Materials

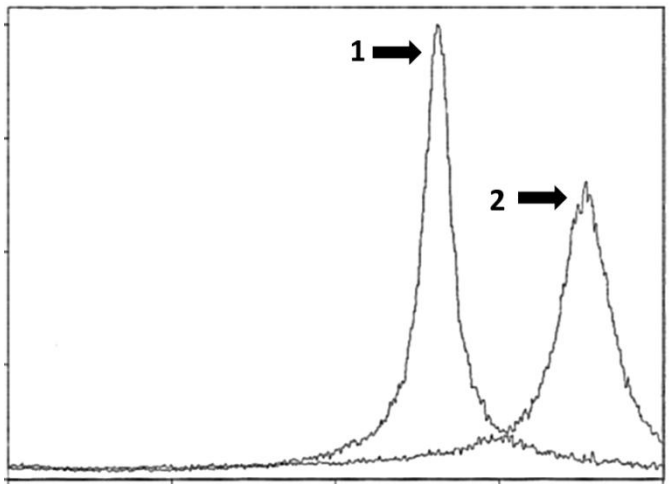
^{111}In-indium chloride (in 0.05 N HCl) was purchased from Covidien. Buffer to be used for labeling was purified from metal contamination using Chelex 100 resin (Bio-Rad Laboratories). Ketalar (ketamine, 50 mg/mL, Pfizer), Rompun (xylazin, 20 mg/mL, Bayer), and heparin (5000 IE/mL, Leo Pharma) were obtained commercially. Distribution of radioactivity along the thin layer chromatography strips and SDS-PAGE gels was measured on a Cyclone Storage Phosphor System and analyzed using the OptiQuantTM image analysis software (Perkin Elmer). The radioactivity was measured using an automated gamma-counter with 3-inch NaI(Tl) detector (1480 WIZARD, Wallac).

Analysis of radiolabeled Affibody molecules

Labeling yield and radiochemical purity was determined by radio-ITLC (150-771 DARK GREEN strips, Biodex Medical Systems), eluted with 0.2 M citric acid. In this system, labeled Affibody molecules remain at the origin while free ^{111}In migrates with the solvent front. Radio-ITLC was cross-validated by SDS-PAGE and size-exclusion chromatography.

For radioSDS-PAGE, a sample of ^{111}In-DOTA-Z09591 was incubated with a four-fold bigger volume of NuPAGE LDS Sample Buffer (Life Technologies Corporation) at 70 ^{o}C for 10 min. A control sample containing ^{111}In-indium acetate was treated in the same way.

The samples were loaded in different wells of NuPAGE Novex® 4-12% Bis-Tris Gel (Life Technologies Corporation) and analyzed at 200 V constant. The distribution of radioactivity on the gel is shown in Supplemental Figure 1.

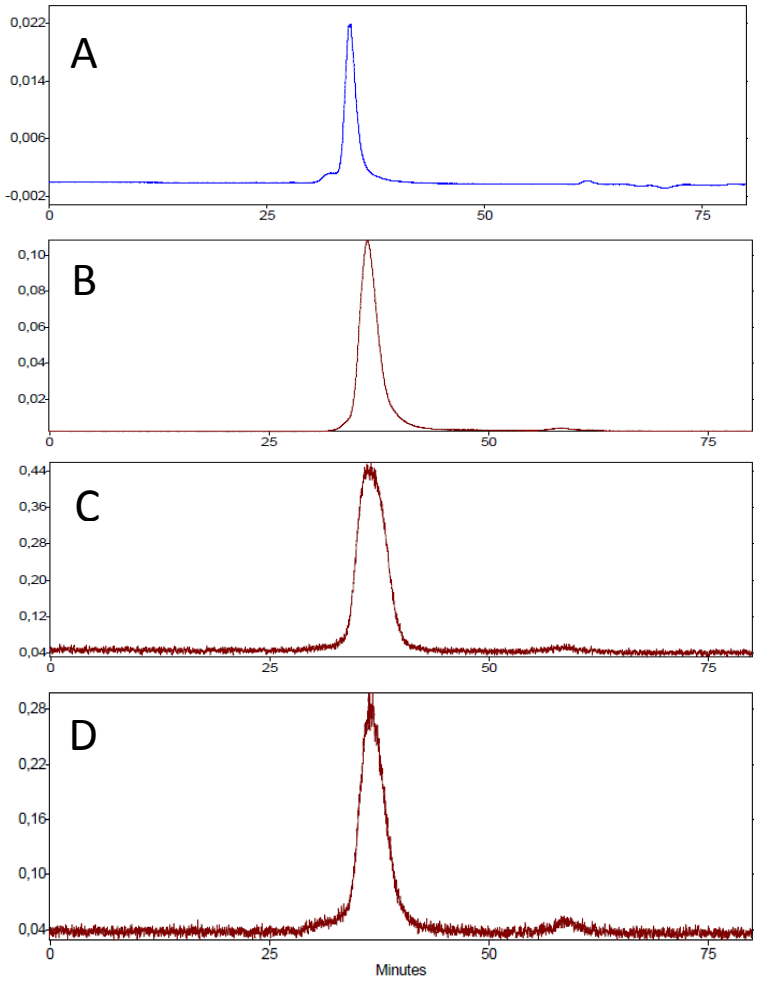


Supplemental Figure 1. SDS PAGE analysis of ^{111}In-DOTA-Z09591. Distribution of radioactivity along lanes was visualized and quantified using Cyclone Storage Phosphor System. Lane 1. ^{111}In-DOTA-Z09591.The migration path of the radioactivity peak corresponds to migration of DOTA-Z09591. Lane 2. ^{111}In-indium acetate was used as a marker for low molecular weight compounds

Stability in human serum

To evaluate stability in human serum, 0.15 µg of ^{111}In-DOTA-Z09591 was mixed with 300 µL of human serum to mimic concentration of ^{111}In-DOTA-Z09591 in blood immediately after injection. The samples were incubated at 37°C. Size-exclusion chromatography of freshly labeled samples, as well as the mixture after 1h and 4 h incubation was performed. The experiment was repeated twice.

High-performance liquid chromatography (HPLC) analysis was performed using a system from Beckman consisting of a 126 pump, a 166 UV detector and a radiation detector (Bioscan) coupled in series. UV detection was performed at 220 nm. Data acquisition and handling were performed using the Beckman System Gold Nouveau Chromatography Software Package. The Superdex Peptide 10/300GL (GE Healthcare) column was eluted with 0.05 M phosphate buffer, pH 7.0 containing 0.15 M NaCl at 0.3 mL/min. The column was calibrated using monomeric and dimeric forms of Affibody molecules.



Supplemental Figure 2. Size-exclusion chromatograms of ^{111}In-DOTA-Z09591. UV (A) and radioactivity (B) of freshly labeled ^{111}In-DOTA-Z0959. Panels C and D show radiochromatograms after 1- and 4-h incubation in human serum.

In the freshly labeled samples, 99.6±0% of the radioactivity (average ±maximum error) were associated with Affibody molecules. After 1- and 4-h incubation, the Affibody-associated fraction of radioactivity was 99.2±0.6% and 97.3±1.1%, respectively (Supplemental Figure 2).

In vitro cell binding and processing of ^{111}In-labeled Affibody molecules

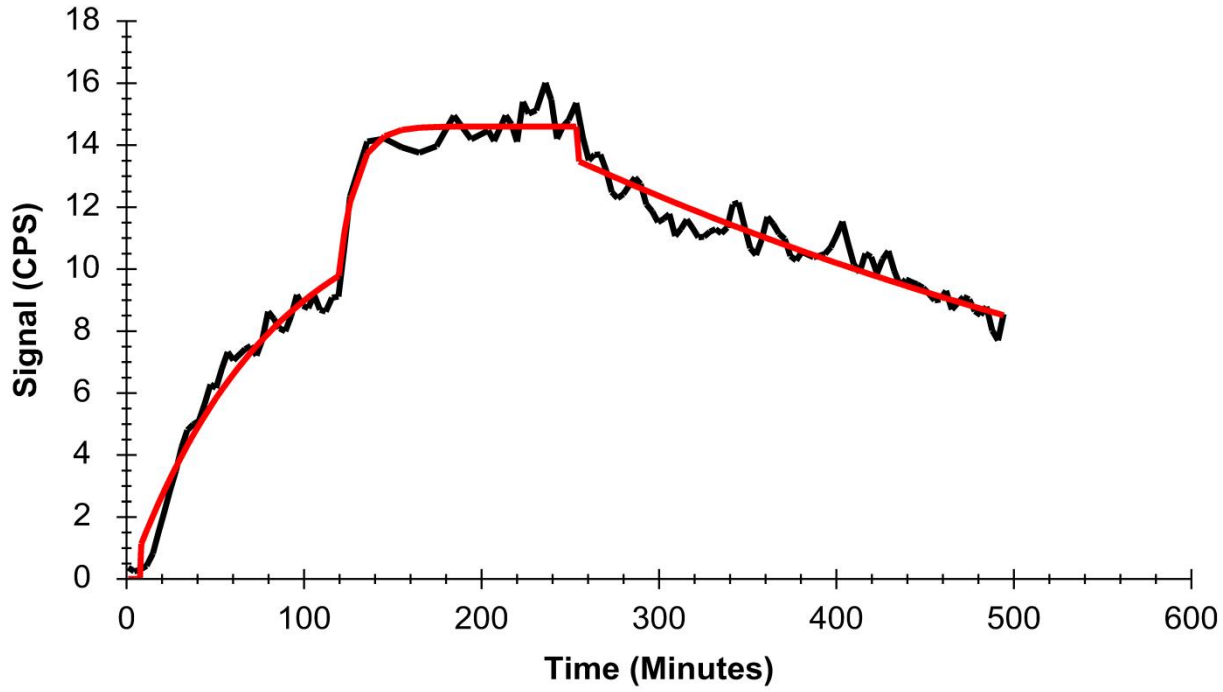
PDGFRβ-expressing U-87 MG glioma cell line (American Type Culture Collection, ATCC) was used for binding specificity and cellular processing studies. For culturing, DMEM media (Lonza, Belgium) supplemented with 10% fetal bovine serum and penicillin (100 U/mL)–streptomycin (100 μg/mL) (both from Biochrom AG) was used for culturing. For binding and cellular processing experiments, the cells were seeded one day before the experiment in Petri dishes (diameter 3.5 cm). At the day of the experiment there were approximately 10^6 cells/dish with 80% confluence. For the LigandTracer experiments, 4×10^6 cells were seeded in a defined area of the dish one day before the experiment.

An in vitro specificity test was performed according to the method described earlier (*28*). A solution of ^{111}In-DOTA-Z09591 was added to six cell-containing (ca. 10^6 cells) Petri dishes to obtain a final concentration of 0.5 nM and the total volume of 1 mL. A 200-fold excess of non-labeled conjugate was added to three dishes 15 min before radiolabeled conjugates to saturate the receptors. The cells were incubated during four hours in a humidified incubator at 37°C. Thereafter, the media was collected, the cells were washed six times with 1mL of ice-cold medium and detached by a trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA, Biochrom AG), and the radioactivity in cells and media was measured, to determine cell-bound radioactivity fraction.

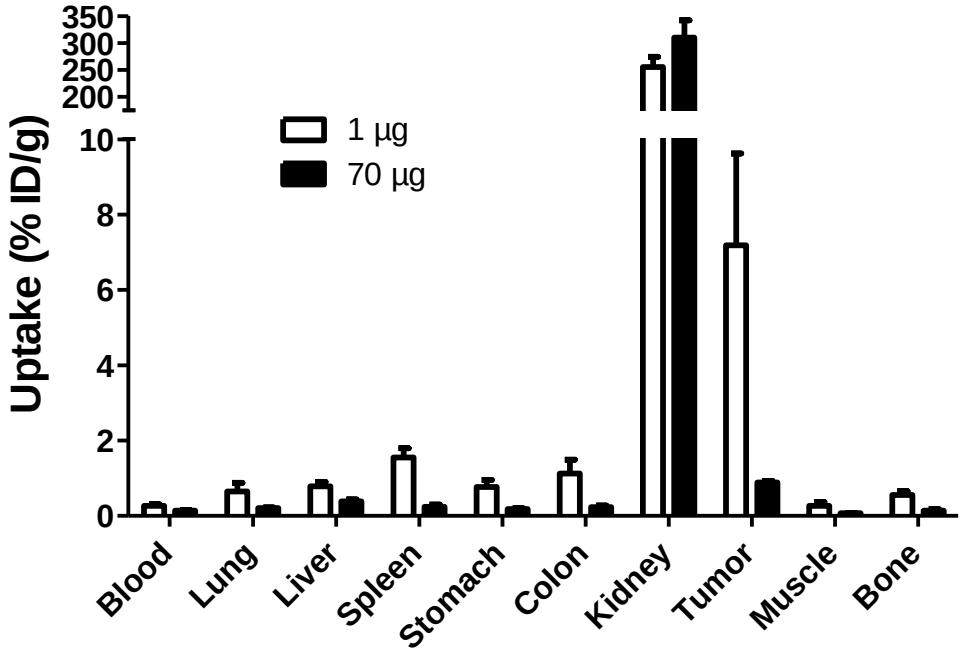
The rate of internalization of ^{111}In-DOTA-Z09591 by U-87 MG cells was measured according to the method described earlier (*31*). Briefly, the cells (approx. 10^6 per dish) were incubated with the labeled compound (0.5 nM, volume 1 mL) at 37°C. At pre-determined time points (1, 4, 8 and 24 h after incubation start), 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, treatment with 0.5 mL of 1 M NaOH at 37°C for 0.5 h was performed. Dishes were additionally washed with 0.5 mL NaOH solution followed by 1 mL PBS, and the alkaline fractions were pooled. The percentage of internalized radioactivity was calculated for each time point. To assess if the internalization is energy dependent, one set of dishes was incubated at 4 ^{o}C for 4 h, and the internalized radioactivity was determined as described above.

Affinity determination using LigandTracer

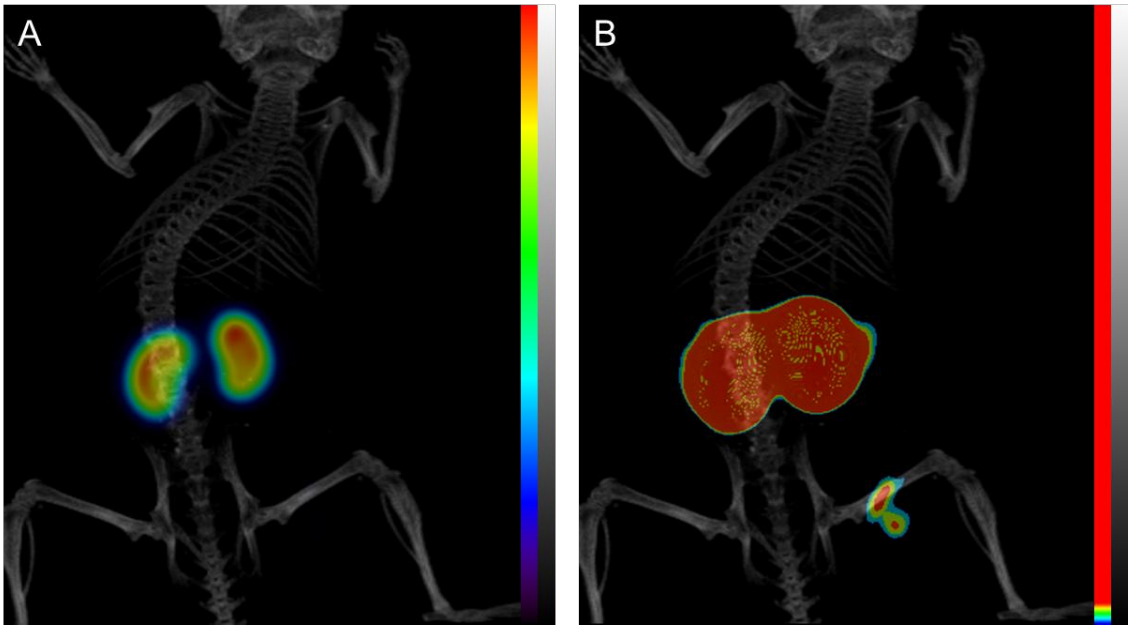
Briefly, a Petri dish (Nunclon, diameter 100 mm, containing 3mL culture medium) with U-87 MG cells was attached to the rotating table of the instrument. After 5-min baseline run, ^{111}In-DOTA-Z09591 was added to the medium to obtain a ligand concentration of 0.5 nM, and the uptake curve was recorded for 120 min. Thereafter, the ligand concentration was increased to 5 nM, and the uptake curve was recorded for another 120 min. Then the ^{111}In-DOTA-Z09591-containing medium was aspirated, 3 mL of fresh medium was added and the dissociation curve was measured. Interaction analysis and calculation of equilibrium dissociation constant (K_D) was performed with TracerDrawer software (Ridgeview Instruments AB).The measurements were repeated six times at room temperature. Two additional measurements were performed in a cold room at 4^{o}C to exclude influence of ligand internalization.



Supplemental Figure 3. Sensorgram of LigandTracer measurement of ^{111}In-MMA-DOTA-Z9591 binding to U-87 MG cells at room temperature (black) and curve fitting results (red). After 5-min base line measurement, ^{111}In-MMA-DOTA-Z9591was added to obtain a concentration of 0.5 nM and the binding kinetics was measured for 120 min. Thereafter, the concentration of ^{111}In-MMA-DOTA-Z9591 was increased to 5 nM, and the binding kinetics was measured for another 120 min. The medium was replaced, and the dissociation kinetics was measured. Association (k_a) and dissociation (k_d) rate constants were determined by curve fitting using TracerDrawer software , and dissociation constant (K_D) was determined as $K_D = k_a / k_d$.



Supplemental Figure 4. Targeting specificity of ^{111}In-DOTA-Z09591 in U-87 MG xenografts in vivo at 2 h p.i. Results are presented as percentage of injected dose per gram of tissue (% ID/g). Uptake in all organs and tissues, except kidneys, after injection of 70 µg ^{111}In- DOTA-Z09591 was significantly lower.



Supplemental Figure 5. Imaging of PDFGRβ expression in U-87 MG xenograft in BALB/C nu/nu mouse using microSPECT/CT (Maximum Intensity Projections). Image was acquired 3 h after injection. A. SPECT scale 0.02-1.00. B. SPECT scale 0.02-0.04. The CT scale bar display 400-2000 HU in both images.