of the amount of 7 on labeling yield is shown in Figure 2. Between 0.01 μg (0.02 nmol) to 1 μg (2 nmol) the labeling yield increased proportionally with the amount of added precursor, from 31 ± 10% up to 71.9 ± 0.4%. An additional amount of precursor up to 12 μg (~24 nmol) did not further improve the labeling yield significantly. In a blank experiment where only solvent (5% acetic acid in methanol) was used, no labeling yield could be determined (Figure 3).

Iododestannylation of 7 was apparently rapid (Figure 4). When 3 μg (6 nmol) of 7 was used, a yield of 73 ± 3% was obtained already after 1 min, and further prolongation of the reaction time did not increase the yield significantly.

For further labeling experiments, the following conditions were selected: 2 μg (4 nmol) of 7, 40 μg (140 nmol) of Chloramine-T, and a reaction time of 2 min.
Conjugation of IPEM (8) to ZHER2:2395

The conjugation of $^{125}$I-IPEM to a reduced ZHER2:2395 was performed using a one-pot two-step approach, that is, in the same reaction vial as iododestannylation without purification of 2. The coupling yield of $^{125}$I-IPEM to ZHER2:2395 was measured by varying Affibody:IPEM molar ratios (1:1, 2:1, and 3:1). The samples were analyzed at 20, 40, and 60 min. The overall labeling yield (i.e., incorporation of radioiodine into Affibody molecules) after 20 min incubation was 21 ± 2% and 43 ± 4% for Affibody:IPEM ratios of 1:1 and 2:1, respectively. The highest overall yield, 53 ± 1%, was obtained at the ratio of 3:1 (Figure 5). Since the difference between ratios 2:1 and 3:1 was small, the ratio of 2:1 was used for preparation of conjugates for in vivo and in vitro experiments in order to keep a higher specific activity of the conjugate. Given that the yield remained reasonably constant for the three time points, the incubation time was set to 40 min. According to instant thin-layer chromatography (ITLC) analysis, purification of $^{125}$I-IPEM-ZHER2:2395 using an AP-5 size-exclusion column provided a radiochemical purity of 98.9 ± 0.5%.

The radioiodination of $^{125}$I-IHPEM-ZHER2:2395 resulted in an overall labeling yield of 45 ± 3% at an Affibody:HPEM molar ratio of 2:1 and a radiochemical purity of 99% after separation on an NAP-5 size exclusion column.

Stability test

To ensure that radioiodine is attached to the Affibody molecules via a stable covalent bond, samples of $^{125}$I-IPEM-ZHER2:2395 were incubated up to 4 h in 2 m of sodium iodide to replace noncovalently bound $^{125}$I and in 30% ethanol to displace a noncovalently bound 2. Incubation in sodium iodide resulted in a radiochemical purity of 98.5 ± 0.1%, and incubation in 30% ethanol in a purity of 99.4 ± 0.1%. The purity of control samples, which were kept in phosphate-buffered saline (PBS) was 99.4 ± 0.1%, that is, the difference was within experimental accuracy. Keeping samples in PBS for 24 h at 4 °C and 37 °C resulted in a purity of 97.3 ± 2.1% and 99.3 ± 0.3%, respectively.

In vitro studies

To verify that the binding of the labeled ZHER2:2395 was specific to HER2 receptors, the labeled Affibody conjugates were incubated with a HER2-overexpressing human ovarian carcinoma cell line (SKOV-3) presaturated with nonlabeled ZHER2:2395. Saturation of HER2 caused significant (p < 0.00005) decrease of cell-bound activity, from 53.7 ± 0.9% to 2.6 ± 0.1% for $^{125}$I-IPEM-ZHER2:2395 and from 56.8 ± 0.8% to 2.8 ± 0.1% for $^{125}$I-IHPEM-ZHER2:2395 (Figure 6). This demonstrates that the binding of the conjugates to HER2-expressing cells is saturable, which suggests its receptor specificity.

Data concerning binding and cellular processing of $^{125}$I-IPEM-ZHER2:2395 and $^{125}$I-IHPEM-ZHER2:2395 by HER2-expressing SKOV-3 cells are shown in Figure 7. The two compounds showed simi-
lar binding and processing patterns. A rapid increase in cell-associated activity was followed by a slower increase phase, a maximum, and then a decrease in cell-bound activity. The maximum value of cellular uptake was obtained at an earlier blood concentration was significantly (p < 0.05) lower for 125I-IPEM-ZHER2.2395 in at least one time point. Although 125I-IPEM-ZHER2.2395 had higher uptake in liver at 1 h pi (4.1 ± 0.7% IA g⁻¹) than by the decrease in retention activity delivered by Affibody molecules.

The decrease in renal radioactivity might be achieved, in principle, by the decrease in renal reabsorption of targeting proteins or by the decrease in retention of radiocatabolites in proximal tubuli cells. Previous studies have demonstrated that the scavenger receptor megalin is not involved in renal reabsorption of Affibody molecules. Therefore, common methods for the decrease in renal reabsorption of small proteins and peptides in kidneys, e.g. saturation

**Table 1. Comparison of the biodistribution of 123I-IPEM-ZHER2.2395 and 123I-HPEM-ZHER2.2395 in NMRI mice.**

| Organs          | 1 h Uptake [% IA g⁻¹] | 4 h Uptake [% IA g⁻¹] | 24 h Uptake [% IA g⁻¹] |
|-----------------|-----------------------|-----------------------|------------------------|
| Blood           | 2.8 ± 0.84²          | 2.2 ± 0.70             | 0.81 ± 0.06             |
| Liver           | 2.8 ± 0.5             | 2.6 ± 0.3              | 0.57 ± 0.04             | 0.54 ± 0.06 |
| Spleen          | 4.1 ± 0.7²            | 2.7 ± 0.1              | 1.4 ± 0.1               | 1.4 ± 0.3   |
| Stomach         | 1.0 ± 0.2             | 0.9 ± 0.1              | 0.34 ± 0.04             | 0.29 ± 0.05 |
| Kidney          | 1.1 ± 0.1³            | 2.1 ± 0.4              | 0.40 ± 0.05             | 0.40 ± 0.05 |
| Salivary gland  | 24 ± 2²               | 50 ± 9                 | 5.7 ± 0.3⁴              | 12 ± 2      |
| Muscle          | 0.6 ± 0.1             | 1.1 ± 0.9              | 0.11 ± 0.01             | 0.12 ± 0.04 |
| GI tract        | 9 ± 1²                | 12 ± 1                 | 9.8 ± 0.9²              | 18 ± 3      |
| Carcass         | 14 ± 2                | 14 ± 1                 | 3.1 ± 0.3               | 3.8 ± 1.0   |

(a) Significant (p < 0.05) difference between 123I-IPEM-ZHER2.2395 and 123I-HPEM-ZHER2.2395 at the same time point. (b) Data from gastrointestinal (GI) tract with content and carcass are presented as % of injected radioactivity per whole sample. Data are presented as mean ± S.D. (n = 4 mice).

**Discussion**

Engineered scaffold proteins such as Affibody molecules, are emerging alternative to Mabs in radionuclide tumor targeting for diagnostics and therapy. A precondition for successful clinical application of scaffold proteins is maximizing the delivery and retention of radionuclides in tumors and minimizing their delivery and retention in normal tissues. In the case of imaging, this provides higher contrast and, therefore, sensitivity. In the case of radionuclide therapy, this ensures destruction of tumors while sparing normal tissues. Hence, a decrease in uptake and retention of radionuclides in normal tissues is as important as an increase in tumor uptake. This study focused on the decrease of renal radioactivity delivered by Affibody molecules.
of scavenger receptors by co- or preinjection of cationic amino acids or Gelofusine, do not work. Therefore, minimization of retention of radiocatabolites in proximal tubuli is the only viable alternative at the moment. Our previous studies have demonstrated that this goal might be achieved for both radiohalogens \(^{125}\)I-IPEM and \(^{125}\)I-IPEM-Z\(_{HER2:2395}\) for the labeling of Fab fragments was reported by Hylarides and co-workers in 1991.\(^{30}\) Factors influencing labeling efficiency were described in the initial report. Unfortunately, we did not find any follow-up studies concerning this interesting precursor for radioidination. 125\(^{I}\)-IPEM attracted our attention because it is suitable for thiol-directed site-specific labeling of cysteine-containing Affibody molecules and its hydrophobicity (log\(_P\) = 2.85) is higher than that of 125\(^{I}\)-IHPEM (log\(_P\) = 2.46).

Iododeestannylation of 7 was efficient, enabling a yield in excess of 70% when 1–3 \(\mu\)g (2–6 nmol) was used for labeling (Figure 2). The reaction was rapid, and the high yield was achieved within 1 min (Figure 4), when 3 \(\mu\)g of 7 was used. The efficient labeling of a small amount of precursor removed the need for the intermediate purification of 2, i.e. we can perform the whole conjugation “in one pot”. This approach minimizes the handling of radioactive compounds, the associated dose burden to personnel, the losses during transfer of labeled precursor, and the probability of human error.\(^{30}\) The overall labeling yield of the two-step process was a modest –20% when an Affibody:precursor molar ratio of 1:1 was used (Figure 5). Increasing the ratio to 2:1 doubled the overall yield, but a further increase in relative protein amount was relatively inefficient. The Affibody:precursor ratio of 2:1 was selected for further labeling to keep a reasonably high specific activity. The conjugation time of 20–30 min was sufficient to reach the maximum overall yield (Figure 5). Importantly, the binding of 125\(^{I}\)-IPEM-Z\(_{HER2:2395}\) to HER2-expressing SKOV-3 cells was saturable (Figure 5A), which demonstrates that the binding specificity of the Affibody molecule was preserved after labeling. Binding specificity of the alternative, 125\(^{I}\)-IHPEM-Z\(_{HER2:2395}\), has also been confirmed (Figure 5B).

The internalization rate of anti-HER2 Affibody molecules by cancer cells is rather slow and reaches 30–40% per day in the case of residualizing labels.\(^{27}\) This internalization rate corresponds to daily cell-surface renewal. In this study the internalized fraction of radioactivity was below 10% at all time points which reflects nonresidualizing properties of radiocatabolites. After binding to a cell-surface receptor, a fraction of Affibody molecules is internalized, transferred to the lysosomal compartment, and degraded by proteolytic enzymes. Lipophilic radiometabolites diffuse through lysosomal and cellular membranes and leak out from cells. A maximum cell-associated activity is achieved when the binding rate is equal to the rate of radiocatabolite leakage. In the case of a higher leakage rate, the maximum is reached earlier. A comparison of binding to SKOV-3 cells (Figure 7) shows that the maximum cell-associated activity for 125\(^{I}\)-IPEM-Z\(_{HER2:2395}\) was reached earlier (4 h) than for 125\(^{I}\)-IHPEM-Z\(_{HER2:2395}\) (8 h). This suggests that 125\(^{I}\)-IPEM provides more “leaky” radiometabolites. It has to be noted that internalization of anti-HER2 Affibody molecules by cancer cells is rather slow,\(^{20,21,28}\) and the difference is therefore relatively small. This complicates in vitro evaluation of an internalization rate, but is favorable for in vivo tumor targeting, as leakage of radiocatabolites has a small effect on tumor-associated radioactivity.\(^{37}\) On the other hand, the internalization rate in kidneys is high, and the difference in radiocatabolite leakage rate has to be much more pronounced.

Direct in vivo comparison of 125\(^{I}\)-IPEM-Z\(_{HER2:2395}\) and 125\(^{I}\)-IHPEM-Z\(_{HER2:2395}\) biodistribution in NMRI mice confirmed the main hypothesis of this study (Table 1). The renal radioactivity was two times lower for 125\(^{I}\)-IPEM-Z\(_{HER2:2395}\) than for 125\(^{I}\)-IHPEM-Z\(_{HER2:2395}\), at 1 h and 4 h after injection. It has to be noted that the label nature has a clear influence in uptake in other organs. For example, hepatic uptake of 125\(^{I}\)-IPEM-Z\(_{HER2:2395}\) (4.1 ± 0.7 %IA g\(^{-1}\)) at 1 h pi was significantly higher than uptake of 125\(^{I}\)-IHPEM-Z\(_{HER2:2395}\) (2.7 ± 0.1 %IA g\(^{-1}\)). This might be because of the higher overall lipophilicity of 125\(^{I}\)-IPEM-Z\(_{HER2:2395}\) as higher lipophilicity is generally associated with higher liver uptake.\(^{38}\) However, the difference disappeared at 4 h pi, a time point relevant for imaging. More disturbing was the 1.5-fold higher blood level of 125\(^{I}\)-IPEM-Z\(_{HER2:2395}\). As this could negatively affect imaging contrast, this phenomenon might be associated with the release of radiometabolites from kidneys to blood or with higher adhesion to blood proteins. Another interesting feature of 125\(^{I}\)-IPEM-Z\(_{HER2:2395}\) was lower radioactivity uptake in the stomach and salivary gland. As both these organs express the Na\(^+\)/\(\beta\) symporter, it is likely that the metabolism of 125\(^{I}\)-IPEM results in the decreased release of free radiiodide. Overall, this
study demonstrated the very pronounced influence of a prosthetic group for radiiodination on the distribution of radioactivity after injection of Affibody molecules.

This information might also be helpful in the development of other imaging probes based on engineered scaffold proteins. It has to be noted that this approach is justified when internalization of an imaging agent by cancer cells is slow but internalization by tubuli cells is rapid. In the case of rapid internalization by cancer cells, the use of a prosthetic group providing lipophilic radiometabolites would decrease tumor accumulation of radioactivity. In this case, radiiodination using prosthetic groups providing charged radiometabolites would be recommended. [39]

Conclusions

The use of a more lipophilic radiodiode label at the C-terminus of Affibody molecules is associated with lower renal retention of radioactivity. This suggests that a careful selection of the labeling strategy could be used in the modification and optimization of the biodistribution profile of radiohalogenated imaging agents.

Experimental Section

Materials

125I was purchased from PerkinElmer (Waltham, USA). Organic solvents were purchased from Merck (Darmstadt, Germany). Chloramine-T (CAT) and Na2S2O4 were from Sigma (St. Louis, USA). Buffers, including 0.1 M PBS (pH 7.5) and 0.2 M NH4OAc (pH 6.3), were prepared using common methods from chemicals supplied by Merck (Darmstadt, Germany). High-quality Milli-Q water (resistance higher than 18 MΩ cm) was used for preparing the solutions and buffers.

Solutions of CAT, Na2S2O4, iodophenethylmaleimide, and hydroxylphenethylmaleimide were prepared immediately before use. TLC plates were developed in 1,2-dichlorethane (BDH chemicals, Poole, England). Ketalar (ketamine, 50 mg mL−1) was purchased from Pfizer (New York, USA). NMR spectra were recorded on a Varian Mercury Plus spectrometer (Santa Clara, USA) at ambient temperature. Chemical shifts are referenced via the residual solvent signals of CDCl3 at 7.26 ppm; 1H NMR (400 MHz, 25 °C, CDCl3); δ = 7.85–7.64 (m, 4H), 7.44–7.32 (m, 2H), 7.26–7.20 (m, 2H), 3.94–3.89 (m, 2H), 2.98–2.94 ppm (m, 2H); 13C NMR (100 MHz, 25 °C, CDCl3); δ = 168.1, 136.9, 134.0, 131.6, 132.0, 130.6, 123.3, 120.5, 38.9, 34.0 ppm; MS (ESI): 330–332 [M + H]+.

Synthesis of N-(4-bromophenethyl)phthalimidine (4)

To a stirred solution of 3 (1.0 g, 5 mmol in 50 mL diethyl ether) was added phthalic anhydride (741 mg, 5 mmol). The mixture was stirred for 2 h. Afterwards, a white precipitate (carbamoylbenzoic acid) was collected after filtration and washed with cold diethyl ether (3 × 30 mL). The acid was dissolved in glacial CH3COOH (15 mL) and heated at 65 °C for 3 h. The mixture was cooled to rt and was allowed to precipitate overnight. Filtration and washing with cold CH3COOH (3 × 20 mL) provided 4 as a white powder (81%, 1.33 g); 1H NMR (400 MHz, 25 °C, CDCl3); δ = 7.86–7.83 (m, 2H), 7.73–7.71 (m, 2H), 7.41–7.43 (m, 2H), 7.14–7.12 (m, 2H), 3.93–3.89 (m, 2H), 2.98–2.94 ppm (m, 2H); 13C NMR (100 MHz, 25 °C, CDCl3); δ = 168.1, 136.9, 134.0, 131.6, 132.0, 130.6, 123.3, 120.5, 38.9, 34.0 ppm; MS (ESI): 330–332 [M + H]+.

Synthesis of N-[(tri-n-butylstannyl)phenethyl]phthalimidine (5)

A pressure-resistant 50 mL glass tube was loaded with 4 (500 mg, 1.5 mmol), bis(triethyltin) (2.63 g, 4.5 mmol, 3 equiv), tetrakis(tributylstannyl)phenethylamine (6) and anhydrous toluene (15 mL). The tube was flushed with nitrogen and, after capping, heated at 115 °C for 48 h. After cooling to rt, the crude dark mixture was evaporated and purified by column chromatography yielding compound 5 as a clear viscous oil (83%, 675 mg); Rf = 0.2 (isohexane/EtOAc 9:1); 1H NMR (400 MHz, 25 °C, CDCl3); δ = 7.85–7.64 (m, 4H), 7.44–7.32 (m, 2H), 7.26–7.20 (m, 2H), 3.94–3.89 (m, 2H), 2.99–2.93 ppm (m, 2H), 1.65–1.25 (m, 12H), 1.10–0.98 ppm (m, 6H), 0.92–0.80 ppm (m, 9H); 13C NMR (100 MHz, 25 °C, CDCl3); δ = 168.2, 139.8, 137.6, 136.7, 135.9, 132.1, 128.5, 123.2, 39.2, 34.6, 29.0, 27.4, 13.7, 9.5 ppm; MS (ESI): 537–545 [M + H]+.

Synthesis of 4-(tri-n-butylstannyl)phenethylamine (6)

To a pressure-resistant 50 mL glass tube containing 5 (600 mg, 1.1 mmol), CH3NH2 (10 mL, 33% in absolute EtOH) was added. The tube was capped and heated to 60 °C for 2 h. After cooling, the solvent was removed leaving a white solid. NaOH (20 mL, 2.5 M) was added and then extracted with diethyl ether (3 × 30 mL). The organic phases were combined, dried over MgSO4, and evaporated leaving the desired product; [39] compound 6, as a clear low viscous oil (94%, 426 mg); 1H NMR (400 MHz, 25 °C, CDCl3); δ = 7.39 (t, J = 7.8 Hz, 2H), 7.17 (t, J = 7.8 Hz, 2H), 2.97 ppm (t, J = 7.0 Hz, 2H), 2.73 ppm (t, J = 7.0 Hz, 2H), 1.64–1.26 ppm (m, 12H), 1.16–0.84 ppm (m, 15H); 13C NMR (100 MHz, 25 °C, CDCl3); δ = 139.5, 139.1, 136.6, 128.5, 43.5, 40.2, 29.0, 27.4, 13.7, 9.5 ppm; MS (ESI): 408–416 [M + H]+.

Synthesis of N-[(4-tri-n-butylstannyl)phenethyl]maleimide (7)

Compound 6 (400 mg, 0.98 mmol) was dissolved in diethyl ether (25 mL) where, after, maleic anhydride (115 mg, 1.2 mmol,
1.2 equiv) was added, and the reaction was left stirring for 2 h yielding crude maleimimidic acid. After removal of solvent in vacuo, acetic anhydride (30 mL) was added together with KOAc (295 mg, 3 mmol). The mixture was stirred at 90 °C for 2 h and then cooled to rt. To the mixture was added diethyl ether (30 mL) and then washed with H₂O (3 × 30 mL) and aq Na₂CO₃ (1 × 100 mL, 5%). The organic phase was then dried with MgSO₄, evaporated, and purified with column chromatography providing compound 7 as a clear viscous oil (30%, 140 mg); Rf = 0.2 (isohexane:EtOAc:Et₃N 20:1:0.2); ¹H NMR (400 MHz, 25 °C, CDCl₃); δ = 7.38 (t, J = 7.8 Hz, 2H), 7.17 (t, J = 7.8 Hz, 2H), 6.66 (s, 2H), 3.78–3.72 (m, 2H), 2.90–2.85 (m, 2H), 1.61–1.27 (m, 12H), 1.12–0.86 ppm (m, 15H); ¹³C NMR (100 MHz, 25 °C, CDCl₃); δ = 170.5, 139.9, 136.7, 134.0, 128.4, 39.1, 31.0, 29.0, 27.4, 13.7, 9.5 ppm; MS (ESI): 487–495 [M + H⁺].

Synthesis of N-(4-iodophenethyl)-maleimide (9)

To a stirred solution of 8 (124 mg, 0.5 mmol) in diethyl ether (10 mL), maleic anhydride (57 mg, 0.6 mmol, 1.2 equiv) was added, and the reaction was left stirring for 2 h. The precipitate was collected and dissolved in glacial CH₂COOH (10 mL) and heated at 65 °C for 3 h. The mixture was cooled to rt and was allowed to precipitate overnight. The precipitate was filtered and washed with cold CH₂COOH (3 × 10 mL) resulting in compound 9 as a white powder (38%, 62 mg); ¹H NMR (400 MHz, 25 °C, CDCl₃); δ = 7.61 (d, J = 8.3 Hz, 1H), 7.19 (d, J = 5.6 Hz, 1H), 6.99 (d, J = 8.0 Hz, 1H), 6.39 (d, J = 5.6 Hz, 1H), 3.87 (t, J = 7.2 Hz, 1H), 2.92 ppm (t, J = 7.2 Hz, 1H); ¹³C NMR (100 MHz, 25 °C, CDCl₃); δ = 166.7, 142.2, 139.1, 137.6, 134.2, 131.0, 128.8, 91.7, 50.8, 36.3 ppm; MS (ESI): 378 [M + H⁺].

Radioiodination of 4-iodophenethylmaleimide (IPEM)

The precursor was radioiodinated using CAT as an oxidant. Typically ¹²⁵I (10 mL, 1 MBq µL⁻¹) was added to the precursor 7 (3 µL, dissolved in EtOH at a predetermined concentration) and solvent (10 µL, 5% HOAc in MeOH). To the mixture, CAT (10 µL, 4 µg µL⁻¹ in Milli-Q H₂O) was added. The reaction proceeded for a predetermined time and then quenched by a double molar excess of Na₂S₂O₄ (6 µg µL⁻¹ in Milli-Q H₂O).

The dependence of labeling yield on the amount of precursor and reaction time was determined by varying one parameter at a time. The amount of 7 varied between 0.01–15 µg and the reaction time varied between 1–10 min. When the amount of 7 varied, the reaction time was set at 10 min. When the reaction time was varied, the amount of 7 remained constant (3 µg).

Silica gel 60 F254 TLC plates (20 × 100 mm, elution path 80 mm) (Merck) were used for analysis. As eluent, 1,2-dichloroethane was used. In this system, iodoimidic acid remains at the origin while IPEM has an Rf of 0.4. The reaction mixture (1.8–2 µL) was applied to the ITLC plate, which was then left to evaporate spontaneously before being developed in the eluent. All experiments were done at least in duplicate. Blank experiments were performed where 5% HOAc in MeOH (3 µL) was used instead of 7.

Conjugation of IPEM (2) to prereduced ZHER2:2395

Before labeling, the Affibody molecule solution in PBS was treated with diithiothreitol (DTT, Merck) in order to reduce spontaneously formed disulfide bonds. Typically, the stock Affibody solution (50 µL, 10 µg µL⁻¹) in PBS was mixed with DTT (350 µL) in PBS to get a final concentration of 50 mM DTT. The mixture was incubated at 40 °C for 3 h at rt. Thereafter the mixture was applied to NAP-5 size-exclusion columns, pre-equilibrated, and eluted with well-degassed 0.2 M NH₄OAc (pH 6.3), containing 0.04 M sodium ascorbate. The first 900 µL of the high molecular weight (HMW) fraction was used for labeling. The reduced Affibody was used for labeling approximately 10–15 min after reduction. Compound 7 was radioiodinated as described above. To get the best conjugation conditions, the Affibody/IPEM molar ratio was varied between 1:1, 2:1, and 3:1. ZHER2:2395 was added in varying amounts to the ¹²⁵I-IPEM solution (2 µg of 7) and incubated at rt. Small fractions (~1.5 µL) were analyzed on ITLC silica gel strips developed in 80% acetone in H₂O, at 20, 40, and 60 min. In this system, the Affibody molecule remains at the origin, and IPEM and iodide have an Rf of 1.0. After conjugation, ¹²⁵I-IPEM-ZHER2:2395 was purified using an NAP-5 size-exclusion column pre-equilibrated with PBS. The mixture (1.6 µL) was analyzed using radio-ITLC to determine radiochemical purity of the final product. All experiments were done in duplicate.

Stability test

The stability of radioiodine coupling to the ZHER2:2395 was assessed by incubating the solution for at 4 °C for 24 h and thereafter at rt for 4 h. Stability was further analyzed by incubating the radioiodinated ZHER2:2395 with 2 mM NaI or 30% EtOH, both for 1 h. The labeling was done as described above and purified via NAP-5 size-exclusion chromatography. ¹²⁵I-IPEM-ZHER2:2395 was added in equal volume (50 µL) to Eppendorf tubes was incubated for 1 h. For the stability test in EtOH, 95% EtOH (30 µL) was added to the sample (70 µL) in Eppendorf tubes, and the solution was incubated for 1 h. All experiments were done in duplicate and analyzed using ITLC strips.

Labeling of HPEM

Labeling of HPEM was performed as previously described.[26] In brief, HPEM precursor solution(5 µg) in 5% HOAc in MeOH was mixed with ¹²⁵I (10 µL). To the mixture, aq CAT solution (10 µL, 4 µg µL⁻¹) was added, and the solution was incubated for 5 min. The reaction was terminated by adding aq Na₂S₂O₄ (10 µL, 6 µg µL⁻¹), given in double molar excess to CAT. Conjugations of ¹²⁵I-IPEM (1) to ZHER2:2395 was done as described for ¹²⁵I-IPEM with an Affibody/HPEM molar ratio of 2:1.

Binding specificity and cellular processing of labeled conjugates to HER2-expressing cells

The binding specificity of the ¹²⁵I-IPEM- and ¹²⁵I-IPEM-ZHER2:2395 conjugates to HER2 were evaluated using HER2-expressing SKOV-3 ovarian cancer cells that are described previously.[27] Briefly, 30% of ¹²⁵I-IPEM-ZHER2:2395 or ¹²⁵I-IPEM-ZHER2:2395 (1.99 ng/dish, 27 pM) was added to a set of two groups of petri dishes (1 × 10⁶ cells/dish). To block the receptors, an excess of nonlabeled ZHER2:2395 was added 5 min before applying the labeled conjugate to one group of dishes. The cells were incubated in 37 °C for 1 h in a humidified incubator; thereafter, the cell media was collected, and the cells were detached with trypsin-EDTA solution (0.5 mL, 0.5% trypsin, 0.02% EDTA in buffer, Biochrom AG, Berlin, Germany). Then the complete medium (0.5 mL) was added to every dish, and cells were resuspended and collected. The percentage of cell-bound radioactivity was calculated by measuring the radioactivity in the media and the cells.

Processing of ¹²⁵I-IPEM-ZHER2:2395 and ¹²⁵I-IPEM-ZHER2:2395 by SKOV-3 cells was studied according to a method previously described and validated.[27] The labeled compounds (1.99 ng/dish, 27 pM) were added to each dish, with three dishes per time point per conjugate (1 × 10⁶ cells/dish). The cells were incubated at 37 °C, 5% CO₂. At predetermined time points (1, 2, 4, 8, and 24 h after the start of incubation), the media from the dishes was collected, and the cells were washed with ice-cold serum-free medium. The cells were then treated with 0.2 M glycine buffer containing 4 M urea, pH 2.5,
Biodistributions study in NMRI mice

All animal experiments were planned and performed according to the Swedish national legislation on the protection of laboratory animals, and the study plans were approved by the Uppsala committee for animal research ethics (Uppsala djurförsöksetisknämnd, permision C224/10 given to A. Orlova). Female NMRI mice (10–12 weeks old at arrival) were acclimatized for one week at the Rudbeck Laboratory animal facility. Groups of four animals per time point were used for the biodistribution study. Animals were intravenously injected with 30 kBq of either $^{125}$I-PEM-Z$_{400}$2250 (4 µg) or $^{125}$I-HPEM-Z$_{400}$2250 (4 µg) dissolved in PBS (100 µL). At 1 h, 4 h, and 24 h after injection, a mixture of Ketala-Rompun (20 mg/mL solution per gram body weight, Ketalar:10 mg/mL, Rompun:1 mg/mL) was injected intraperitoneally. The mice were sacrificed by heart puncture. Blood was collected, and organ samples were excised. The samples were put in preweighed plastic vials. The samples were weighed, and their radioactivity was measured. The uptake in tissue and organ were calculated as percent injected activity per gram tissue (% IA g$^{-1}$). For the gastrointestinal tract and the carcass, injected activity per whole sample was calculated (% IA g$^{-1}$).

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