# Potent Bombesin-like Peptides for GRP-Receptor Targeting of Tumors with $^{99m}$Tc: A Preclinical Study

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Four open chain tetraamine-functionalized bombesin (BB) analogues were synthesized [parent tetradecapeptide-based Demobesin 3 and 4 and BB(7−14)-based Demobesin 5 and 6]. Labeling with $^{99m}$Tc afforded high-purity and high specific activity radiotracers. Peptides showed high affinity for the human GRP-R (GRP-R = gastrin releasing peptide receptor) expressed in PC-3 cells. In human tumors preferentially expressing single bombesin receptor subtypes, they showed high affinity for the GRP-R, less affinity for the NMB−R (NMB-R = neuromedin B receptor) and no affinity for the orphan $BB_3$-R (bombesin subtype 3 receptor). [$^{99m}$Tc]Demobesin 3−6 efficiently internalized in a time- and dose-dependent manner in PC-3 cells and showed a high and specific uptake in human PC-3 xenografts and the pancreas of nude mice. [$^{99m}$Tc]-Demobesin 3 and 4 were rapidly excreted via the kidneys while the truncated analogues were predominantly processed by the hepatobiliary system. Patient studies are scheduled for validating the suitability of [$^{99m}$Tc]Demobesin 3 and 4 in the GRP-R-targeted imaging of tumors.

## Introduction

The advent of radiolabeled somatostatin analogues in the targeted imaging and radionuclide therapy of somatostatin receptor expressing tumors has revolutionized the field of nuclear medicine.[1−5] The prototypic radiopharmaceutical [$^{111}$In−DTPA$^0$]octreotide ([$^{111}$In]-OctreoScan) has been successfully used in the clinic for the scintigraphic detection of carcinoids and neuroendocrine tumors.[2,5] Furthermore, several other somatostatin analogues modified with DOTA (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) for stable binding of trivalent metallic radionuclides, such as $^{111}$In$^{3+}$, $^{90}$Y$^{3+}$, and radiolanthanides, have been proposed as candidates in the targeted radionuclide therapy of somatostatin-receptor-positive tumors.[3−5] Today multicenter clinical trials for the evaluation of such therapeutic somatostatin-based radiopharmaceuticals are in progress. The clinical usefulness of radiolabeled somatostatin analogues, however, remains restricted by the fact that several frequently occurring tumors do not express somatostatin receptors. In these cases, the expression of alternative peptide receptors in neoplastic cells in sufficient density may provide the molecular basis for effective tumor targeting employing radiolabeled peptides other than those based on somatostatin.[3,5]

In this respect, the expression of GRP-Rs (GRP-R = gastrin releasing peptide receptor) documented in several human cancer cell lines, such as in prostate, breast cancer, SCLC (SCLC= small-cell lung cancer), and colon carcinoma,[6−10] is of clinical relevance. In fact, the role of GRP and its interaction with the GRP-R operating via autocrine and paracrine loops in promoting tumor growth has been reported for these cancer cell lines in culture and in nude mice xenografts.[6−12] Most interestingly, however, GRP-Rs are overexpressed in biopsy samples from frequently occurring human carcinomas, while their expression in surrounding noncancerous tissue is minimal.[13−20] Thus, high-density GRP-R expression has been documented in primary prostatic invasive carcinoma with >50% of androgen-independent bone metastases still expressing the GRP-R.[14] Furthermore, recent evidence reports on the high-density GRP-R-expression in primary breast cancer (∼60% in incidence) and infiltrated lymph nodes (∼100% incidence).[17,18] Finally, gastrointestinal stromal tumors have been reported to express extremely high amounts of GRP-Rs.[20] These data are very convincing as far as the clinical impact of radiolabeled bombesin analogues is concerned for application in the targeted imaging, staging, and internal radiotherapy of GRP-R-positive prostate and breast carcinomas, two of the most frequently occurring human cancers.

Bombesin (BB) is a tetradecapeptide first isolated from the skin of the European frog *Bombina bombina*.[21,22] Amphibian BB and its mammalian analogues GRP and NMB (NMB = neuromedin B) elicit their effects after interaction with protein receptors located on the cell membrane of target cells. Bombesin receptors belong to the superfamily of G-protein-coupled receptors characterized by the typical seven transmembrane domain configuration and comprise four subtypes.[23] Three of the four bombesin receptor subtypes are found in humans, the GRP-R, the NMB-R, and the orphan $BB_3$-R, for which a native ligand has not yet been identified.[23] Radiolabeling of bombesin analogues for

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nuclear oncology applications requires modification of the parent peptide after covalent coupling of a suitable chelating agent for stable binding of the radionuclide, which as a rule is a metal.[3−5,24] Several bombesin analogues derivatized with DTPA (DTPA= diethylenetriaminepenteacetic acid) for binding $^{111}In^{3+}$ or DOTA for binding $^{68}Ga^{3+}$, $^{64}Cu^{2+}$ (for positron emission tomography—PET—applications), $^{111}In^{3+}$, $^{90}Y^{3+}$, and radiolanthanides, such as $^{177}Lu^{3+}$, $^{149}Pm^{3+}$, and $^{53}Sm^{3+}$, (for internal radiotherapy) have been developed over the past few years.[24−33] A few of these analogues are currently being clinically tested and more promising results are expected soon from this effort with the final goal to upgrade the arsenal of nuclear oncology with new potent diagnostic and therapeutic tools.

A special category of bombesin-based radiopeptides are those labeled with $^{99m}$Tc, which still remains the gold standard of nuclear medicine diagnosis.[27,34] The preeminence of $^{99m}$Tc is based on its almost ideal nuclear properties (monoenergetic $\gamma$ photons of 141 keV, $t_{1/2}$ of 6 h, lack of particulate emission) that lead to high-quality images and minimize the radiation burden both to patients and personnel. Furthermore, its cost effectiveness and wide availability through commercial generators along with the possibility to prepare the $^{99m}$Tc tracer very rapidly upon demand from freeze-dried technetium kits that can be stored in the clinic for a long time offer unique logistic and economic advantages for using this radionuclide in a clinical setting.[34] Several attempts have been made for the development of an effective $^{99m}$Tc-based bombesin radiotracer for GRP-R-targeted imaging that involves a wide variety of chelators, like $N_3S$, $P_2S_2$ (forming $^{99m}Tc^{V}(O)_x$ chelates, $x = 1$ and 2, respectively), and carbonyls in combination with several tridentate coligands (forming organometallic $^{99m}Tc^{I}$-chelates).[35−42] In most of these cases, however, cumbersome labeling protocols are described restricting routine application in a clinical environment and/or the radiotracers show high hepatobiliary excretion, thereby preventing reliable detection of lesions in the abdomen.

We have previously reported on [$^{99m}$Tc]Demobesin 1 ([$^{99m}$Tc−$N_4$-Bzdig$^0$,(D)Phe$^6$,Leu-NHEt$^{13}$,*des*-Met$^{14}$]BB-(6−14)),[43] a GRP-R-seeking radiotracer based on a potent bombesin antagonist modified at the N-terminal by coupling of an open chain tetraamine framework for effective binding of $^{99m}$Tc.[43−46] Despite its minimal internalization in human prostate cancer cells, [$^{99m}$Tc]-Demobesin 1 showed a very high and persistent tumor uptake in a nude mice model.[43] In this work, we present four additional bombesin analogues functionalized at the N-terminal with open chain tetraamines for labeling with $^{99m}$Tc that are based on agonists instead, Demobesin 3−6. The first two analogues are based on the parent bombesin tetradecapeptide sequence after minor modifications, while the remaining two are truncated peptides based on the essential residues needed for receptor interaction, the BB(7−14) motif. Demobesin 4 and 6 have undergone substitution of the oxidation-sensitive Met$^{14}$ by Nle$^{14}$.[47] Several biological properties of new analogues are studied in cells, resected human tumor samples, and nude mice bearing human GRP-R-positive xenografts with the aim to evaluate the suitability of [$^{99m}$Tc]Demobesin 3−6 for application in the GRP-R-targeted tumor imaging in man.

## Experimental Section

**General.** Unless otherwise stated, chemicals were reagent grade and used without further purification. The synthesis of the tetraamine precursors *N*,*N*′,*N*″,*N*‴-tetrakis(*tert*-butoxycarbonyl)-6-{*p*-[carboxymethoxy)acetyl]aminobenzyl}-1,4,8,11-tetraazaundecane and *N*,*N*′,*N*″,*N*‴-tetrakis(*tert*-butoxycarbonyl)-6-(*p*-carboxy)-1,4,8,11-tetraazaundecane will be reported elsewhere. The L-amino acid precursors used in solid-phase peptide synthesis, Fmoc-Met-OH, Fmoc-Leu-OH, Fmoc-His(Trt)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Trp(Boc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Pro-OH, were purchased from CBL (Patras, Greece), while Fmoc-Nle-OH was provided by Neosystem (Strasbourg, France). [Tyr$^4$]BB ([Tyr$^4$]bombesin = Pyr-Gln-Arg-Tyr-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-$NH_2$) was purchased from Bachem (Bubendorf, Switzerland). Solid-phase peptide synthesis (SPPS) was performed on an automated synthesizer (Advanced ChemTech Med 90, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Separations were performed on a semipreparative high-performance liquid chromatography (HPLC) system from Amersham Pharmacia Biotech AB (Uppsala, Sweden) coupled to a UV/vis detector from Amersham Pharmacia Biotech AB (Uppsala, Sweden) on a RP C-18 support (Phase Sep C-18 S10 ODS2 column) eluted with a linear gradient (vide infra). Product analysis by HPLC was conducted on a RP column (Techsphere 5 ODS2 C-18 250 mm × 4.6 mm) using the conditions given in the text. Electrospray mass spectra (ES-MS) were registered after infusion of test peptide solution into an electrospray interface mass spectrometer (AQA Navigator, Finnigan) using a Harvard Syringe pump. Hot nitrogen gas (Dominic-Hunter UHPLCMS-10) was used for desolvation. The charge of each ion and the molecular mass of test peptide were determined by deconvolution algorithms.

Iodine-125 was obtained from MDS Nordion, SA (Fleurus, Belgium). Technetium-99m was eluted as [$^{99m}$Tc]$NaTcO_4$ from a $^{99}$Mo/$^{99m}$Tc generator from Cis International, while $^{99g}$Tc was provided by Oak Ridge National Laboratories in the form of $NH_4{}^{99g}TcO_4$ powder. All manipulations with $\gamma$- and $\beta$-emitting radionuclides and their solutions were performed behind suitable shielding in dedicated laboratories by authorized and trained personnel and in compliance with national and international radiation safety guidelines. Radiochemical HPLC analyses were performed on a Waters Chromatograph (Waters, Vienna, Austria) with a 600 solvent delivery system coupled to twin detection instrumentation, namely a Waters 996 photodiode array UV detector and a Gabi $\gamma$-detector from Raytest (RSM Analytische Instrumente GmbH, Germany). Data processing and chromatographic control were conducted using Millennium Software (Waters). Analyses were performed on a RP-8 XTerra (5 $\mu$m, 4.6 mm × 150 mm) cartridge column (Waters, Germany) by applying the gradient systems given in the text. For instant thin-layer chromatography (ITLC) ITLC-SG strips from Gelman Sciences (Ann Arbor, MI) were used. All solvents used in analyses were HPLC grade and were previously filtered through 0.22 $\mu$m membrane filters (Millipore, Milford, CN) and degassed by helium flux. Radioactivity measurements were conducted in an automated well-type $\gamma$-counter [NaI(Tl) crystal, Canberra Packard Auto-Gamma 5000 series model] calibrated for $^{99m}$Tc or $^{125}$I.

The human androgen-insensitive prostate cancer cell line PC-3 was provided by LGC Promochem (Teddington, UK). All culture media were purchased from Gibco BRL, Life Technologies (Grand Island, NY), while supplements were provided by Biochrom KG Seromed (Berlin, Germany). For protein measurements the Bio-Rad protein assay kit (based on the Bradford procedure) by Bio-Rad Laboratories GmbH (Munich, Germany) was employed. The Brandel M-48 Cell Harvester (Adi Hassel Ingenieur Büro, Munich, Germany) was used for filtrations during binding assays. Animal experiments were carried out in compliance with European and national regulations. For metabolism experiments, in-house male Swiss albino mice (30 ± 5 g) were used. For experimental tumor models, female Swiss nu/nu mice of 7 weeks of age on the day of arrival

**Table 1.** Analytical Data for Demobesin 3−6

| peptide conjugate | sequence | % purity | MW expected, $m/z$ | ES-MS[a] | $t_R$, min: UV trace sys 1[b] | $t_R$, min: UV trace sys 2[c] | $t_R$, min: $\gamma$-trace[d] sys 2[c] |
|---|---|---|---|---|---|---|---|
| Demobesin 3 | [$N_4^0$,Pro$^1$,Tyr$^4$]BB | >98 | 1842.17<br>[M + 2H$^+$]/2 = 922.08 | 921.9 (100) | 15.0 | 18.6 | 19.6 |
| Demobesin 4 | [$N_4^0$,Pro$^1$,Tyr$^4$,Nle $^{14}$]BB | ≥98 | 1824.14<br>[M + 2H$^+$]/2 = 913.07<br>[M + 3H$^+$]/3 = 609.05 | 912.9 (100)<br>609.0 (25) | 15.2 | 20.2 | 21.2 |
| Demobesin 5 | [($N_4$-Bzdig)$^0$]BB(7−14) | 99 | 1303.61<br>[M + H$^+$] = 1304.61, [M + 2H$^+$]/2 = 652.80 | 1304.0 (42)<br>652.6 (100) | 16.0 | 17.8 | 21.3 |
| Demobesin 6 | [($N_4$-Bzdig)$^0$,Nle$^{14}$]BB(7−14) | ≥98 | 1285.57<br>[M + H$^+$] = 1286.57, [M + 2H$^+$]/2 = 643.78 | 1286.0 (45)<br>643.7 (100) | 16.5 | 19.3 | 22.9 |

[a] Peaks found in the ES-MS spectra of bioconjugates with intensities in parentheses. [b] RP-HPLC with UV detection of Demobesin 3−6. Column, Techsphere 5 ODS2 C-18 (250 mm × 4.6 mm); solvents, A = 0.1% TFA (v/v) and B = MeCN containing 0.1% TFA (v/v); linear gradient, 10% B to 60% B in 30 min; flow rate, 1 mL/min. [c] RP-HPLC with UV detection of Demobesin 3−6. Column: Waters XTerra RP-8, 5 $\mu$m, (150 mm × 4.6 mm) coupled to the respective 2 cm guard column; solvents, A = 0.1% TFA (v/v) and B = pure MeCN; linear gradient, 10% B to 40% B in 30 min; flow rate, 1 mL/min. [d] RP-HPLC of [$^{99m}$Tc]Demobesin 3−6 using the conditions of sys2; detection of radiolabeled peptides was achieved by a $\gamma$-detector coupled to the HPLC system.

(Charles Rivier Laboratories) were employed. The animals were kept under aseptic conditions until the day of biodistribution. For imaging, a small field of view $\gamma$-camera was employed.[48] The system was based on a position-sensitive photomultiplier tube (Hamamatsu R2486), a pixelized CsI(Tl) scintillation crystal, and CAMAC electronics.

**Synthesis of Conjugates.** SPPS was performed using standard 9-fluorenylmethyloxycarbonyl (Fmoc)/*tert*-butyl ($^t$Bu) methodology on an automated synthesizer. The peptide amides (i) Pro-Gln-Arg-Tyr-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-$NH_2$ (for Demobesin 3), (ii) Pro-Gln-Arg-Tyr-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Nle-$NH_2$ (for Demobesin 4), (iii) Gln-Trp-Ala-Val-Gly-His-Leu-Met-$NH_2$ (for Demobesin 5), and (iv) Gln-Trp-Ala-Val-Gly-His-Leu-Nle-$NH_2$ (for Demobesin 6) were assembled on Rink-Bernatowicz-2-chlorotrityl resin (0.5 mequiv/g substitution), purchased from CBL (Patras, Greece). The coupling reaction was achieved with a 3-fold excess of Fmoc-amino acid, using 1-hydroxybenzotriazol (HOBt) (4.5 molar excess) and *N*,*N*′-diisopropylcarbodiimide (DIC) (3.3 molar excess) as activating agents, in *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), or mixtures thereof. After a coupling time of 2−2.5 h, the completeness of the reaction was monitored by the standard ninhydrin test. In cases where incomplete coupling was found, the coupling procedure was repeated prior to $N^\alpha$-Fmoc protecting group removal. Fmoc deprotection was performed by the addition of 25% piperidine in DMF for 5 min, followed by a 25 min treatment. After Fmoc removal, resin samples were treated with a mixture of acetic acid (AcOH):trifluoroethanol (TFE):DCM (1:2:7 v/v/v) for 5 min, and the intermediate peptides released from the resin were checked for purity and complete deprotection by TLC in MeCN:$H_2O$ (6:1 v/v). After assembling the desired amino acid sequence, final coupling of the tetraamine chelator precursor was performed by employing ∼150 mg of each protected peptide−resin. The *N*,*N*′,*N*″,*N*‴-tetrakis(*tert*-butoxycarbonyl)-6-(carboxy)-1,4,8,11-tetraazaundecane precursor ($N_4$(Boc)$_4$-COOH, for Demobesin 3 and 4) or/and *N*,*N*′,*N*″,*N*‴-tetrakis-(*tert*-butoxycarbonyl)-6-{*p*-[carboxymethoxy)acetyl]aminobenzyl}-1,4,8,11-tetraazaundecane ($N_4$(Boc)$_4$-Bzdig, for the truncated peptides Demobesin 5 and 6) was coupled at the N-terminal of the amino acid chain using *o*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) as coupling reagent in the presence of *N*,*N*-diisopropylethylamine (DIEA) in DMF (3-fold excess of protected chelator, 3.3-fold excess of HATU, and 7-fold excess of DIEA, 2.5 h coupling). Removal of lateral chain protecting groups and cleavage from the resin was performed by 3.5 h incubation in a cleavage cocktail comprising TFA:anisole:1,2-ethanedithiol:$H_2O$:DCM 85:3:2:3:7 v/v/v/v/v. The resin was then filtered and washed with the above mixture, the cleavage mixture was evaporated, and the free peptide conjugates were precipitated with diethyl ether, filtered, washed with $Et_2O$, redissolved in $H_2O$, and lyophilized. The yield of crude products ranged from 70 to 80%. The crude products were subjected to gel chromatography on Sephadex G-15 using 0.2 M acetic acid as eluent. Further purification was carried out by semipreparative HPLC on a RP C-18 support using a linear gradient from 10% to 60% MeCN (+0.1% TFA, v/v) for 30 min at a 2 mL/min flow rate. Eluted peptides were lyophilized immediately.

**Radiolabeling.** Labeling of Demobesins 3−6 with $^{99m}$Tc was performed according to a published protocol.[43] Briefly, each lyophilized conjugate was dissolved in 50 mM acetic acid:EtOH 8:2 v/v to a final 1 mM peptide concentration. Each bulk solution was distributed in 50 $\mu$L aliquots in Eppendorf tubes and stored at −20 °C. Labeling was conducted in an Eppendorf vial, wherein the following solutions were consecutively added: (i) 0.5 M phosphate buffer pH 11.5 (50 $\mu$L), (ii) 0.1 M sodium citrate (5 $\mu$L), (iii) [$^{99m}$Tc]$NaTcO_4$ generator eluate (415 $\mu$L, 10−20 mCi), (iv) peptide conjugate stock solution (15 $\mu$L, 15 nmol), and (v) fresh $SnCl_2$ solution in EtOH (30 $\mu$g, 15 $\mu$L). After reaction for 30 min at ambient temperature, the pH was brought to ∼7 by adding 1 M HCl (10 $\mu$L).

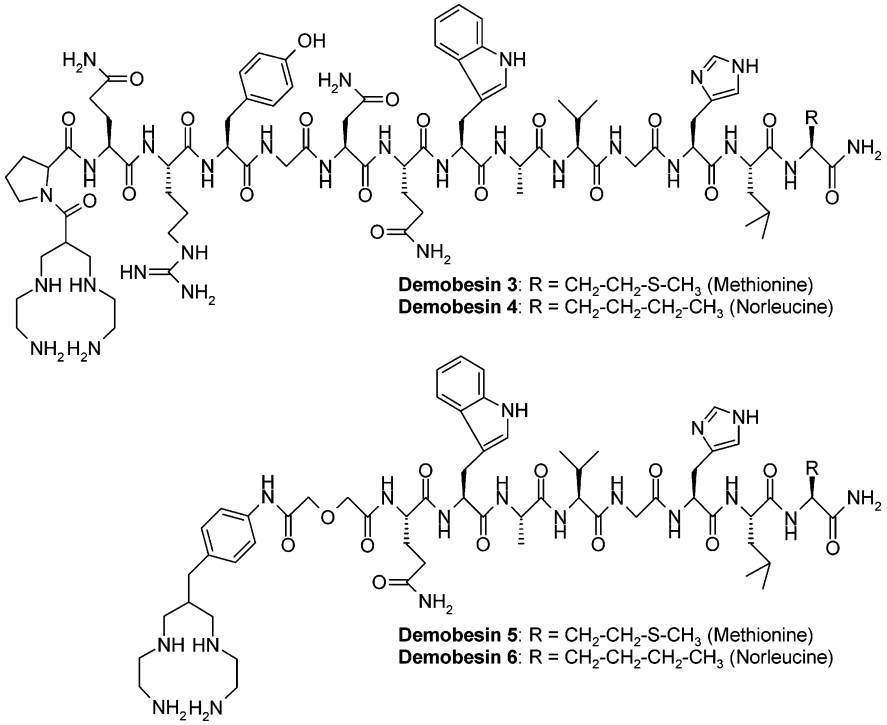
A similar procedure was applied for the preparation of the corresponding [$^{99g}$Tc/$^{99m}$Tc]peptides at carrier-added level. [$^{99g}$Tc]$NH_4TcO_4$ was used as $^{99g}$Tc source (0.9 $\mu$g, 5 nmol) that was "spiked" with $^{99m}$Tc found in the generator eluate. Complete reduction to Tc(V) was accomplished by addition of a higher amount of $SnCl_2$ (1 $\mu$mol/mL). The resulting [$^{99g}$Tc/$^{99m}$Tc]peptides were isolated in a highly pure form by HPLC. For saturation binding experiments triplicates in the $10^{-12}$−$10^{-16}$ M concentration range were prepared, as previously described.[43,44]

Radioiodination of [Tyr$^4$]BB was performed by following a published protocol and [$^{125}$I−Tyr$^4$]BB was obtained in a highly pure form after HPLC purification.[49]

**Quality Control.** For quality control of radiolabeled products both RP-HPLC and ITLC were applied. In a typical HPLC experiment, an aliquot (1 $\mu$L) of test peptide labeling mixture was injected on the column. Elution at a 1.0 mL/min flow rate followed a linear gradient pattern with 10% solvent B at start reaching to 40% solvent B within the next 30 min (solvent A = 0.1% aqueous TFA (v/v) and solvent B = acetonitrile). Under these conditions, [$^{99m}$Tc]citrate elutes at 2.0 min, $^{99m}TcO_4^-$ at 2.6 min, and the radiolabeled peptides with a $t_R$ > 19 min (Table 1). For detection of reduced hydrolyzed technetium traces ($^{99m}TcO_2{\cdot}xH_2O$), an aliquot of test peptide labeling mixture was positioned on the ITLC strip. For development up to 10 cm from the origin, 1 M ammonium acetate:MeOH 1:1 (v/v) was used. Strips were left to dry in the open before they were cut into two sections: origin + 0.5−1 cm, giving $^{99m}TcO_2{\cdot}xH_2O$, and the remainder of the strip, giving $^{99m}TcO_4^-$, [$^{99m}$Tc]citrate, and [$^{99m}$Tc]peptide conjugate.[43,44] Counts of each piece were measured in the $\gamma$-counter. For stability studies, tests were conducted at predetermined time points up to 6 h postlabeling.

**Cell Culture.** Human androgen-independent prostate cancer PC-3 cells were cultured in DMEM GLUTAMAX-I supple-

**Chart 1.** Chemical Structures of Demobesin 3−6




mented by 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin. Cells were kept in a controlled humidified atmosphere containing 5% $CO_2$ at 37 °C. Passages were performed weekly using a trypsin/EDTA (0.05%/0.02% w/v) solution.

**Competition Binding Experiments.** Competition binding assays were conducted in PC-3 cell membranes harvested as previously described,[43,44] employing [$^{125}$I−Tyr$^4$]BB as the radioligand[49] and [Tyr$^4$]BB as reference compound. In brief, ∼40 000 cpm of radioligand was used per assay tube along with 50 $\mu$g of protein and tested peptide in increasing concentrations in a total volume of 300 $\mu$L of 50 mM HEPES (pH 7.6, 1% BSA, 5.5 mM $MgCl_2$, 35 $\mu$M bacitracin). Triplicates of each concentration point were incubated for 1 h at 37 °C in an Incubator-Orbital Shaker unit, (MPM Instr. SrI, Italy). After completion of the assay, samples were counted for their radioactivity content in a $\gamma$-counter and $IC_{50}$ values were extracted by nonlinear regression according to a one-site model applying the PRISM 2 program (Graph Pad Software, San Diego, CA).

**Saturation Binding Assays.** Saturation binding assays were conducted for [$^{99g}$Tc/$^{99m}$Tc]Demobesin 5 and 6 in PC-3 membrane homogenates, as detailed previously.[43,44] Two sets of triplicates of the following concentrations were prepared: 0.06, 0.18, 0.42, 0.6, 1.8, 4.2, 6, 9, and 12 nM (Tc total peptide concentration = $^{99g}$Tc test peptide + $^{99m}$Tc test peptide concentration) for total and nonspecific binding each. Each assay tube contained 50 $\mu$L of binding buffer (50 mM HEPES, pH 7.6, containing 1% BSA, 5.5 mM $MgCl_2$, 35 $\mu$M bacitracin), 50 $\mu$L of radioligand solution of the corresponding concentration, and 200 $\mu$L of PC-3 membrane homogenate containing 50 $\mu$g of protein. For the nonspecific series, the 50 $\mu$L binding buffer used contained [Tyr$^4$]BB at a 6 $\mu$M concentration. Tubes were incubated for 1 h at 37 °C in an Incubator-Orbital Shaker unit and a similar protocol was followed as in competition binding experiments. Nonspecific binding was defined as the amount of activity binding in the presence of 1 $\mu$M [Tyr$^4$]BB. The Scatchard plot was then drawn and the $K_d$ values were calculated by employing the PRISM 2 program (GraphPad Software, San Diego, CA).

**Receptor Autoradiography in Human Tumors.** Competition binding assays using Demobesin 3−6 in human tumors evaluated previously for their expression of either GRP-Rs, NMB-Rs, or $BB_3$-Rs were conducted utilizing [$^{125}$I-(D)Tyr$^6$,($\beta$)Ala$^{11}$,Phe$^{13}$,Nle$^{14}$]BB(6−14) as universal radioligand[50] according to a described protocol.[50,51]

**Internalization.** Confluent PC-3 cells were seeded in six-well plates [(1.0−1.5) × $10^6$ cells per well] 24 h before conducting the internalization experiment. On the day of the study, cells were rinsed twice with ice-cold internalization buffer containing DMEM GLUTAMAX-I supplemented by 1% (v/v) FBS, and fresh medium was added (1.2 mL). Approximately 300 000 cpm of test peptide (∼200 fmol total peptide in 150 mL of PBS/0.5% BSA buffer) was added, and the experiment was performed according to a published protocol.[43] Discrimination of the surface-bound radioactivity from that internalized was achieved by washing the cells at low pH (50 mM glycine buffer, pH 2.8, 0.1 M NaCl). Considering that the total activity comprises membrane-bound plus internalized activity, the percent internalized activity versus the selected time intervals could be plotted by applying Microsoft Excel.

**Metabolism. In Plasma**. Blood (∼1 mL) was collected in heparinized polypropylene tubes from the heart of male Swiss albino mice while under ether anesthesia and centrifuged at 5000 rpm at 4 °C. The plasma was collected and incubated fresh with test radiopeptide at 37 °C. Fractions withdrawn at 15, 30, 60, and 120 min were mixed with ethanol in a 2:1 EtOH:aliquot v/v ratio and centrifuged at 35 000 rpm for 10 min. Supernatants were filtered through Millex GV filters (0.22 $\mu$m) and analyzed by HPLC by applying a different gradient system as the one described in the section "Quality Control". Thus, elution at a 1.0 mL/min flow rate started with 100% solvent A and declined to 60% solvent A within the next 40 min (solvent A = 0.1% aqueous TFA (v/v) and solvent B = acetonitrile). ITLC-SG was performed in parallel using acetone as the eluent to detect traces of $TcO_4^-$ release ($TcO_4^-$ $R_f$ = 1.0).

**In the Urine.** To detect metabolites excreted in the urine, each test peptide was injected as a 100 $\mu$L bolus (0.5−1 mCi) via the tail vein of male Swiss albino mice. Animals were kept in metabolic cages for 30 min with free access to water and food. They were then sacrificed by cardiac puncture under a mild ether anesthesia and urine was immediately collected from their bladder with a syringe. This urine was mixed with that collected in the metabolic cages, filtered through a Millex GV filter (0.22 $\mu$m), and analyzed by HPLC (conditions as in

the section "In Plasma"). ITLC-SG was performed as well with the strips developed with acetone to reveal traces of $TcO_4^-$ release ($TcO_4^-$ $R_f = 1.0$).

**Biodistribution in Human PC-3 Xenograft-Bearing Mice.** A ~150 $\mu$L bolus containing a suspension of (1.5−2) × $10^7$ freshly harvested human PC-3 cells in PBS buffer was subcutaneously injected in the right flank of each female nu/nu mouse. The animals were kept under aseptic conditions and 2−3 weeks later developed well-palpable tumors at the inoculation site (80−150 mg). Biodistribution was conducted as previously described.[43] Briefly, each test radiopeptide was injected as a 100 $\mu$L bolus containing 4−5 $\mu$Ci (corresponding to ~10 pmol total peptide) in PBS buffer, pH 7.4, through the tail vein of each xenograft-bearing mouse. Animals were sacrificed in groups of four or five by cardiac puncture under a mild ether anesthesia at 1, 4, and 24 h time intervals. Additional sets of four animals received intravenously (iv) 100 $\mu$g [Tyr$^4$]BB along with tested radiopeptide (blocked animals). These animals were sacrificed at 4 h postinjection (pi). Samples of urine, blood, and tissues of interest were immediately collected, weighed, and measured for radioactivity in a $\gamma$-counter. Stomach and intestines were not emptied of their contents, but used as collected. Biodistribution data were calculated as percent injected dose per gram tissue (%ID/g) using an in-house computer program and employing appropriate standards.

**Imaging.** Two additional PC-3 xenograft-bearing mice were injected each via the tail vein with a 200 $\mu$L bolus containing 0.5−1 mCi [$^{99m}$Tc]Demobesin 3 alone or together with 100 $\mu$g [Tyr$^4$]BB (blocked animal). The mice were sacrificed by ether asphyxiation at 90 min pi. They were appropriately positioned for planar imaging under the head of a small field of view experimental $\gamma$-camera. Decay correction for $^{99m}$Tc was conducted during processing of projection data. Animals were dissected soon after imaging and scintigraphic data were correlated to anatomical and tissue distribution findings.

## Results

**Synthesis.** The respective Demobesin 3−6 sequences (Table 1) were built on the solid support, and the corresponding Boc-protected chelator, [(Boc-N)$_4$-6-R-1,4,8,11-tetraazaundecane, R = COOH (for Demobesin 3 and 4) or *p*-$CH_2C_6H_4NHCOCH_2OCH_2COOH$ (for Demobesin 5 and 6)] was coupled at the N-terminal. The $N_4$-peptide conjugates (Demobesin 3−6, Chart 1) were purified by chromatographic methods and obtained as white solids after freeze-drying. Lyophilized products were obtained in 50−60% yields. Analytical data for synthesized bioconjugates, comprising HPLC, UV/vis, and ES-MS spectroscopy results, are presented in Table 1.

**Radiopeptides.** Labeling was achieved by 30 min incubation at ambient temperature of $^{99m}TcO_4^-$ generator eluate, peptide conjugate, and tin chloride in alkaline aqueous medium in the presence of citrate anions. This procedure resulted in complete incorporation of $^{99m}$Tc by the tetraamine framework and formation of respective radiopeptides, [$^{99m}$Tc]Demobesin 3−6, in a >96% purity and a typical specific activity of 1 Ci/$\mu$mol total peptide. As revealed by combined HPLC and ITLC analysis, the expected radiochemical impurities ($^{99m}TcO_4^-$, [$^{99m}$Tc]citrate, and $^{99m}TcO_2{\cdot}xH_2O$)[43−45] were detectable by <2%, while radiopeptides comprised a single radiochemical species. A typical HPLC radiochromatogram of [$^{99m}$Tc]Demobesin 4 labeling mixture is presented in Figure 1. The radiopeptides remained intact within the open labeling reaction vial for at least 6 h after labeling.

**Conjugate Affinities for the Human GRP-R.** Determination of Demobesin 3−6 affinities was achieved during competition binding experiments in human prostate cancer PC-3 cells, preferably expressing the human GRP-R.[52] [$^{125}$I−Tyr$^4$]BB was the radioligand[43,49] employed while [Tyr$^4$]BB was used as reference hormone. All four conjugates were able to displace [$^{125}$I−Tyr$^4$]BB from bombesin binding sites in PC-3 cell membranes in a monophasic and dose-dependent manner, as shown by the displacement curves of Figure 2A. All $IC_{50}$ values were found in the sub-nanomolar range (Demobesin 3, 0.06 ± 0.04 nM; Demobesin 4, 0.15 ± 0.04 nM; Demobesin 5, 0.08 ± 0.05 nM; and Demobesin 6, 0.60 ± 0.05 nM) and were comparable or better than the respective value of [Tyr$^4$]BB ($IC_{50}$= 0.85 ± 0.29 nM). These findings suggest that introduction of the tetraamine moiety at the N-terminal of the peptides was well-tolerated by the GRP-R. Demobesin 4 and its

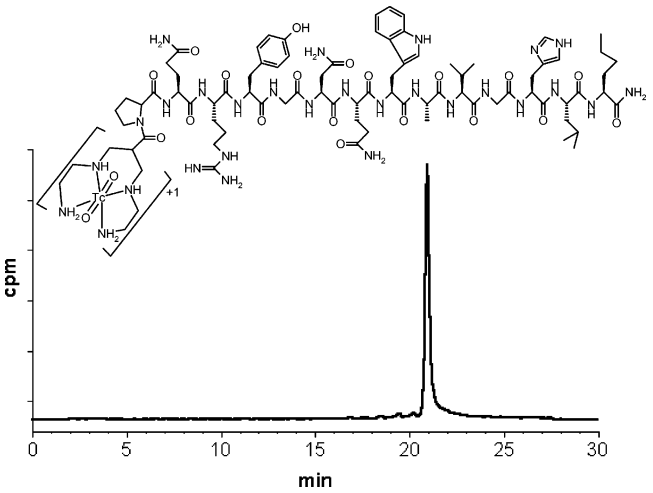



**Figure 1.** $\gamma$-Trace of [$^{99m}$Tc]Demobesin 4 elution during RP-HPLC analysis.

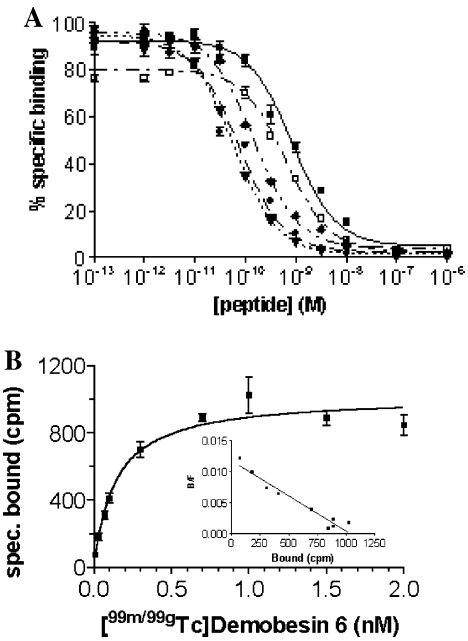



**Figure 2.** (A) Displacement of [$^{125}$I-Tyr$^4$]BB from human GRP-Rs in PC-3 cell membranes by increasing concentrations of test peptide: ■, [Tyr$^4$]BB ($IC_{50}$ = 0.85 ± 0.29 nM); ▼, Demobesin 3 ($IC_{50}$ = 0.06 ± 0.04 nM); ◆, Demobesin 4 ($IC_{50}$ = 0.15 ± 0.04 nM); ●, Demobesin 5 ($IC_{50}$ = 0.08 ± 0.05 nM); and □, Demobesin 6 ($IC_{50}$ = 0.60 ± 0.05 nM). (B) Saturation binding of [$^{99m}$Tc/$^{99g}$Tc]Demobesin 6 ($K_d$ = 0.14 ± 0.03 nM) in PC-3 cell membranes with Schatchard plot shown in the inset.

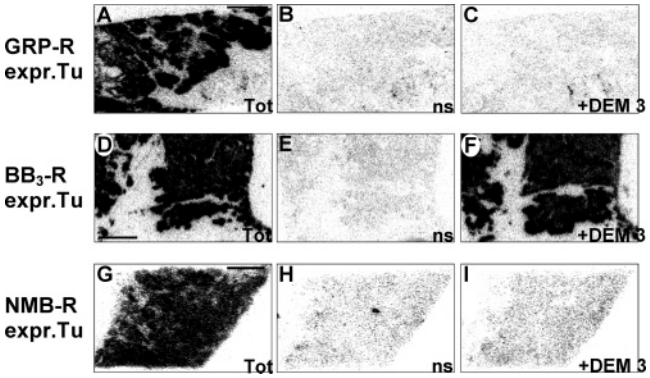



**Figure 3.** Receptor autoradiography showing the displacement by Demobesin 3 of the universal radioligand in GRP-R-expressing human prostate cancer (A−C) and in NMB-R-expressing ileal carcinoids (G−I), while no displacement by Demobesin 3 was observed in $BB_3$-R-expressing lung carcinoids (D−F). (A, D, G) Autoradiograms showing the total binding of [$^{125}$I-(D)Tyr$^6$,($\beta$)Ala$^{11}$,Phe$^{13}$, Nle$^{14}$]BB(6−14) with all three tumors labeled with this universal radioligand (bars = 1 mm). (B, E, H) Autoradiograms showing nonspecific binding (in the presence of $10^{-7}$ M [(D)Tyr$^6$,($\beta$)Ala$^{11}$,Phe$^{13}$,Nle$^{14}$]BB(6−14)). (C, F, I) Autoradiograms showing displacement of [$^{125}$I-(D)Tyr$^6$,-($\beta$)Ala$^{11}$,Phe$^{13}$,Nle$^{14}$]-BB(6−14) with $10^{-7}$ M Demobesin 3. Full displacement is seen in the GRP-R-expressing tumor (C), almost full displacement in the NMB-R-expressing tumor (I), and no displacement in the $BB_3$-R-expressing tumor (F).

truncated counterpart Demobesin 6, having undergone substitution of Met$^{14}$ by Nle,$^{14}$ showed a slight drop of affinity for the human GRP-R in comparison to the corresponding Met$^{14}$ conjugates.

**Saturation Binding Tests.** Saturation binding assays were performed in PC-3 cell membranes for the truncated and more lipophilic analogues [$^{99m}$Tc/$^{99g}$Tc]-Demobesin 5 and 6, which could be well-isolated by HPLC as a single (radio)metalated peptide species.[43−46] A typical curve for [$^{99m}$Tc/$^{99g}$Tc]Demobesin 5 is presented in Figure 2B. Both (radio)metalated peptide conjugates demonstrated a strong and dose-dependent interaction with a single class of high-affinity binding sites in the PC-3 cell membranes with equilibrium binding constants ($K_d$) values 0.07 ± 0.02 and 0.14 ± 0.03 for [$^{99m}$Tc/$^{99g}$Tc]Demobesin 5 and 6, respectively. These $K_d$ values illustrate the high affinity of new radiopeptides for the human GRP-R and are in the same range of values reported for other bombesin-like radioligands.[27,43]

**In Vitro Receptor Autoradiography on Human Tumor Biopsy Samples.** The selective affinities of Demobesin 3−6 conjugates for each of the three bombesin receptor subtypes found in humans, namely the NMB-Rs, the GRP-Rs, and the orphan $BB_3$-Rs, were studied in human tumor samples each preferentially expressing one of these receptor subtypes.[20,50,51] Resected cancer specimens included NMB-R-expressing ileal carcinoids, GRP-R-expressing prostate cancer, and $BB_3$-R-expressing lung carcinoids. Representative autoradiograms for Demobesin 3 in sections of three human tumors expressing each a distinct subtype are depicted in Figure 3, whereas $IC_{50}$ values for all four peptides versus the three human bombesin receptor subtypes are summarized in Table 2. These data demonstrate that Demobesin 3−6 show high affinity for the human GRP-R, less affinity for the NMB-R, and no affinity for the $BB_3$-R. Similarly to what was found during competition binding assays in PC-3 cell membranes, the Nle$^{14}$-containing peptides exhibited slightly reduced affinity for the GRP-R as compared to their unmodified Met$^{14}$ analogues.

**Table 2.** Affinities of Demobesin 3−6 for the Three Human Bombesin Receptor Subtypes[a]

| | $IC_{50}$ in nM | | |
|---|---|---|---|
| peptide conjugate | GRP-R[b] | NMB-R[c] | BB3-R[d] |
| universal ligand[e] | 0.72 ± 0.07 (3) | 0.96 ± 0.07 (3) | 1.1 ± 0.07 (3) |
| Demobesin 3 | 0.47 (0.2; 0.6) | 1.6 (1.2; 2) | >100 |
| Demobesin 4 | 1.94 (2.3; 1.4) | 11.2 (10; 11.4) | >100 |
| Demobesin 5 | 0.65 (0.7; 0.6) | 0.9 (0.4; 1.4) | 37 (37; 37) |
| Demobesin 6 | 1.3 (1.7; 0.9) | 2.0 (1.7; 2.5) | >100 |

[a] The data represent the mean (±SEM; $n$ = 3) for the non-radioactive universal ligand and the mean ($n$ = 2; individual values in parentheses) for Demobesin 3−6. [$^{125}$I−(D)Tyr$^6$,($\beta$)Ala$^{11}$,Phe$^{13}$,Nle$^{14}$]BB(6−14) was used as radioligand in all experiments. [b] Expressed in human prostate cancer. [c] Expressed in human ileal carcinoids. [d] Expressed in human lung carcinoids. [e] [(D)Tyr$^6$,($\beta$)Ala$^{11}$,Phe$^{13}$,Nle$^{14}$]BB(6−14) was used as cold universal ligand.

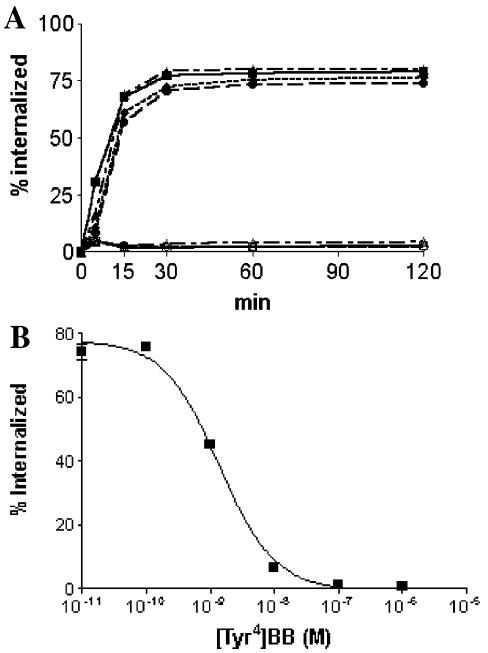



**Figure 4.** (A) Internalization rate curves of [$^{99m}$Tc]Demobesin 3−6 in PC-3 cells by incubation at 37 °C with nonspecific internalization curves (in the presence of 1 $\mu$M [Tyr$^4$]BB in the medium) indicated with empty bullets; ■, [$^{99m}$Tc]Demobesin 3; ▲, [$^{99m}$Tc]Demobesin 4; ●, [$^{99m}$Tc]Demobesin 5; and ◆, [$^{99m}$Tc]Demobesin 6. (B) Concentration-dependent internalization of [$^{99m}$Tc]Demobesin 3 in PC-3 cells after 1 h incubation at 37 °C.

**Internalization.** The curves of [$^{99m}$Tc]Demobesin 3−6 internalization in PC-3 cells at 37 °C as a function of time are summarized in Figure 4 A. Rapid internalization is observed, reaching a 75% plateau of cell-associated activity within the first 30 min for all tested radiopeptides and remaining at this level till 2-h incubation. The fact that internalization was negligible in the presence 1 $\mu$M [Tyr$^4$]BB suggests a receptor-mediated process. This fact is more evident by the additional concentration-dependent internalization curve presented for [$^{99m}$Tc]Demobesin 3 in Figure 4B demonstrating that migration of [$^{99m}$Tc]Demobesin 3 into PC-3 cells after 1 h incubation at 37 °C follows a [Tyr$^4$]BB dose-dependent fashion and is receptor-mediated. The high internalization capacity of [$^{99m}$Tc]Demobesin 3−6

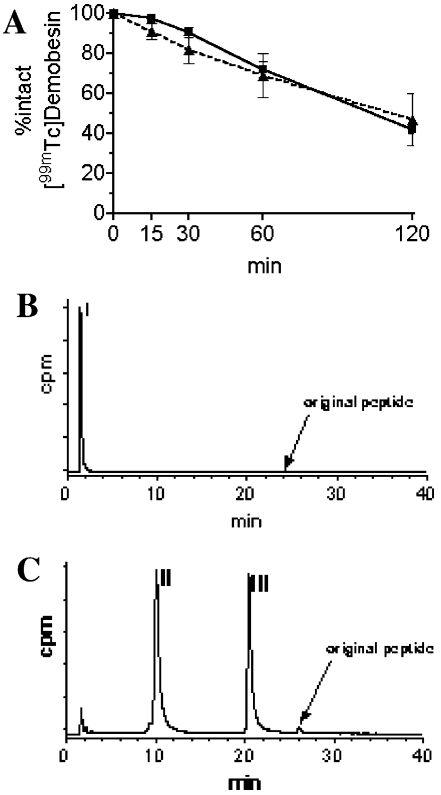



**Figure 5.** (A) Degradation rates of [$^{99m}$Tc]Demobesin 3−6 by incubation in mouse plasma at 37 °C: ■, [$^{99m}$Tc]Demobesin 3 and 4; ▲, [$^{99m}$Tc]Demobesin 5 and 6. (B) $\gamma$-Trace of HPLC analysis of mouse urine collected 30 min after iv injection of [$^{99m}$Tc]Demobesin 3 and 4; the position of original peptide elution is indicated by the arrow. (C) $\gamma$-Trace of HPLC analysis of mouse urine collected 30 min after iv injection of [$^{99m}$Tc]-Demobesin 5 and 6; the position of original peptide elution is indicated by the arrow.

contrasts the low internalization ability of the antagonist-based [$^{99m}$Tc]Demobesin 1.[43] Similar to [$^{99m}$Tc]Demobesin 3−6, analogues coupled to other radiometal−chelate entities have shown comparable GRP-R-mediated internalization profiles in PC-3 and/or other GRP-R-positive cell lines.[27,40,52]

**Metabolic Studies.** The metabolic fate of [$^{99m}$Tc]-Demobesin 3−6 was studied by incubation of each radiopeptide in murine plasma at 37 °C, and data are presented in Figure 5A. It is shown that radiopeptide catabolism is slow in mouse plasma during the initial critical time intervals with >90% radiopeptide remaining intact within 10 min incubation. Analysis by HPLC of urine collected 30 min after iv injection of [$^{99m}$Tc]-Demobesin 3−6 in mice revealed that all four radiopeptides were excreted in the urine completely converted to more hydrophilic metabolites. As demonstrated in Figure 5, the decatetrapeptide analogues produce hydrophilic metabolite I ($t_R$= 1.8 min; Figure 5B), whereas the truncated compounds generate two metabolic species, II and III (B $t_R$= 10.2 min and C $t_R$= 21 min; Figure 5C). A previously reported negligible protein binding of the [$^{99m}$Tc/$^{188}$Re$O_2$−$N_4$]$^+$ metal chelate in rodent and human serum, as well as excretion of intact complex in the urine after in vivo administration,[45,46,53,54] is a strong indication that metabolites I, II, and III are not related to in vivo breakdown of the metal chelate. This assumption is further supported by our own findings from urine analysis after injection of [$^{99m}$Tc$O_2$−$N_4$]$^+$-conjugated somatostatin analogues in rodents and patients that showed radioactivity to be excreted only in the form of original radiopeptide.[44,55] Furthermore, parallel analysis of urine or plasma [$^{99m}$Tc]Demobesin 3−6 samples by ITLC ruled out any release of $^{99m}$Tc$O_4^-$ deriving from decomposition of the tetraamine complex. Therefore, [$^{99m}$Tc]Demobesin 3−6 metabolic products seem to be generated by cleavage of amide bond(s) either within the peptide chain or (and) between the metal chelate and the peptide. It is characteristic that metabolite I is not only distinct but also substantially more hydrophilic than the metabolic products II and III of the truncated radiopeptides. This may be a result of (i) the presence of the polar and hydrophilic Arg$^3$ and Tyr$^4$ residues only in the tetradecapeptide analogues and (ii) the extra benzyl group in the spacer of truncated radiopeptides. It is interesting to see how the distinct metabolic fate of [$^{99m}$Tc]Demobesin 3−6 influences their respective biodistribution profiles (vide infra).

**Biodistribution in Mice Bearing Human PC-3 Xenografts.** Comparative data of [$^{99m}$Tc]Demobesin 3−6 biodistribution in female athymic mice bearing human PC-3 xenografts are summarized in Figures 6 and 7. Data are given as %ID/g for the 1, 4, and 24 h pi time intervals. The radiopeptides are rapidly cleared from blood via the liver and the kidneys, showing low background activity (Figure 6). While [$^{99m}$Tc]Demobesin 3 and 4 are excreted predominantly via the kidneys into the urine, the truncated and more lipophilic peptides [$^{99m}$Tc]Demobesin 5 and 6 show a high percentage of hepatobiliary excretion (Figure 6, liver; Figure 7, intestines). Furthermore, the intestinal radioactivity for [$^{99m}$Tc]Demobesin 3 and 4 at 4 h pi could be blocked by 85% by co-injection of 100 $\mu$g [Tyr$^4$]BB, implying a receptor-mediated uptake in the intestinal wall. In fact, the presence of bombesin binding sites has been documented in the whole intestinal tract, including the submucosal layer of the small intestine and the longitudinal and circular muscle and the submucosal layer of the colon.[57] In contrast, [$^{99m}$Tc]Demobesin 5 and 6 show a much higher intestinal uptake that was not possible to block by [Tyr$^4$]BB treatment. This increased uptake could be attributed to the higher lipophilicity of the truncated radiopeptides originating from the extra benzyl group of the spacer as well as the lack of the polar and hydrophilic Arg$^3$ and Tyr$^4$ residues present in [$^{99m}$Tc]Demobesin 3 and 4. This property in combination with the formation of more lipophilic metabolic products from the truncated analogues (see "Metabolic Studies") are suspected to have favored excretion via the hepatobiliary system.

Uptake of all four radiopeptides in the pancreas, wherein the major part of bombesin binding sites are located, is very high, slowly declining at 4−24 h pi (Figure 7). Treatment with [Tyr$^4$]BB caused a >97% drop in pancreas uptake at 4 h pi, suggesting a receptor-specific process. Uptake of radiopeptides in the PC-3 human prostate cancer xenograft was high, especially for [$^{99m}$Tc]Demobesin 3 and 4 (9−11%ID/g at 1 h pi), remaining high (7−9%ID/g) at 4 h pi. Tumor values were significantly reduced by co-injection of a high amount of the blocker (0.2−0.8%ID/g), demonstrating that accumulation in the tumor is receptor-mediated (Figure 7). This is also evident in the static images of

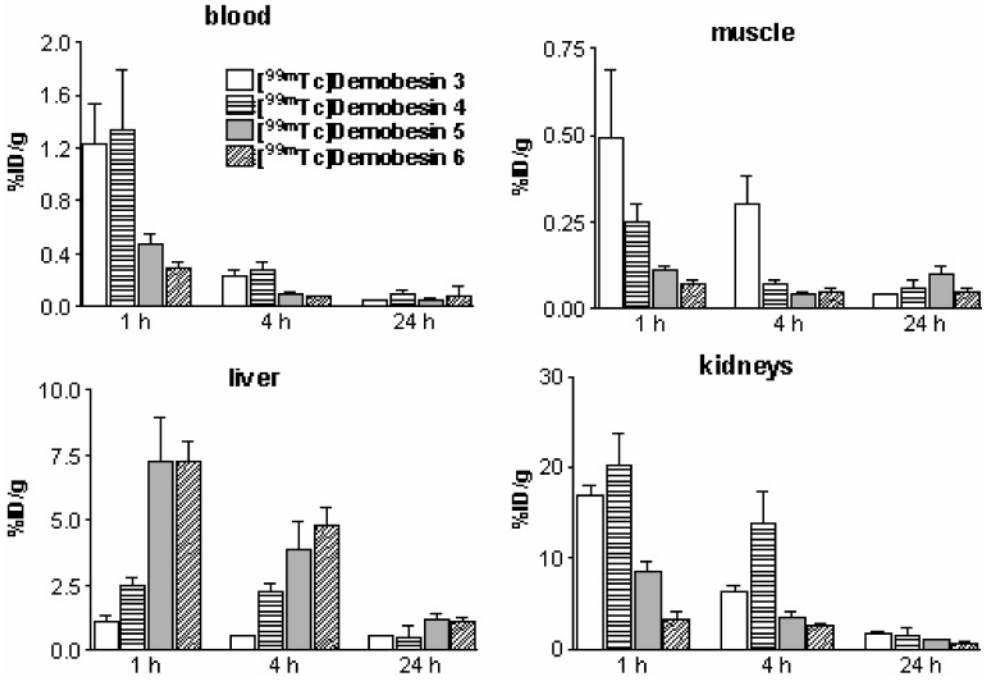



**Figure 6.** Comparative data of [$^{99m}$Tc]Demobesin 3−6 biodistribution in PC-3 xenograft-bearing nude mice selectively shown for blood, muscle, liver, and kidneys; data are given as %ID/g for the 1, 4, and 24 h time intervals.

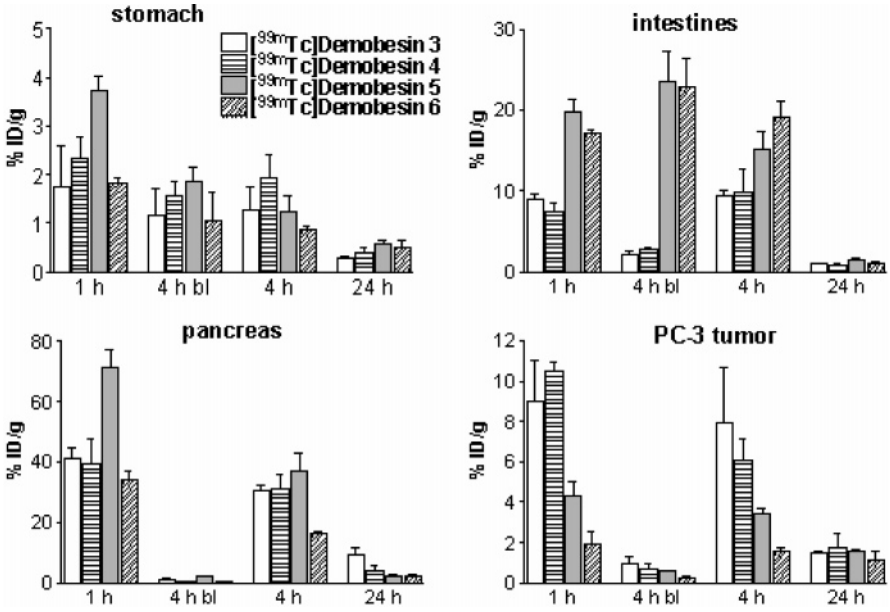



**Figure 7.** Comparative data of [$^{99m}$Tc]Demobesin 3−6 biodistribution in PC-3 xenograft-bearing nude mice selectively shown for stomach, intestines, pancreas, and experimental tumor; data are given as %ID/g for the 1, 4, and 24 h time intervals with bl indicating values from animals co-injected with 100 $\mu$g [Tyr$^4$]BB.

two nude mice bearing human PC-3 xenografts shown in Figure 8. Images are taken 1.5 h after iv administration of [$^{99m}$Tc]Demobesin 3 alone or together with 100 $\mu$g [Tyr$^4$]BB (blocked mouse). In the first mouse the tumor is clearly delineated with a very low background activity clearing via the kidneys, while in the blocked animal the tumor is no longer detectable by the camera.

## Discussion

The search for radiolabeled bombesin analogues that are able to target GRP-R-positive tumors in vivo, thereby permitting effective diagnosis, staging, and radionuclide therapy of cancer, has been very intensive recently.[24−43] Research has been triggered mainly by the fact that frequently occurring neoplasms, like prostate and breast cancer, which along with lung cancer constitute the predominant cancer population, massively express GRP-Rs at a high incidence.[13−20,51] Most agents proposed so far for GRP-R-targeted imaging and radionuclide therapy are based on bombesin agonists given that agonists are expected—as a rule—to internalize into cancer cells and thus enhance the radionuclide ratio between target and nontarget tissues with clear advantages both for imaging and radiotherapy.[13,25,27] However, bombesin agonists are known to elicit potent pharmacological effects after iv administration, and special care should be taken during injection to patients.[26] For this reason, we selected a potent antagonist as a motif for

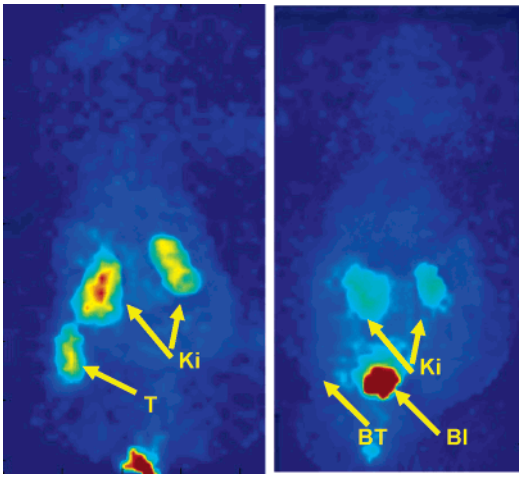



**Figure 8.** Static images of human PC-3 xenograft-bearing mice 1.5 h after iv injection of [$^{99m}$Tc]Demobesin 3 alone (mouse on the left) or together with 100 $\mu$g [Tyr$^4$]BB (mouse on the right, blocked animal); Ki = kidneys, T = PC-3 tumor, BT = blocked PC-3 tumor, and Bl = bladder.

developing our first tetraamine-functionalized GRP-R-seeking radiopeptide, [$^{99m}$Tc]Demobesin 1.[43] The open chain tetraamine system was selected due to previous studies reporting the easy formation of hydrophilic and in vivo stable monocationic $[^{99m}Tc(O)_2(N_4)]^+$ complexes in specific activities suitable for receptor targeting applications[44,45] but also due to previous experience with somatostatin analogues ([$^{99m}$Tc]Demotate 1 = [$^{99m}$Tc–$N_4^0$,Tyr$^3$]octreotate) showing excellent qualities for targeted-tumor imaging applications.[44,55,56] [$^{99m}$Tc]Demobesin 1 demonstrated a very high and persistent uptake in human PC-3 xenografts in nude mice, despite its minimal internalization in human prostate cancer cells.[43]

In this work we have developed four tetraamine-functionalized bombesin analogues based instead on agonists, Demobesin 3–6. The first two analogues are BB tetradecapeptides wherein Pyr$^1$ has been replaced by Pro$^1$ to allow for N-terminal modification, as previously proposed.[25] Further substitution of Met$^{14}$ by Nle$^{14}$ in Demobesin 4 was intended to produce a more oxidation-resistant analogue.[47] The last two peptides are truncated at position 6–7 of the mother sequence and lack the polar Arg$^3$ and Tyr$^4$ residues. Due to the vicinity of the metal chelate with the peptide receptor binding site, a spacer containing an extra benzyl group has been introduced in between.[27,38] As a result, Demobesin 5 and 6 are expected to be more lipophilic than the tetradecapeptide analogues. The effects of these modifications on the biological profile of new analogues are discussed and their performance compared with that of [$^{99m}$Tc]Demobesin 1.

As in the case of [$^{99m}$Tc]Demobesin 1, labeling with $^{99m}$Tc was straightforward, providing high-purity and high specific activity radiopeptides. The affinities of Demobesin 3–6 and (radio)metalated analogues thereof for the human GRP-R were found to be high during binding assays in PC-3 cell membranes, demonstrating that the presence of the tetraamine framework as well as of the respective metal chelate was well-tolerated by the GRP-R. In addition, the affinities of Demobesin 3–6 versus the three human bombesin receptor subtypes were compared during competition binding experiments in resected human cancer samples. Thus, the peptides showed high affinity for the human GRP-R, less affinity for the NMB-R, and no affinity for the $BB_3$-R. In a previously reported study, Demobesin 1, showed high affinity selectively for the GRP-R while its affinity for the other two human bombesin receptor subtypes was negligible.[43] Up to now, it is not clear how the affinity profile of the continuously emerging new radiolabeled bombesin analogues versus the three bombesin receptor subtypes affects biodistribution, tumor uptake, and accumulation in critical organs in man, especially in the pancreas.[36,37,51,58–60] More extensive studies are certainly warranted to elucidate the role and interplay of bombesin receptor subtypes in human healthy and cancerous tissue in order to design more effective bombesin-based radiopharmaceuticals.[20,50]

As expected, [$^{99m}$Tc]Demobesin 3–6 internalized very rapidly and in a high efficiency in PC-3 cells in contrast to [$^{99m}$Tc]Demobesin 1, which showed very minimal internalization in the same cell line.[43] Unexpectedly, the higher internalization efficiency of the agonist-based [$^{99m}$Tc]Demobesin 3–6 did not translate in a higher tumor and target-organ localization in vivo in comparison to the antagonist-based [$^{99m}$Tc]Demobesin 1. Indeed, after injection in nude mice bearing human PC-3 xenografts, high uptake was observed in the experimental tumor, especially for [$^{99m}$Tc]Demobesin 3 and 4, and the natural in vivo bombesin binding sites, namely the pancreas and the intestinal tract. However, the tumor uptake of [$^{99m}$Tc]Demobesin 3 and 4 was slightly lower than the values reported for the antagonist-based [$^{99m}$Tc]Demobesin 1 at all time intervals.[43] Nevertheless, the PC-3 tumor values still remain very high in comparison to those reported for other radiolabeled bombesin agonist-based analogues.[27] Furthermore, despite the lower pancreatic uptake of [$^{99m}$Tc]Demobesin 3 and 4 in the initial time intervals as compared to [$^{99m}$Tc]-Demobesin 1, pancreatic activity of the agonist-based peptides declined at a much slower pace. The most striking difference in the biodistribution profile of these peptides is their excretion pathway. Thus, while the more hydrophilic [$^{99m}$Tc]Demobesin 3 and 4 were excreted mainly via the kidneys into the urine, showing a lower and receptor-mediated intestinal uptake, [$^{99m}$Tc]-Demobesin 5 and 6 showed excessive hepatobiliary excretion as a combined result of their higher lipophilicity and their in vivo conversion to metabolites that are more lipophilic than the product of [$^{99m}$Tc]Demobesin 3 and 4 metabolism. This excessive hepatobiliary excretion of the truncated peptides should be given particular attention, given that several proposed GRP-R-seeking radiotracers are based on the BB(7–14) lipophilic motif and are reported to lead to excessive intestinal radioactivity.[27,36,37] This effect is even more pronounced when less hydrophilic metal chelates are employed[61] and eventually hampers detection of tumor deposits in the abdomen. Recent reports, however, explore the possibility to use hydrophilic modifiers for directing excretion to the kidneys.[42]

## Conclusion

Four new bombesin analogues labeled with $^{99m}$Tc are presented herein, [$^{99m}$Tc]Demobesin 3–6. The radiopeptides are all agonist-based and are modified with open

chain tetraamines for effective binding of the radiometal. They show high affinity for the human GRP-R both in GRP-R-positive cells and in human tumor samples while internalizing very efficiently and very rapidly in GRP-R-positive cells. After injection in PC-3 xenograft-bearing nude mice, they show distinct in vivo profiles. Thus, the tetradecapeptides [$^{99m}$Tc]Demobesin 3 and 4 show a very high and rapid localization in the experimental tumor combined with a rapid clearance from nontarget tissues predominantly via the kidneys and the urinary system. In contrast, the truncated peptides [$^{99m}$Tc]Demobesin 5 and 6 show a lower tumor uptake and an excessive hepatobiliary excretion. Their favorable in vivo profiles validate [$^{99m}$Tc]Demobesin 3 and 4 for further studies in man, and pilot/phase I studies in a small number of prostate cancer patients involving these analogues have been scheduled for the near future.

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