Materials and Methods

All reagents were obtained from commercial sources and used without further purification. Rink amide 4-methyl-benzhydrylalanine (MBHA) resin and all amino acids are available from NovaBiochem (Laeufelfingen, Switzerland) or NeoMPS (Strasbourg, France). DOTA-tris(^{t}Bu ester) and NODAGA-tris(^{t}Bu ester) were purchased from CheMatech (Dijon, France). The synthesis of CB-TE1A1A(^{t}Bu ester) will be reported elsewhere. The $N_4(Boc)_4$-COOH was synthesized according to the protocol described recently (1). $^{111}InCl_3$, $^{67}GaCl_3$ and $^{99}Mo/^{99m}Tc$ generator from which ^{99m}Tc was eluted as Na[^{99m}Tc]TcO4 were purchased from Covidien Medical (Petten, Netherlands). ^{68}Ga was eluted and purified from a commercially available generator (Obninsk, Russia) according to Zhernosekov, et al. (2). $^{64}CuCl_2$ was purchased from University Hospital of Tübingen (Germany). BIM26226 was provided by Ipsen Biotech (Paris, France). Electrospray ionization mass spectroscopy was carried out with a Finnigan SSQ-7000-spectrometer (Bremen, Germany). Analytical high-performance liquid chromatography (HPLC) was performed on a Hewlett Packard 1050-HPLC-system with a multi-wavelength detector and a flow-through Berthold LB-506-Cl γ-detector using a Macherey-Nagel Nucleosil 120 C_{18}-column (Oensingen, Switzerland) (eluents: A=0.1% TFA in water and B=acetonitrile; gradient: 0-30 min, 95%-30% A; flow: 0.750 mL/min). Preparative HPLC was performed on a Metrohm HPLC-system LC-CaDI 22–14 (Herisau, Switzerland) with a Macherey-Nagel VP 250/21 Nucleosil 100–5 C_{18}-column (gradient: 0-20 min, 90%-50% A; flow: 10 mL/min). Quantitative γ-counting was performed on a COBRA 5003 γ-system well counter from Packard Instruments (Packard, Meriden, CT, USA).

Radiolabeling of chelator-peptide conjugates

^{111}In-DOTA-AR*:* This radioligand was prepared by dissolving 10 μg peptides in 250 μL sodium acetate buffer (0.4 mol/L, pH 5.0), followed by incubation with $^{111}InCl_3$ (100-180 MBq) for 30 min at 95°C.

$^{67/68}$Ga-NODAGA-AR*:* Purified ^{68}Ga(III) (250-300 MBq) was used directly for the labeling of NODAGA-AR-02 (10 μg) in NH_4-acetate buffer (0.2 mol/L, pH 4.0) followed by incubation for 10 min at RT. ^{67}Ga-NODAGA-AR was obtained using the same labeling conditions and 100-180 MBq of $^{67}GaCl_3$.

^{64}Cu-CB-TE2A-AR*:* The labeling was performed by modifying and optimizing the protocol published previously (*32*). In brief, 10 μg of the peptide was dissolved in 250 μL of NH_4-acetate buffer (0.1 mol/L, pH 8.0) followed by incubation with $^{64}CuCl_2$ (100-120 MBq) at 95°C.

^{99m}Tc-N4-AR: A similar protocol as published recently was used (*33*). A stock solution of N4-AR (1 mmol/L) was prepared by dissolution in a mixture (8:2 v/v) of acetic acid (50 mmol/L) and ethanol. A mixture of phosphate buffer (0.5 mol/L, pH 11.5; 50 μL) and sodium citrate (0.1 mol/L, 5 μL) was taken up in an Eppendorf tube. $Na^{99m}TcTcO_4$ generator eluate (650–750 MBq, 700 μL), N4-AR solution (31 μg, 20 μL) and freshly prepared $SnCl_2$ solution in ethanol (25 mg, 25 μL) were added to this mixture. The reaction mixture was incubated at room temperature for 30 min.

Supplemental Table 1: Analytical data of the purified conjugates

Compound	Purity (%)	MS (ESI): m/z (%)	Rt (min)	RP-HPLC Gradient
DOTA-AR	98.1	1747.7 (100) $[M+H]^+$	19.1	0-30 min, 95%-30% A
NODAGA-AR	97.4	1718.9 (100) $[M+H]^+$	19.4	0-30 min, 95%-30% A
CB-TE2A-AR	98.5	1685.1 (45) $[M+H]^+$, 843.3 (100) $[M+H]^{2+}$	20.1	0-20 min, 95%-30% A
N4-AR	98.1	1546.9 (25) $[M+H]^+$, 773.9 (100) $[M+H]^{2+}$	15.9	0-20 min, 95%-50% A

DOTA-AR: purity 98.1%; R_t = 19.1 min (analytical RP-HPLC; 0-30 min, 95%-30% A);: 1747.7 (100) $[M+H]^+$.

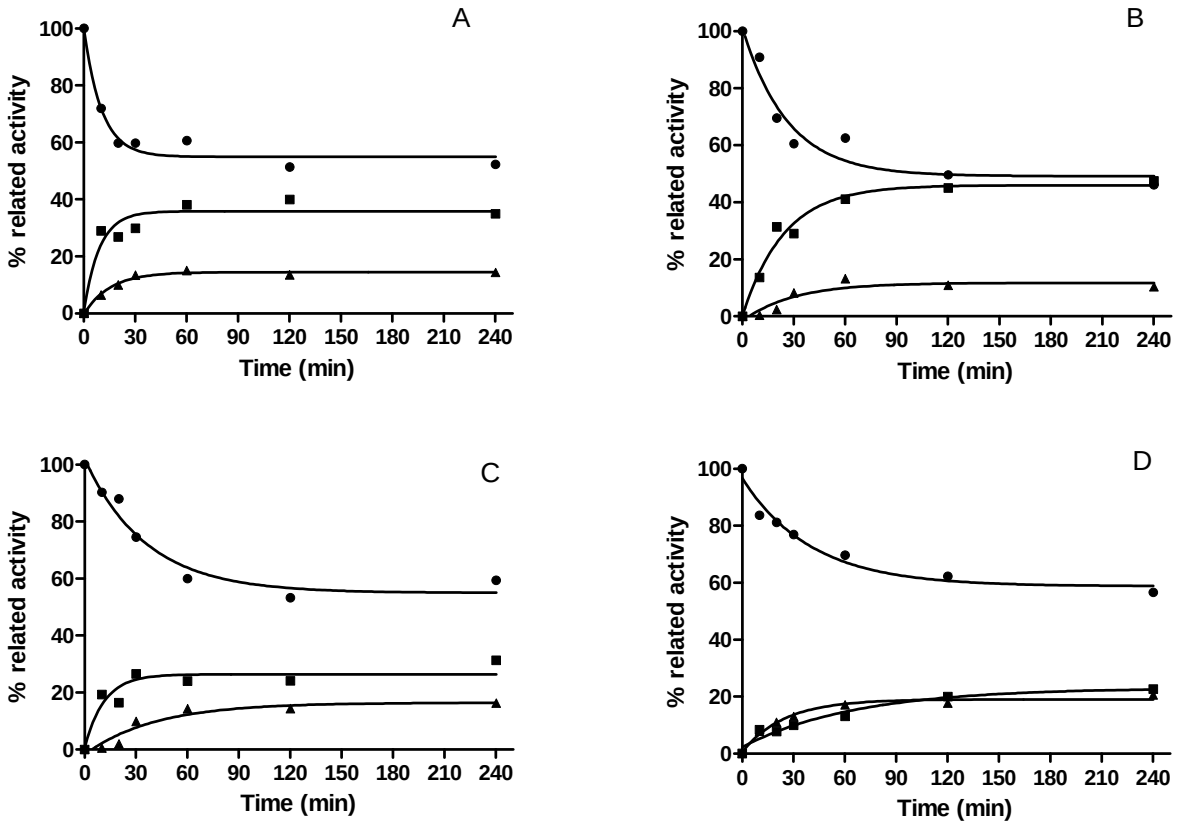
NODAGA-AR: purity 97.4%; R_t = 19.4 min (analytical RP-HPLC; 0-30 min, 95%-30% A); MS (ESI): *m/z* (%): 1718.9 (100) $[M+H]^+$.

CB-TE2A-AR: purity 98.5%; R_t = 20.1 min (analytical RP-HPLC; 0-20 min, 95%-30% A); MS (ESI): *m/z* (%):1685.1 (45) $[M+H]^+$, 843.3 (100) $[M+H]^{2+}$.

N4-AR: purity 98.1%; R_t = 15.9 min (analytical RP-HPLC; 0-20 min, 95%-50% A); MS (ESI): *m/z* (%):1546.9 (25) $[M+H]^+$, 773.9 (100) $[M+H]^{2+}$.

Supplemental Table 2: Biodistribution studies of ^{111}In-DOTA-AR, ^{68}Ga-NODAGA-AR, ^{99m}Tc-N4-AR and ^{64}Cu-CB-TE2A-AR in PC-3 tumor-bearing mice in presence of a large excess of cold peptide. Data are expressed as IA%/g (percentage of injected activity per gram tissue) and are presented as mean ± SD (n = 4-6).

	^{111}In-DOTA-AR (%ID/g tissue ± SD)	**^{68}Ga-NODAGA-AR (%ID/g tissue ± SD)**	**^{99m}Tc-N4-AR (%ID/g tissue ± SD)**	**^{64}Cu-CB-TE2A-AR (%ID/g tissue ± SD)**
Organ	**4h blocked**	**1h blocked**	**4h blocked**	**4h blocked**
Blood	0.01 ± 0.00	0.47 ± 0.27	0.21 ± 0.01	0.51± 0.05
Tumor	0.45 ± 0.06	0.18 ± 0.06	0.43 ± 0.07	0.69± 0.25
Kidneys	1.40 ± 0.29	0.76 ± 0.60	7.08 ± 1.00	5.56± 2.94
Pancreas	0.02 ± 0.00	0.30 ± 0.22	0.15 ± 0.02	0.18± 0.04
Muscle	0.0 ± 0.00	0.61 ± 0.42	0.07 ± 0.02	0.07± 0.10
Intestine	0.05 ± 0.04	0.06 ± 0.04	0.26 ± 0.04	1.88± 0.23
Liver	0.09 ± 0.01	0.15 ± 0.01	2.59 ± 0.56	5.77± 1.17
Stomach	0.03 ± 0.01	0.05 ± 0.02	0.32 ± 0.08	1.61± 0.49
Bone	0.02 ± 0.01	0.08 ± 0.002	0.57 ± 0.08	0.10± 0.04



SUPPLEMENTAL FIGURE 1. Fate of the receptor bound ^{111}In-DOTA-AR (A), ^{67}Ga-NODAGA-AR (B), ^{64}Cu-CB-TE2A-AR (C) and ^{99m}Tc-N4-AR (D) as measured with PC-3 cells. The percentage of bound (●), internalised (▲) and dissociated (■) radioligand was measured with respect to the total receptor bound radioligand in 2h at 4 ºC (100%). Values and standard deviations are the result of two independent experiments with triplicates in each experiment.

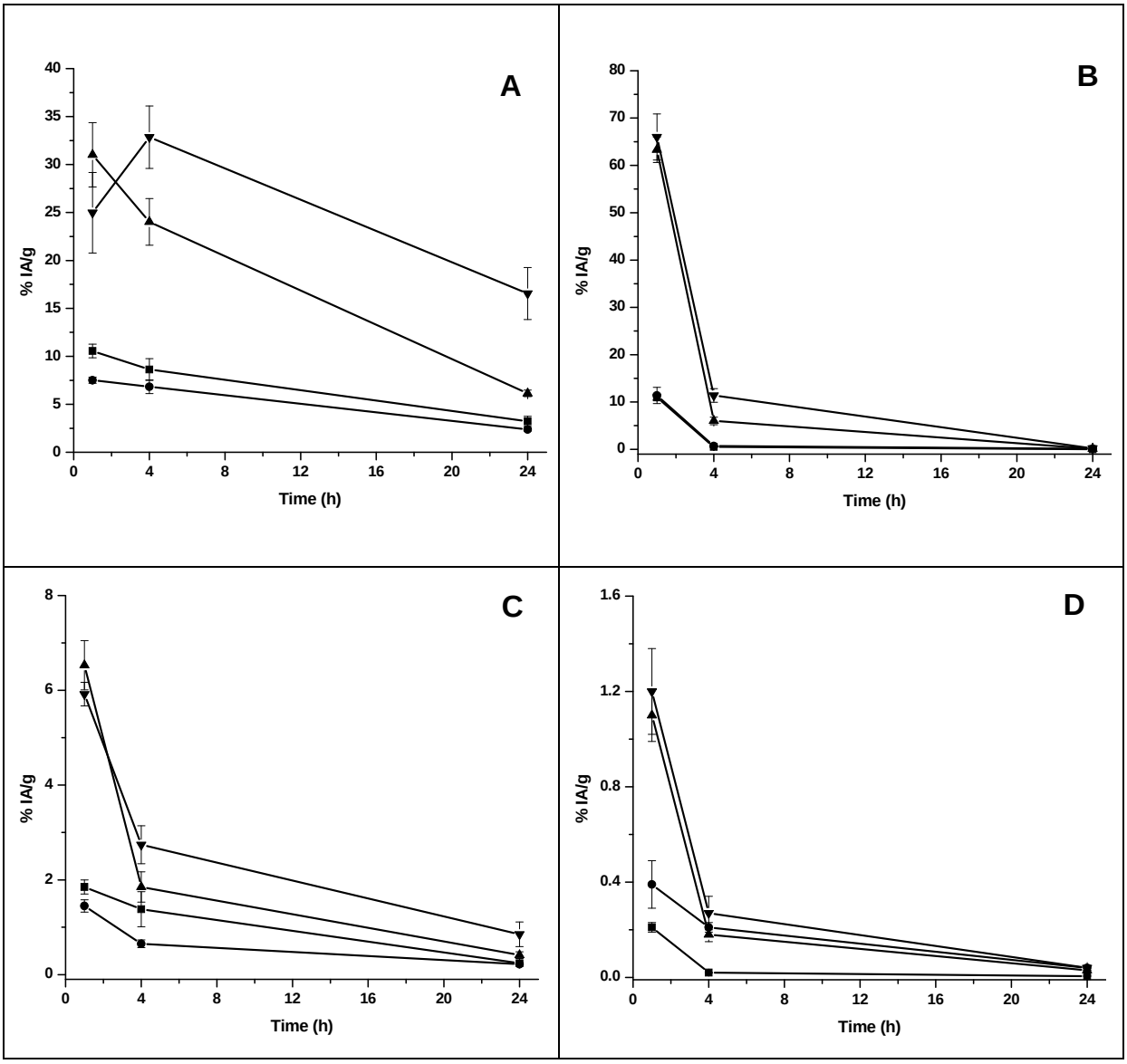
k_{obs} (N4-AR)= 0.025±0.006 min^{-1}; $T_{1/2}$= 27.8 min

k_{obs} (CB-TE2A-AR)= 0.028±0.007 min^{-1}; $T_{1/2}$= 24.9 min

k_{obs} (DOTA-AR)= 0.097±0.022 min^{-1}; $T_{1/2}$= 7.1 min

k_{obs} (NODAGA-AR)= 0.037±0.011 min^{-1}; $T_{1/2}$= 18.3 min

The equation used is one-phase decay :Y=(Y0 - Plateau)*exp(-K*X) + Plateau



SUPPLEMENTAL FIGURE 2: Comparison of uptake and washout kinetics of the radioligands in selected organs as determined by biodistribution studies in PC-3 tumor bearing nude mice. The accumulation/ retention of ^{111}In-DOTA-AR (■), ^{67}Ga-NODAGA-AR (●), ^{64}Cu-CB-TE2A-AR (▲) and ^{99m}Tc-N4-AR (▼) in tumor (A), pancreas (B), kidney (C) and blood (D) are compared at 1, 4 and 24 after injection. Data are expressed as %IA/g (percentage of injected activity per gram tissue) and are presented as mean ± SD (n = 4-6). Note differences in Y-axis scale.

References

1. Abiraj K, Mansi R, Tamma ML, et al. Tetraamine-derived bifunctional chelators for technetium-99m labelling: synthesis, bioconjugation and evaluation as targeted SPECT imaging probes for GRP-receptor-positive tumours. *Chemistry.* Feb 15;16(7):2115-2124.

2. Zhernosekov KP, Filosofov DV, Baum RP, et al. Processing of generator-produced ^{68}Ga for medical application. *J Nucl Med.* Oct 2007;48(10):1741-1748.

3. Rogers BE, Manna DD, Safavy A. In vitro and in vivo evaluation of a 64Cu-labeled polyethylene glycol-bombesin conjugate. *Cancer Biother Radiopharm.* Feb 2004;19(1):25-34.