MATERIALS AND METHODS:

$^{111}InCl_3$ was purchased from Mallinckrodt Medical BV (Petten, Holland). The affibody molecule $Z_{HER2:2395}$ was obtained from Affibody AB. All chemicals were purchased from Sigma-Aldrich. The synthesis of DOTA-PEG_{10}-tetrazine and the NHS-ester of (*E*)-2-(cyclooct-4-enyloxy)acetic acid has been described elsewhere (*1-3*). High-quality Milli-Q water (resistance higher than 18 MΩ cm) was used for preparing solutions. The HER2-expressing ovarian cancer cell line SKOV-3 and breast cancer cell line BT-474 were used in cell studies. Both cell lines were obtained from American Type Culture Collection (ATCC). Cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and PEST (penicillin 100 IU/ml and 100 μg/ml streptomycin). An automated γ-spectrometer with a ~7.6-cm (3-in) NaI(Tl) detector (1480 WIZARD; Wallac Oy) was used to measure radioactivity. Data on cellular uptake and biodistribution were analyzed by unpaired 2-tailed t test using GraphPad Prism (version 4.00 for Windows; GraphPad Software) to determine significant differences ($p < 0.05$).

Synthesis of maleimido-PEG_4-TCO

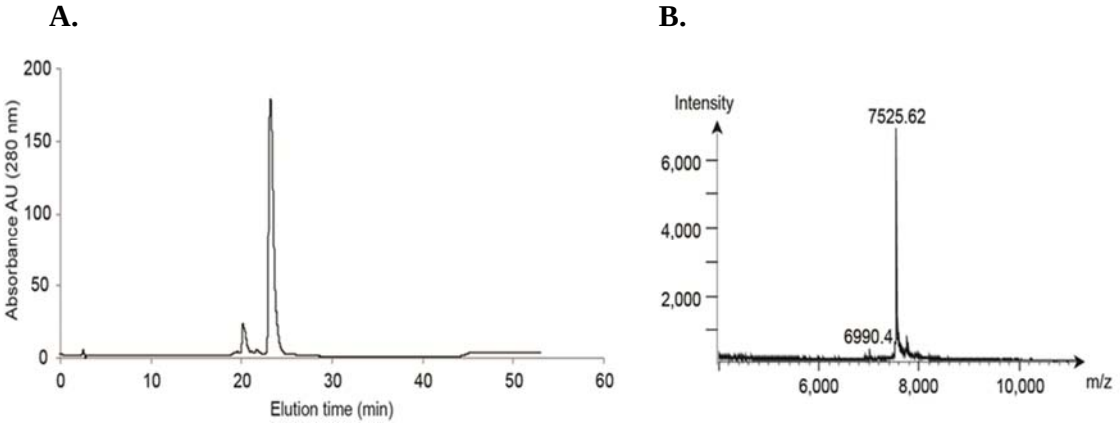
A solution of the NHS-ester of (*E*)-2-(cyclooct-4-enyloxy)acetic acid (minor isomer) in 25 mL dichloromethane was added over a few minutes to a solution of 1,11-diamino-3,6,9-trioxaundecane (2.0 g, 10.4 mmol) in 10 mL dichloromethane. The solution was stirred for 2 days, 20 mL dichloromethane was added and the mixture was washed with 3 × 10 mL water. Drying and rotary evaporation gave a residue, which was chromatographed on 20 g silica using dichloromethane / methanol (up to 20%) as the eluent. The desired product was subsequently eluted with dichloromethane / 20% methanol / 20% triethylamine. This gave 220 mg of the amine derivative as an oil.
^1H NMR (300 MHz, $CDCl_3$): 7.05 (bs, 1H), 5.60 - 5.45 (m, 2H), 3.95 (d, J = 15 Hz, 2H), 3.85 (d), 3.7 - 3.5 (m), 2.95 (m), 2.4 - 1.15 (m).
The amine derivative (110 mg) was dissolved in 15 mL dichloromethane and the NHS-ester of 4-maleimidobutyric acid (135 mg, 0.48 mmol) was added. The solution was stirred for 2 days at room temperature, then rotary evaporated at 55 °C. The residue was chromatographed on 12 g silica using dichloromethane as the eluent with a gradual increase of the methanol content. The starting maleimide eluted with 1% methanol, the desired product (35 mg) eluted with slightly higher methanol content.
^1H NMR (300 MHz, $CDCl_3$): 6.6 (s, 2H), 6.35 (bs, 1H), 5.6 - 5.45 (m, 2H), 3.95 (d, J = 15 Hz, 2H), 3.85 (d), 3.7 - 3.5 (m), 3.45 (m), 2.4 -1.15 (m). HPLC-MS (ESI, *m/z*): 524.29 [$M+H^+$].

Coupling of maleimido-PEG_4-TCO to Z_{2395}

Initial attempts to create an affibody-TCO conjugate were performed using non-PEGylated maleimido-TCO. The poor solubility of this reagent in aqueous solution led to formation of two-phase system. Attempts to solve this issue by using water-miscible organic solvents (e.g. DMSO, DMF) were unsuccessful. The overall yield of conjugation was only of 8.6%.

The PEG_4-containing maleimido derivative of TCO was conjugated to the $Z_{HER2:2395}$ Affibody molecule containing a C-terminal cysteine using site-specific thiol-directed chemistry. For this purpose, freeze-dried $Z_{HER2:2395}$ (5 mg, 0.714 μmol) was dissolved in 1 mL PBS. $Z_{HER2:2395}$ was reduced by adding a 10-fold molar excess of TCEP solution (50 μmol dissolved in 100 μL Milli-Q water). The reaction vial was incubated for 30 min at 37°C. Immediately before use, 10 mg (27.8 μmol) of maleimido-PEG_4-TCO was dissolved in 150 μL DMSO. 18.6 μmol (26-fold molar excess) of maleimido-PEG_4-TCO was added to the

reduced protein sample dropwise and the mixture was carefully vortexed after each addition (total added volume was 100 μL). An extra 10 μL of DMSO was added to the reaction vial and the mixture was continuously agitated for 2 h at room temperature. Purification and determination of the degree of coupling were performed using an HPLC system (Agilent 1200 series) as described earlier for affibody molecules (*5*). Collected fractions were analyzed with MALDI-TOF LT3 plus instrument mass spectrometry (SAI) in order to verify the correct product, further designated as Z_{2395}-TCO.



Supplemental Figure 1. Characterization of the TCO-Z_{2395} Affibody molecule. **(A)** Reversed-phase HPLC chromatogram of purified conjugate. **(B)** Obtained mass spectrum of purified conjugate.

Labelling

Labeling of Z_{2395}-TCO *with* ^{125}I. For indirect radioiodination of Z_{2395}-TCO, ^{125}I (25 MBq) was dissolved in 10 μL of 0.1% acetic acid in water. N-Succinimidyl-*p*-(trimethylstannyl)benzoate (5 μL, 1 mg/mL in 5% acetic acid in methanol) was added. Iodination was initiated by the addition of chloramine-T (10 μL, 4 mg/mL in water), and the solution was incubated for 5 min at room temperature. The reaction was quenched by adding sodium meta-bisulfite (10 μL, 8 mg/mL in water). Z_{2395}-TCO (50 μg in 0.07 M sodium borate, pH 9.3) was then added. After 1 h of incubation at room temperature, the radiolabeled conjugate was purified on NAP-5 columns eluted with PBS.

Labeling of DOTA-PEG_{10}-tetrazine *with* ^{111}In. A solution of 50 μg in 70 μL 0.2 M ammonium acetate, pH 5.5, was mixed with 80 μL (60 MBq) ^{111}In-chloride solution. The mixture was incubated at 60°C for 30 min. Thereafter, a small aliquot (1 μL) of reaction mixture was taken and analyzed by radio-ITLC eluted with 0.2 M citric acid, pH 2.0. ^{111}In-DOTA-PEG_{10}-tetrazine was further designated as ^{111}In-tetrazine.

Labeling of DOTA-PEG_{10}-tetrazine *with* ^{177}Lu. A solution of 50 μg in 80 μL 0.2 M ammonium acetate, pH 5.5, was mixed with 74 μL (50 MBq) ^{177}Lu-chloride solution. The mixture was incubated at 60°C for 30 min. Thereafter, a small aliquot (1 μL) of reaction mixture was taken and analyzed by radio-ITLC eluted with 0.2 M citric acid, pH 2.0. ^{111}In-DOTA-PEG_{10}-tetrazine was further designated as ^{177}Lu-tetrazine.

In vitro studies

In vitro pretargeting specificity. The feasibility of specific pretargeting of HER2-expressing cancer cells was tested using ovarian carcinoma SKOV-3 (1.6×10^6 receptors/cell) and breast carcinoma BT474 (2×10^6 receptors/cell) cell lines. Briefly, 12 petri dishes (for each cell line) containing 5×10^5 cells were divided into groups of three. In one set of dishes, cells were pre-incubated with 4 nM Z_{2395}-TCO for 60 min and dishes then were washed with serum free medium. Thereafter, ^{111}In-tetrazine (4 nM) was added to the cells. After incubation for 60 min at 37°C, the media were collected, cells were detached by trypsin and cell-associated radioactivity was measured. The following control experiments were performed. In one set of dishes, cells were pretreated with a 500-fold molar excess of parental anti-HER2 $Z_{HER2:342}$ affibody molecules to prevent Z_{2395}-TCO binding. In another set, a 10-fold molar excess of non-labeled tetrazine was added to compete with the specific binding of ^{111}In-tetrazine. Other manipulations were as described above. To the third set, ^{111}In-tetrazine (4nM) was added directly to the cells without the addition of Z_{2395}-TCO and incubated for 1 h. Media and cells were collected and samples were measured.

Affinity determination using LigandTracer. SKOV-3 or BT474 cells were seeded on a local area of a cell culture dish (NunclonTM, Size 100620, NUNC A/S, Roskilde, Denmark), as described previously (5). The binding of the primary targeting agent Z_{2395}-TCO to living cells was monitored in real-time at room temperature using LigandTracer-Yellow (Ridgeview Instruments AB, Sweden) , using both ^{125}I-Z_{2395}-TCO and pre-reacted ^{111}In-tetrazine-Z2395-TCO. The affinity of radiolabeled conjugates was determined using TraceDrawer software (3). In order to cover the concentration span needed for proper affinity estimation, two concentrations of 153 pM and 306 pM (selected based on previous K_D values for parental $Z_{HER2:342}$ obtained using Biacore) of each variant were added for each affinity assay.

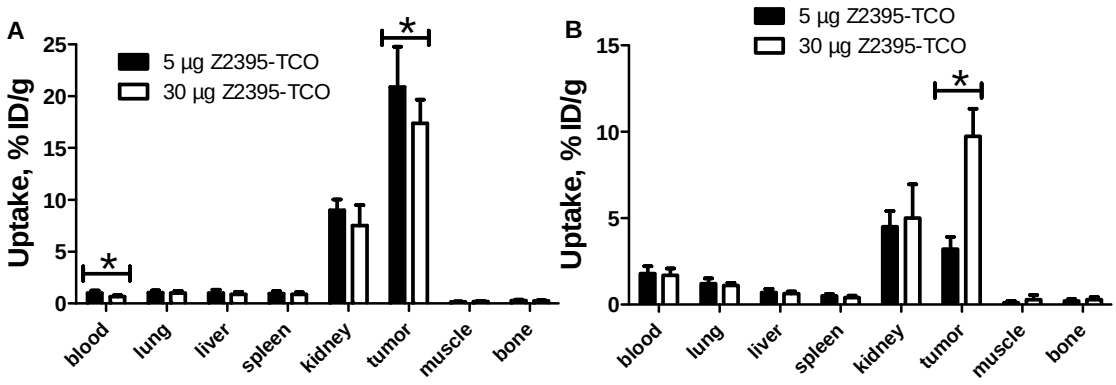
Cellular retention of the primary targeting agent Z_{2395}-TCO. Cellular retention of ^{125}I-Z_{2395}-TCO was measured, after interrupted incubation, without discrimination between internalized and membrane-bound activity, as internalization would be underestimated for the non-residualizing radioiodine label.
Both SKOV-3 and BT474 cells (1×10^6 cells/dish) were incubated with a 4 nM solution of ^{125}I-Z_{2395}-TCO at 4°C. After 1 h incubation, the medium with the labeled compound was removed and cells were washed with serum-free medium. One mL of complete medium was added to each dish and cells were further incubated at 37°C in an atmosphere containing 5% CO_2. At 1, 4, 8 and 24 h, one group of three dishes was removed from the incubator, the medium was collected and cells were washed with serum-free medium. Thereafter, cells were detached by trypsin-EDTA solution and the radioactivity in cells and media was measured to calculate the percentage of cell-bound radioactivity.

Cellular processing and internalization of ^{111}In-tetrazine-TCO-Z_{2395} adduct. After cells were incubated for 1 h at 4 °C with a 4 nM of pre-reacted ^{111}In-tetrazine-TCO-Z_{2395}, the incubation media was discarded and cells were washed and 1 mL fresh complete medium was added. The cells were incubated further at 37 °C. At 1, 4, 8 and 24 h, one group of three dishes was analyzed for cell-associated radioactivity. Medium was collected. Cells were washed with serum-free medium and treated with 0.5 mL 4 M urea solution in 0.2 M glycine buffer, pH 2.5, for 5 min on ice. The acid fraction was collected, and cells were washed with an additional 0.5 mL acid solution. The radioactivity in the acid wash fraction was considered as membrane-bound radioactivity. After addition of 0.5 mL 1 M NaOH, the cells were

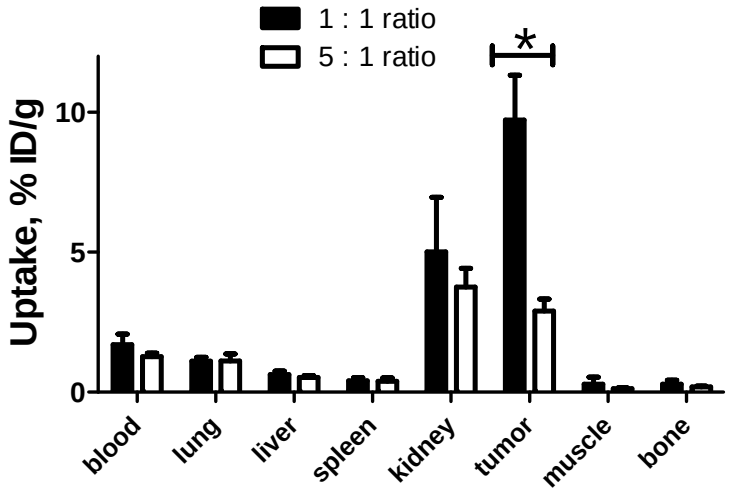
incubated at 37 °C for at least 0.5 h, and basic solution was collected. Dishes were washed with an additional 0.5 mL basic solution. The radioactivity in the alkaline fractions was considered internalized radioactivity.

In vivo studies:

Imaging using SPECT/CT. A mouse was injected with ^{111}In-tetrazine (5 µg/4 nmol) in 120 µL PBS, ca. 2.6 MBq) 4 h after the primary agent Z_{2395}-TCO injection (30 µg/4.1 nmol in 100 µL PBS). Immediately before imaging, the animals were sacrificed and the bladder was excised. SPECT/CT studies were performed in a Triumph Trimodality system (Gamma Medica, Inc.), a fully integrated SPECT/PET/CT hardware and software platform optimized for small animals in preclinical applications.



Supplemental Figure 2. Influence of Z_{2395}-TCO injected dose on biodistribution in mice bearing SKOV-3 xenografts. (**A**) Biodistribution of ^{125}I-Z_{2395}-TCO, 5 h after injection of 5 (0.67 nmol) and 30 µg (4.1 nmol) ^{125}I-Z_{2395}-TCO. (**B**) Biodistribution of ^{111}In-tetrazine (1 h after injection at 1:1 (1 ug/0.7 nmol and 5.2 µg/4.1 nmol) molar ratio, 5 h after injection of 5 and 30 µg Z_{2395}-TCO. Data represent the mean with one SD (n=5). Asterisk marks a significant difference ($p<0.05$).



Supplemental Figure 3. Biodistribution of ^{111}In-tetrazine 5.2 μg/4.1 nmol, tetrazine-to-Z_{2395}-TCO ratio 1:1 (**solid bars**) and 26 μg/20.5 nmol, tetrazine-to-Z_{2395}-TCO ratio 5:1 (**empty bars**) in BALB/C nu/nu mice bearing SKOV-3 xenografts at 1 h p.i. when mice were pre-injected with 30 μg (4.1 nmol) ^{125}I-Z_{2395}-TCO 4 h prior to ^{111}In-tetrazine injection. Data represent the mean with one SD (n=5). Asterisk marks a significant difference ($p<0.05$).

Supplemental Table 1. Biodistribution of ^{177}Lu-tetrazine in BALB/C nu/nu mice bearing HER2-expressing SKOV3 xenografts. Mice were pre-injected with 30μg/4.1 nmol Z_{2395}-TCO 4 h prior to ^{177}Lu-tetrazine (5.2 μg/4.1 nmol) injection.

Uptake*					
Time (h)	1	4	24	72	168
Blood	0.17±0.02	0.034±0.002	0.013±0.002	0.006±0.002	NM
Heart	0.11±0.05	0.041±0.007	0.03±0.02	0.017±0.009	NM
Lung	0.41±0.09	0.13±0.03	0.06±0.01	0.06±0.04	NM
Salivary glands	0.14±0.04	0.08±0.04	0.03±0.02	0.020±0.004	NM
Bladder	38±31	0.23±0.07	0.2±0.1	0.12±0.01	NM
Liver	0.23±0.09	0.108±.007	0.09±0.01	0.060±0.005	0.029±0.005
Spleen	0.10±0.01	0.06±0.01	0.05±0.01	0.035±0.004	NM
Pancreas	0.07±0.01	0.024±0.006	0.012±0.007	0.008±0.008	NM
Stomach	0.12±0.04	0.049±0.006	0.022±0.009	0.03±0.02	NM
Small intestine	0.09±0.01	0.049±0.009	0.03±0.02	0.011±0.006	NM
Large intestine	0.08±0.01	0.06±0.01	0.021±0.006	0.02±0.01	NM
Kidney	2.0±0.4	1.42±0.07	1.2±0.2	0.71±0.06	0.19±0.06
Tumor	3.7±1.2	2.47±0.64	1.6±0.2	0.9±0.3	0.22±0.02
Skin	0.29±0.02	0.10±0.01	0.07±0.01	0.041±0.002	NM
Muscle	0.08±0.02	0.021±0.006	0.013±0.003	0.009±0.002	NM
Bone	0.11±0.05	0.034±0.004	0.023±0.008	0.02±0.01	NM
Brain	0.020±0.017	0.005±0.001	0.005±0.002	0.002±0.002	NM
Body**	5±3	1.1±0.4	0.41±0.10	0.18±0.03	0.08±0.01

*The uptake is expressed as %ID/g and presented as an average value from 4 animals±SD.
** data of the body is presented as %IA per whole sample.
NM= not measurable (below detection limit).

Dosimetry. To make a preliminary dose estimate for different organs, the areas under curves (AUC) [% ID/g * h] were calculated. We assumed that relative uptake in organs and tissues can be scaled up from mice to humans; the tumors are bulky enough to absorb completely beta-particles emitted by ^{177}Lu; dose after 168 h pi can be neglected; and that a dose contribution from low-abundance gamma-radiation can be neglected. The organ uptake values from the biodistribution study were re-calculated to take into account the decay of ^{177}Lu. The values were time integrated from 0 to 168 h by the trapezoidal methods using GraphPad Prism software version 5.00 for Windows (GraphPad Software, San Diego, USA, www.graphpad.com). The results are presented in Supplemental Table 2.

Supplemental Table 2. Areas under curve for ^{177}Lu-tetrazine in BALB/C nu/nu mice bearing HER2-expressing SKOV3 xenografts. Data were obtained by trapezoidal integration of time-activity curves (non-decay-corrected) from 0 h to 168 h.

	AUC (%ID x h/g)	Ratio of AUC for tumour and AUC for organ
blood	1.4	102
liver	8.3	17
kidney	102	1.4
tumor	141	

Absorbed doses in humans were estimated using OLINDA/EXM 1.0. Uptake values were extrapolated from animal data according to the percent kg/g method, as recommended by OLINDA user's guide

$$(\%IA/organ)human = [(\%IA/g)animal \times (kgTBweight)animal \times (gorgan/(kgTBweight)human]$$

Organ time-activity curves were calculated using organ weights of the 73 kg reference adult male (ICRP publication 23). Residence times were calculated as the area under the curve of bi-exponential fits to the animal organ time-activity curves. Remainder of the body residence time was based on radioactivity in carcass. Red marrow activity concentrations were conservatively assumed to be equal to whole blood concentrations. Absorbed doses were estimated using OLINDA/EXM 1.0. The OLINDA output data are presented in Supplemental Table.3.

Supplemental Table 3. Calculated absorbed dose in (mGy/MBq) for pretargeted ^{177}Lu-tetrazine in humans using OLINDA/EXM 1.0.

	Organ absorbed doses (mGy/MBq)
Adrenals	0.005
Brain	0.000
Breasts	0.005
Gallbladder wall	0.005
Large intestine wall	0.006
Small intestine	0.006
Stomach wall	0.005
Heart wall	0.001
Kidneys	0.021
Liver	0.002
Lungs	0.002
Muscle	0.001
Pancreas	0.001

Red marrow	0.004
Osteogenic cells	0.016
Skin	0.005
Spleen	0.001
Testes	0.005
Thymus	0.005
Thyroid	0.005
Urinary bladder wall	0.008
Total body	0.005
Effective dose (mSv/MBq)	0.005

Supplemental References

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