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Breast-Cancer Diagnosis by Neuropeptide Y Analogues: From Synthesis to Clinical Application**

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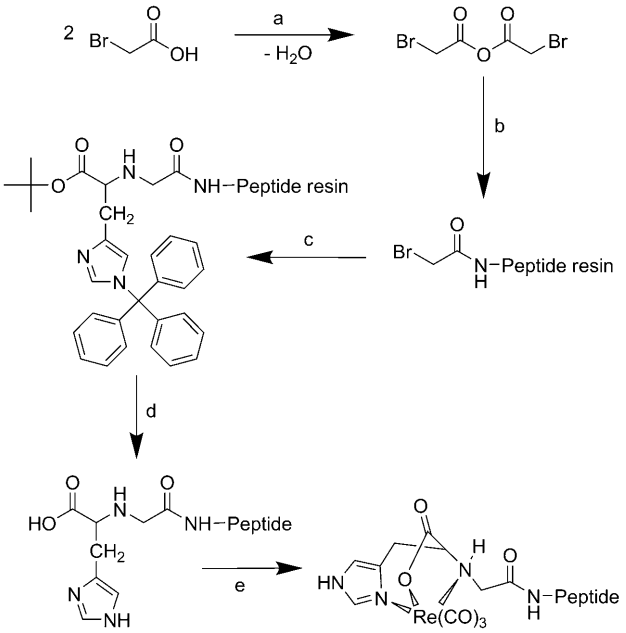
In memory of Rainer Rudolph

Breast cancer is still one of the most frequently occurring tumors in women. Severe and often therapy-limiting side effects are a major obstacle in chemotherapy. New delivery concepts that reduce systemic side effects are needed to optimize anticancer therapies, and selective targeting concepts are required for early and selective tumor diagnosis. Neuropeptide Y (NPY), a member of the pancreatic polypeptide family, is a C-terminal amidated peptide hormone consisting of 36 amino acid residues.[1,2] NPY-mediated functions are transmitted by so-called Y receptors, named Y_1, Y_2, and Y_5 receptors, which bind NPY with nanomolar affinity. All Y receptors are members of the class A of heptahelix receptors, that signal through heterotrimeric G proteins.[3,4]

Reubi et al. have recently described Y-receptor expression in human breast cancer. They have shown that over 90% of all breast tumors and 100% of the examined metastases express Y_1 receptors.[5] Interestingly, a shift of the receptor subtype from Y_2 receptors in healthy tissue to Y_1 receptors during neoplasm was found, which is potentially related to reduced differentiation. Based on NPY and the known structure–activity relationships for Y_1-receptor binding,[6] we designed, synthesized, and characterized two analogues for tumor labeling that vary in the position of the chelator to conjugate ^{99m}Tc. Peptides **1a** and **2a** were synthesized with a N^{α}-histidinyl acetyl (N^{α}His-ac) chelator[7] at the N terminus, whereas peptides **1b** and **2b** were modified at the N^{ε} side chain of Lys^4. The tridentate ligand N^{α}His-ac is able to form stable and biologically active complexes.[8,9] Modification of the resin-bound peptide was performed by an efficient strategy (Scheme 1). In the first step, bromoacetic acid was activated by diisopropylcarbodiimide to form the corresponding anhydride. His(Trt)-O*t*Bu was then added and the NH–CH bond was formed by HBr elimination. Cleavage of the peptide yielded His-acetyl peptides either at the N terminus or at the N^{ε} side chain of Lys^4. Rhenium was used as a cold surrogate for ^{99m}Tc and introduced for in vitro studies (peptides **1c**, **1d**, **2c**, **2d**). The (N^{α}His-ac) peptides were labeled with rhenium by using $(NEt_4)_2[Re(CO)_3Br_3]$ at pH 4.3 and incubation of the reaction mixture for 2 h at room temperature. $Re(CO)_3$-(N^{α}His-ac)–NPY was separated from its non-labeled counterpart by preparative HPLC in a yield of 55%. Displacement binding assays using [^{3}H-propionyl]–NPY and increasing concentrations of each peptide were performed at Y_1- (SK-N-MC (human neuroblastoma) and MCF-7 (human breast adenocarcinoma)), Y_2- (SMS-KAN



Scheme 1. The synthesis of NPY analogues with reagents and conditions for rhenium conjugation (see also Table S1 in the Supporting Information). a) Diisopropylcarbodiimide in dichloromethane, shaking time 20 min. b) NH_2-peptide resin, diisopropylethylamide (DIPEA), incubation time 2 h, washing with dimethylformamide (DMF). c) His-(Trt)-O*t*Bu in DMF, DIPEA, incubation time 24 h, washing with routine resin-washing solvents, dried in vacuo. d) Cleavage by 95% trifluoroacetic acid/5% thioanisol/thiocresole. e) 5% excess $(NEt_4)_2[Re(CO)_3Br_3]$, pH 4.3, heating at 37°C, in nitrogen atmosphere, incubation time 2 h.

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(human neuroblastoma)) and Y_5- (HEC-1b-hY_5 (human endometrial carcinoma)) receptor-expressing cells.[10] Peptides **1c** and **1d** showed similar IC_{50} values at Y_1- and Y_2-receptor-expressing cells and a slightly reduced affinity for Y_5 receptors, as expected, whereas peptides **2c** and **2d** showed selective, nanomolar receptor binding only at Y_1 receptors (Table 1). Accordingly, no significant influence of the Re(CO)$_3$-(N$^{\alpha}$His-ac) moiety was found for in vitro peptide receptor binding.

Table 1: In vitro binding data of the NPY analogues.

		IC_{50} [nM]			
		SK-N-MC	MCF-7	SMS-KAN	HEC-1b-hY_5
1c	Re(CO)$_3$-(N$^{\alpha}$His-ac)-NPY	3.9 ± 0.3	17.0 ± 6.5	3.2 ± 1.3	29.8 ± 1.9
1d	Lys^4(Re(CO)$_3$-(N$^{\alpha}$His-ac))-NPY	10.5 ± 3.9	8.5 ± 6.5	6.1 ± 2.6	27.3 ± 5.1
2c	Re(CO)$_3$-(N$^{\alpha}$His-ac)-[Phe^7, Pro^{34}]NPY	11.8 ± 2.6	26.9 ± 5.2	106.3 ± 22.2	> 1000
2d	Lys^4(Re(CO)$_3$-(N$^{\alpha}$His-ac))-[Phe^7, Pro^{34}]NPY	1.3 ± 0.1	5.2 ± 1.0	97.5 ± 11.9	208.4 ± 8.0

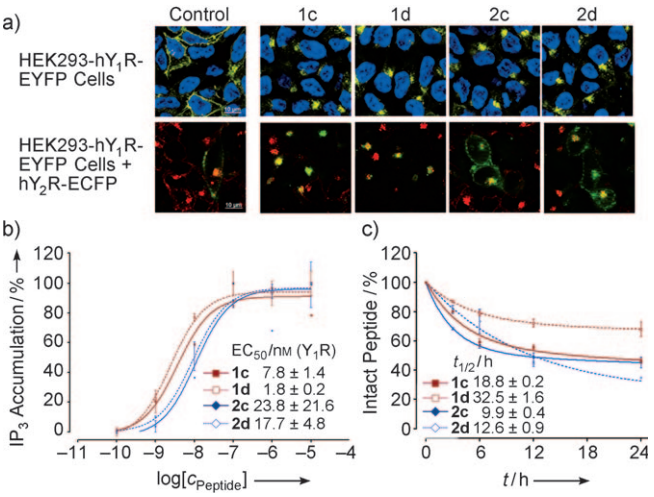

Figure 1. Rhenium-labeled NPY analogues internalize into Y_1-receptor-expressing cells and are slowly degraded in human blood plasma. a) HEK293 cells stably transfected with hY_1R-EYFP (upper panel, receptors: yellow) and transiently co-transfected with hY_2-ECFP receptors were incubated with 1 μM of the peptide **1c**, **1d**, **2c**, and **2d** for 60 minutes (Y_1 receptors: red, Y_2 receptors: green), confirming selective internalization of peptide **2c** and **2d**. b) EC_{50} curves and EC_{50} values determined by a ^{3}H-based IP_3 signal-transduction assay using COS7 cells transiently coexpressing hY_1 and Gα_{qi}. c) Metabolic stability curves and values of the peptides **1c**, **1d**, **2c**, and **2d** in human blood plasma. All the values are average ± standard deviation of three experiments.

Agonist stimulation leads to Y-receptor internalization,[11] which can significantly increase the labeling efficiency. Signal transduction measurements were performed after co-transfection of human Y_1 receptors and a chimeric Gα_{qi} protein in COS7 cells (African green monkey, kidney).[12,13] The EC_{50} values of **1c**, **1d**, **2c**, and **2d** confirmed the results from the displacement binding assays and showed a strong agonistic activity of all four peptides (Figure 1b). To further support our hypothesis, we followed the internalization of **1c**, **1d**, **2c**, and **2d** by microscopic studies. After stimulation with 1 μM of each peptide, complete peptide mediated Y_1-receptor internalization was found in HEK293-hY_1-EYFP receptor cells. To investigate the selectivity of the internalization, we used cells that co-express hY_1-EYFP and hY_2-ECFP receptors. As shown in Figure 1a, peptides **1c** and **1d** lead to internalization of both receptor subtypes, whereas peptides **2c** and **2d** induce selectively the internalization of the addressed Y_1 receptor.

The metabolic stability of **1c**, **1d**, **2c**, and **2d** was studied in vitro by the determination of their half-lives in human blood plasma. The peptides were labeled with carboxyfluorescein (CF) at the N-terminus or at the respective N$^{\varepsilon}$ side chain of Lys^4.[14] Time-dependent incubation showed half-lives of 9.9 to 32 h; these values are in the same range or slightly reduced for the Y_1 receptor selective peptides compared to NPY ($t_{1/2} = 24.7 \pm 1.4$ h) (Figure 1c).[15,16]

For radiolabeling of the highly selective peptides **2a** and **2b**, an approved protocol[17–19] was used, yielding in ^{99m}Tc^{V} species, which are denoted ^{99m}Tc(core)$^{3+}$. Because the interaction between plasma proteins and peptides directly and indirectly affects different pharmacokinetic parameters, such as volume of distribution, metabolism and excretion of the drugs, and accordingly the dosage,[20] protein binding of ^{99m}Tc(core)$^{3+}$-labeled peptides **2a** and **2b** were studied in vitro in human blood at several time intervals and showed high stability of the formed ^{99m}Tc(core)$^{3+}$–peptide complex. Initially, slower protein binding was observed for peptide **2a** compared to peptide **2b**, although an increased protein binding of peptide **2a** was found after 3 h of incubation (Figure 2c).

In vivo uptake of ^{99m}Tc(core)$^{3+}$-labeled peptides **2a** and **2b** was then studied in various organs of normal rabbits after regular time intervals. Peptide **2a** showed a slower uptake compared to peptide **2b**, whilst the retention time was significantly higher (Figure 2a,b). Accordingly, peptide **2a** has sufficient renal excretion time to allow absorption into the tumor, but body clearance is fast enough to avoid unspecific peptide accumulation.

Neither analogues of NPY nor NPY itself have been previously used in clinical studies in human breast-cancer patients. Our preliminary in vitro and in vivo studies led to the most promising peptide, the ^{99m}Tc(core)$^{3+}$-labeled peptide **2a**, which then was used in imaging studies with four patients showing different stages of disease along with metastases spreading from the originating tumor (Figure 3). For control, ^{99m}Tc-MDP bone scan was performed for each patient. In the process, the radiolabeled ligand methylene diphosphate (MDP) can preferentially be taken up by bones and the radioactivity attached to bones by the hydroxyapatite can be used for imaging.[21,22] Therefore, it was used to detect the possibility of skeletal metastases. Another control was the treatment of a healthy volunteer with peptide **2a**. Whole body scintimammography showed no peptide uptake, and in particular not in breasts (Figure 3a).

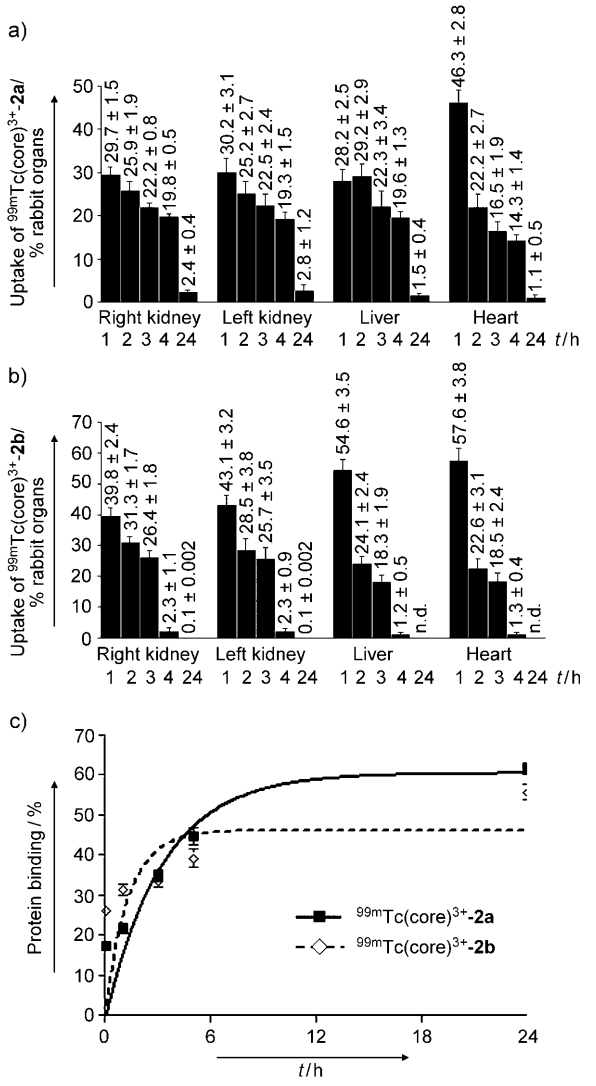

Figure 2. Good renal excretion and fast body clearance in rabbits, and adequate protein binding of the $^{99m}Tc(core)^{3+}$-labeled peptides **2a** and **2b**. a,b) In vivo uptake (%) with 3–5 mCi of each peptide in rabbits after different time intervals. c) Protein binding (%) with 2 mCi of each peptide in human blood after different time intervals. All values are average ± standard deviation of two or three experiments (n.d.: not detected).

Whole-body scintimammography of patient 1 at 60 min post-injection resulted in a clear uptake in a right breast tumor (Figure 3b,c). An image of the skeleton showed normal and symmetrical tracer uptake (Figure 3d). Similar results were observed for patient 2, ruling out the possibility of metastases cells in bone, because of the normal and symmetrical tracer uptake (Figure 3e–g). A tumor uptake in left breast was detected in patient 3 (Figure 3h,i). Treatment with the $^{99m}Tc(core)^{3+}$-labeled peptide **2a** of patient 4 and whole body scintimammography at 60 min post-injection resulted in a distinct accumulation of radioactivity at metastases sites, predominantly at the right side. The observed hot spots suggest that this patient has metastasis lumps originating from the right breast (Figure 3k). This was further confirmed in ^{99m}Tc-MDP bone scan by an increased uptake in bones only at

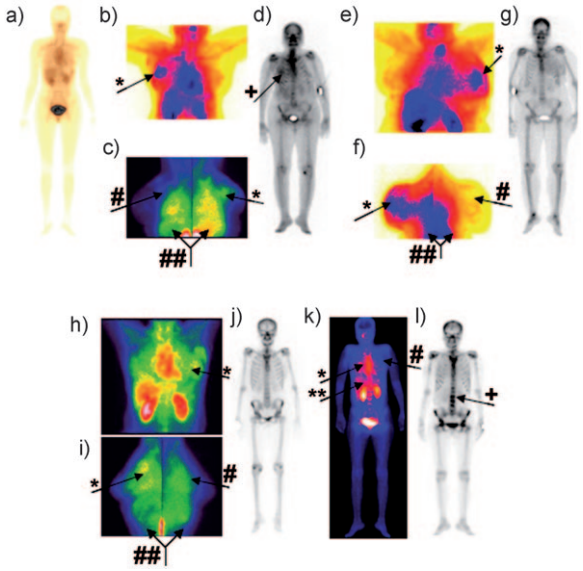

Figure 3. Clear uptake of the $^{99m}Tc(core)^{3+}$-labeled peptide **2a** in breast-cancer patients shown by whole-body scintimammography. a) No significant peptide uptake in the healthy volunteer; b–d) Patient 1; e–g) Patient 2; h–j) Patient 3; k,l) Patient 4. b,e,h,k) Detection of radioactivity in the cancer bearing breast at 60 min post injection; c,f,i) Lateral view of both breasts showing clear uptake in only one breast (⋆ uptake, # no uptake, ## blood pool formation in both breasts, ⋆⋆ uptake by metastases); d,g,j,m) Bone scan shows mild tracer uptake in bone adjacent to tumor site at 2.5 h post injection (+ mild tracer uptake).

the sites of bone metastases. These findings agree with the clinical history of patient 4 (Figure 3l; see also the Supporting Information). Our studies demonstrate for the first time a clear uptake of a $^{99m}Tc(core)^{3+}$-labeled Y_1-receptor-selective ligand into the tumor, whereas normal tissues and organs only show background radiation. Interestingly, we found that metastases spread in other organs that originated from breast cancer could also be detected. No radioactivity was found in the brain, which indicates that the peptide cannot cross the blood–brain barrier. Minimized adverse effects during peptide treatment can therefore be expected.

In conclusion, NPY-derived Y_1-receptor ligands were developed and investigated in vitro and in vivo. These preclinical and first clinical data clearly indicate that the overexpression of the Y_1 receptor in human breast cancers offers a unique possibility for tumor targeting by using NPY-derived Y_1-receptor ligands. This approach might be further extended to a tumor-specific targeting by using NPY derivatives for carrier of chemotherapeutic agents and might not be limited to breast cancer, because Y-receptor expression has been identified in other tumors, such as neuroblastoma, glioblastoma, ovarian adenocarcinoma, gastrointenstinal stromal tumor, nephroblastomas, renal cell carcinoma, and pheochromocytoma.[5,23,24]

Experimental Section

See Supporting Information for details. All animal and human studies were done at the Institute of Nuclear Medicine and Oncology

(Lahore, Pakistan) according to the local rules and regulations of the country (NMOL 53/07). Human studies were performed by their informed written consent about the new drug.

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[1] K. Tatemoto, *Proc. Natl. Acad. Sci. USA* **1982**, *79*, 5485 – 5489.
[2] T. Pedrazzini, F. Pralong, E. Grouzmann, *Cell. Mol. Life Sci.* **2003**, *60*, 350 – 377.
[3] M. C. Michel, A. Beck-Sickinger, H. Cox, H. N. Doods, H. Herzog, D. Larhammar, R. Quirion, T. Schwartz, T. Westfall, *Pharmacol. Rev.* **1998**, *50*, 143 – 150.
[4] P. M. Rose, P. Fernandes, J. S. Lynch, S. T. Frazier, S. M. Fisher, K. Kodukula, B. Kienzle, R. Seethala, *J. Biol. Chem.* **1995**, *270*, 22661 – 22664.
[5] J. C. Reubi, M. Gugger, B. Waser, J. C. Schaer, *Cancer Res.* **2001**, *61*, 4636 – 4641.
[6] R. M. Soll, M. C. Dinger, I. Lundell, D. Larhammer, A. G. Beck-Sickinger, *Eur. J. Biochem.* **2001**, *268*, 2828 – 2837.
[7] P. A. Schubiger, L. Allemann-Tannahill, A. Egli, R. Schibli, R. Alberto, N. Carrel-Remy, M. Willmann, P. Blauenstein, D. Tourwe, *Q. J. Nucl. Med.* **1999**, *43*, 155 – 158.
[8] D. R. van Staveren, S. Mundwiler, U. Hoffmanns, J. K. Pak, B. Spingler, N. Metzler-Nolte, R. Alberto, *Org. Biomol. Chem.* **2004**, *2*, 2593 – 2603.
[9] D. R. van Staveren, N. Metzler-Nolte, *Chem. Commun.* **2002**, 1406 – 1407.
[10] C. Moser, G. Bernhardt, J. Michel, H. Schwarz, A. Buschauer, *Can. J. Physiol. Pharmacol.* **2000**, *78*, 134 – 142.
[11] I. Bohme, J. Stichel, C. Walther, K. Mörl, A. Beck-Sickinger, *Cell. Signaling* **2008**, *20*, 1740 – 1749.
[12] I. Bohme, K. Morl, D. Bamming, C. Meyer, A. G. Beck-Sickinger, *Peptides* **2007**, *28*, 226 – 234.
[13] N. Merten, D. Lindner, N. Rabe, H. Römpler, K. Mörl, T. Schöneberg, A. Beck-Sickinger, *J. Biol. Chem.* **2007**, *282*, 7543 – 7551.
[14] P. J. Weber, J. E. Bader, G. Folkers, A. G. Beck-Sickinger, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 597 – 600.
[15] I. U. Khan, R. Reppich, A. G. Beck-Sickinger, *Biopolymers* **2007**, *88*, 182 – 189.
[16] D. Zwanziger, I. U. Khan, I. Neundorf, S. Sieger, L. Lehmann, M. Friebe, L. Dinkelborg, A. G. Beck-Sickinger, *Bioconjugate Chem.* **2008**, *19*, 1430 – 1438.
[17] G. Ferro-Flores, C. Arteaga de Murphy, J. Rodriguez-Cortés, M. Pedraza-López, M. T. Ramírez-Iglesias, *Nucl. Med. Commun.* **2006**, *27*, 371 – 376.
[18] R. Schibli, R. La Bella, R. Alberto, E. Garcia-Garayoa, K. Ortner, U. Abram, P. A. Schubiger, *Bioconjugate Chem.* **2000**, *11*, 345 – 351.
[19] E. G. Eising, C. Reiners, *Nucl. Med. Commun.* **1998**, *19*, 241 – 250.
[20] P. E. Rolan, *Br. J. Clin. Pharmacol.* **1994**, *37*, 125 – 128.
[21] G. Subramanian, J. G. McAfee, *Radiology* **1971**, *99*, 192 – 196.
[22] R. Bares, *Q. J. Nucl. Med.* **1998**, *42*, 43 – 48.
[23] M. Korner, B. Waser, J. C. Reubi, *Lab. Invest.* **2004**, *84*, 71 – 80.
[24] M. Korner, B. Waser, J. C. Reubi, *Int. J. Cancer* **2005**, *115*, 734 – 741.

