LABELING AND STABILITY

^{188}Re was obtained as perrhenate by elution of a ^{188}W/^{188}Re generator (29.6 GBq) with 0.9% sodium chloride (Polatom). Elution efficiency was 90% when 4 mL of eluent were used.

For animal studies, the content of 1 freeze-dried kit was dissolved in100 μL of 1.25 M sodium acetate, pH 4.2, and added to 100 μg of freeze-dried $Z_{HER2:V2}$. To the reaction mixture, 14–100 μL of ^{188}Re-containing generator eluate were added under argon gas. An equivalent of 220 μg of ascorbic acid (2 mg/mL in 1.25 M sodium acetate buffer, pH 4.2) was added to the reaction vial. The mixture was incubated at 90°C for 60 min and then cooled at room temperature for 5 min. Thereafter, the total amount of ascorbic acid in the reaction vial was adjusted to 1 mg with a 5 mg/mL solution of ascorbic acid in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). ^{188}Re-$Z_{HER2:V2}$ was purified using disposable NAP-5 columns (GE Healthcare) preequilibrated and eluted with PBS containing 2% BSA. The final solution was diluted with an extra 100 μL of PBS containing 2% BSA and 500 μg of ascorbic acid to a final volume of 1 mL.

In up-scaling experiments, the content of a freeze-dried labeling kit vial (4 mg tin(II) chloride dihydrate, 400 μg of disodium EDTA, and 20 mg of sodium α-D-gluconate) was reconstituted in 400 μL of 1.25 M sodium acetate, pH 4.2, containing 5 mg/mL acid, and vortexed. The content of the vial was transferred to another vial containing 400 μg of freeze-dried $Z_{HER2:V2}$ and vortexed. To this mixture, 1 mL (∼7 GBq) of ^{188}Re-containing generator eluate was added, and the labeling mixture was vortexed carefully and incubated at 95°C for 60 min. Further processing was performed as described above.

For measurement of the labeling yield and radiochemical purity, samples of ^{188}Re-$Z_{HER2:V2}$ were analyzed using ITLC SG strips eluted with PBS. For measurement of reduced hydrolyzed rhenium colloid levels, a pyridine:acetic acid:water (5:3:1.5) mobile phase was

used. The ITLC analysis was cross-calibrated by SDS-PAGE (Novex 4%–12% Bis-Tris Gel, MES buffer, 200 V constant).

To estimate the shelf-life, the purity of ^{188}Re-$Z_{HER2:V2}$ was measured at 1, 2, and 4 h after purification using ITLC in duplicates.

IN VITRO EVALUATION

In vitro specificity testing was performed using SKOV-3 cells. Briefly, a solution of ^{188}Re-$Z_{HER2:V2}$ (0.015 ng of protein per dish, 2 nM) was added to 6 Petri dishes ($\sim 10^6$ cells in each). For blocking, an excess of nonlabeled recombinant $Z_{HER2:342}$ (7.4 μg) was added 10 min before ^{188}Re-$Z_{HER2:V2}$ to saturate the receptors. The cells were incubated during 1 h in a humidified incubator at 37°C. Thereafter, the medium was collected, the cells were detached by trypsin-EDTA solution, and the radioactivity in cells and medium was measured to calculate a percentage of cell-bound radioactivity.

For cellular processing, SKOV-3 cells (1×10^6 cells/dish) were incubated with a 2 nM solution of labeled Affibody at 4°C. After 1 h of incubation, the medium with the labeled compound was removed and the cells were washed 3 times with ice-cold serum-free medium. One milliliter of complete medium was added to each dish, and the cells were further incubated at 37°C in an atmosphere containing 5% CO_2. At designated time points (0, 1, 4, 8, and 24 h), a group of 3 dishes was removed from the incubator, the medium was collected, and the cells were washed 3 times with ice-cold serum-free medium. Thereafter, the cells were treated with 0.5 mL of 0.2 M glycine buffer, pH 2, containing 4 M urea, for 5 min on ice. The acidic solution was collected, and the cells were additionally washed with 0.5 mL of glycine buffer. The acidic fractions were pooled. The cells were then incubated with 0.5 mL of 1 M NaOH at 37°C for 30 min. The cell debris was collected, and the dishes were additionally washed with 0.5 mL of NaOH solution. The alkaline solutions were pooled. The

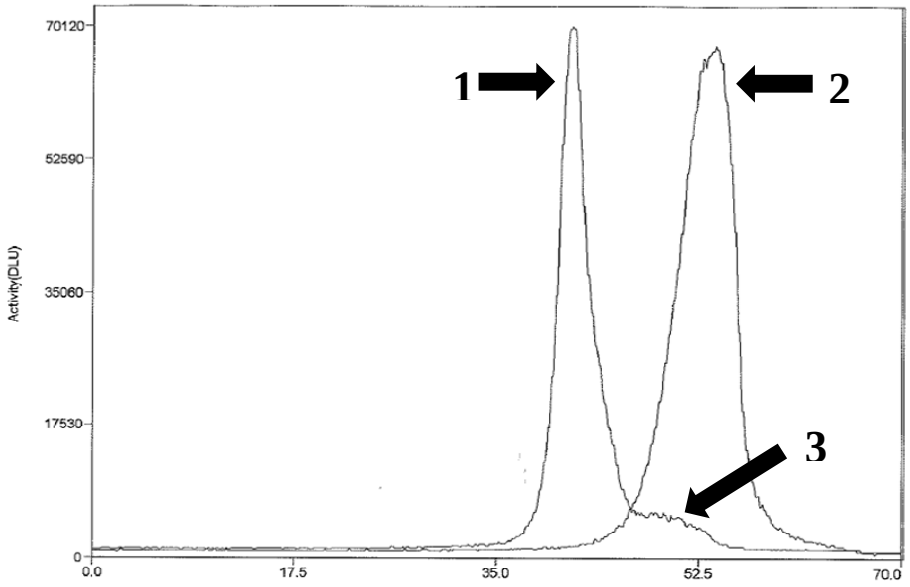
radioactivity was considered as membrane-bound in the acidic solution and as internalized in the alkaline fractions.

AFFINITY DETERMINATION USING LIGANDTRACER

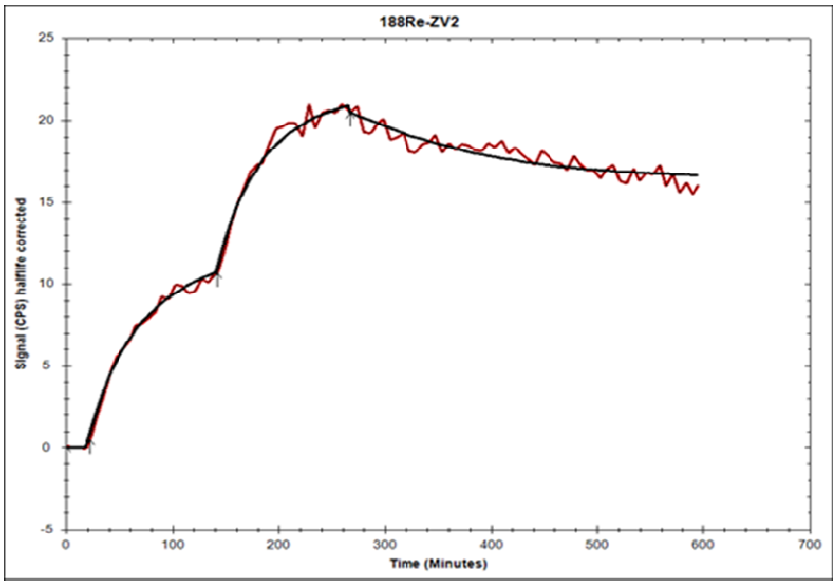
SKOV-3 cells were seeded on a local area of a cell culture dish (Nunclon, size 100620; NUNC A/S), as described previously (*1*). The binding of ^{188}Re-labeled anti-HER2 Affibody molecules to living cells was monitored in real time at 4°C using LigandTracer Yellow, using established methods described by Björkelund et al (*1*). In brief, the LigandTracer records the real-time kinetics of binding and dissociation of radiolabeled tracer in living cells. By using the TraceDrawer software, which allows the calculation of both association and dissociation rate, it becomes possible to determine the affinity of radiolabeled conjugate (*1*). To cover the concentration span needed for proper affinity estimation, 2 increasing concentrations of 49 and 98 pM (selected on the basis of previous K_D values obtained using Biacore) of each variant were added in each affinity assay (Supplemental Fig. 2).

REFERENCE

1. Björkelund H, Gedda L, Barta P, Malmqvist M, Andersson K. Gefitinib induces epidermal growth factor receptor dimers which alters the interaction characteristics with ^{125}I-EGF. *PLoS One.* 2011;6:e24739.



SUPPLEMENTAL FIGURE 1. SDS–PAGE analysis of the stability of ^{188}Re-$Z_{HER2:V2}$ in murine serum. 1. Incubated in murine serum at 37°C for 1 h; 2. $^{188}ReO_4^-$ used as a marker for low-molecular-weight compounds. Signal, measured as digital light units, is in proportion to radioactivity in given point of lane in SDS-PAGE gel. DLU = digital light units.



SUPPLEMENTAL FIGURE 2. LigandTracer sensor gram of interaction of ^{188}Re-$Z_{HER2:V2}$ with HER2-expressing SKOV-3 cells. Concentrations of ^{188}Re-$Z_{HER2:V2}$ were 49 pM and 98 pM.