Supplemental data

Reagents and Instrumentation

The pro-chelators NODA-GA(tBu)$_3$ ester and NOTA-bis(tBu) ester are commercially available from CheMatech, Dijon, France. All reagents were of the best grade available and were purchased from common suppliers. ^{68}Ge/^{68}Ga-generator IGG100 was available from Eckert & Ziegler (Berlin, Germany). $^{64}CuCl_2$ was purchased from Acom (Italy). All culture reagents were from Gibco BRL, Life Technologies (Grand Island, NY).

The quality control of the radiolabeled compounds was performed by analytical RP-HPLC on an analytical 120-5 C18 Nucleosil column (250 x 4.5 mm) applying a linear gradient of 15-90% solvent B in 25 min at a flow rate of 1 mL/min. (solvent A, 0.1% TFA/H_2O; solvent B, .1%TFA/Acetonitrile). Ultraviolet detection was performed using a Knauer detector at 280 nm. For radioactivity measurement, a Na(TI) well-type scintillation Gina star was used. The radiotracer solutions were prepared by dilution with 0.9% NaCl.

Quantitative γ-counting was performed with a COBRA 5003 γ-system well counter from Packard Instrument (USA). All experiments were carried out 2 times in triplicate.

For PET studies a dedicated small-animal PET scanner (Focus 120 microPET scanner; Concorde Microsystems Inc.) was used.

Radiochemistry

^{68}Ga-NODAGA-MJ9 and ^{68}Ga-NOTA-MJ9: For the evaluation of the 68Ga-labeling efficiency, the new radiotracers ^{68}Ga-NODAGA-MJ9 and ^{68}Ga-NOTA-MJ9 were prepared within 10 min either at room temperature or at 95 °C, using the Modular-Lab PharmTracer module by Eckert & Ziegler (Berlin, Germany). Briefly, the ^{68}Ge/^{68}Ga-generator was eluted with 7 mL HCl 0.1 N and the eluate (~ 450 MBq) was loaded onto a cation exchange column (Strata-XC, Phenomenex). ^{68}Ga was eluted with 800 µL of a mixture of acetone/HCl (97.6%/0.02 N) directly in a vial containing 2 mL sodium acetate buffer (0.2 M, pH 4.0) and minimum 5-20 µg of the conjugates, without any purification step.

For animal studies the same procedure as described before was performed using 10 µg of NODAGA- and NOTA-MJ9 respectively, followed by SepPak C-18 purification to remove uncomplexed radiometal. All radiopeptides were analyzed with (analytic) RP-HPLC. The radiotracer solutions were prepared by dilution with 0.9% NaCl.

^{nat}Ga-NODAGA-MJ9 and ^{nat}Ga-NOTA MJ9

A mixture of NODAGA-MJ9 (1.1 mg, ~0.00068 mmol) in sodium acetate buffer pH 4.0 (0.2 M, 500 µL) and an aliquot (5.7 µL, ~0.0017 mmol) of an aqueous solution of $^{nat}Ga(NO_3)_3$ x H_2O 0.3 M, was

allowed to react at room temperature for 30 min. By then, the complex formation checked by analytical HPLC was complete. The elution time difference between the new product and the starting material was 35 sec using the same gradient. Excess Ga^{3+} was removed by SepPak C-18 purification (Waters). The metallopeptide was eluted with ethanol, followed by evaporation to dryness, dissolution in water, and lyophilization. Quality control of the ^{nat}Ga-containing complex was carried out by analytical HPLC following the conditions described above where only one single peak was detected. The ^{nat}Ga-complex was also identified by ESI-MS (Table 1, manuscript).

A mixture of NOTA-MJ9 (1.9 mg, ~0.0023 mmol) in sodium acetate buffer pH 4.0 (0.2 M, 500 μL) and an aliquot (10.25 μL, ~0.00307 mmol) of an aqueous solution of $^{nat}Ga(NO_3)_3$ x H_2O 0.3 M, was heated at 95 °C for 30 min and exactly the same procedure as before was followed for the preparation of ^{nat}Ga-NOTA-MJ9.

^{nat}Cu-NODAGA-MJ9 and ^{nat}Cu-NOTA MJ9

A mixture of NODAGA-MJ9 (1.0 mg, ~0.62 μmol) in ammonium acetate buffer pH 8.0 (0.1 mol/L, 500 μL) and an aliquot (5.3 μL, ~1.6 μmol) of an aqueous solution of $^{nat}CuCl_2$ x $2H_2O$ 0.3 M, was allowed to react at room temperature for 30 min.

A mixture of NOTA-MJ9 (1.5 mg, ~0.00097 mmol) in ammonium acetate buffer pH 8.0 (0.1 mol/L, 500 μL) and an aliquot (8 μL, ~0.0024 mmol) of an aqueous solution of $^{nat}CuCl_2$ x $2H_2O$ 0.3 M was heated at 95 °C for 30 min. Both ^{nat}Cu-MJ9 conjugates purified following the same procedure as described above for the corresponding ^{nat}Ga-complexes.

Determination of Binding Affinity

The binding affinity profile of NODAGA-MJ9 was determined by in vitro GRPr autoradiography on cryostat sections of well-characterized prostate carcinomas as described in the manuscript. NODAGA-MJ9 exhibited high affinity towards GRPr with an IC_{50} value of 6.2 ± 0.3 nM.

IC_{50} values for displacement of GRP receptor-bound [^{125}I-Tyr^4]BN by increasing concentration of BN analogs (*1*).

Code No.	Peptide structure	Charge	GRP receptor		
			human	mouse	Human/ mouse
[Cu^{II}]-BZH4	Cu^{II}-DOTA-GABA-[D-Tyr^6, βAla^{11}, Nle^{14}]BN(6-14)	-1	26.3±3.5	1.1±0.3	24
[In^{III}]-BZH4	In^{III}-DOTA-GABA-[D-Tyr^6, βAla^{11}, Nle^{14}]BN(6-14)	0	41.5±2.5	0.8±0.4	52
[Cu^{II}]-BZH5	Cu^{II}-CPTA-[D-Tyr^6, βAla^{11}, Nle^{14}]BN(6-14)	+2	3.2±0.5	0.6±0.2	5.3
[Cu^{II}]-BZH6	Cu^{II}-CPTA-[D-Tyr^6, βAla^{11}]BN(6-14)	+2	1.0±0.2	0.22±0.07	1.25
[Cu^{II}]-BZH7	Cu^{II}-CPTA-[βAla^{11}]BN(6-14)	+2	0.42±0.13	26.3±3.5	1.9
[Cu^{II}]-BZH8	Cu^{II}-CPTA-[Gly^6, βAla^{11}]BN(6-14)	+2	1.8±0.6	0.8±0.2	2.25

IC_{50} values (nM ± SD) are in triplicates
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Biodistribution Studies

Ten pmol / 0.3-0.4 / 100 μL MBq of ^{68}Ga-NOTA-MJ9 or ^{68}Ga-NODAGA-MJ9 in 100 μL. NaCl 0.9 % were injected intravenously into the tail vein of PC3 mice. Animals were sacrificed by isoflurane anesthesia at 1 and 2 h after injection of the ^{68}Ga-labeled vectors.

The organs of interest were dissected and weighted, and the radioactivity in tissue samples was counted in a γ-counter. Biodistribution data are given as percent of injected activity per gram of tissue (% IA/g) and are means ± SD (n = 4).

To demonstrate the specificity of binding, PC3 mice were co-injected with 10 pmol of ^{68}Ga-NOTA-MJ9 or ^{68}Ga-NODAGA-MJ9 along with 20 nmol of the respective ^{nat}Me-peptides. Animals were sacrificed at 1 h after injection by isoflurane anesthesia.

100 pmol 2.5-3.0 MBq of ^{64}Cu-NOTA-MJ9 or ^{64}Cu-NODAGA-MJ9 in 100 μL. NaCl 0.9 % were injected intravenously into the tail vein of PC3 mice. Animals were imaged at 1, 4 and 24 h p.i. and sacrificed by isoflurane anesthesia at 24 h after injection of the ^{64}Ga-labeled vectors and biodistribution studies were performed. The biodistribution data are presented in Supplemental Table 1.

Supplemental Table 1: Biodistribution data of ^{64}Cu-NOTA-MJ9 and ^{64}Cu-NODAGA-MJ9 in PC3 xenografts.

	^{64}Cu-NOTA-MJ9	**^{64}Cu-NODAGA-MJ9**
Organ	**24 h**	**24 h**
Blood	0.09 ±0.01	0.04 ±0,00
Heart	0.2 ±0.05	0.1 ±0.01
Liver	0.7 ±0.04	0.4 ±0,00
Spleen	0.3 ±0.07	0.2 ±0.06
Lung	0.3 ±0.04	0.1 ±0.01
Kidney	0.6 ±0.1	0.6 ±0.01
Stomach	0.3 ±0.08	0.1 ±0,01
Intestine	0.3 ±0.03	0.1 ±0.00
Adrenal	0.5 ±0.3	0.2 ±0.1
Pancreas	0.1±0.03	0.1 ±0.01
Muscle	0.05±0.00	0.02 ±0.00
Bone	0.2 ±0.07	0.1 ±0.00
PC3-tumor	7.1 ±0.7	3.0 ±0.2
Tumor/Blood	79±2	75±1
Tumor/Liver	10±1	8±0.5
Tumor/Kidney	12±1	5±1
Tumor/Pancreas	71±2	30±1
Tumor/Muscle	143±1	150±1

Data are expressed in percentage of injected activity per gram of tissue (% IA/g) and are presented as the mean ± SD (n = 2).

Small-Animal PET Studies

PET images were obtained upon injection of 100 pmol of the radioligands (^{68}Ga-NOTA-MJ9 or ^{68}Ga-NODAGA-MJ9 : 1.6-1.8 MBq / 100 µL, ^{64}Cu-NOTA-MJ9 or ^{64}Cu-NODAGA-MJ9 : 2.5-3.0 MBq / 100 µL) on PC3 tumor bearing mice. For the ^{68}Ga-labaled compounds static imaging was acquired for a time period of 30 min at 1 h post injection while for the ^{64}Cu-radiotracers static imaging was

acquired for a time period of 30 min at 1, 4 and 24 h post injection. To visualize the extent of GRP-specific tumour uptake of the ^{68}Ga and ^{64}Cu NOTA-MJ9 and NODAGA-MJ9 radioconjugate, PET blocking studies were performed as described above and static scans were obtained as previously described. PET-images were corrected for ^{68}Ga or ^{64}Cu decay and reconstructed with an ordered-subset expectation maximization algorithm provided by the manufacturer. For the quantitative analysis of the ^{64}Cu-images, the images counts per pixel per second were calibrated to activity concentrations (Bq/mL) by measuring a 3.5-cm cylinder phantom filled with a known concentration of radioactivity. To determine tracer concentration in the tumor, kidney, liver, pancreas and muscles, ellipsoid regions of interest were placed in the area exhibited the highest radioactivity as determined by visual inspection on micro-PET images generated by the AMIDE software (*2*).

Tracer uptake is expressed as percentage of decay-corrected %IA/g, with a color scale set from 0% to 20% for qualitative comparison among the images.

References

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