Supplementary data

Radionuclide Imaging of VEGFR2 in Glioma Vasculature using Biparatopic Affibody Conjugate: Proof-of-Principle in Murine Model

Mitran B, Güler R, Roche FP, Lindström E, Selvaraju RK, Fleetwood F, Rinne SS, Claesson-Welsh L, Tolmachev V, Ståhl S, Orlova A, Löfblom J

Production, characterization

The gene encoding HEHEHE-Z_{VEGFR2}-Bp_2-Cys in the pET26b (+) vector was designed and ordered from Biobasic Inc. (Amherst, New York, USA). To facilitate purification through immobilized metal ion affinity (IMAC) and alter the biodistribution profile, a HEHEHE-sequence was introduced at the N-terminus. A C-terminal cysteine was introduced to enable site specific labeling of maleimide-NODAGA (CheMatech, Dijon, France). The plasmid was transformed to E. coli BL21 Star (DE3) cells (Invitrogen, Carlsbad, Ca) via heat shock. One colony was inoculated to Tryptic Soy Broth media supplemented with yeast extract (TSB + Y; Merck, Darmstadt, Germany) and 30 μg/mL kanamycin (Sigma-Aldrich Company Ltd, Dorset, UK) and cultivated for 18 hours at 37°C and 150 rpm. 150 μL overnight culture was re-inoculated to 100 mL TSB + Y supplemented with 30 μg/mL kanamycin and incubated at 37°C and 150 rpm. When OD600 of 0.8-1 was reached, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the culture was incubated at 25°C at 150 rpm for 16 hours. Cultures were harvested by centrifugation (8 minutes at 4°C and 4000 rpm). The pellet was re-suspended in 10 mL equilibration buffer and lysed by French press cell disruption. Lysate was heat-treated at 90°C for 10 minutes followed by centrifugation (20 min at 4°C 8000 rpm) and filtered through 0.45-μm filter prior to IMAC purification using Ni-sepharose resin and ÄKTA system (GE Healthcare). Eluted protein was buffer-exchanged to 20 mM NH_4Ac using PD-10 size exclusion column (GE Healthcare) according to manufacturer's recommendations.

Purity and size of purified protein was analyzed by SDS-PAGE and liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) on a 6520 Accurate Q-TOF LC/MS.

A maleimide derivative of NODAGA was site-specifically conjugated onto the unique C-terminal cysteine of the protein. 1.5 mg/mL HEHEHE-Z_{VEGFR2}-Bp_2-Cys was incubated with 20 mM dithiothreitol for 30 minutes at 40°C. The reduced protein was buffer-exchanged to 20

mM NH_4Ac pH 5.5 using a PD-10 column according to manufacturer's recommendations. Maleimide-NODAGA was added in 3-fold molar excess and incubated for 1 hour at 40°C. Conjugated protein was purified by semi-preparative RP-HPLC (Agilent Technologies). Correct labeling was confirmed by LC/ESI-MS. Purity of conjugated protein was determined by analytical RP-HPLC (Agilent Technologies).

Secondary structure and thermal stability of HEHEHE-Z_{VEGFR2}-Bp_2-Maleimide-NODAGA was analyzed using a JASCO J-810 spectropolarimeter (JASCO, Tokyo, Japan). Protein secondary structure and refolding capability was analyzed by measuring ellipticity between 250-195 nm at 25°C before and after applying a temperature gradient. Melting temperature was analyzed by applying a temperature gradient between 25-90°C and fitting the ellipticity at 221 nm to a sigmoidal equation.

Biosensor assay on HEHEHE-Z_{VEGFR2}-Bp_2-NODAGA interaction with murine VEGFR2

Binding analysis experiments were performed using a surface plasmon resonance (SPR)-based biosensor assay on a Biacore 3000 instrument (GE Healthcare). Phosphate-buffered saline supplemented with 0.05% Tween 20 was used as running buffer and 10 mM HCl was used for regeneration. Monomeric murine His-VEGFR2 (Sino Biological Inc. Beijing, China) was immobilized by amine coupling on a CM5 sensor chip surface (GE Healthcare). Conjugate was injected over the chip surface at 500, 100 and 10 nM concentrations, respectively. ABD-containing conjugate Z_{VEGFR2_BP2}-ABD was included for comparison. The experiment was performed in duplicates.

Flow cytometric analysis

Cells (MS1, PC3, and gl261) were harvested by trypsinization, centrifuged at 210g for 5 min, pellets were washed with PBSP (PBS + 0.1% Pluronic) and resuspended in 100 nM of Z_{VEGFR2}-Bp_2 or positive control antibody (Ramucirumab or DC101). Cells were pelleted by centrifugation at 210g for 5 min and re-suspended in 150 nM HSA (human serum albumin; Sigma-Aldrich) conjugated to Alexa Fluor 647 (Invitrogen) or 2 μg/ml Alexa Fluor 647 Goat Anti-Human IgG (H + L) antibody (Thermofisher) for Ramucirumab or 2 μg/ml Alexa Fluor 647 Goat Anti-Rat IgG (H + L) antibody (Thermofisher) for DC101. After incubation for 30 min on ice in the dark, cells were pelleted by centrifugation and washed and re-suspended in PBSP. Cell binding of the Affibody construct and positive control antibodies were detected by

flow-cytometric analysis using a Gallios flow cytometer (Beckman Coulter, Indianapolis, IN, USA).

Radiolabeling, identity, stability

All buffers used for labeling were purified from metal contamination using Chelex 100 resin (Bio-Rad Laboratories). [^{111}In]$InCl_3$ was purchased from Mallinckrodt. All chemicals were from Sigma-Aldrich.

The radiochemical yield of the conjugate in the crude mixture and radiochemical purity of the product were determined by instant thin layer chromatography (radio-ITLC, 150-771 DARK GREEN, Tec-Control Chromatography strips, Biodex Medical Systems) eluted with citric acid (0.2 M, pH 2.0) and verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using polyacrylamide NuPAGE 10 % Bis–Tris gel (Life Technologies) and MES buffer (950 mL MQ H2O and 50 mL Nu-PAGE buffer) as running buffer. Radio-ITLC and SDS-PAGE gels were measured on a Cyclone Storage Phosphor System on radioactivity distribution and analyzed with the OptiQuant image analysis software (both from Perkin Elmer Sweden AB). Purification of radiolabeled conjugate was performed using size exclusion NAP5-columns (Amersham Biosciences). The radiolabeled compound was diluted by addition of PBS (pH 7.3) and stored frozen.

In vitro characterization: binding specificity, cellular processing, affinity

The pancreatic islet endothelial cells MS1 (Mus EC MS1 mouse endothelial) were cultured in DMEM growth media (Dulbecco's Modified Eagle Medium) with a low L-glutamine level, supplemented with 10 % FBS (Fetal bovine serum, Sigma-Aldrich) and 1 % PEST (Penicillin 100 UI/mL, streptomycin 100 µg/mL, all from Biochrom AG) in a 37°C incubator with 5 % CO_2. Murine glioblastoma gl261 cells were grown in DMEM GlutaMAXTM medium (ThermoScientific) supplemented with 10% FCS. PC-3 human prostate cancer cells (ATCC) were cultured in RPMI media complemented with 10% fetal calf serum, 2 mM L-glutamine and PEST (penicillin 100 IU/ml). Harvesting of cells was performed by treatment with 0.25 % trypsin, 0.02 % EDTA solution (Biochrom AG). Change of media was done twice a week, and the cells were seeded two days prior to experiments. Experiments were done in triplicate.

In vitro specificity test

[^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (500 µL, 1.2 nM) was added to a set of dishes. Additional sets were pre-incubated with 180 nM of unlabeled compound (Z_{VEGFR2}-Bp_2), or VEGFA or

anti-mVEGFR2 antibody DC101 for 10 min at room temperature. Dishes were incubated for 1 h at 37°C. After cell detachment with trypsin, measurements of cell-associated activity were performed against standards in an automated gamma well counter (3-inch NaI(Tl) detector, 2480 Wizard^2, PerkinElmer).

Cellular processing

[^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (1000 µL, 0.6 nM) was added to all dishes and incubated at 37°C (5 % CO_2). At 1, 2, 4, 8, and 24 h, one set of cell dishes was incubated on ice for 5 min with 2 mL acid buffer (4 M urea in 0.2 M glycine buffer, pH 2.0). The acid wash solution was collected in vials and the radioactivity of the samples was considered to represent the membrane bound fraction. A base solution (NaOH, 1 M, 2 mL) was added to the cells and incubated for 30 min at 37°C. The base solution was collected and the radioactivity of these samples was considered as the internalized fraction. Activity in samples was measured as described above.

In vitro real time study of affibody-receptor interaction

Association to and dissociation of radiolabeled affibody conjugate from VEGFR2 were investigated by quantitative real time binding experiments using LigandTracer Yellow Instrument (Ridgeview Instruments AB). Briefly, a Petri dish (Nunclon, diameter 100 mm, containing 3 mL culture medium) with living MS1 cells covering a sector of the dish was attached to the rotating table of the instrument. After an initial 15 min baseline run, ligand/receptor interaction was studied at increasing concentrations and the uptake curves were recorded for 120 min for each concentration. Then, [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2-containing medium was removed, 3 mL of fresh medium were added, and the dissociation was followed overnight. Measurements were done in duplicate for two added concentrations (1 and 5 nM) and for three added concentrations (0.33, 1 and 5 nM). The resulting data were analyzed in TraceDrawer™ (Ridgeview Instruments AB). Values of association- (k_a) and dissociation (k_d) constants and equilibrium dissociation constant (K_D) were calculated.

Influence of treatment with HSP90 inhibitor 17-DMAG on binding of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 to MS1 endothelial cells in vitro

To assess if MS1 cells respond to treatment with 17-DMAG (HSP90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin, Alvespimycin), MS1 cells were seeded in cell culture flasks (50 000 cells/cm^2) and allowed to attach overnight. Thereafter, cells were incubated with 25 nM 17-DMAG continuously during 14 days.

To evaluate if treatment with 17-DMAG causes changes in [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 binding, the MS1cells were seeded in cell culture dishes (500 000 cell per dish). Cells were kept overnight. To a set of three dishes, 17-DMAG was added to a concentration of 25 nM, and cells were incubated for two hours. Thereafter, the medium with 17-DMAG was withdrawn, and fresh medium was added. [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (500 µL, 1.2 nM) was added to both treated cells and to untreated control cells and cells were incubated for 1 h. After incubation, cells were detached by trypsin treatment, and the cell-associated radioactivity was measured.

The experimental in vitro therapy was performed as described earlier (Anderson 2014). Cells were seeded in cell culture flacks as described above and incubated in the culture medium containing 25 M 17-DMAG for 48 h, while control cells were incubated in the culture medium only. Thereafter, the media containing 17-DMAG was withdrawn, cells were detached, and counted.

Andersson J, Rosestedt M, Asplund V, Yavari N, Orlova A. In vitro modeling of HER2-targeting therapy in disseminated prostate cancer. Int J Oncol. 2014 Nov;45(5):2153-8.

Small animal experiments

The mice were sacrificed by intraperitoneal injection of a Ketalar-Rompun solution (10 mg/mL Ketalar and 1 mg/mL Rompun; 20 µL of solution per gram of body weight). The blood, lung, liver, spleen, pancreas, kidneys, muscle, skin, bone, tumor, large intestine, brain, and eyes were collected for radioactivity measurements. The gastrointestinal tract with content and the rest of carcass were also collected. Activity of samples was measured together with injection standard.

In vivo stability

Female NMRI mice (n=2) were injected with [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 intravenously and sacrificed 15 min pi. Blood was collected with heparinized syringe and centrifuged. Blood plasma was analyzed using ITLC (for detection of free indium) and SDS PAGE (for detection of fragmentation).

Two groups of NMRI mice (n=4) were intravenously injected with [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (4 µg, 30 kBq, 100 µl) or [^{111}In]In-acetate in PBS (30 kBq, 100 µl). Mice were

sacrificed 2 h pi and activity was measured in blood, liver, spleen, kidneys, muscle, bones, GI tract with content and remain carcass.

Tumor targeting and specificity

MS-1 model:

1. Animals were iv injected with 1, 4, or 20 µg of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (30 kBq, 100 µl) per animal, sacrificed 2 h pi, biodistribution.
2. Animals were iv injected with 4 µg of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (30 kBq, 100 µl) per animal, sacrificed 2, 6 or 24 h pi, biodistribution.
3. Animals (n=2) were iv injected with 4 µg of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (30 kBq, 100 µl) per animal, sacrificed 2 h pi, SPECT/CT acquisition, tissue samples for ARG.

PC-3 model:

Animals were iv injected with 4 µg of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (30 kBq, 100 µl) per animal, sacrificed 2 h pi, biodistribution.

Gl261 model:

Intracerebral glioma studies were carried out in female C57Bl/6 mice aged 8-10 weeks. Briefly, an incision was made in the scalp and a small hole drilled at coordinates corresponding to the caudate nucleus (+1mm anteroposterior, -2.3 medial-lateral from bregma). GL261 cell inoculation was carried out by injection of 7,500 GL261 cells, in a volume of 0.5ul, into the caudate nucleus.

In vivo MRI scans were performed on a 3T nanoScan PET/MRI instrument (Mediso Medical Imaging Systems Ltd., Hungary) every third day starting from day 12 after tumor inoculation under anesthesia (0,06% sevoflurane; 50%/50% medical oxygen:air at 5 bars). Whole body multislice T2-weighted Fast spin echo coronal scans were performed at the following parameters: echo time = 71.84; repetition time = 2 s; matrix = 512x256; 25 slices; slice thickness = 1 mm; total imaging time = 13 min.

1. Animals with 2 mm tumors in diameter were iv injected with 0.5, 1, 2, 4, or 40 µg of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (30 kBq, 100 µl) per animal, sacrificed 2 h pi, SPECT/CT acquisition, brain sections for ARG (one animal per dose).

2. Animals with 4 mm tumors in diameter were iv injected with 4 or 40 µg of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 or 4 µg of [^{111}In]In-NODAGA-Z_{taq}-Z_{taq} (30 kBq, 100 µl) per animal, sacrificed 2 h pi, SPECT/CT acquisition, brain sections for ARG and immunostaining (three animals per data point).

SPECT/CT imaging

Whole body SPECT/CT scans were carried out using nanoScan SPECT/CT (Mediso Medical Imaging Systems Ltd., Hungary) with the following parameters: CT-energy peak of 50 keV, 670 µA, 480 projections, 2.29 min acquisition time; SPECT-^{111}In energy peaks of 245.4 keV and 171.3 keV, window width of 20 %, matrix of 256 × 256, 1 h acquisition time. CT raw files were reconstructed in real time using Nucline 2.03 Software (Mediso Medical Imaging Systems, Hungary). SPECT raw data were reconstructed using Tera-Tomo™ 3D SPECT reconstruction technology.

In vivo co-localization of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 uptake with glioma

Whole extracted brains were freshly isolated and the tumor excised using a Zeiss Discovery.V12 stereomicroscope. For sectioning, brains were snap-frozen in dry ice-cooled isopentane and 50 µm sections cut through the brain using a Microm HM500 cryostat (ThermoScientific).

macroAutoradiography

On autoradiography images, the region of interest (ROI) corresponding to tumor tissue on hematoxylin treated slides was delineated. The ROI was copied to an area of the brain image, which corresponded to an intact brain. The signal intensities in both ROIs were compared. Following overnight exposure, sections were processed for H&E staining. Haemotoxylin (Mayers', Histolab) and Eosin (0.2%, Histolab) staining was carried out followed by dehydration and mounting in Pertex® (Histolab).

VEGFR2 and CD31 Immunofluorescence

Crysosections were thawed, incubated in PBS for 10 minutes and blocked in blocking buffer (3% bovine serum albumin, 5% normal donkey serum, 0.25% Triton-X in PBS) for 4 hours at RT. VEGFR2 was probed with anti-VEGFR2 antibody (2479S, Cell Signalling) at a 1:200 dilution and CD31 - with anti-CD31 antibody (Rat Anti-Mouse CD31 (PECAM-1), BD Pharmingen) at a 1:50 dilution overnight at 4 °C. Sections were washed four times in PBS

with 0.5% Tween-20 (PBS-T) followed by incubation of Alexa Fluor®-labelled anti-rabbit antibody (1:500 dilution) in 0.5x blocking buffer. Sectioned were washed, stained with DAPI and mounted with Flouromount-G (SouthernBiotech, USA). Images were acquired using a LSM 700 Confocal Microscope (Zeiss, Germany).

Suppl. Table 1 Labeling and stability of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2

	% activity associated with conjugate	
Radiochemical yield	96.9±1.3	
Radiochemical purity (after NAP5)	100	
	1 h	**4 h**
Incubation in in the presence of excess EDTA *	99.3±0.9	96.9±1.2
Incubation in PBS	98.8±0.9	98.3±1.0

* Stability was checked after 1 h incubation at room temperature in the presence of 1000x molar excess of EDTA. Data are presented as average ± standard deviation.

Suppl. Table 2 Comparative biodistribution of different injected protein doses (1, 4 and 20 µg/animal) of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 in MS1 xenografted BALB/C nu/nu mice at 2 h pi. The organ uptake values are expressed as a percentage of injected dose per gram of tissue weight (% ID/g). Tumor-to-normal-tissue ratios of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 in MS1 xenografted BALB/C nu/nu mice at 2 h pi (1, 4, 20 µg).

Organ	Uptake, % ID/g			T/O		
	1µg	4µg	20µg	1µg	4µg	20µg
Blood	0.39±0.05	0.5±0.3	0.12±0.02 [b]	8.0±0.8	11±7	7±2
Lung	4.7±0.7	3±2	0.64±0.09 [b, d]	0.7±0.2	1±1	1.3±0.3
Liver	56±2	51±8	47.6±0.7 [b]	0.06±0.01	0.07±0.04	0.017±0.003 [b]
Spleen	20±5	16±3	10±1 [b, d]	0.17±0.06	0.2±0.1	0.08±0.03
Pancreas	1.0±0.2 [a]	0.6±0.2	0.31±0.02 [b, d]	3±1	6±3	2.6±0.6
Large intestine	0.9±0.2	0.8±0.2	0.57±0.05	3.6±0.6	5±2	1.4±0.3 [b, d]
Kidneys	87±12	97±18	126±12 [c, e]	0.04±0.01	0.04±0.02	0.0064±0.0009 [b, d]
Tumor	3.1±0.7	4±2	0.8±0.2 [b, d]	-	-	-
Skin	0.82±0.06	1.0±0.5	0.50±0.06 [b]	4±1	4±2	1.6±0.3 [b]
Muscle	0.4±0.1	0.3±0.1	0.17±0.05 [b]	9±4	15±7	5±2
Bone	2.5±1.6	3±1	2.8±0.3	2±2	2±1	0.28±0.04
Brain	0.074±0.004	0.1±0.2	0.03±0.01 [b]	43±10	78±51	33±11
Eyes	0.38±0.05	0.3±0.2	0.22±0.04 [b]	8±2	15±10	3.8±0.9 [b]
GI tract*	0.71±0.06	2.8±3.6	0.8±0.2	-	-	-
Carcass*	11.3±0.8	7.2±3.8	6.2±0.3	-	-	-

* values for GI (gastrointestinal) tract and carcass are given as %ID per whole sample.

Data were assessed by an unpaired, two-tailed t-test in order to determine significant differences ($p<0.05$).

[a]Higher than 4 µg; [b]lower than 1 µg; [c]higher than 1 µg; [d]lower than 4 µg; [e]higher than 4 µg.

Suppl. Table 3 Biodistribution of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (4 µg) in MS1 xenografted BALB/C nu/nu mice at 2, 6 and 24 h pi. The organ uptake values are expressed as a percentage of injected dose per gram of tissue weight (% ID/g). Tumor-to-normal-tissue ratios of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 in MS1 xenografted BALB/C nu/nu mice at 2, 6 and 24 h pi (4 µg).

Organ	**Uptake, % ID/g**			**T/O**		
	2 h p.i.	**6 h p.i.**	**24 h p.i.**	**2 h p.i.**	**6 h p.i.**	**24 h p.i.**
Blood	0.5±0.3	0.21±0.03 [a]	0.13±0.02	11±7	9.0±0.6 [b]	14.7±0.9
Lung	3±2	2.0±0.1	1.8±0.3	1±1	0.9±0.1	1.04±0.06
Liver	51±8 [a]	36±2 [a]	30±4	0.07±0.04	0.05±0.01	0.06±0.01
Spleen	16±3 [a]	11.3±1.0	12±1	0.2±0.1	0.17±0.02	0.16±0.04
Pancreas	0.6±0.2	0.79±0.09	0.80±0.10	6±3	2.4±0.6	2.3±0.4
Large intestine	0.8±0.2 [a]	1.1±0.1	0.9±0.1	5±2 [a]	1.7±0.4	2.0±0.4
Kidneys	97±18	109±8	116±7	0.04±0.02	0.017±0.002	0.016±0.004
Tumor	4±2	1.9±0.2	1.9±0.3	-	-	-
Skin	1.0±0.5	0.99±0.08	0.90±0.05	4±2	1.9±0.3	2.1±0.4
Muscle	0.3±0.1	0.40±0.02	0.38±0.07	15±7 [a]	4.7±0.4	4.9±0.9
Bone	3±1	2.6±0.2 [b]	3.3±0.5	2±1	0.73±0.09	0.6±0.2
Brain	0.1±0.2	0.070±0.006	0.071±0.005	78±51	27±2	26±4
Eyes	0.3±0.2	0.45±0.09	0.53±0.07	15±10	4.4±0.9	3.6±0.7
GI tract*	2.8±3.6	2.8±1.5	2.7±0.9	-	-	-
Carcass*	7.2±3.8	9.9±0.5	5±4	-	-	-

* values for GI (gastrointestinal) tract and carcass are given as %ID per whole sample.

Data were assessed by an unpaired, two-tailed t-test in order to determine significant differences ($p<0.05$).

[a]Value is significantly higher than next time point; [b]lower than next time point;

Suppl. Table 4 Biodistribution of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (4 µg) in PC-3 xenografted BALB/C nu/nu mice at 2 h pi. The organ uptake values are expressed as a percentage of injected dose per gram of tissue weight (% ID/g). Tumor-to-normal-tissue ratios of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 in PC-3 xenografted BALB/C nu/nu mice at 2 h pi (4 µg).

Organ	**PC3**	
	Uptake, % ID/g	**T/O**
Blood	0.5±0.1	3±1
Lung	4±2	0.4±0.1
Liver	58±8	0.021±0.006
Spleen	19±3	0.06±0.02
Pancreas	0.77±0.06	1.6±0.5
Large intestine	1.2±0.2	1.0±0.2
Kidneys	130±12	0.009±0.003
Tumor	1.2±0.5	-
Skin	1.1±0.3	1.1±0.2
Muscle	0.4±0.2	3±2
Bone	4.2±0.6	0.29±0.09
Brain	0.057±0.006	22±10
Eyes	0.45±0.05	2.7±0.9
GI tract*	1.4±0.2	-
Carcass*	10±1	-

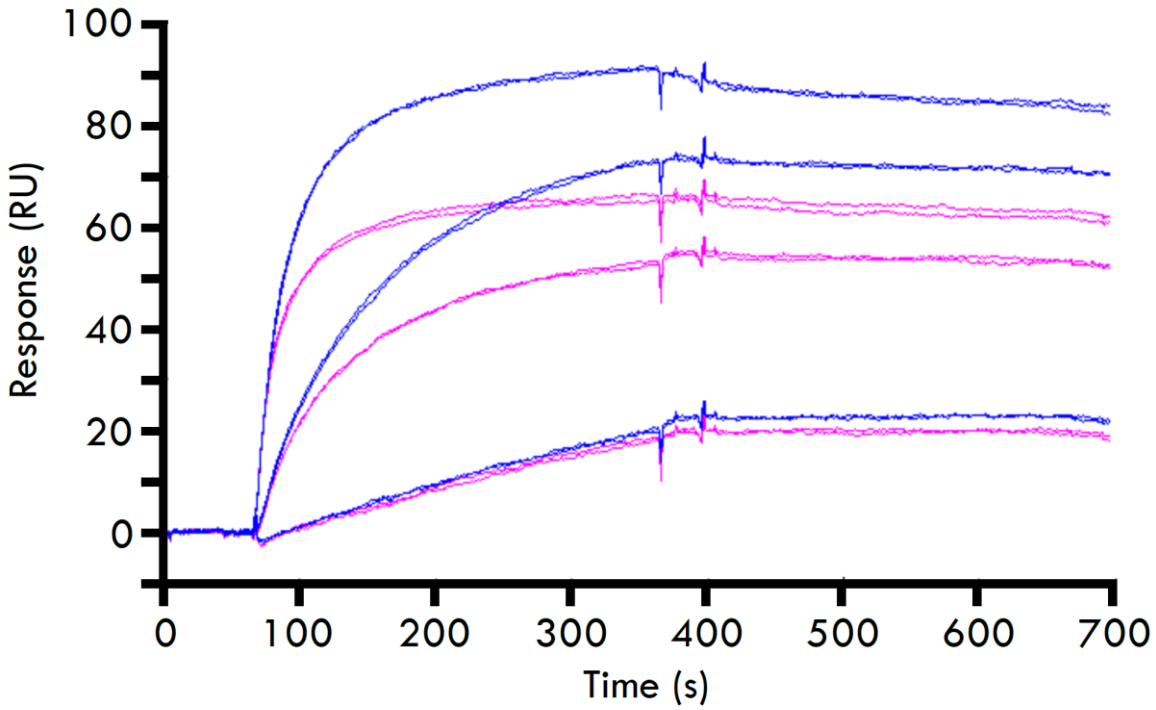

Figure S1. Representative sensorgrams from SPR analysis of NODAGA-Z_{VEGFR2}-Bp2 and Z_{VEGFR2}-BP2-ABD binding murine monomeric VEGFR2. Sensorgrams were obtained by injection of 10, 100 and 500 nM NODAGA-Z_{VEGFR2}-Bp2 (shown in magenta) and Z_{VEGFR2}-BP2-ABD, respectively, over immobilized monomeric murine VEGFR2 (10, 100 and 500 nM). Please note that the higher responses for Z_{VEGFR2}-BP2-ABD are due to the higher molecular weight compared to NODAGA-Z_{VEGFR2}-Bp2. The experiment was performed in duplicates. The data for the NODAGA-Z_{VEGFR2}-Bp2 was fitted to a 1:1 binding model (Chi2 = 0.81), which resulted in a k_{on} of 1.9±0.01 x 10^5 $M^{-1}s^{-1}$ and a k_{off} of 5.4±0.2 x 10^{-5} s^{-1} that was used for calculation of the equilibrium dissociation constant (K_D) = 288±6 pM, which is in accordance with previously reported affinities for Z_{VEGFR2}-Bp2 (Fleetwood et al. Novel affinity binders for neutralization of vascular endothelial growth factor (VEGF) signaling. Cell Mol Life Sci. 2016; 73: 1671-1683.).

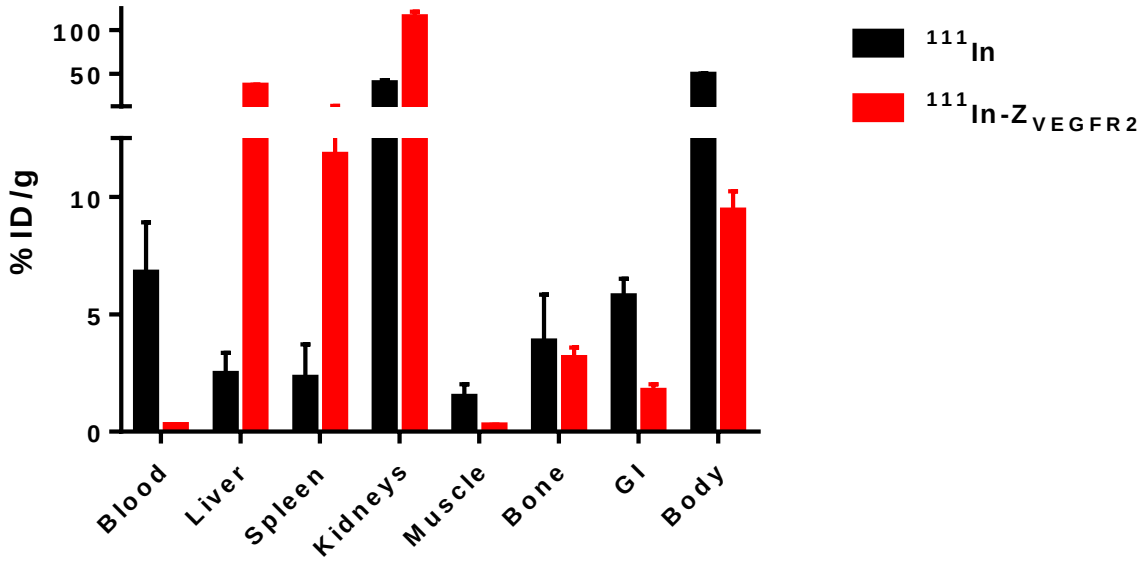

Figure S2. Comparative biodistribution in NMRI mice of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 (4 µg) and [^{111}In]In-acetate 2 h pi.

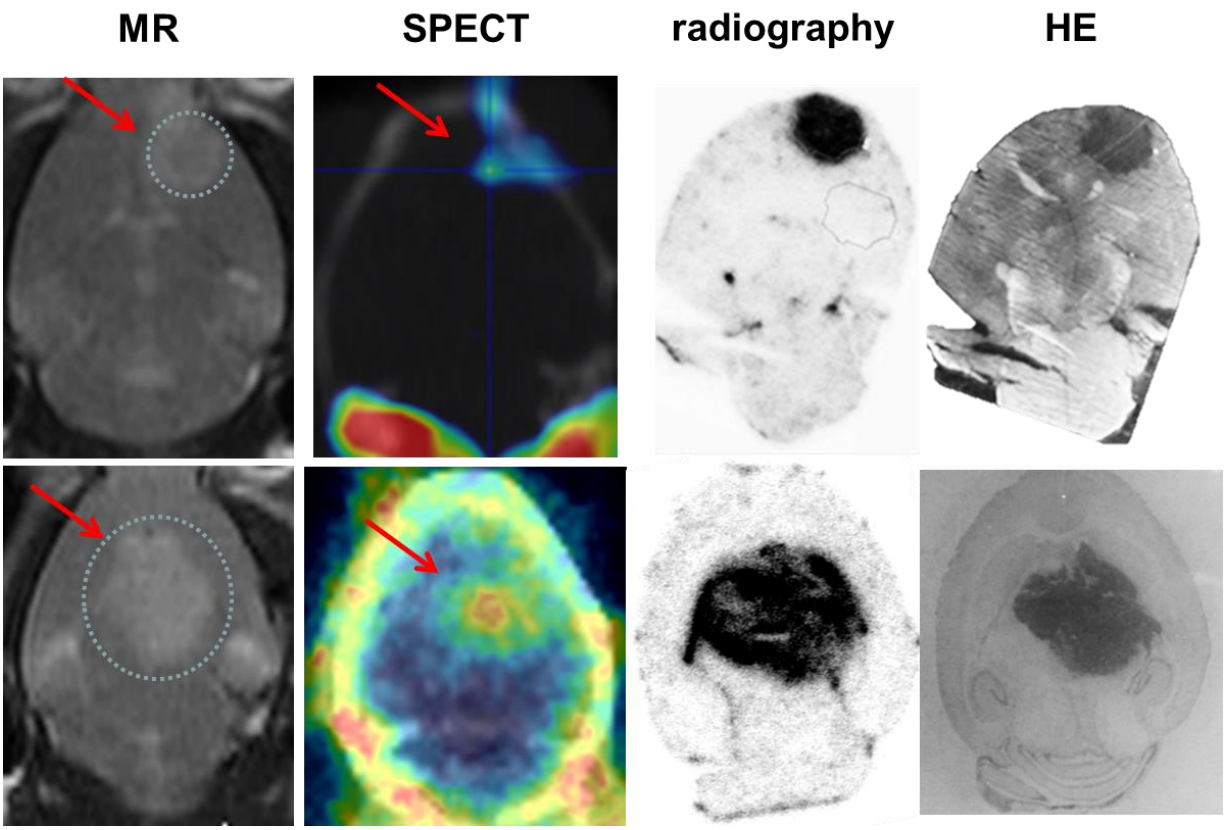

Figure S3. In vivo MRI (prior to the injection) and SPECT/CT coronal imaging, macroautoradiography and H&E staining of brain slices of mice bearing 2 mm (upper row) or 4 mm (bottom row) intracranial GL261 tumors. Animals were injected with 4 µg of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 and imaged 2 h pi. Whole body SPECT/CT scans were carried out using Triumph™ Trimodality system (Gamma Medica) for mice bearing 2 mm (upper row) and nanoScan SPECT/CT for mice bearing 4 mm (bottom row).

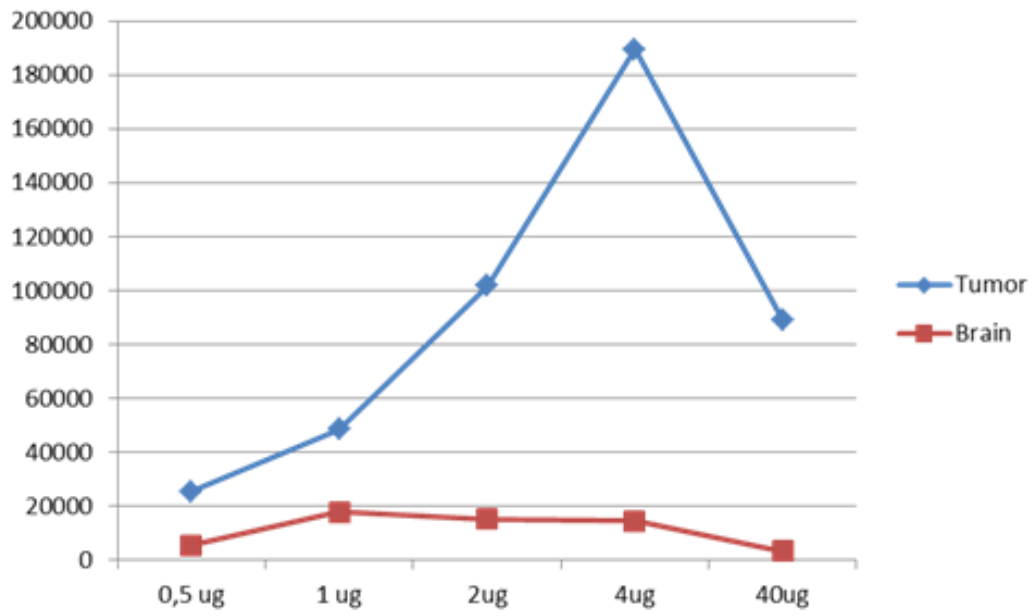

Figure S4. Radioactivity uptake in 2 mm intracranial GL261 tumor and normal brain based on analyses of macroautoradiography of brain slices. Animals were injected with [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 and sacrificed 2 h pi.

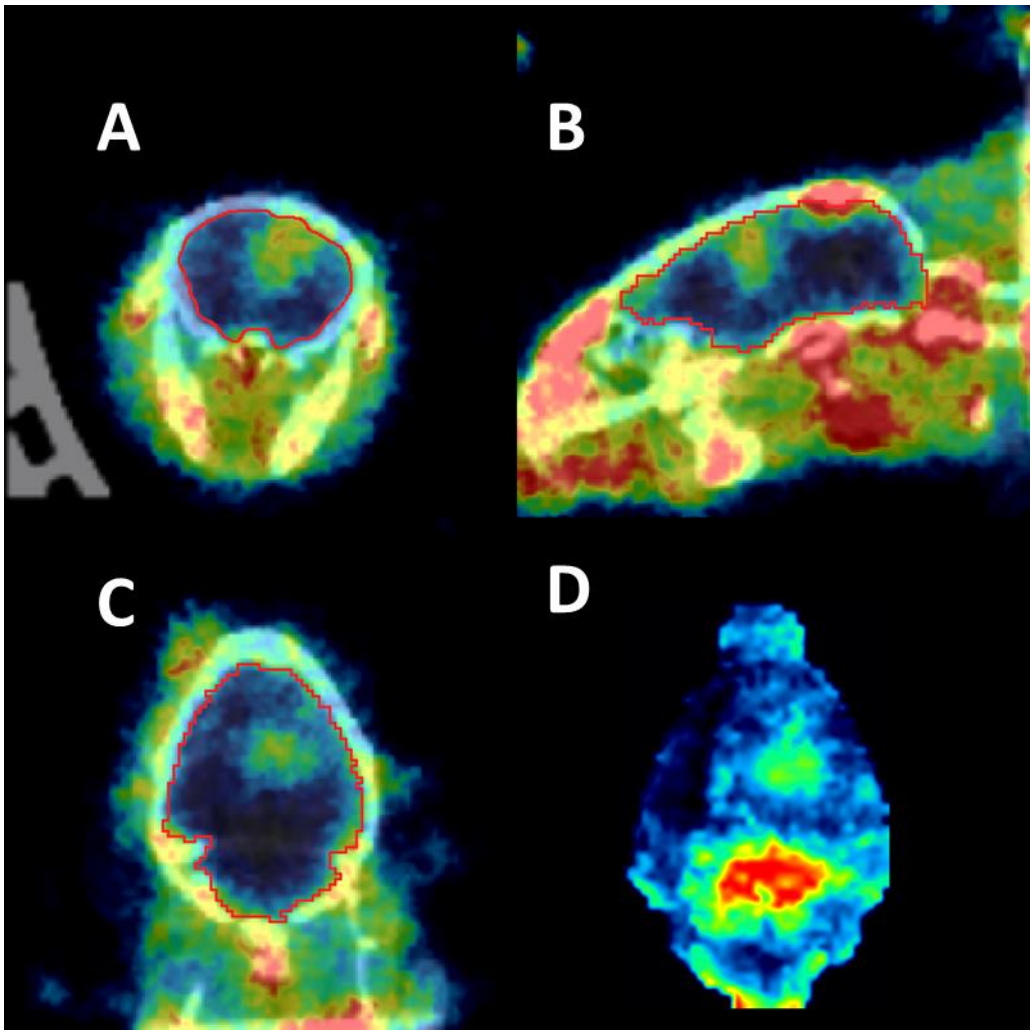

Figure S5. (A-C) VOI drawn on fused SPECT-CT image, along trans-axial plane to delineatethe whole brain. (D) Coronal MIP SPECT image obtained after excluding pixels outside selected area. Animals were injected with 4 μg of [^{111}In]In-NODAGA-Z_{VEGFR2}-Bp_2 and imaged 2 h pi.