[CANCER RESEARCH 64, 6707–6715, September 15, 2004]

Synthesis and Evaluation of Bombesin Derivatives on the Basis of Pan-Bombesin Peptides Labeled with Indium-111, Lutetium-177, and Yttrium-90 for Targeting Bombesin Receptor-Expressing Tumors

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ABSTRACT

Bombesin receptors are overexpressed on a variety of human tumors like prostate, breast, and lung cancer. The aim of this study was to develop radiolabeled (Indium-111, Lutetium-177, and Yttrium-90) bombesin analogues with affinity to the three bombesin receptor subtypes for targeted radiotherapy. The following structures were synthesized: diethylenetriaminepentaacetic acid-γ-aminobutyric acid-[D-Tyr^6, β-Ala^{11}, Thi^{13}, Nle^{14}] bombesin (6–14) (BZH1) and 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid-γ-aminobutyric acid-[D-Tyr^6, β-Ala^{11}, Thi^{13}, Nle^{14}] bombesin (6–14) (BZH2). [^{111}In]-BZH1 and in particular [^{90}Y]-BZH2 were shown to have high affinity to all three human bombesin receptor subtypes with binding affinities in the nanomolar range. In human serum metabolic cleavage was found between β-Ala^{11} and His^{12} with an approximate half-life of 2 hours. The metabolic breakdown was inhibited by EDTA and β-Ala^{11}-His^{12} (carnosine) indicating that carnosinase is the active enzyme.

Both ^{111}In-labeled peptides were shown to internalize into gastrin-releasing peptide-receptor–positive AR4–2J and PC-3 cells with similar high rates, which were independent of the radiometal. The biodistribution studies of [^{111}In]-BZH1 and [^{111}In]-BZH2 ([^{177}Lu]-BZH2) in AR4–2J tumor-bearing rats showed specific and high uptake in gastrin-releasing peptide-receptor–positive organs and in the AR4–2J tumor. A fast clearance from blood and all of the nontarget organs except the kidneys was found. These radiopeptides were composed of the first pan-bombesin radioligands, which show great promise for the early diagnosis of tumors bearing not only gastrin-releasing peptide-receptors but also the other two bombesin receptor subtypes and may be of use in targeted radiotherapy of these tumors.

INTRODUCTION

The development of ligand-targeted therapeutics in anticancer therapy including drug-ligand conjugates has gained momentum in recent years (1). Systemic cytotoxic chemotherapy shows little selectivity and is limited by potentially serious side effects. One strategy to improve the lack of selectivity is to couple therapeutics to vectors like monoclonal antibodies, their fragments, or even smaller molecules (2). The cytotoxic drug part of conjugates used in ligand-targeted therapeutics is often composed of a therapeutic radiometal encapsulated by its bifunctional chelator.

A very promising group of small targeting ligands is composed of regulatory peptides (3). A high number of peptide receptors were shown to be overexpressed in various human tumors (4). They are promising targets for molecular imaging and targeted therapy of cancer, because they are located on the plasma membrane and, upon binding of a ligand, the receptor-ligand complex is internalized. These findings were the basis for the development of diagnostic and therapeutic radiopeptides useful in peptide receptor scintigraphy and targeted radiotherapy (5–10). Among the most relevant peptide receptors, the bombesin receptors are of major interest, because they were found to be overexpressed in various important cancers like prostate (11, 12), breast (13, 14), and small cell lung cancer (15). The human counterparts of bombesin, namely gastrin-releasing peptide (16) and neuromedin B (17), have been found in mammalian tissue. They bind to different bombesin receptor subtypes, such as the neuromedin B preferring receptor (BB1 receptor; ref. 18), the gastrin-releasing peptide preferring receptor (BB2; ref. 19), as well as the orphan bombesin receptor subtype-3 (BB3 receptor; ref. 20) and the BB4 receptor (21). The BB1, BB2, and BB3 receptors have been shown recently to be overexpressed on different human tumors (22). Gastrin-releasing peptide receptors were predominantly expressed in human prostate cancer (100%), gastrinoma (100%), and breast cancer (70%), whereas concomitant expression of gastrin-releasing peptide receptor (33%) and BB3 receptor (40%) were found in small cell lung cancer. Also gastrin-releasing peptide receptor (40%) and BB3 (25%) were found concomitantly in renal cell carcinoma. Preferential expression of BB1 was found in intestinal carcinoids (11 of 24), and bronchial carcinoids had preferential BB3 receptor expression (9 of 26).

These findings provide a possibility to apply bombesin-like peptides as a vehicle for delivering cytotoxic drugs (23–25) into tumor cells. In addition, radiolabeling may allow us to diagnose and treat these tumors (10, 26–37). The sequence bombesin(7–14) was regarded to be sufficient for the specific binding interaction with the gastrin-releasing peptide receptor (38, 39). Therefore, most radiolabeled bombesin-like peptides are based on the sequence bombesin (7–14) (10, 28–31, 33–35). For example, different conjugates were developed using bifunctional chelators for labeling with ^{99m}Tc, like N_2S_2 (29), N_3S (31), N_α-histidinyl acetate (35), and diaminopropionic acid (36), using the carbonyl approach. Also, diethylenetriaminepentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) were coupled to this sequence for labeling with hard Lewis acid radiometals like ^{111}In, $^{67,\ 68}$Ga, ^{90}Y, and the lanthanides. Some ^{99m}Tc-labeled peptides have been or are currently being investigated in gastrin-releasing peptide receptor-positive tumors in patients (30, 31, 33).

Recently, a universal ligand, (D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}) bombesin (6–14), has been developed by Mantey *et al.* (40) and Pradhan *et al.* (41), which has high affinity to all of the bombesin receptor subtypes. The finding that not only the gastrin-releasing peptide receptor is overexpressed on human tumors but in some cases also neuromedin B and BB3 receptor subtypes prompted us to develop conjugates based on the slightly modified (Thi^{13} *versus* Phe^{13}) universal bombesin ligand [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}] bombesin (6–14) that can be labeled with hard Lewis acid-type metallic

Received 12/09/03; revised 4/22/04; accepted 7/8/04.

Grant support: Supported analytically by Novartis and financially by the Swiss National Science Foundation (Grant Nr. 3100A0–100390), by the Amt für Ausbildungsbeiträge (H. Zhang), the Commission for Technology and Innovation (KTI-project 4668–1 EUS), and by Mallinckrodt Med.

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radionuclides like ^{111}In, ^{90}Y, and ^{177}Lu. The Thi^{13} *versus* Phe^{13} modification was done because preliminary data from our laboratory have shown an increased metabolic stability of this peptide over the Phe^{13} analogue. We studied these (radio)metallopeptides with regard to the bombesin receptor subtype profile. The internalization rate of the two chelated peptides, labeled with the indicated radiometals, into AR4–2J (rat pancreatic tumor cells bearing the gastrin-releasing peptide receptor) and PC-3 (human prostate cancer cell line) cells was studied as well. We also report on the metabolic stability in human blood serum and the identification of metabolites. In addition, the biodistribution of the ^{111}In-labeled peptides was studied. This work is the first one of a pan-bombesin ligand aimed at radiotargeted diagnosis and therapy.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from commercial sources and used without additional purification. DOTA-tris(tBu ester) was commercially available (Macrocyclics, Dallas, TX) or was synthesized according to Heppeler *et al.* (42). DTPA(tris-tBu) was received from Mallinckrodt Medical (Dr. Ananth Srinivasan, St. Louis, MO). Rink amide MBHA resin and all of the Fmoc-protected amino acids were commercially available from NovaBiochem (Läufelfingen, Switzerland). [^{111}In]Cl_3 was purchased from Mallinckrodt Medical (Petten, the Netherlands), [^{90}Y]Cl_3 from Perkin-Elmer Life Sciences Inc. (Boston, MA), and [^{177}Lu]Cl_3 from I.D.B. (Petten, the Netherlands). Electrospray ionization mass spectroscopy was carried out with a Finnigan SSQ 7000 spectrometer or fast atom bombardment mass spectroscopy with a VG 70SE spectrometer and matrix-assisted laser desorption ionization-mass spectrometry measurements on a Voyager sSTR equipment with a Nd:YAG laser (Applied Biosystems, Framingham, NY). Analytical high-performance liquid chromatography (HPLC) was performed on a Hewlett Packard 1050 HPLC system with a multiwavelength detector and a flow-through Berthold LB 506 Cl γ-detector using a Macherey-Nagel Nucleosil 120 C_{18} column. Preparative HPLC was performed on a Metrohm HPLC system LC-CaDI 22–14 with a Macherey-Nagel VP 250/21 Nucleosil 100–5 C_{18} column. Quantitative gamma counting was performed on a COBRA 5003 γ-system well counter from Packard Instruments. Solid-phase peptide synthesis was performed on a semiautomatic peptide synthesizer commercially available from Rink Combichem (Bubendorf, Switzerland). The cell culture medium was DMEM with 10% FCS from Bioconcept.

Synthesis. The peptide synthesis was performed on a semiautomatic peptide synthesizer according to a general procedure described previously (ref. 43; Fig. 1). Standard Fmoc chemistry was used throughout (44); the peptide was assembled on a Rink amide MBHA resin. Trt and tBu were used as protecting groups of His and D-Tyr, respectively, and Boc for Trp. The chelators were coupled as follows. Three equivalents DTPA(tBu)$_3$ were preincubated with *N*,*N*′-diisopropylcarbodiimide in 1-methyl-2-pyrrolidone for 30 min and incubated with the resin-assembled peptide until the 2,4,6-trinitrobenzenesulfonic acid test was negative (~3 hours). DOTA(tBu)$_3$ was coupled as described (43). The peptide chelator conjugates were cleaved from the resin and deprotected by incubation with trifluoroacetic acid—thioanisole–water 92:6:2 for 4 to 6 hours at room temperature and precipitated in isopropyl ether–petroleum ether (1:1). The crude peptide-chelator conjugate was purified by preparative HPLC (Macherey-Nagel Nucleosil 100–5 C_{18}, flow: 15 mL/min; eluents: A = 0.1% trifluoroacetic acid in water and B = acetonitrile; nonlinear gradient: 0 min, 70% A; 10 min, 50% A). Mass spectrometry [(−)electrospray ionization, matrix-assisted laser desorption ionization] was used to determine the composition of the conjugates.

The Potential Metabolites. Diethylenetriaminepentaacetic acid-γ-aminobutyric acid (DTPA-GABA), DTPA-GABA-D-Tyr, DTPA-GABA-D-Tyr-Gln, DTPA-GABA-D-Tyr-Gln-Trp, DTPA-GABA-D-Tyr-Gln-Trp-Ala-Val, DTPA-GABA-D-Tyr-Gln-Trp-Ala-Val-β-Ala, and DTPA-GABA-D-Tyr-Gln-Trp-Ala-Val-β-Ala-His were synthesized in parallel using the same protocol as described above.

[In^{III}]-BZH1. The metal complex was synthesized according to the methods described previously (42). A mixture of DTPA-GABA-[D, Tyr^6, β-Ala^{11}, Thi^{13}, Nle^{14}] BN(6–14) (BZH1; 0.5 μmol) in 500 μL of 0.4 mol/L sodium acetate buffer (pH 5.0) was incubated with 1.5 μmol $InCl_3 \cdot 5H_2O$ in 0.04 mol/L HCl for 1 h at room temperature and purified over a SepPak C_{18} cartridge preconditioned with 10 mL of EtOH and 10 mL of water. The cartridge was eluted with 10 mL of water followed by 3 mL of methanol resulting in [^{111}In]-BZH1 after evaporation of the methanol. The final product was analyzed by analytical HPLC; the purity was ≥97%. Mass spectrometry [matrix-assisted laser desorption ionization, *m*/*z* (%)]: 1711.0 (100, [M+H]$^+$), 1732.9 (25, [M+Na]$^+$).

[Y^{III}]-DOTA-GABA-[D-Tyr^6, β-Ala^{11}, Thi^{13}, Nle^{14}] BN (6–14) (BZH2) was synthesized using $Y(NO_3)_3 \cdot 5H_2O$ as described above except that the incubation was at 95°C for 20 to 25 minutes. The final product was analyzed by analytical HPLC; the purity was >95%. Mass spectrometry (matrix-assisted laser desorption ionization; *m*/*z* (%)):1696.8 (100, [M+H]$^+$), 1718.8 (20, [M+Na]$^+$), 1734.8 (10, [M+K]$^+$).

Preparation of the Radiotracer. [^{111}In]-BZH1 was prepared by dissolving 10 μg of BZH1 (6.25 nmol) in sodium acetate buffer [300 μL and 0.4 mol/L (pH 5.0)] and by incubation with $^{111}InCl_3$ (3 to 6 mCi) for 1 hour at room temperature. A 1.5 molar excess of $InCl_3 \cdot 5H_2O$ was added and the final solution incubated again at room temperature for 1 hour. Subsequently, radiometalated peptides were purified using a SepPak C_{18} cartridge as described above affording a very pure ^{111}In/^{nat}In-labeled ligand for internalization studies. For biodistribution and serum stability studies, the labeling was performed accordingly without the addition of cold $InCl_3 \cdot 5H_2O$. For injection the solution was prepared by dilution with 0.9% NaCl (0.1% bovine serum albumin) to afford the radioligand solution.

[^{90}Y/^{111}In/^{177}Lu]-BZH2 were prepared and purified accordingly by heating at 95°C for 20 to 25 minutes; $Y(NO_3)_3 \cdot 5H_2O$, $Lu(NO_3)_3 \cdot 6H_2O$, and $InCl_3 \cdot 5H_2O$ were used.

Serum Stability and Identification of Metabolites. To 1 mL of freshly prepared human serum, previously equilibrated in a 5% CO_2 (95% air) environment at 37°C, we added 0.6 nmol ^{111}In-labeled BZH1 or BZH2 standard solution. The mixture was incubated in a 5% CO_2, 37°C environment. At different time points, 50-μL aliquots (in triplicate) were removed and treated with 60 μL of EtOH. Samples were then cooled (4°C) and centrifuged for 15 min at 500 × *g* and 4°C to precipitate serum proteins. Fifty μL of supernatant were removed for activity counting in a γ-well counter, the sediment was washed twice with 1 mL of EtOH and counted, and the activity in the supernatant was compared with the activity in the pellet to give the percentage of peptides not bound to proteins or radiometal transferred to serum proteins. The supernatant was analyzed with HPLC (eluents: A = 0.1% trifluoroacetic acid in water and B = acetonitrile; gradient: 0 to 45 minutes, 95% to 60% A; 45 to 46 minutes, 100% B; 46 to 49 minutes, 100% B; 50 minutes, 95% A) to determine relative amounts of metabolites.

The data points were fitted using origin 6 (Microcal Software, Inc., Northampton, MA), assuming a consecutive reaction (Eq. A) and Eq. B and C to calculate the disappearance of intact peptide A as well as the formation and disappearance of metabolite B, respectively.

$$A \xrightarrow{k1} B \xrightarrow{k2} C + D \tag{A}$$

$$[A] = 100 \cdot e^{-k1t} \tag{B}$$

$$[B] = 100 \cdot \frac{k1}{k1 - k2} \cdot (e^{-k2t} - e^{-k1t}) \tag{C}$$

To identify the metabolites, [^{111}In]-BZH1 was used as the leading peptide. The extracted supernatant, obtained as described above, was co-injected with the potential "metabolites," synthesized as described above. The metabolism in serum was studied by the addition of EDTA (2.4 mmol/L) or carnosine (22.4 mmol/L) to [^{111}In]-BZH1 serum solutions.

Binding Affinity and Receptor Subtype Profile. The binding affinity profiles of [In^{III}]-BZH1 and [Y^{III}]-BZH2 for the three bombesin receptor subtypes were determined *in vitro* using receptor autoradiography. Human tumors were selected that had been shown previously to express predominantly one single bombesin receptor subtype, namely either neuromedin B receptor, gastrin-releasing peptide receptor or BB_3 receptor. IC_{50} were measured in

competitive binding experiments performed with increasing concentrations of [In^{III}]-BZH1, [Y^{III}]-BZH2 and [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14; as reference) in successive tissue sections containing tumors expressing either gastrin-releasing peptide-receptors, neuromedin B receptors or BB3 receptors, using [^{125}I-D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]bombesin(6–14) as universal radioligand, as described in detail previously (22, 45).

Cell Culture. AR4–2J rat pancreatic tumor cells and PC-3 cells were cultured in Dulbecco's minimal essential medium (DMEM). DMEM was supplemented with vitamins, essential and nonessential amino acids, L-glutamine, antibiotics (penicillin/streptomycine), fungicide (amprotencine) and 10% fetal calf serum (FCS) as described elsewhere (46).

Internalization and Externalization (Efflux) Studies. Internalization and externalization experiments were performed in 6-well plates as indicated in a previous publication (46). The procedure was the same for both cell lines. Briefly, the cells were washed twice with the internalization medium and allowed to adjust to the medium for 1 h at 37°C. Approximately 1.8 kBq (0.25 pmol) of radioligand were added to the medium and the cells (10^6 cells per well) and incubated (in triplicates) for 0.5, 1, 2, 4 and 6 h at 37°C, 5% CO_2, with or without excess of cold BZH2 (150 μL of a 5.8 μmol/L solution, final concentration of cold BZH2 was 0.58 μmol/L) to determine nonspecific internalization. The final volume was 1.5 ml. At appropriate time points the internalization was stopped by removal of the medium followed by washing the cells with ice-cold solution composed of 0.9% NaCl/0.01 mol/L Na_2HPO_4/0.01 M KH_2PO_4 (pH 7.2). Cells were then treated 5 min (twice) with glycine buffer (0.05 mol/L glycine solution, pH adjusted to 2.8 with 1 mol/L HCl) to distinguish between cell surface-bound (acid releasable) and internalized (acid resistant) radioligand. Finally, cells were detached from the plates by incubation with 1 mol/L NaOH for 10 min at 37°C, and the radioactivity was measured in a γ-counter.

For externalization studies, the AR4–2J cells were allowed to internalize the radioligands for periods of 2 h and were then exposed to an acid wash, as described in the previous section, to dissociate cell-surface-bound radioligand. One ml of culture medium was added to each well, cells were incubated at 37°C in a 5% CO_2 environment and externalization of the cell-incorporated radioactivity was studied at different times. The culture medium was collected and measured for radioactivity.

Biodistribution Experiments in AR4–2J Tumor Bearing Lewis Rats. Lewis male rats were implanted subcutaneously with 10 millions AR4–2J tumor cells, which were freshly expanded in a sterilized solution of 0.9% NaCl/0.01 mol/L Na_2HPO_4/0.01 mol/L KH_2PO_4 (pH 7.2).

Fourteen days after inoculation the tumors weighed 0.3–1.2 g and the rats were injected into the back leg vein with 0.1 μg radiolabeled peptides (about 0.5 MBq ^{111}In or 0.9 MBq ^{177}Lu), diluted in NaCl (0.1% bovine serum albumin, pH 7.4, total injected volume = 200 μL). For the determination of nonspecific uptake in tumor or receptor positive organs, a group of 4 animals was injected with a mixture of 0.1 μg radiolabeled peptide/50 μg BZH2 in 0.9% NaCl solution (injected volume, 225 μL). At 4 h, 24 h, 48 h, and 72 h rats (in groups of 4–11 rats) were sacrificed, and organs of interest were collected, rinsed of excess blood, blotted, weighed and counted in a γ-counter. The percentage of injected dose per gram (% ID/g) was calculated for each tissue. The total counts injected per animal were determined by extrapolation from counts of an aliquot taken from the injected solution as a standard.

All animal experiments were performed in compliance with the Swiss regulation for animal treatment (Bundesamt für Veterinärwesen, approval no. 789).

RESULTS

Synthesis and Labeling. BZH1 and BZH2 (Fig. 1) were synthesized using Fmoc strategy affording an overall yield of approximately 30% based on the removal of the first Fmoc group; the purity analyzed by HPLC was $\geq$ 97%. BZH1 was labeled with ^{111}In by incubation at room temperature (1 h incubation, pH 5, 0.4 mol/L sodium acetate buffer). BZH2 was labeled with ^{111}In, ^{90}Y, and ^{177}Lu at elevated temperature (95°C, 20–25 min). In all cases, labeling yields of $\geq$ 98% at specific activities of $>$37 GBq μmol^{-1} were achieved.

Receptor Binding Affinity and the Receptor Subtype Profile. Table 1 shows the bombesin receptor subtype binding profile of the 2 new metallopeptides. As reference peptide [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}] bombesin(6–14) was used, which binds with high affinity to all 3 bombesin receptor subtypes. Although less potent than the reference peptide, the 2 new bombesin analogues still have retained high affinity to all three receptor subtypes. The IC_{50} values of [In^{III}]-BZH1 are 3.47 $\pm$ 0.32 nM to the gastrin-releasing peptide receptor, 10.5 $\pm$ 3.03 nM to the neuromedin B receptor, and 41.7 $\pm$ 22.2 nM to the BB3 receptor. The respective values for [Y^{III}]-BZH2 are 1.40 $\pm$ 0.10 nM, 4.93 $\pm$ 1.03 nM, and 10.7 $\pm$ 4.2 nM.

Stability in Human Serum and Identification of Metabolites. After incubation of the radiolabeled peptides with fresh serum, three metabolites were determined using HPLC and radiometric analysis. The proteic fraction obtained as pellet contained $<$5% of radioactivity. [^{111}In]-BZH1 was metabolized to [^{111}In]-DTPA-GABA-D-Tyr-Gln-Trp-Ala-Val-β-Ala (corresponding to B) due to the cleavage between β-Ala^{11} and His^{12}, and then this metabolite decomposed to [^{111}In]-DTPA-GABA-D-Tyr-Gln (C) and [^{111}In]-DTPA (D; Fig. 2). These metabolites were identified using reverse-phase HPLC by co-injection of presynthesized chelator-conjugated peptides. The similarity of HPLC elution times indicates that [^{111}In]-BZH2 is metabolized to the same breakdown products. There was no indication of radiometal transfer to serum proteins during incubation studies.

Fig. 3 shows the kinetics of metabolic degradation of [^{111}In]-BZH1 and [^{111}In]-BZH2. Curve fitting data gave rate parameters for the disappearance of intact peptides (A, A′) and the built-up of metabolites (B, C, D; B′, C′, D′).

The respective half-lives for [^{111}In]-BZH1 and [^{111}In]-BZH2 were determined to 2.0 hours and 2.3 hours; however, the first metabolite B′ of [^{111}In]-BZH2 was more stable than B of [^{111}In]-BZH1, and their half-lives of disappearance are 26 hours and 46 hours, respectively (Table 2). When carnosine or EDTA were incubated together with [^{111}In]-BZH1 in serum, [^{111}In]-BZH1 is significantly more stable than without these inhibitors (Fig. 4).

Internalization and Externalization Studies. Both ^{111}In-labeled radiopeptides showed a fast cell uptake, which did not reach a plateau within 6 hours of incubation at 37°C in AR4–2J cells (Figs. 5 and 6). Internalization was strongly reduced in the presence of 0.57 μmol/L unlabeled BZH2 (data not shown). Nonspecific internalization was $<$1% of the added activity. The surface bound peptide (acid removable) was $<$7% of the added activity (data not shown). BZH2 labeled with different radioisotopes (^{111}In, ^{177}Lu, and ^{90}Y) exhibited the same internalization rates (Fig. 5). [^{111}In]-BZH1 was internalized by AR4–2J and PC-3 cells with a similar kinetics (Fig. 4). Preliminary studies using *in vitro* receptor autoradiography showed that AR4–2J and PC-3 tumors express predominantly the gastrin-releasing peptide receptor.[3]

The kinetics of externalization of both peptides was studied with cells exposed for 2 hours to the radioligand as described for internalization. Within 2 hours, 45% of [^{111}In]-BZH1 and 30% of [^{111}In]-BZH2 were released from the AR4–2J cells. The efflux of [^{111}In]-BZH1 was faster than that of [^{111}In]-BZH2 (Fig. 7). To identify the composition of externalized peptide, [^{111}In]-BZH1 was used as a leading peptide. The externalized radioactivities corresponded to the intact peptide and one metabolite [^{111}In(DTPA)]$^{2-}$, identified by HPLC using co-injection.

Upon 2 hours of internalization and acid wash, the externalized radioactivity already after 30 min consisted of $\sim$50% [^{111}In-(DTPA)]$^{2-}$ as the only metabolite and 50% intact radiolabeled pep-

[3] J. C. Reubi, unpublished observations.

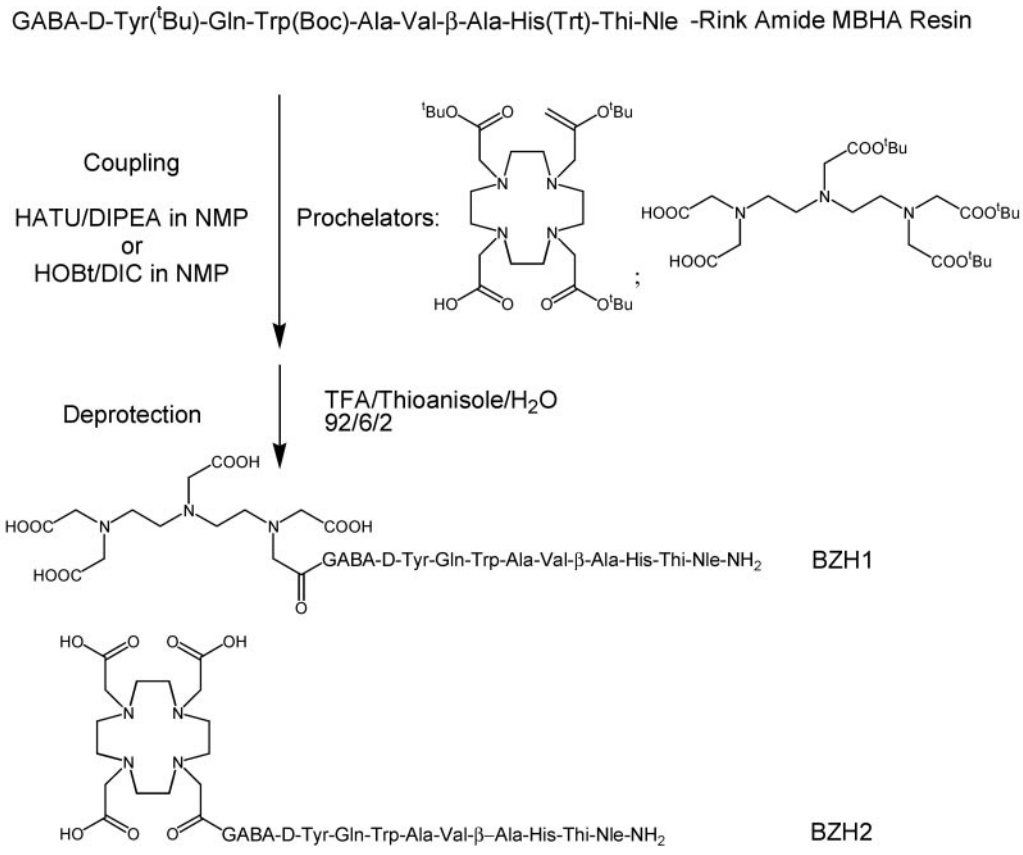

Fig. 1. GABA-D-Tyr(tBu)-Gln-Trp(Boc)-Ala-Val-β-Ala-His(Trt)-Thi-Nle -Rink Amide MBHA Resin.

tide. At 4 hours, the externalized radioactivity consisted of the metabolite $[^{111}In(DTPA)]^{2-}$ (87.5%) and intact peptide (12.5%), whereas at 24 hours, only the metabolite was found.

Animal Biodistribution Studies. Results from biodistribution studies using the ^{111}In-labeled peptides performed with Lewis rats bearing the AR4–2J pancreatic tumor are presented in Table 3 as the percentage of injected dose per gram of tissue (% ID/g). Tumor uptake and retention of [^{111}In]-BZH2 is also shown in Fig. 8.

Both [^{111}In]-BZH1 and [^{111}In]-BZH2 displayed rapid blood clearance with <0.02% ID/g and 0.01% ID/g at 4 hours, respectively. Fast clearance from the gastrin-releasing peptide receptor-negative tissues except the kidneys was found as well. [^{111}In]-BZH1 and [^{111}In]-BZH2 show high uptake values in the AR4–2J tumor and in the gastrin-releasing peptide receptor-positive organs, *e.g.*, at 4 hours: tumor, 1.71 ± 0.51% ID/g *versus* 0.79 ± 0.07% ID/g; and pancreas, 3.92 ± 0.86% ID/g *versus* 2.63 ± 0.59% ID/g. The tumor uptake of [^{111}In]-BZH2 dropped from 0.79 ± 0.07% ID/g at 4 hours to 0.44 ± 0.07% ID/g at 24 hours, 0.42 ± 0.05 at 48 hours, and 0.33 ± 0.08 at 72 hours. The respective pancreas values are 2.63 ± 0.59 at 4 hours, 2.13 ± 0.68 at 24 hours, 2.07 ± 0.22 at 48 hours, and 1.65 ± 0.32 at 72 hours.

In vivo competition experiments using 50 μg of BZH2 co-injected with [^{111}In]-BZH1 (data for [^{111}In]-BZH2 in brackets) resulted in a >93% (89%) reduction of tumor uptake and also in a reduction of the uptake in normal gastrin-releasing peptide receptor-positive organs (>97% for both radiopeptides in the pancreas). Also, the uptake in other organs could be blocked to a high degree: stomach 84% (65%), bowel 72% (82%), and adrenals 73% (66%). The injection of the blocking dose had no significant influence on the uptake in nontarget organs except the kidneys where the uptake decreased by a factor of 1.5 on blocking. Due to the fast clearance of both peptides, high tumor–background ratios were found (Table 3).

^{111}In was used as a surrogate of ^{90}Y for studying the pharmacokinetics of BZH2, because the latter is a pure β-emitter. This is the strategy most often used in such studies, although there may be some differences between the two due to structural differences (47, 48). Therefore, we also studied [^{177}Lu]-BZH2 as a radiotherapeutic peptide radiopharmaceutical. ^{177}Lu is a low energy β-emitter that has two γ-lines at 133 keV (7%) and 208 keV (11%) allowing convenient localization. The organ uptake values of [^{177}Lu]-BZH2 were very similar to those of [^{111}In]-BZH2. The values for the most important organs are: tumor 0.67 ± 0.04% ID/g (4 hours) and 0.42 ± 0.03% ID/g (24 hours); pancreas 2.39 ± 0.24% ID/g (4 hours) and 1.92 ± 0.25% ID/g (24 hours); and kidneys 1.17 ± 0.37% ID/g (4 hours) and 0.54 ± 0.07% ID/g (24 hours).

Table 1 *Affinity profiles (IC_{50}) for human BB1-BB3 receptors of the bombesin analogues [In^{III}]-BZH1 and [Y^{III}]-BZH2*

Peptide	BB1 (NMB-R)	BB2 (GRP-R)	BB3-R
[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]BN (6–14)	1.01 ± 0.06 (5)	0.68 ± 0.05 (5)	1.73 ± 0.64 (3)
[In^{III}]-BZH1	10.5 ± 3.03 (3)	3.47 ± 0.32 (3)	41.7 ± 22.2 (3)
[Y^{III}]-BZH2	4.93 ± 1.03 (3)	1.40 ± 0.10 (3)	10.7 ± 4.2 (3)

NOTE. The IC_{50} values (nM ± SE) are in triplicates. The number of independent studies are in brackets.

Abbreviations: BN, bombesin; NMB, neuromedin B; R, receptor; GRP, gastrin-releasing peptide.

DISCUSSION

This study describes two very promising pan-bombesin peptidic ligands for radiolabeling with diagnostic and therapeutic radiometals.

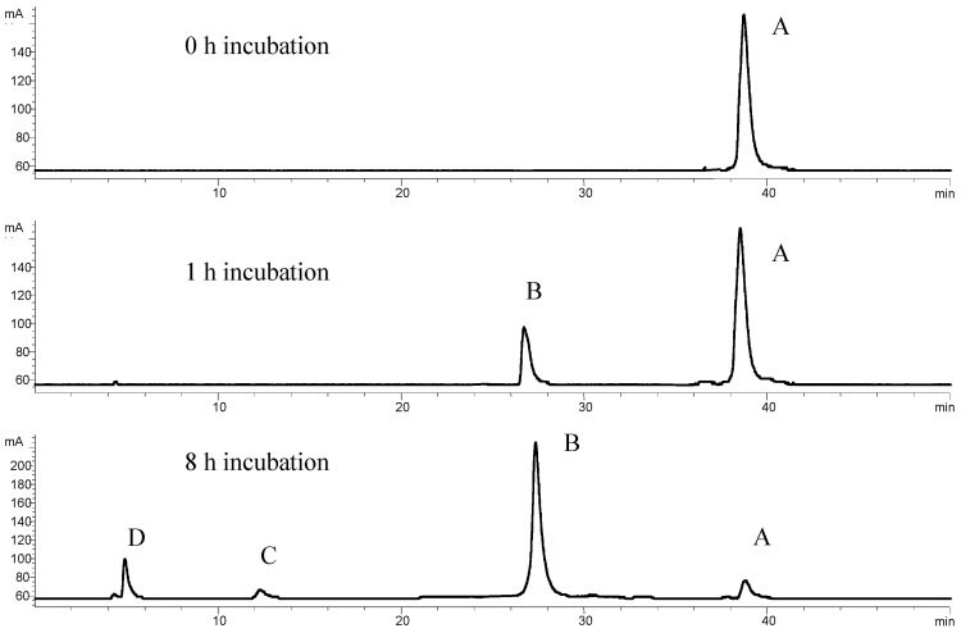

Fig. 2. HPLC elution profile of [^{111}In]-BZH1 after incubation with fresh human serum, immediately after incubation, t_R = 38.7 min; 1 hour after incubation, t_R ([^{111}In]-BZH1) = 38.6 minutes, t_R (metabolite B) = 27.3 min; 8 hours after incubation, t_R ([^{111}In]-BZH1) = 38.7 minutes, t_R (metabolite B) = 27.5 minutes, t_R (metabolite C) = 12.3 minutes, t_R (metabolite D) = 5.1 minutes.

They may be used for targeted diagnosis and radiotherapy of bombesin receptor-positive tumors like prostate and breast cancer.

A variety of radiopeptides are currently being developed for the targeting of tumors (5–10). Those based on bombesin are of interest, because bombesin receptors were shown to be overexpressed on a variety of frequently occurring tumors like breast cancer and prostate cancer. In addition, Markwalder and Reubi (11) not only found a massive gastrin-releasing peptide receptor overexpression in invasive prostate cancer tissue but also in the early stage of the disease, *i.e.*, the prostatic intraepithelial neoplasia, which may open the possibility to localize an early event in prostate carcinogenesis.

Many reports on bombesin-based radiopeptides have been published recently (26–36), but there has been no study thus far on radiopeptides based on pan-bombesin ligands. These may be of interest, because not only the gastrin-releasing peptide (BB2)-receptor but also BB1-receptor and BB3-receptor were found to be overexpressed on human tumors as well (22).

In our present study we evaluated the slightly modified pan-bombesin ligand [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}] bombesin(6–14) developed by the Jensen group (40, 41), by attaching DTPA(BZH1) and DOTA(BZH2) via a GABA spacer to the octapeptide. The chelators allow a high specific activity labeling with ^{111}In (DTPA). DOTA chelates a large number of radiometals with extremely high kinetic stability like ^{68}Ga for PET-studies; ^{111}In for SPECT; and ^{90}Y, ^{177}Lu, and other lanthanides for therapeutic applications.

The receptor binding profiles of [In^{III}]-BZH1 and [Y^{III}]-BZH2 to the three bombesin receptor subtypes BB1, BB2, and BB3 were tested on human tumor specimen preferentially expressing each of the three receptor subtypes. Both metallopeptides show high binding affinity to the gastrin-releasing peptide receptor and slightly

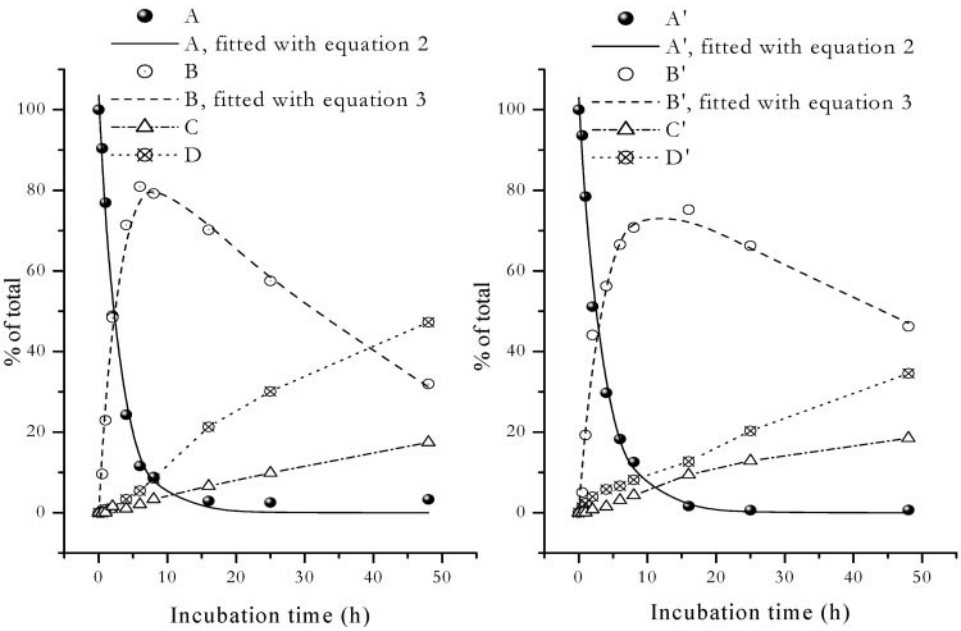

Fig. 3. Comparison of kinetic stability of [^{111}In]-BZH1 (*left*) and [^{111}In]-BZH2 (*right*) in fresh human serum. Percentage of total peptide after different incubation times with serum under 37°C, 5% CO_2, the concentration is 0.6 nmol radiolabeled peptide per mL serum.

Table 2 *Calculation of serum stabilities according to the equations 2 and 3; A means intact radiolabeled peptide; B represents their first metabolites*

	Equation 2 (A) $= 100 \cdot e^{-k1t}$		Equation 3 (B) $= 100 \cdot k1/k1 - k2 \cdot (e^{-k2t} - e^{k1t})$		
Radioligand	k_1 (h^{-1})	$t^1_{1/2}$ (h)	k_1 (h^{-1})	k_2 (h^{-1})	$t^2_{1/2}$ (h)
[^{111}In]-BZH1	0.35 ± 0.02	2.0	0.32 ± 0.03	0.026 ± 0.001	26
[^{111}In]-BZH2	0.30 ± 0.02	2.3	0.27 ± 0.04	0.014 ± 0.003	46

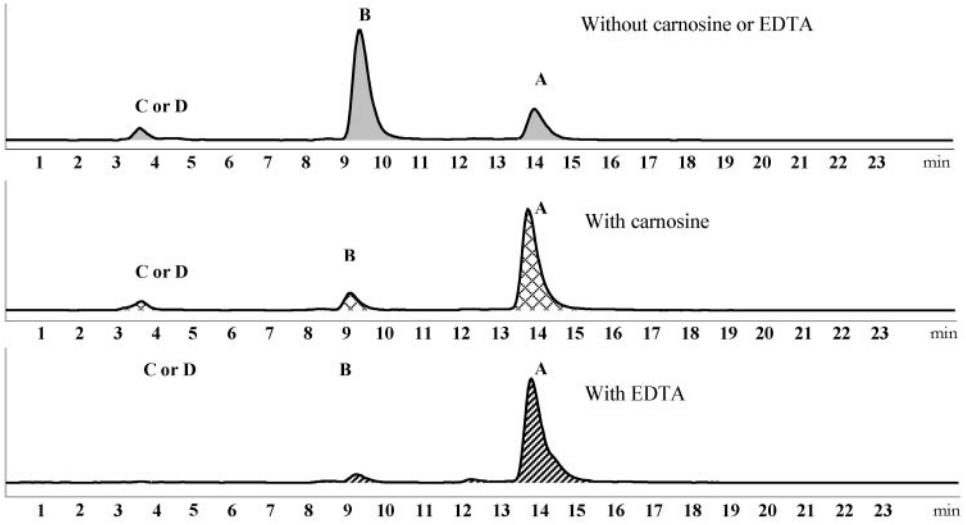

Fig. 4. HPLC elution profile of [^{111}In]-BZH1 after 4-hour incubation with fresh human serum and in the presence of carnosine and EDTA, respectively.

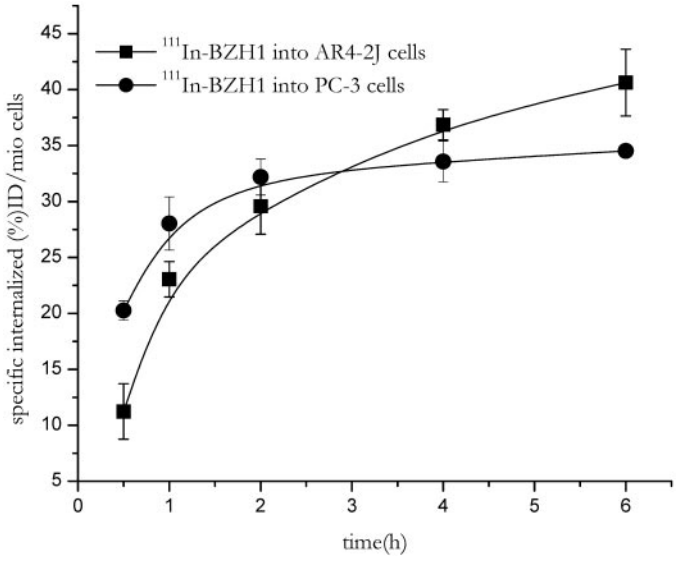

Fig. 5. Comparison of the internalization rate of [^{111}In]-BZH1 into rat pancreatic tumor AR4–2J (■) cells and human PC-3 (●) cells. Data result from two independent experiments with triplicates in each experiment and are expressed as specific internalization; *bars,* ±SD.

lower binding affinities to BB1 receptor and BB3 receptor. The [Y^{III}]-DOTA-derivative shows a distinctly and significantly higher binding affinity to all three of the receptor subtypes compared with the [In^{III}]-DTPA-analogue. We assume that this is due to the extra negative charge at the NH_2 terminus, which was shown to lower the binding affinity of bombesin-based radiopeptides.[4]

Both radiopeptides internalize rapidly, and no significant difference could be found between the two when labeled with ^{111}In. In addition, no significant difference was observed when different radionuclides ([^{111}In/^{90}Y/^{177}Lu]-BZH2) were used with the DOTA-based peptide, indicating that there are no structural differences among these three radiopeptides.

For an optimized use of radiopharmaceuticals in targeted radiotherapy, not only an efficient internalization is of importance but also the trapping (residualization) of the radioactivity adds to the potential success of the treatment via targeted radiotherapy. Therefore, we studied the externalization of [^{111}In]-BZH1 as well as the identity of the externalized radioactivity. The rate of efflux of both compounds was shown to be rather fast, even faster for [^{111}In]-BZH1 than for [^{111}In]-BZH2. The externalized radioactivity consisted preferentially of [^{111}In(DTPA)]$^{2-}$ as the only metabolite. This is in contrast to the metabolites found when incubated in human serum, and it is also in

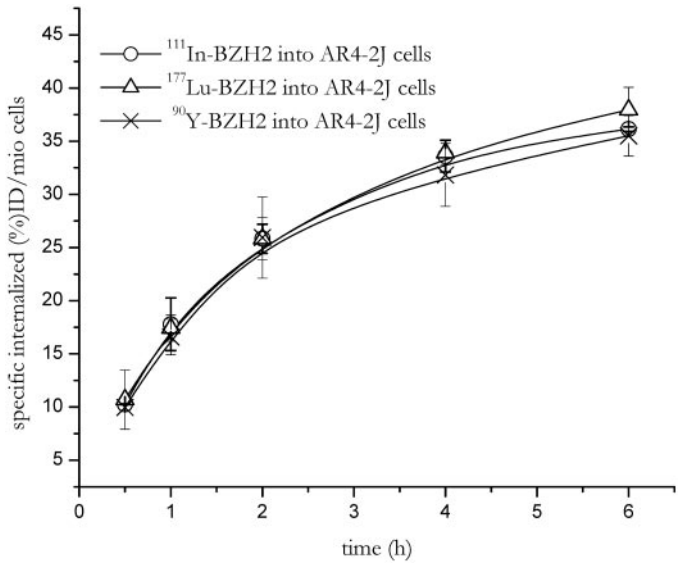

Fig. 6. Comparison of the internalization rate of [^{111}In]-BZH2 (○), [^{90}Y]-BZH2 (×) and [^{177}Lu]-BZH2 (△) into AR4–2J cells. Data result from two independent experiments with triplicates in each experiment and are expressed as specific internalization; *bars,* ±SD.

[4] Unpublished observations.

contrast to the chelator-conjugated somatostatin analogues that show only intact externalized peptide at least within the first 4 hours of externalization (43). At present we have no information on the particular enzymes responsible for the metabolism inside the cell.

An additional important aspect for the suitability of a radiopeptide used in targeted radiotherapy is its metabolic stability in human serum. High metabolic stability allows the radiopeptide to reach the target intact and in optimal concentrations. Both peptides were studied in the form of their ^{111}In-labeled versions in fresh human serum. Fig. 2 shows the disappearance kinetics of the two radiopeptides and the build-up and decay of the first metabolite (B). The metabolic stability of both radiopeptides is relatively low with half-lives of 2.0 hours for [^{111}In]-BZH1 and 2.3 hours for [^{111}In]-BZH2, respectively. The curve fitting procedure gave similar results for k_1 (k_1') independent of whether it was derived from the disappearance of A or the build-up and decay of the first intermediate B. Whereas the k_1-values show little difference between the two radiopeptides, the rate of metabolic decay of the intermediate is influenced by the chelate. Reverse-phase HPLC analysis of the metabolites along with co-injection of the predefined metabolites allowed an identification of the decay products.

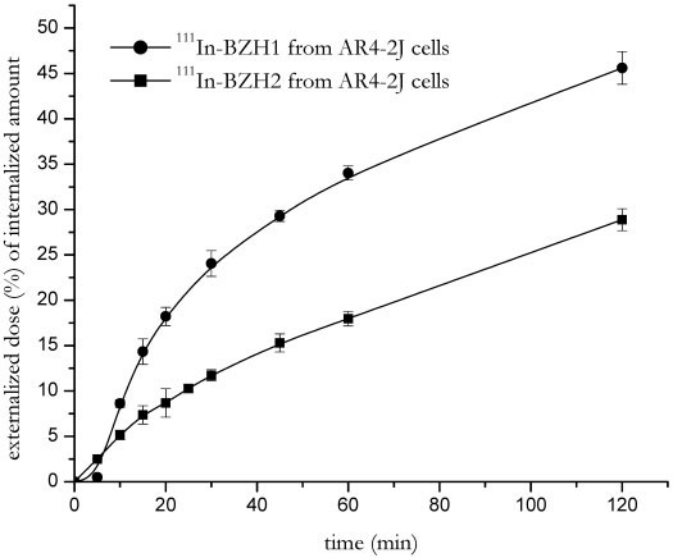

Fig. 7. Comparison of the externalization rate from AR4–2J after 2 hours of internalization and acid wash of [^{111}In]-BZH1(●) and [^{111}In]-BZH2(■); *bars,* ±SD.

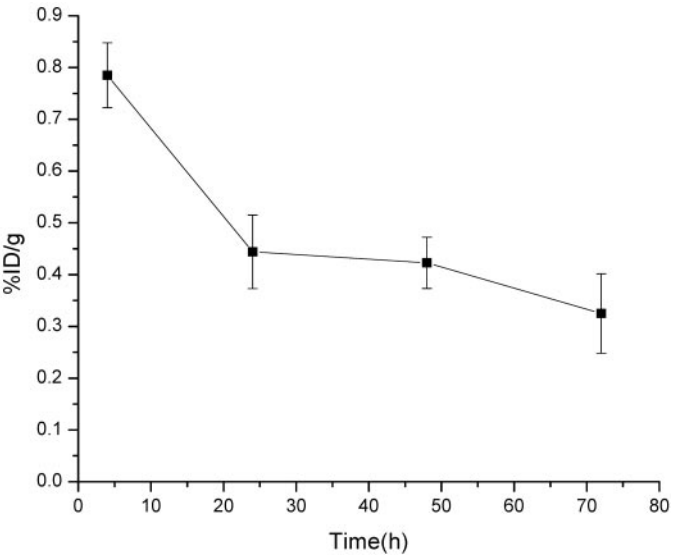

Fig. 8. Tumor retention of ^{111}In-BZH2 inAR4–2J tumor-bearing rats. The uptake is given in percentage of injected dose per g (*%ID/g*) tumor at 4 hours, 24 hours, 48 hours, and 72 hours.

The definition of the cleavage sites and the characterization of the metabolites are of importance, because this knowledge allows us to define and synthesize peptides of enhanced metabolic stability. Because of lack of reliable access to liquid chromatography/mass spectrometry, we mainly relied on the synthesis of potential metabolites and their co-injection using reverse-phase HPLC and radiometric detection. This procedure allowed us to define the first degradation site of both peptides between β-Ala^{11} and His^{12} and the second degradation site between Gln^{7} and Trp^{8}. The question arises as to which enzyme may be responsible for the processing of this amino acid sequence. A first experiment adding EDTA to the human serum/radiopeptide mixture slowed down the metabolic degradation distinctly indicating that the responsible enzyme is a metalloenzyme. The addition of a large excess of carnosine (β-Ala-His), a dipeptide, which

Table 3 *Biodistribution analyses [% ID/g ± SD, n = 4] and tissue ratios of [^{111}In]-BZH1 and [^{111}In]-BZH2 in AR4-2J tumor-bearing Lewis rats*

	[^{111}In]-BZH1 (4h)		[^{111}In]-BZH2 (4h)			
Site	Unblocked	Blocked*	Unblocked	Blocked*	[^{111}In]-BZH1 (24h)†	[^{111}In]-BZH2 (24h)†
Blood	0.016 ± 0.003	0.010 ± 0.003	0.008 ± 0.001	0.007 ± 0.001	0.005 ± 0.001	0.002 ± 0.000
Muscle	0.010 ± 0.001	0.007 ± 0.003	0.008 ± 0.001	0.008 ± 0.001	0.008 ± 0.002	0.005 ± 0.000
Pancreas	3.92 ± 0.86	0.09 ± 0.02	2.63 ± 0.59	0.05 ± 0.01	2.43 ± 0.34	2.13 ± 0.68
Bowel	0.33 ± 0.09	0.09 ± 0.05	0.22 ± 0.04	0.04 ± 0.00	0.18 ± 0.06	0.09 ± 0.03
Spleen	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.00
Liver	0.05 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.06 ± 0.00	0.03 ± 0.01	0.03 ± 0.01
Stomach	0.21 ± 0.06	0.04 ± 0.02	0.11 ± 0.01	0.04 ± 0.01	0.12 ± 0.02	0.06 ± 0.02
Adrenals	0.08 ± 0.01	0.02 ± 0.01	0.07 ± 0.01	0.02 ± 0.00	0.06 ± 0.01	0.06 ± 0.01
Kidney	1.15 ± 0.22	0.77 ± 0.21	1.19 ± 0.29	0.90 ± 0.09	0.79 ± 0.12	0.78 ± 0.16
Lung	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Heart	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Bone	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Tumor	1.71 ± 0.51	0.12 ± 0.02	0.79 ± 0.06	0.09 ± 0.00	0.72 ± 0.22	0.44 ± 0.07
			Tumor—normal tissue radioactivity ratios			
Tumor/blood	107		99		144	220
Tumor/muscle	171		99		90	88
Tumor/liver	34		11		24	15
Tumor/kidney	1.5		0.66		0.91	0.56

NOTES. Results are the mean of groups of 4 animals (except for the tumor, pancreas, and kidney of [^{111}In]-BZH2, where 11 animals were studied).
* Blocked by injecting 50 μg BZH2 together with radiopeptide.
† Unblocked.

is cleaved by carnosinase (49), competitively inhibits the metabolic process (Fig. 3). Therefore, we assume that carnosinase, which is present in human serum, is the enzyme cleaving the peptide at β-Ala^{11}-His^{12}. This result also supports the finding that the first enzymatic cleavage occurs between these 2 amino acids.

In biodistribution studies, a strong accumulation of all of the radiotracers in bombesin receptor-positive tissues and the xenografted tumor was observed. As a tumor model the AR4–2J rat pancreatic carcinoma cell line was used, which is known to express high levels of bombesin receptors (50). Lewis rats bearing the AR4–2J solid tumor showed a high and specific uptake of both ^{111}In-labeled peptides as well as [^{177}Lu]-BZH2 in the tumor (and other bombesin receptor-bearing organs and tissues like the pancreas, the stomach, and the intestines). The blood clearance of both radiopeptides is very fast with $<$0.015% ID/g remaining in the blood at 4 hours. Somewhat unexpectedly, the uptake in receptor-positive organs was higher for [^{111}In]-BZH1 compared with [^{111}In]-BZH2 by almost a factor of 2, although the latter has a higher binding affinity to all three of the receptor subtypes, and the rate of internalization is comparable for the two. BZH2 was developed for radiolabeling with therapeutic radionuclides like ^{90}Y and the lanthanides. ^{111}In was used as a surrogate of ^{90}Y, because it can be followed and measured with more certainty than the pure β-emitter ^{90}Y. The two show similar chemistry; nevertheless, some differences in the pharmacokinetics of radiopharmaceuticals labeled with the two and studied in comparison have been reported. ^{177}Lu, another therapeutic radiometal [β_{energy} = 0.49 MeV; γ-emissions 133 keV (7%); 208 keV (11%)], shows great promise in targeted radiotherapy. Therefore, we studied the biodistribution of [^{177}Lu]-BZH2. There was no significant difference between [^{111}In]-BZH2 and [^{177}Lu]-BZH2 indicating that we may expect similar results with [^{90}Y]-BZH2.

The competitive binding studies with cold peptides clearly demonstrated that the uptake of both radiopeptides in relevant target tissues is specific and receptor mediated. The residence times of both peptides in the tumor are not very long; this may originate from a somewhat low metabolic stability in the respective cells and may parallel the relatively low serum stability. Relatively fast tumor washout was also found for other radiometal-labeled, bombesin-based radiopeptides, for instance, ^{99m}Tc-labeled bombesin(7–14) derivatives (34, 35). The identification of the "weakest" peptide bond at β-Ala^{11}-His^{12} will help to improve the stability in future developments.

Tumor–normal tissue radioactivity ratios were very high for both radiopeptides. Ratios of tumor–blood of and tumor–muscle of $>$100 indicate that an early scintigraphic detection with a low background should be feasible. These ratios are higher than for other bombesin-based radiopeptides published recently using the PC-3 (33, 34, 36) and AR4–2J (51) tumor mouse model and argue for an early human use of these compounds. For instance, the tumor–muscle ratio for ^{99m}Tc-RP527 [*N*,*N*-dimethyl-Gly-Ser-Cys-Gly-5-aminovaleric acid-bombesin(7–14)] in a AR4–2J mouse tumor model was 23.5 at 4 hours and 73 at 24 hours. Our values of 171 (for ^{111}In-BZH1) and 103 (for ^{111}In-BZH2) at 4 hours and 90 (98) at 24 hours compare well with these data. In addition, both radioligands appear to interact with the human gastrin-releasing peptide receptor (PC-3 cell line; $2.5 \pm 0.6 \times 10^5$ gastrin-releasing peptide receptor-binding sites per cell; ref. 52) in a way very similar to that with the rat gastrin-releasing peptide receptor ($\sim 1.5 \times 10^5$ gastrin-releasing peptide receptor binding sites per cell; ref. 50). Indeed, first clinical applications in breast and prostate cancer patients, if labeled with ^{68}Ga or ^{111}In, showed very promising tumor localizations. Because we do not expect that the performance with the therapeutic radionuclides is much different, [^{90}Y, ^{177}Lu]-BZH2 may already be good candidates for targeted radiotherapy in patients. However, because the localization of neuromedin B receptor and BB3 receptor in normal human tissues is virtually unexplored, one cannot yet exclude the appearance of unwanted side effects related to yet unknown physiologic bombesin targets. In addition, one may argue that ^{177}Lu with its long physical half-life of 6.65 days may not be the ideal therapeutic radionuclide considering the relatively low metabolic stability of the two radiopeptides. ^{90}Y (half-life = 64 hours) and other radiolanthanides like ^{166}Ho (half-life = 27 hours) or ^{149}Pm (half-life = 53 hours), which also can be labeled to the DOTA-modified peptides, may be more suitable.

ACKNOWLEDGMENTS

We thank Evelyn Peter for reviewing the English grammar.

REFERENCES

1. Allen TM. Ligand-targeted therapeutics in anticancer therapy. Nat Rev Cancer 2002;2:750–63.
2. Maison W, Frangioni JV. Improved Chemical Strategies for the Targeted Therapy of Cancer. Angew Chem Int Ed Engl 2003;42:4726–8.
3. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. Endocr Rev 2003;24:389–427.
4. Reubi JC. Neuropeptide receptors in health and disease: the molecular basis for in vivo imaging. J Nucl Med 1995;36:1825–35.
5. Behr TM, Behe M, Becker W. Diagnostic applications of radiolabeled peptides in nuclear endocrinology. Q J Nucl Med 1999;43:268–80.
6. Breeman WA, de Jong M, Kwekkeboom DJ, et al. Somatostatin receptor-mediated imaging and therapy: basic science, current knowledge, limitations and future perspectives. Eur J Nucl Med 2001;28:1421–9.
7. Heppeler A, Froidevaux S, Eberle AN, Maecke HR. Receptor targeting for tumor localisation and therapy with radiopeptides. Curr Med Chem 2000;7:971–94.
8. Liu S, Edwards DS. ^{99m}Tc-Labeled Small Peptides as Diagnostic Radiopharmaceuticals. Chem Rev 1999;99:2235–68.
9. Okarvi SM. Recent developments in ^{99m}Tc-labelled peptide-based radiopharmaceuticals: an overview. Nucl Med Commun 1999;20:1093–112.
10. Hoffman TJ, Quinn TP, Volkert WA. Radiometallated receptor-avid peptide conjugates for specific in vivo targeting of cancer cells. Nucl Med Biol 2001;28:527–39.
11. Markwalder R, Reubi JC. Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. Cancer Res 1999;59:1152–9.
12. Sun B, Halmos G, Schally AV, Wang X, Martinez M. Presence of receptors for bombesin/gastrin-releasing peptide and mRNA for three receptor subtypes in human prostate cancers. Prostate 2000;42:295–303.
13. Gugger M, Reubi JC. Gastrin-releasing peptide receptors in non-neoplastic and neoplastic human breast. Am J Pathol 1999;155:2067–76.
14. Halmos G, Wittliff JL, Schally AV. Characterization of bombesin/gastrin-releasing peptide receptors in human breast cancer and their relationship to steroid receptor expression. Cancer Res 1995;55:280–7.
15. Toi-Scott M, Jones CL, Kane MA. Clinical correlates of bombesin-like peptide receptor subtype expression in human lung cancer cells. Lung Cancer 1996;15:341–54.
16. McDonald TJ, Jornvall H, Tatemoto K, Mutt V. Identification and characterization of variant forms of the gastrin-releasing peptide (GRP). FEBS Lett 1983;156:349–56.
17. Minamino N, Kangawa K, Matsuo H. Neuromedin B: a bombesin like peptide identified in porcine spinal cord. Biochem Biophys Res Commun 1983;114:542–8.
18. Von Schrenck T, Heinz-Erian P, Moran T, Mantey SA, Gardner J, Jensen R. Neuromedin B receptor in esophagus: evidence for subtypes of bombesin receptors. Am J Physiol 1989;256:G747–58.
19. Spindel ER, Giladi E, Brehm P, Goodman RH, Segerson TP. Cloning and functional characterization of a complementary DNA encoding the murine fibroblast bombesin/gastrin-releasing peptide receptor. Mol Endocrinol 1990;4:1956–63.
20. Fathi Z, Corjay MH, Shapira H, et al. BRS-3: a novel bombesin receptor subtype selectively expressed in testis and lung carcinoma cells. J Biol Chem 1993;268:5979–84.
21. Nagalla SR, Barry BJ, Creswick KC, Eden P, Taylor JT, Spindel ER. Cloning of a receptor for amphibian [Phe^{13}]bombesin distinct from the receptor for gastrin-releasing peptide: identification of a fourth bombesin receptor subtype (BB4). Proc Natl Acad Sci USA 1995;92:6205–9.
22. Reubi JC, Wenger S, Schmuckli-Maurer J, Schaer JC, Gugger M. Bombesin receptor subtypes in human cancers: detection with the universal radioligand ^{125}I-[D-TYR^6, beta-ALA^{11}, PHE^{13}, NLE^{14}] bombesin(6–14). Clin Cancer Res 2002;8:1139–46.
23. Schally AV, Nagy A. Cancer chemotherapy based on targeting of cytotoxic peptide conjugates to their receptors on tumors. Eur J Endocrinol 1999;141:1–14.
24. Nagy A, Armatis P, Cai RZ, Szepeshazi K, Halmos G, Schally AV. Design, synthesis, and in vitro evaluation of cytotoxic analogs of bombesin-like peptides containing doxorubicin or its intensely potent derivative, 2-pyrrolinodoxorubicin. Proc Natl Acad Sci USA 1997;94:652–6.
25. Szereday Z, Schally AV, Nagy A, et al. Effective treatment of experimental U-87MG human glioblastoma in nude mice with a targeted cytotoxic bombesin analogue, AN-215. Br J Cancer 2002;86:1322–7.

26. Hu F, Cutler CS, Hoffman T, Sieckman G, Volkert WA, Jurisson SS. Pm-149 DOTA bombesin analogs for potential radiotherapy. in vivo comparison with Sm-153 and Lu-177 labeled DO3A-amide-betaAla-BBN(7–14)NH(2). Nucl Med Biol 2002;29: 423–30.
27. Breeman WA, De Jong M, Bernard BF, et al. Pre-clinical evaluation of [^{111}In-DTPA-Pro^{1}, Tyr^{4}]bombesin, a new radioligand for bombesin-receptor scintigraphy. Int J Cancer 1999;83:657–63.
28. Karra SR, Schibli R, Gali H, et al. ^{99m}Tc-labeling and in vivo studies of a bombesin analogue with a novel water-soluble dithiadiphosphine-based bifunctional chelating agent. Bioconjug Chem 1999;10:254–60.
29. Baidoo KE, Lin KS, Zhan Y, Finley P, Scheffel U, Wagner HN Jr. Design, synthesis, and initial evaluation of high-affinity technetium bombesin analogues. Bioconjug Chem 1998;9:218–25.
30. Scopinaro F, De Vincentis G, Varvarigou AD, et al. ^{99m}Tc-bombesin detects prostate cancer and invasion of pelvic lymph nodes. Eur J Nucl Med Mol Imaging 2003;30: 1378–82.
31. Van de Wiele C, Dumont F, Dierckx RA, et al. Biodistribution and dosimetry of ^{99m}Tc-RP527, a gastrin-releasing peptide (GRP) agonist for the visualization of GRP receptor-expressing malignancies. J Nucl Med 2001;42:1722–7.
32. Gali H, Hoffman TJ, Sieckman GL, Owen NK, Katti KV, Volkert WA. Synthesis, characterization, and labeling with ^{99m}Tc/^{188}Re of peptide conjugates containing a dithia-bisphosphine chelating agent. Bioconjug Chem 2001;12:354–63.
33. Van de Wiele C, Dumont F, Vanden Broecke R, et al. Technetium-99m RP527, a GRP analogue for visualisation of GRP receptor-expressing malignancies: a feasibility study. Eur J Nucl Med 2000;27:1694–9.
34. Nock B, Nikolopoulou A, Chiotellis E, et al. [^{99m}Tc]Demobesin 1, a novel potent bombesin analogue for GRP receptor-targeted tumour imaging. Eur J Nucl Med Mol Imaging 2003;30:247–58.
35. La Bella R, Garcia-Garayoa E, Bahler M, et al. A ^{99m}Tc(I)-postlabeled high affinity bombesin analogue as a potential tumor imaging agent. Bioconjug Chem 2002;13: 599–604.
36. Smith CJ, Sieckman GL, Owen NK, et al. Radiochemical investigations of gastrin-releasing peptide receptor-specific [^{99m}Tc(X)$(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)] in PC-3, tumor-bearing, rodent models: syntheses, radiolabeling, and in vitro/in vivo studies where Dpr = 2,3-diaminopropionic acid and X = H_2O or $P(CH_2OH)_3$. Cancer Res 2003;63:4082–8.
37. Breeman WA, de Jong M, Erion JL, et al. Preclinical comparison of ^{111}In-labeled DTPA- or DOTA-bombesin analogs for receptor-targeted scintigraphy and radionuclide therapy. J Nucl Med 2002;43:1650–6.
38. Broccardo M, Falconieri Erspamer G, Melchiorri P, Negri L, de Castiglione R. Relative potency of bombesin-like peptides. Br J Pharmacol 1975;55:221–7.
39. Girard F, Bachelard H, St-Pierre S, Rioux F. The contractile effect of bombesin, gastrin releasing peptide and various fragments in the rat stomach strip. Eur Pharmacol 1984;102:489–97.
40. Mantey SA, Weber HC, Sainz E, et al. Discovery of a high affinity radioligand for the human orphan receptor, bombesin receptor subtype 3, which demonstrates that it has a unique pharmacology compared with other mammalian bombesin receptors. J Biol Chem 1997;272:26062–71.
41. Pradhan TK, Katsuno T, Taylor JE, et al. Identification of a unique ligand which has high affinity for all four bombesin receptor subtypes. Eur Pharmacol 1998;343:275–87.
42. Heppeler A, Froidevaux S, Maecke HR, et al. Radiometal-labelled macrocyclic chelator-derivatised somatostatin analogue with superb tumor-targeting properties and potential for receptor-mediated internal radiotherapy. Chem Eur J 1999;5:1974–81.
43. Wild D, Schmitt JS, Ginj M, et al. DOTA-NOC, a high-affinity ligand of somatostatin receptor subtypes 2, 3 and 5 for labelling with various radiometals. Eur J Nucl Med Mol Imaging 2003;30:1338–47.
44. Atherton E, Sheppard R. Fluorenylmethoxycarbonyl-polyamide solid phase peptide synthesis. General principles and development. Oxford: Oxford Information Press; 1989.
45. Fleischmann A, Laderach U, Friess H, Buechler MW, Reubi JC. Bombesin receptors in distinct tissue compartments of human pancreatic diseases. Lab Invest 2000;80: 1807–17.
46. Eisenwiener KP, Prata MI, Buschmann I, et al. NODAGATOC, a new chelator-coupled somatostatin analogue labeled with [$^{67/68}$Ga] and [^{111}In] for SPECT, PET, and targeted therapeutic applications of somatostatin receptor (hsst2) expressing tumors. Bioconjug Chem 2002;13:530–41.
47. Liu S, Pietryka J, Ellars CE, Edwards DS. Comparison of yttrium and indium complexes of DOTA-BA and DOTA-MBA: models for ^{90}Y- and ^{111}In-labeled DOTA-biomolecule conjugates. Bioconjug Chem 2002;13:902–13.
48. Maecke HR, Scherer G, Heppeler A, Hennig M. Is In-111 an ideal surrogate for Y-90? If not, why? Eur J Nucl Med 2001;28:967.
49. Teufel M, Saudek V, Ledig JP, et al. Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. J Biol Chem 2003;278:6521–31.
50. Singh P, Draviam E, Guo YS, Kurosky A. Molecular characterization of bombesin receptors on rat pancreatic acinar AR42J cells. Am J Physiol 1990;258:G803–9.
51. Hoffmann T, Simpson S, Smith C, et al. Accumulation and retention of Tc-99m-RP527 by GRP receptor expressing tumors in scid mice. J Nucl Med 1999;40:104P.
52. Rogers BE, Bigott HM, McCarthy DW, et al. MicroPET imaging of a gastrin-releasing peptide receptor-positive tumor in a mouse model of human prostate cancer using a ^{64}Cu-labeled bombesin analogue. Bioconjug Chem 2003;14:756–63.