[^{99m}Tc]Demobesin 1, a novel potent bombesin analogue for GRP receptor-targeted tumour imaging

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Abstract. Demobesin 1 is a potent new GRP-R-selective bombesin (BN) analogue containing an open chain tetraamine chelator for stable technetium-99m binding. Following a convenient labelling protocol, the radiopeptide, [^{99m}Tc]Demobesin 1, formed in nearly quantitative yields and with high specific activities. Both unlabelled and labelled peptide demonstrated high-affinity binding in membrane preparations of the human androgen-independent prostate adenocarcinoma PC-3 cell line. The IC_{50} values determined for Demobesin 1 and [Tyr^4]BN were 0.70±0.08 n*M* and 1.5±0.20 n*M*, respectively, while the K_d defined for [^{99m}Tc/^{99g}Tc]Demobesin 1 was 0.67±0.10 n*M*. [^{99m}Tc]Demobesin 1 was rather stable in murine plasma, whereas it degraded rapidly in kidney and liver homogenates. After injection in healthy Swiss albino mice, [^{99m}Tc]Demobesin 1 accumulated very efficiently in the target organs (pancreas, intestinal tract) via a GRP-R-mediated process, as shown by in vivo receptor blocking experiments. An equally high and GRP-R-mediated uptake was exhibited by [^{99m}Tc]Demobesin 1 after injection in PC-3 tumour-bearing athymic mice. The initial high radioligand uptake of 16.2±3.1%ID/g in the PC-3 xenografts at 1 h p.i. remained at a similar level (15.61±1.19%ID/g) at 4 h p.i. Even after 24 h p.i., when the radioactivity had cleared from all other tissues, a value of 5.24±0.67%ID/g was still observed in the tumour. The high and prolonged localization of [^{99m}Tc]Demobesin 1 at the tumour site and its rapid background clearance are very promising qualities for GRP-R-targeted tumour imaging in man.

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Introduction

Bombesin (BN) is a tetradecapeptide that was first isolated from the skin of the European frog *Bombina bombina* [1]. Bombesin and its mammalian counterparts gastrin-releasing peptide (GRP) and neuromedin B (NMB) elicit a wide spectrum of biological responses in mammalian tissues, including the central nervous system and the gut [2, 3, 4]. Bombesin-like peptides exert their effects on target cells by binding to surface G protein-coupled receptors characterized by the typical configuration of seven transmembrane domains [5]. The physiological role of GRP, in particular, is mediated through the GRP receptor (GRP-R) [6, 7] and includes stimulation of enzyme secretion from exocrine glands or stimulation of release of a series of gastrointestinal peptide hormones [8]. In addition to this physiological role, it has been established by numerous studies over the past two decades that GRP and its interaction with the GRP-R promotes tumour growth in a number of normal and human cancer cell lines both in culture and in nude mice xenografts [8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23]. Most interestingly, GRP-Rs have been identified in human lung cancers [24, 25] and are also frequently expressed in high numbers in primary and metastatic prostate [26, 27, 28] and breast cancers [29, 30, 31, 32]. These findings stimulated the search for potent synthetic bombesin/GRP antagonists for the hormonal treatment of GRP-R-positive tumours, and candidates showing prom-

ising antiproliferative effects in human cell lines and nude mice xenografts have already been identified [15, 20, 33, 34, 35]. Furthermore, the use of bombesin/GRP antagonists as carrier molecules for targeting cytotoxic drugs to tumour cells has recently been proposed [36, 37].

In another approach, bombesin/GRP analogues may be used as carriers to direct diagnostic or therapeutic radionuclides to GRP-R-expressing neoplastic cells with high specificity. This approach has been competently explored for the peptide hormone somatostatin and its radiolabelled analogues, which are used today in the diagnostic imaging and treatment of neuroendocrine tumours [38]. Thus, following the successful paradigm of radiolabelled somatostatin analogues, use of GRP-R-specific radiotracers in combination with single-photon emission tomography (SPET) may provide a valuable non-invasive tool in the early diagnosis and staging of GRP-R-expressing malignant disease. Furthermore, this diagnostic modality may allow prediction and follow-up of the responsiveness of GRP-R-positive neoplasms to treatment with bombesin/GRP antagonists. Eventually, the first diagnostic bombesin-based radiotracers are expected to provide the critical mass for the design of second-generation radiopharmaceuticals for GRP-R-targeted internal radiotherapy of cancer.

So far, several bombesin analogues labelled with the diagnostic metallic radionuclides ^{111}In [39] and ^{99m}Tc have been proposed for the scintigraphic detection of GRP-R-positive lesions [40, 41, 42, 43, 44, 45, 46], while first research efforts toward the development of ^{111}In- [39] and ^{188}Re-based radiotherapeutic agents have been reported [47]. Due to the dominance of ^{99m}Tc in nuclear medicine diagnosis, most attempts have been focussed on ^{99m}Tc-based GRP-R-seeking radiotracers involving a wide range of chelators, such as the N_3S [40, 41, 42, 43], the P_2S_2 [44] or the carbonyl [45, 46] containing frameworks. Drawbacks encountered by these systems include (a) excessive hepatobiliary excretion, which impairs imaging quality in the abdominal area, (b) cumbersome labelling protocols that are unsuitable for routine application in a clinical environment, and (c) poor in vivo stability, preventing sufficient localization at the target site.

Demobesin 1 is a novel tetraamine derivatized potent bombesin analogue of potential relevance in this setting. Following a convenient labelling protocol, Demobesin 1 binds ^{99m}Tc with formation of a single radiopeptide species, [^{99m}Tc]Demobesin 1, in nearly quantitative yields and with sufficiently high specific activities for receptor-targeted applications. According to previous reports, the open chain tetraamine framework wraps around the equatorial plane of the $Tc(V)O_2^+$ core, forming a monocationic polar complex in an octahedral configuration [48, 49]. In addition to its advantages of easy formation at ambient temperatures and stability in the biological milieu, this metal chelate imparts considerable hydrophilicity to the originally lipophilic alkylamidated peptide moiety of [^{99m}Tc]Demobesin 1 [50, 51] and is therefore expected to favour excretion via the kidneys and the urinary system [52]. Several aspects of the new peptide's behaviour in cells, animal models and resected human biopsy specimens are discussed in detail below. The preclinical data presented in this study are instrumental for assessment of [^{99m}Tc]Demobesin 1 as a candidate for GRP-R-targeted diagnosis of malignant tumours in patients.

Materials and methods

General

Unless otherwise stated, all chemicals were reagent grade and were used without further purification. Synthesis of the Boc-protected tetraamine precursor *N*,*N*′,*N*″,*N*‴-tetra-(*tert*-butoxycarbonyl)-6-{*p*-[(carboxymethoxy) acetyl]amino-benzyl}-1,4,8,11-tetraazaundecane will be reported elsewhere. [Tyr^4]BN (Pyr-Gln-Arg-Tyr-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) and the potent bombesin antagonist [(D)Phe^6,Leu-NHEt^{13},*des*-Met^{14}]BN(6–14) were purchased from Bachem (Bubendorf, Switzerland). Iodine-125 was provided by MDS Nordion, SA (Fleurus, Belgium). Radioiodination of [Tyr^4]BN was performed according to a published protocol [53]. Technetium-99m in the form of [^{99m}Tc]$NaTcO_4$ in physiological saline was eluted from a commercial ^{99}Mo/^{99m}Tc generator (Cis International, France). Technetium-99g was purchased from Oak Ridge National Laboratories, USA, as $NH_4{}^{99g}TcO_4$. The impure black solid was purified prior to use by overnight treatment with H_2O_2 and NH_4OH in MeOH. Evaporation of the solvent afforded $NH_4{}^{99g}TcO_4$ as a white powder. Solvents for high-performance liquid chromatography (HPLC) were HPLC grade; they were filtered through 0.22-µm membrane filters (Millipore, Milford, USA) and degassed by helium flux.

Analyses and separations by HPLC were performed on a Waters Chromatograph with a 600 solvent delivery system and coupled to both a Waters 996 photodiode array UV detector (Waters, Vienna, Austria) and a Gabi gamma detector from Raytest (RSM Analytische Instrumente GmbH, Germany). The Millennium Software by Waters was applied to control the HPLC system and process the data. Separations were performed on a Waters RadialPak cartridge (µBondapak, 10 µm, 8 mm×100 mm) eluted as described in the text. For analyses we used the Reverse Phase (RP-18) Symmetry Shield cartridge column from Waters (5 µm, 3.9 mm×150 mm) or a XTerra cartridge column from Waters (5 µm, 4.6 mm×150 mm) applying the elution systems described in the text. Instant thin-layer chromatography (ITLC) was conducted on ITLC-SG strips from Gelman Science (Gelman, Ann Arbor, Michigan, USA). For radioactivity measurements an automatic well-type gamma counter calibrated for either ^{125}I or ^{99m}Tc was used [NaI(Tl) crystal, Canberra Packard Auto-Gamma 5000 series instrument]. A Brandel M-48 Cell Harvester (Adi Hassel Ingenieur Büro, Munich, Germany) was employed in binding experiments. For imaging, a small field of view experimental gamma camera, suitable for both planar and tomographic imaging, was employed. The system is based on a position-sensitive photomultiplier tube (Hamamatsu R2486), a pixelized CsI(Tl) scintillation crystal and CAMAC electronics [52]. For the ESI mass spectral analysis, test peptide solution was infused into an electrospray interface mass spectrometer (AQA Navigator, Finnigan) using a Ha-

rvant Syringe pump. Hot nitrogen gas (Dominic-Hunter UHPLCMS-10) was used for desolvation. The charge of each ion and the molecular mass of the peptide were determined by deconvolution algorithms.

The human prostate carcinoma cell line PC-3 was kindly provided by Dr. E. García-Garayoa (Paul Scherrer Institute, Villigen, Switzerland) or was alternatively purchased from LGC Promochem (Teddington, UK). All culture media were supplied by Gibco BRL, Life Technologies (Grand Island, N.Y.) and all supplements by Biochrom KG Seromed (Berlin, Germany). For protein measurements the protein microdetermination kit (procedure No. P 5656) by Sigma Diagnostics (St. Louis, USA) was utilized. Animal experiments were carried out in compliance with European and national regulations. For biodistribution and metabolism experiments, in-house male Swiss albino mice (30±5 g) were used. For experimental tumour models, female Swiss nu/nu mice aged 7 weeks upon arrival (Iffa Credo, France) were employed, and the animals were kept under aseptic conditions until biodistribution was performed.

Synthesis of bombesin analogues

Coupling step. The bifunctional tetraamine precursor, *N*,*N*′,*N*″,*N*‴-tetra-(*tert*-butoxycarbonyl)-6-{*p*-[(carboxymethoxy) acetyl]-aminobenzyl}-1,4,8,11-tetraazaundecane (18.1 mg, 23.1 µmol), was dissolved in CH_2Cl_2 (~400 µl) and *O*-(7-azabenzotriazolyl-1,1,3,3-tetramethylammonium hexafluorophosphate (HATU) (9.5 mg, 25 µmol) dissolved in MeCN (200 µl) was added to the solution, followed by *N*-ethyldiisopropylamine (Hünig's base) (6.46 mg, 50 µmol, 8.6 µl). After a ca. 3-min incubation this mixture was added to a solution of the bombesin antagonist [(D)Phe^6,Leu-NHEt^{13},*des*-Met^{14}]BN(6–14) (*H*-(D)Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHEt) in *N*-methylpyrrolidone (10 mg, 7.7 µmol) in a final volume <1 ml and left to react for 90 min. After dilution of the reaction mixture with H_2O (10 ml), the peptide conjugate was extracted in ethyl acetate and the organic solvent evaporated under vacuum. The residue was redissolved in $CHCl_3$ and purified over a small SiO_2 column (8 mm×100 mm) eluted with $CHCl_3$/MeOH 100/15. The collected fraction was evaporated to dryness by a gentle N_2 stream at 35–40°C and stored in the freezer.

t_R (retention time) Boc-Demobesin 1 (Symmetry Shield column, flow rate: 1 ml/min, from 100% 0.1% trifluoroacetic acid (TFA) to 10% 0.1% TFA/90% MeCN within 45 min)=30.5 min (tetraamine precursor at 34.7 min and free peptide at 18.1 min); R_f Boc-Demobesin 1 (SiO_2; $CHCl_3$/MeOH 10/5)=0.7 (tetraamine precursor at 0.4); ES-MS, m/z, Boc-Demobesin 1=1,771.7 ($M+Na^+$, 100), 1,749.8 ($M+H^+$, 43).

Deprotection step. Water (25 µl), thioanisol (20 µl) and TFA (500 µl) were added to the protected peptide conjugate and the mixture was incubated for 30 min at ambient temperature. It was then transferred to an ice-cooled 0.5 *M* NaOH solution (12 ml) and the aqueous phase was rinsed with $CHCl_3$ and concentrated to a small volume (2 ml) by rotary evaporation. The aqueous phase was loaded on a RadialPak cartridge from Waters and purified by gradient elution starting with 0.1% TFA H_2O/0.1% TFA MeCN 100/0 and reaching 0.1% TFA H_2O/0.1% TFA MeCN 40/60 in 60 min at a 1 ml/min flow rate. Fractions containing the peak at 36 min and corresponding to Demobesin 1 were collected. The organic solvent was expelled under vacuum and the aqueous solution lyophilized.

Yield=70%; t_R Demobesin 1 (Symmetry Shield column, flow rate: 1 ml/min, from 80% 0.1% TFA to 70% 0.1% TFA/30% MeCN within 20 min)=13.1 min; ES-MS, m/z, Demobesin 1=1,348.2 ($M+H^+$, 20), 674.8 ($(M+2H^+)/2$, 100).

Radiolabelling of Demobesin 1 with ^{99m}Tc

The lyophilized peptide analogue was dissolved in 50 m*M* acetic acid/EtOH 8/2 v/v at a final 1 m*M* concentration. The bulk solution of Demobesin 1 was distributed in 50-µl aliquots in Eppendorf vials that were stored at –20°C. To an Eppendorf vial containing 0.5 *M* phosphate buffer pH 11.5 (50 µl), 0.1 *M* sodium citrate solution (5 µl) was added followed by pertechnetate generator eluate (415 µl, 370–740 MBq or 10–20 mCi), Demobesin 1 stock solution (15 nmol) and finally a freshly prepared ethanolic $SnCl_2$ solution (30 µg, 15 µl). The mixture was left to react for 30 min at ambient temperature and then was brought to pH 7 by the addition of 1 *M* HCl (10 µl).

Radiochemical analysis

Aliquots (1 µl) of the labelling solution were analysed by HPLC on a XTerra cartridge column from Waters applying a linear gradient system at a 1.0 ml/min flow rate from 20% B to 60% B in 20 min, where solvent A = 0.1% TFA in water and solvent B = pure acetonitrile. Under these conditions, [^{99m}Tc]Demobesin 1 elutes at 11.1 min, [^{99m}Tc]citrate at 2.0 min and $^{99m}TcO_4^-$ at 2.6 min. For detection of traces of reduced hydrolysed technetium ($^{99m}TcO_2$), ITLC was performed. An aliquot (<1 µl) of the labelled product was applied on the ITLC strip that was developed up to 10 cm from the origin with 1 *M* ammonium acetate/MeOH 1/1 (v/v). The strip was left to dry in the open and then cut into two pieces: 1st=Start (origin +0.5–1 cm): $^{99m}TcO_2$ and 2nd=Front (the rest of the strip): $^{99m}TcO_4^-$, [^{99m}Tc]citrate and [^{99m}Tc]Demobesin 1. The radioactivity content of each piece was measured in the gamma counter. Stability tests of the radiolabelled product were conducted up to 6 h post labelling.

Preparation of [^{99g}Tc]Demobesin 1

The [^{99g}Tc]Demobesin 1 was prepared by following a protocol similar to that for its [^{99m}Tc]Demobesin 1 counterpart at tracer level. Due to the higher mass of $[^{99g}Tc]NH_4TcO_4$ utilized (0.9 µg, 5 nmol), an increased amount of $SnCl_2$ (1 µmol/ml) was required for complete reduction. The peptide was purified by RP-HPLC applying the conditions described above. Triplicates in the 10^{-12}–10^{-6} *M* concentration range were prepared and used for binding experiments, as previously described [52].

Cell culture

Human prostate carcinoma PC-3 cells were cultured in Dulbecco's MEM GLUTAMAX-I supplemented by 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were kept in humidified air containing 5% CO_2 at 37°C. Weekly passages were performed applying a trypsin/EDTA (0.05%/0.02% w/v) solution.

Competition binding assays

Competition binding experiments with Demobesin 1 were performed in cell membrane homogenates of PC-3 cells, harvested as detailed previously [52]. [Tyr^4]BN served as the control peptide and [^{125}I-Tyr^4]BN as the radioligand, prepared according to a published method [53]. Briefly, 40,000 cpm of radioligand was used per assay tube, with 50 µg protein in the presence of increasing concentrations of tested peptide in a total volume of 300 µl 50 m*M* HEPES (pH 7.6, 0.3% BSA, 5 m*M* $MgCl_2$, 14 m*M* bacitracin). Tubes in triplicate for each concentration point were incubated for 30 min at 37°C. The assay was completed and the IC_{50} values calculated as previously described [52].

Receptor autoradiography in human tumours

Competition binding experiments were performed with Demobesin 1 in human tumours expressing either GRP-Rs or NMB receptors (NMB-Rs) or BB_3 receptors (BB_3-Rs), using [^{125}I-(D)Tyr^6,(β)Ala^{11},Phe^{13},Nle^{14}]BN(6–14) as universal radioligand or [^{125}I-Tyr^4]BN as GRP-R preferring ligand, as described in detail previously [54, 55].

Saturation binding assays

Saturation binding experiments for [^{99g}Tc/^{99m}Tc]Demobesin 1 were performed in PC-3 cell membranes as well, according to a previously described protocol [52].

Internalization experiments

For internalization experiments, confluent PC-3 cells were seeded in 35-mm-diameter dishes (Greiner Labortechnik, Germany), wherein they remained overnight (1.0–1.5×10^6 cells per well). On the day of the experiment, cells were rinsed twice with ice-cold internalization medium comprising Dulbecco's MEM GLUTAMAX-I supplemented by 1% (v/v) fetal bovine serum [52]. After addition of fresh medium (1.2 ml), ca. 300,000 cpm [^{99m}Tc]Demobesin 1 (in 150 µl PBS/0.5% BSA buffer, corresponding to ~200 fmol total peptide) was added and the experiment conducted as previously described [52].

Metabolism

Collected urine. Mice were injected with a 100-µl bolus containing 18.5–37 MBq (0.5–1 mCi) of [^{99m}Tc]Demobesin 1 via the tail vein (see "Tissue distribution experiments" below). The animals were kept for 30 min in metabolic cages with water and food available ad libitum. Immediately after sacrifice the urine was collected from their bladder with a syringe and, in parallel to that collected in the cages, it was filtered through a Millex GV filter (0.22 µm). Aliquots were then analysed by HPLC under the conditions described in the section "Radiochemical analysis".

Plasma incubation. Blood collected from mice in heparinized polypropylene tubes was immediately centrifuged for 15 min at 5,000 rpm at 4°C and the plasma collected. Samples of [^{99m}Tc]Demobesin 1 were then incubated with fresh plasma at 37°C and aliquots withdrawn 15 min and 1 h later. Ethanol was added to the latter in a 2:1 EtOH/aliquot v/v ratio, the samples were centrifuged at 35,000 rpm for 10 min and supernatant fractions were passed through a Millex GV filter (0.22 µm) prior to HPLC analysis conducted as described above.

Incubation in kidney and liver homogenates. Kidneys or liver freshly excised from mice was rapidly rinsed and placed in a chilled 50 m*M* TRIS/0.2 *M* sucrose buffer of pH 7.4, wherein they were homogenized in an Ultra-Turrax T25 homogenator for 1 min at 4°C. As described above for the plasma incubates, the radiopeptide was incubated with fresh homogenates at 37°C for 5, 30 and 60 min and then, always following the same procedure, they were analysed by HPLC.

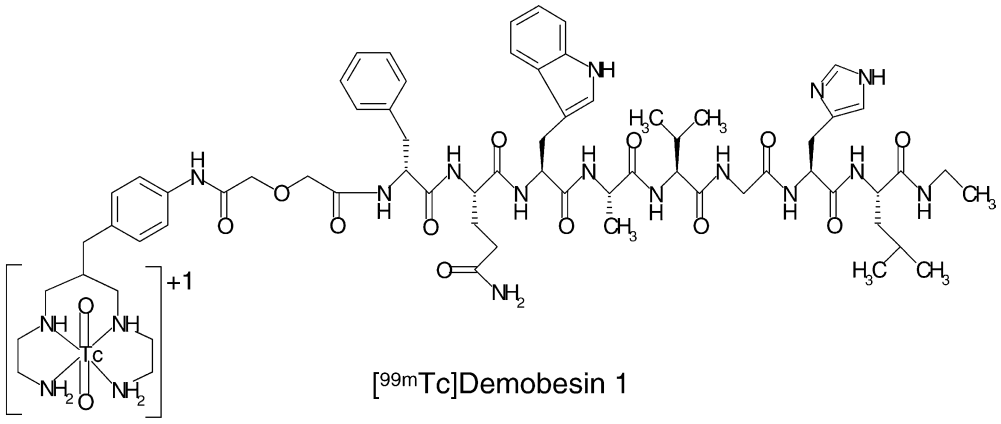
Tissue distribution experiments

Experiments in healthy mice. Male Swiss albino mice (*n*=30) were used for the biodistribution in groups of four to six per time point. Each animal received a 100-µl bolus containing 148–185 kBq (4–5 µCi) of [^{99m}Tc]Demobesin 1 in PBS buffer pH 7.4 (corresponding to ca. 10 pmol of total peptide) via the tail vein. Animals were sacrificed by cardiac puncture under mild ether anaesthesia at 0.5-, 1-, 2- and 4-h time intervals. The animals of an additional group were each treated with 1 mg of [Tyr^4]BN administered intraperitoneally 35 min prior to the injection of the radioligand (blocked animals) [44]. These were sacrificed 30 min post injection (p.i.) of the radioligand. Samples of urine, blood and organs of interest were immediately collected and weighed, and their radioactivity content was measured in a gamma counter. Stomach and intestines were not emptied of their contents during this study. Biodistribution data were calculated as percent injected dose per gram (%ID/g) applying an in-house software program and using appropriate standards.

Experiments in PC-3 tumour-bearing mice. A bolus (<150 µl) containing a suspension of 1.5–2×10^7 PC-3 cells in PBS buffer was inoculated under the skin of the flank of each female Swiss nu/nu mouse. After 2–3 weeks, visible tumours were grown in the location of the implants and biodistribution was performed in a similar manner as described for healthy mice. Time points included 1, 4 and 24 h p.i., while blocking experiments included the 1 and 4 h p.i. time points. Blocked animals received intravenously (i.v.) 250 µg [Tyr^4]BN along with the radioligand or intraperitoneally 1 mg of [Tyr^4]BN 35 min prior to the administration of the radioligand. Each animal group comprised four to six inoculated mice.

Imaging

Two additional mice bearing the implanted PC-3 tumour were injected each with a 200-µl bolus containing 18.5–37 MBq (0.5–1 mCi) of [^{99m}Tc]Demobesin 1 in PBS buffer pH 7.4 via the tail vein, either alone or together with 250 µg [Tyr^4]BN (blocked mouse). The animals were sacrificed by ether asphyxiation 1.5 h p.i. and then placed in a typical position for planar imaging under a small field of view experimental gamma camera suitable for planar imaging [52]. Correction for ^{99m}Tc decay was performed in projection data as well. Imaging was immediately followed by dissection of the animals for correlation of scintigraphic findings with anatomical and biodistribution data.



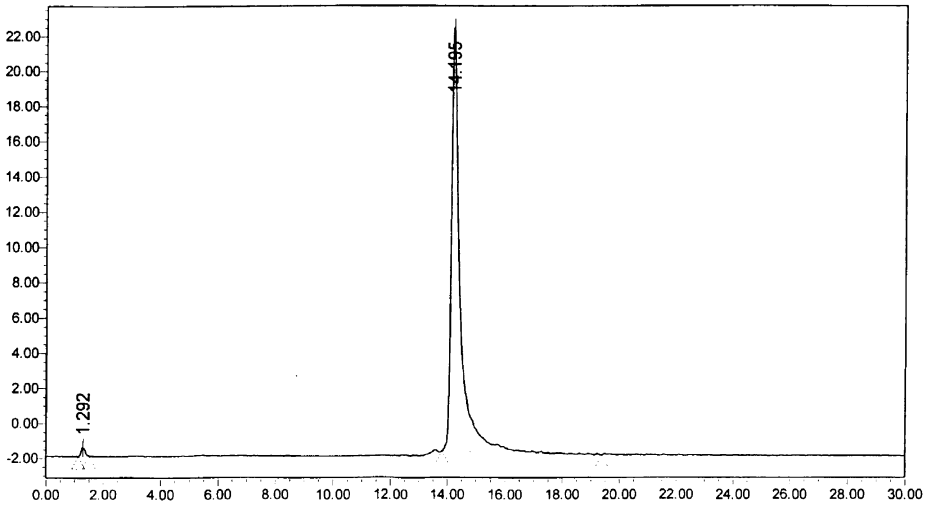


Fig. 1. [99mTc]Demobesin 1 formula and typical HPLC gamma trace of related labelling reaction mixture

Results

Synthesis of Demobesin 1

Synthesis of Demobesin 1 was performed in solution in two consecutive steps. In the first step coupling of the Boc-protected tetraamine chelator precursor to the N-terminal of the potent bombesin antagonist [(D)Phe^{6},Leu-NHEt^{13},*des*-Met^{14}]BN(6–14) [49, 50] was achieved in nearly quantitative yields using the HATU coupling agent. In the final step, removal of Boc groups was completed by TFA treatment. Purification by semi-preparative HPLC and lyophilization afforded a pure product as white powder in an overall yield of ~70%. Analysis by HPLC confirmed its high purity while ES-MS spectra were in accordance with the expected structure.

Labelling with ^{99m}Tc

A nearly quantitative incorporation of ^{99m}Tc by the tetraamine framework present in Demobesin 1 was easily achieved following a convenient protocol, previously detailed for other tetraamine functionalized peptides [52]. Typical specific activities of 37 GBq/μmol (1 Ci/μmol) Demobesin 1 were easily accessible with fresh generator eluate obtained 3–24 h after previous elution of the generator [52]. This specific activity value is well within the range required for receptor-targeting applications employing ^{99m}Tc [52] and, therefore, no further purification was deemed necessary. As demonstrated by twin RP-HPLC (Fig. 1) and ITLC analysis of the labelling reaction mixtures, [^{99m}Tc]Demobesin 1 formed as single radioactive species and remained stable in the open reaction vial for at least 6 h after labelling (peptide-associated radioactivity >96%).

GRP-R-binding capability of Demobesin 1

The binding capability of Demobesin 1 for the GRP-R was determined in membrane preparations of the human androgen-insensitive PC-3 prostate cancer cell line re-

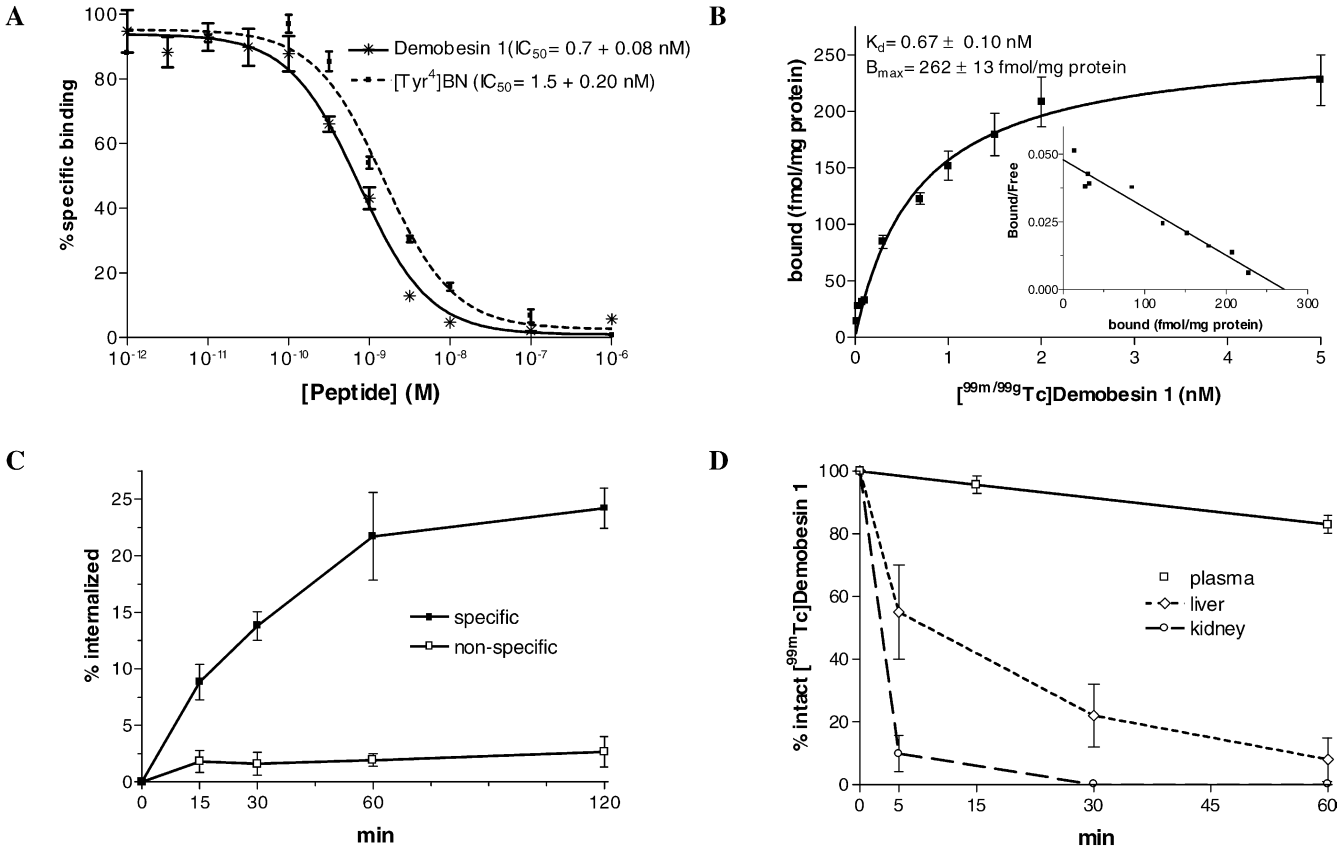


Fig. 2A–D. In vitro tests with Demobesin 1 and [^{99m}Tc]Demobesin 1. **A** Displacement of [^{125}I-Tyr^4]BN from PC-3 membranes by increasing amounts of Demobesin 1 and [Tyr^4]BN. **B** Saturation binding of [^{99m}Tc/^{99g}Tc]Demobesin 1 in PC-3 membranes with Scatchard plot shown in the *inset*. **C** Internalization rate of [^{99m}Tc]Demobesin 1 in live PC-3 cells at 37°C. **D** Rates of enzymatic degradation of [^{99m}Tc]Demobesin 1 by incubation in murine plasma, kidney and liver homogenates at 37°C

ported to predominantly express this particular BN-R-subtype [18, 19, 20, 21, 22]. As shown in Fig. 2A, the radioligand, [^{125}I-Tyr^4]BN, is displaced by Demobesin 1 from bombesin binding sites in a monophasic and dose-dependent manner. The IC_{50} value calculated during this assay for Demobesin 1 is found in the sub-nanomolar range (0.7±0.08 n*M*) and is comparable to that of [Tyr^4]BN (1.5±0.20 n*M*), suggesting that coupling of the tetraamine moiety is well tolerated by the GRP-R.

Autoradiography on resected human biopsy specimens with Demobesin 1

The affinity profile of Demobesin 1 for the three bombesin receptor subtypes GRP-Rs, NMB-Rs and BB_3-Rs was tested in tumours preferentially expressing each of the receptors, namely prostate cancer for GRP-Rs, gut carcinoid for NMB-Rs and lung carcinoid for BB_3-Rs [54]. Demobesin 1 showed high binding affinity in the GRP-R-expressing prostate cancer, but not in the NMB-R- and BB_3-R-expressing tumours, as shown in Fig. 3. IC_{50} values for Demobesin 1 were 2.6±0.2 n*M* (mean±SEM; *n*=5) in GRP-R-positive prostate cancer and over 1,000 n*M* in BB_3-R- or NMB-R-expressing tumours (*n*=3).

Saturation binding of [^{99m}Tc/^{99g}Tc]Demobesin 1

Binding data for [^{99m}Tc/^{99g}Tc]Demobesin 1 were extracted from saturation assays on PC-3 cell membranes. Due to the high specific activity of ^{99m}Tc (19,240 GBq/µmol or 520 Ci/µmol), the required concentration series (1–5 n*M*) comprising a single radioligand species was accessible by the addition of a sufficient and carefully monitored amount of the pseudo-stable beta emitter ^{99g}Tc during preparation of the radiopeptide, as previously described [52]. As depicted in Fig. 2B after fitting the data to a one-site model, [^{99m}Tc/^{99g}Tc]Demobesin 1 demonstrates a strong and dose-dependent interaction with a single class of high-affinity binding sites in the PC-3 cell membranes. The equilibrium dissociation constant K_d of 0.67±0.10 n*M* illustrates the high affinity of [^{99m}Tc/^{99g}Tc]Demobesin 1 for the GRP-R, which is comparable to that of other BN-based radioligands determined in the same cell line [41, 43]. Furthermore, the B_{max} value of 262±13 fmol/mg is well within the range of values reported for the GRP-R expression in this cell line [22, 46].

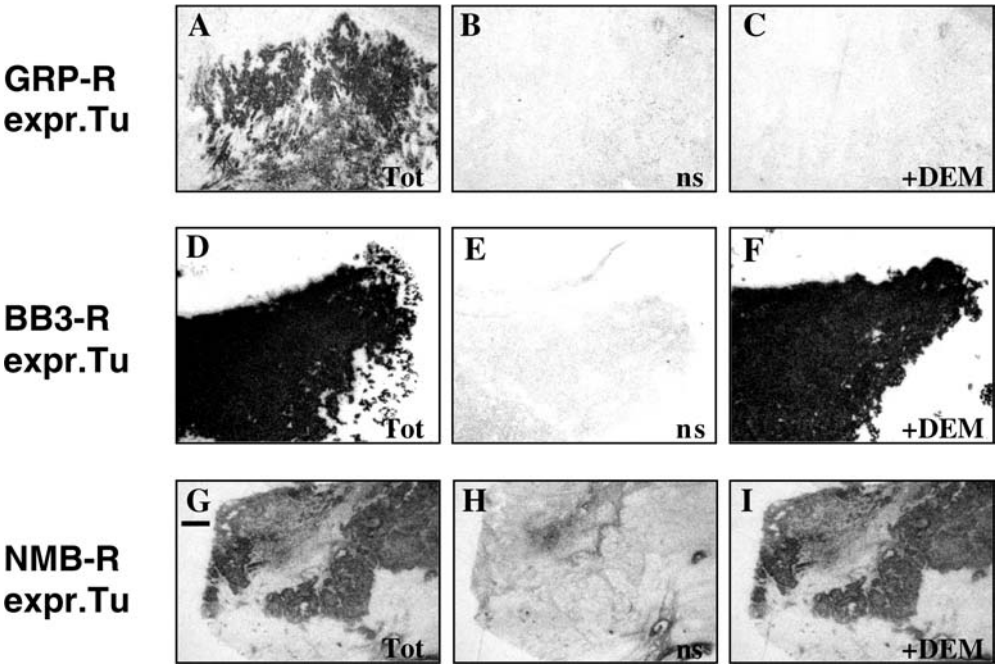


Fig. 3A–I. Receptor autoradiography showing the selective binding of Demobesin 1 to GRP-Rs expressed in human prostate cancer (**A–C**) but not to BB_3-R (**D–F**) or NMB-R (**G–I**)-expressing tumours. **A, D, G** Autoradiograms showing total binding of [^{125}I-(D)Tyr^6,(β)Ala^{11},Phe^{13},Nle^{14}]BN(6–14). *Bars*=1 mm. All three tumours are labelled with this universal radioligand. **B, E, H** Autoradiograms showing non-specific binding (in the presence of 10^{-7} *M*) of [(D)Tyr^6,(β)Ala^{11},Phe^{13},Nle^{14}]BN(6–14). **C, F, I** Autoradiograms showing displacement of the radioligand with 10^{-7} *M* Demobesin 1 (DEM). Full displacement is seen in the GRP-R-expressing tumour (**C**) whereas no displacement is seen in the other two tumours (**F** and **I**)

Internalization of [^{99m}Tc]Demobesin 1 in PC-3 cells

The internalization properties of [^{99m}Tc]Demobesin 1 were studied during incubation in human PC-3 cells at 37°C in the absence or presence of 1 μ*M* [Tyr^4]BN (non-specific series). The curve of percent radioligand internalization plotted over time is shown in Fig. 2C, revealing rapid migration of a small (~25%) but significant portion of radioactivity within the cells. This finding is intriguing considering that [^{99m}Tc]Demobesin 1 is based on the potent antagonist [(D)Phe^6,Leu-NHEt^{13},*des*-Met^{14}]BN(6–14) [50, 51]. However, agonist activity for the latter peptide has been determined during specific rat and guinea pancreatic acinar assays [51]. In this respect, interspecies differences in agonist activity already reported for other *des*-Met^{14} alkylamide BN analogues [51] may be involved. Furthermore, the original peptide motif of the antagonist has been structurally altered in [^{99m}Tc]Demobesin 1, accommodating the bulky and polar moiety of the monocationic metal chelate, and probably affecting internalization to a certain extent.

Metabolism and biodistribution of [^{99m}Tc]Demobesin 1 in healthy mice

Incubation of [^{99m}Tc]Demobesin 1 in mouse plasma at 37°C revealed the presence of >85% intact peptide after 1 h. It is interesting to note that during the critical initial time points of incubation (15 min) the intact peptide fraction exceeded 90% (Fig. 2D). A completely different picture was obtained during incubation of [^{99m}Tc]Demobesin 1 in kidney and liver homogenates. Very rapid degradation of the radiopeptide was evident in the kidney preparation, with less than 5% intact peptide detected after just 5 min of incubation (Fig. 2D). This finding is in good agreement with results from analysis of urine collected 30 min after injection of [^{99m}Tc]Demobesin 1 in mice, which revealed complete degradation of the original peptide. In each assay, two major hydrophilic metabolites were detected; these were, however, unrelated to free $^{99m}TcO_4^-$ as verified by both their t_R values and results from ITLC tests run in parallel.

The slow degradation of [^{99m}Tc]Demobesin 1 in plasma versus its very rapid conversion to hydrophilic daughter compounds in the kidney may be of benefit by favouring rapid body clearance of radioactivity from non-target tissues without affecting localization in bombesin binding sites in vivo. In fact, this is clearly the case during the biodistribution of [^{99m}Tc]Demobesin 1 in healthy mice, as shown by the data summarized in Fig. 4. The compound showed rapid clearance predominantly via the kidneys and the urinary system into the urine, with a small portion of liver uptake. Uptake in the pancreas was very high (58.7±6.1%ID/g at 30 min p.i.) and specific, as demonstrated by the effective in vivo blockade of this organ in the animals pretreated with a high dose of [Tyr^4]BN (2.7±1.1%ID/g). The high pancre-

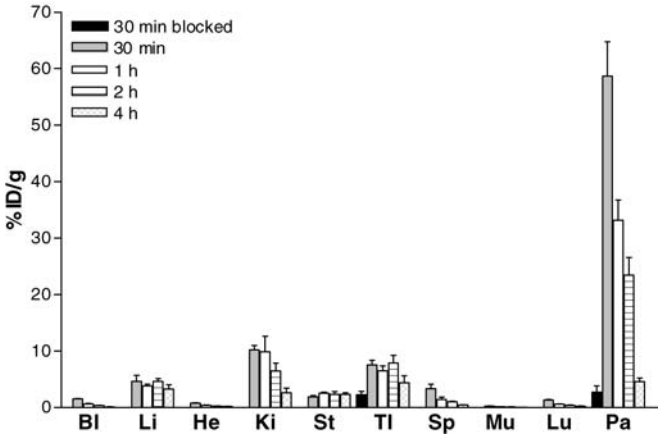


Fig. 4. Tissue distribution data at 30-min and 1-, 2- and 4-h intervals after injection of [^{99m}Tc]Demobesin 1 in healthy male Swiss albino mice. Four to six animals were used per time point and results are given as mean ± standard deviation of %ID/g. For the blocking experiment at 30 min p.i., animals each received intraperitoneally 1 mg [Tyr^4]BN 35 min prior to the injection of the radioligand. *Bl*, Blood; *Li*, liver; *He*, heart; *Ki*, kidneys; *St*, stomach; *TI*, total intestines; *Sp*, spleen; *Mu*, muscle; *Lu*, lung; *Pa*, pancreas

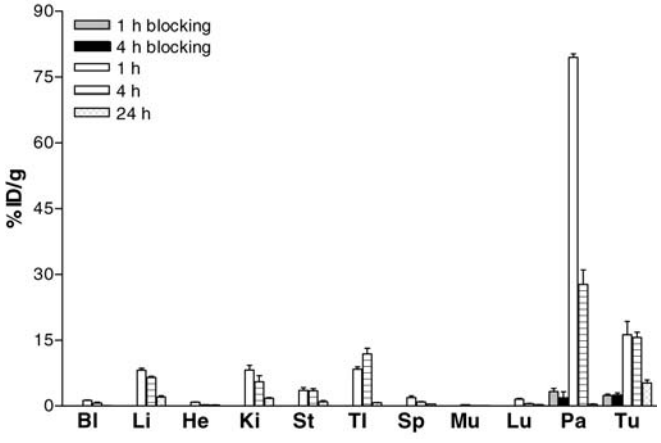


Fig. 5. Tissue distribution data at 1-, 4- and 24-h intervals after injection of [^{99m}Tc]Demobesin 1 in human PC-3 xenograft-bearing female Swiss nu/nu mice. Four to six animals were used per time point and results are given as mean ± standard deviation of %ID/g. For the blocked series at the 1 and 4 h time points, the animals each received intravenously 250 µg [Tyr^4]BN along with the radioligand. *Bl*, Blood; *Li*, liver; *He*, heart; *Ki*, kidneys; *St*, stomach; *TI*, total intestines; *Sp*, spleen; *Mu*, muscle; *Lu*, lung; *Pa*, pancreas; *Tu*, tumour

atic uptake exhibited by [^{99m}Tc]Demobesin 1 is substantially higher than the values previously reported for other radiolabelled BN analogues [39, 40, 41, 44, 45, 46], reflecting the high affinity of this analogue for the GRP-R [56]. The intestinal uptake (7.53±0.85%ID/g at 30 min p.i.) is attributable mainly to the GRP-R expressed in this tissue [57] and not to hepatobiliary excretion of the radioligand, given that it was substantially reduced in the [Tyr^4]BN-pretreated animals (2.26±0.56%ID/g).

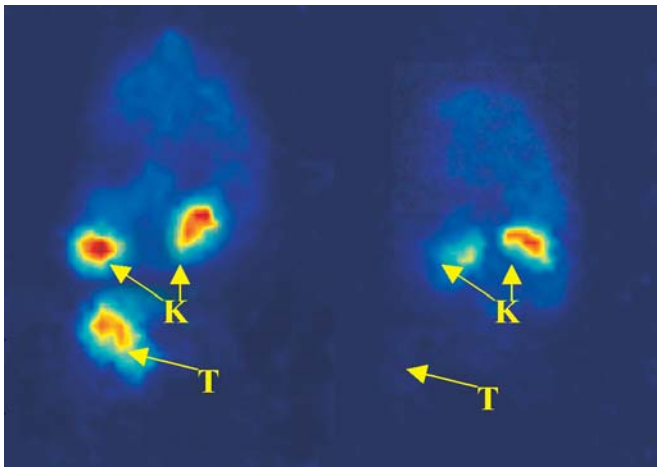


Fig. 6. Static images of PC-3 xenograft-bearing athymic mice 1.5 h after i.v. injection of [^{99m}Tc]Demobesin 1 (~20 MBq) alone (*left*) or together with 250 µg [Tyr^4]BN (*right:* blocked mouse); *K*, kidneys, *T*, tumour

Table 1. Tumour to non-target tissue and tumour to blocked tumour ratios at 1, 4 and 24 h after injection of [^{99m}Tc]Demobesin 1 in female Swiss nu/nu mice

Ratio	1 h	4 h	24 h
Tumour/blood	12.9±3.3	23.1±3.6	71.4±1.2
Tumour/muscle	67.6±13.7	206.0±21.1	81.3±12.1
Tumour/liver	2.0±0.5	2.2±0.3	2.7±0.7
Tumour/kidneys	2.0±0.5	2.7±0.4	3.0±0.5
Tumour/blocked tumour	6.9±1.3	7.7±1.2	–

Four to six animals were used per time point and results are given as mean ± standard deviation. For the blocking experiment animals each received 250 µg [Tyr^4]BN intravenously along with the radioligand

Uptake of [^{99m}Tc]Demobesin 1 in PC-3 xenografts

Tissue distribution data as %ID/g of [^{99m}Tc]Demobesin 1 in female Swiss nu/nu mice bearing human PC-3 xenografts in their flanks are summarized in Fig. 5. Specific uptake of the radioligand was evident in the pancreas (79.5±0.8%ID/g at 1 h p.i.) and the experimental tumour (16.2±3.1%ID/g), which could be blocked by co-injection of a high dose of [Tyr^4]BN (3.26±0.71%ID/g and 2.34±0.33%ID/g, respectively). The high tumour uptake of [^{99m}Tc]Demobesin 1 at 1 h p.i. remained at this level (15.61±1.19%ID/g) up to 4 h p.i. This result, along with the impressively high pancreatic uptake of [^{99m}Tc]Demobesin 1, is well above the values reported for other radiolabelled BN analogues [42, 45, 46] and may again reflect the high affinity of the radioligand. On the other hand, the rapid body clearance of [^{99m}Tc]Demobesin 1, predominantly into the urine (67.5±4.5%ID at 1 h p.i.) via the kidneys, led to increasingly high tumour to background ratios (Table 1). This property is also apparent in

the static images obtained at 1.5 h p.i. that are shown in Fig. 6, where [^{99m}Tc]Demobesin 1 clearly delineates the neoplastic region and at the same time demonstrates rapid body clearance from the PC-3 xenografted mouse via the kidneys (Fig. 6, left). In contrast, the same tumour is not visualized in another athymic mouse which had received a high dose of [Tyr^4]BN together with [^{99m}Tc]Demobesin 1 (Fig. 6, right).

Discussion

Numerous studies have reported on the GRP(BN)/GRP-R interaction as an autocrine tumour growth stimulating pathway for several neoplastic human cell lines in vitro or in nude mice xenografts in vivo [8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 33, 34, 35]. On the other hand, it has been established that GRP-Rs are expressed in resected human biopsy specimens of mainly small cell lung cancers (SCLCs) [24, 25], prostate carcinomas [26, 27, 28] and breast [29, 30, 31, 32] carcinomas, often at much higher densities than in normal surrounding tissue; this provides the molecular basis for GRP-R-targeted diagnostic imaging and/or internal radiotherapy of malignant lesions with radiolabelled bombesin analogues. In addition, the high frequency of these tumours has had a synergic effect in stimulating research on novel bombesin-like radiotracers for effective in vivo targeting of GRP-R-positive neoplastic disease [39, 40, 41, 42, 43, 44, 45, 46, 47]. Most agents reported so far are ^{111}In- or ^{99m}Tc-based radiotracers, wherein the peptide moiety has been modified to accommodate a radiometal binding site, such as DTPA (for ^{111}In) [39], N_3S [40, 41, 42, 43], P_2S_2 [44] and the 2-picolylamine-*N*,*N*-diacetate (PADA)- or the N_α-histidinyl acetate-carbonyl frameworks [45, 46] (for ^{99m}Tc). However, it is clear that, owing to the superior nuclear properties, cost-effectiveness and wide availability of the pre-eminent radionuclide of nuclear medicine, ^{99m}Tc, a ^{99m}Tc-based GRP-R-seeking radiotracer is highly desirable for routine medical use as compared with a ^{111}In-related radioligand. Furthermore, the similarities of technetium and rhenium chemistries raise the possibility of routine application of analogous diagnostic ^{99m}Tc and therapeutic ^{188}Re GRP-R-specific radiopharmaceutical pairs based on bombesin [47].

In view of the above, this study reports on a new bombesin analogue, Demobesin 1, derived from the potent bombesin antagonist [(D)Phe^6,Leu-NHEt^{13},*des*-Met^{14}]BN(6–14) [50, 51], wherein a tetraamine ^{99m}Tc binding unit has been covalently attached to (D)Phe^6 through a benzylaminodiglycolic acid spacer. As reported previously for other tetraamine-functionalized peptides [52], incorporation of the radiometal by the open chain tetraamine framework is straightforward applying a convenient protocol and leads to formation of a single radiopeptide species, [^{99m}Tc]Demobesin 1, in specific activities sufficiently high for receptor-targeted applications [52]. According to previous reports a monocationic octahedral metal chelate containing the $^{99m}Tc(V)O_2^+$ core is involved, showing sufficient stability versus competing ligands in the biological milieu and at the same time imparting hydrophilicity to the final radiopeptide [48, 49, 52]. The easy formation of a defined radiopeptide species provides significant practical advantages as far as routine application in a clinical environment is concerned, and contrasts with the previously reported cumbersome labelling protocols required for other ^{99m}Tc-labelled bombesins [44, 45, 46]. Furthermore, the hydrophilic character of the polar $^{99m}TcO_2^+$-tetraamine metal chelate is expected to favour renal clearance of the final radiopeptide over hepatobiliary excretion. Clearance via the hepatobiliary pathway is a frequently reported drawback of radiolabelled bombesin peptides derivatized with neutral $^{99m}Tc(V)O^{3+}$ [40, 41, 42] or ^{99m}Tc(I)carbonyl chelates [45, 46].

Attachment of the bulky tetraamine chelator is well tolerated by the GRP-R, as demonstrated by the IC_{50} of 0.7±0.08 n*M* determined for Demobesin 1 during competition binding assays in human androgen-insensitive prostate adenocarcinoma PC-3 cell membranes, whereas the respective IC_{50} value for unmodified [Tyr^4]BN was 1.5±0.20 n*M*. Of further importance is the high affinity binding of Demobesin 1 detected in vitro with receptor autoradiography in the putative human targets for this drug, namely in human GRP-R-expressing cancers (IC_{50}=2.6±0.20 n*M*), and the very high selectivity for GRP-Rs as compared with NMB-Rs and BB_3-Rs.

Incorporation of the radiometal seemed also to have a positive effect on receptor affinity, as shown by the K_d value of 0.67±0.10 n*M* calculated for [^{99m}Tc/^{99g}Tc]Demobesin 1 during saturation binding experiments in the same cell line. This value is very comparable to the K_d values of previously reported radiolabelled bombesin analogues [42, 44].

After administration in healthy mice, [^{99m}Tc]Demobesin 1 localizes rapidly in GRP-R-positive tissues, such as the pancreas and the intestines [56, 57]. The high pancreatic uptake of 58.7%ID/g at 30 min p.i. was effectively reduced (2.7%ID/g) in the animals receiving 1 mg [Tyr^4]BN intraperitoneally 35 min prior to the injection of the radioligand, suggesting a specific GRP-R-mediated process. To our knowledge, these are the highest pancreatic values reported so far for radiolabelled bombesin analogues, and they seem to reflect the high affinity of [^{99m}Tc]Demobesin 1 for the GRP-R. The radioactivity is cleared from the pancreas with time (33.13±3.63%ID/g at 1 h p.i., 23.43±3.13%ID/g at 2 h p.i. and 4.59±0.63%ID/g at 4 h p.i.), most probably as a result of the excessive enzymatic activity in this organ. In fact, similar trends have been reported for other ^{99m}Tc-labelled bombesin analogues, such as [^{99m}Tc-P_2S_2]BN(7–14), whereby the pancreatic values declined from 21.3±2.1%ID/g at 30 min p.i. to 8.5±1.25%ID/g at 4 h p.i.

[44]. Significant uptake was also observed in the intestinal tract (7.53±0.85%ID/g at 30 min p.i.). In the animals pretreated with a high dose of [Tyr^4]BN this uptake was significantly reduced (2.26±0.56%ID/g at 30 min p.i.), implying an at least partially specific and GRP-R-associated process. In fact, bombesin binding sites have been identified throughout the intestine, such as in the submucosal layer of the small intestine and in the longitudinal and circular muscle and the submucosal layer of the colon [57].

The predominant excretion pathway of [^{99m}Tc]Demobesin 1 is through the kidneys into the urine, wherein ~70%ID is collected within the first 30 min. All radioactivity found in the urine is in the form of two major metabolites, with no traces of intact [^{99m}Tc]Demobesin 1 present. It is interesting that no trace of free $^{99m}TcO_4^-$ is detected either, revealing the in vivo stability of the ^{99m}Tc-tetraamine chelate, in confirmation of previous findings [52]. [^{99m}Tc]Demobesin 1 is sufficiently stable during incubation in murine plasma, with >90% peptide remaining intact during the first critical 15 min and >85% during the first hour. The metabolites seem to form mainly in the kidneys, as only ~5% [^{99m}Tc]Demobesin 1 survives after 5-min incubation in kidney homogenates.

The combination of plasma stability and fast enzymatic conversion to daughter compounds in the kidney – rapidly forwarded into the urine – is a considerable advantage in that it favours fast clearance from background tissues without affecting the localization of [^{99m}Tc]Demobesin 1 at the target site. This fact is clearly evident during the biodistribution of [^{99m}Tc]Demobesin 1 in athymic mice bearing human PC-3 xenografts. Thus, uptake of [^{99m}Tc]Demobesin 1 in the experimental tumour reached 16.2±3.1%ID/g at 1 h p.i. and remained at this level up to 4 h p.i. (15.61±1.19%ID/g). Exceptionally high tumour uptake was still evident after 24 h (5.24±0.67%ID/g) despite the fact that [^{99m}Tc]Demobesin 1, as a potent bombesin antagonist [50, 51], shows only a small degree of internalization in vitro into PC-3 cells. The mechanism governing the observed prolonged tumour uptake of the antagonist-based [^{99m}Tc]Demobesin 1 is unknown and is currently under investigation. It should be stressed that there is currently very little experience using peptide receptor antagonists for in vivo receptor targeting; for comparison, all commercially available somatostatin analogues are currently agonists. In contrast to the prolonged uptake in the experimental tumour, the radioactivity was cleared from all other tissues after 24 h. It is interesting to note the low pancreatic values at 24 h p.i. (0.38±0.09%ID/g) in contrast to the impressively high uptake in this organ at the initial time points (79.49±0.77%ID/g at 1 h p.i. and 27.71±3.28 at 4 h p.i.), in accordance with the respective values attained in healthy mice. It is worth mentioning that analogous trends of radioactivity washout from the pancreas have been reported for radiolabelled bombesin analogues derived from agonists [44]. In any case, uptake in both the pancreas and the tumour is specific, as demonstrated by appropriate in vivo GRP-R-blocking tests at 1 and 4 h p.i. Furthermore, tumour to background ratios, which are already high at 1 h p.i., increase with time, illustrating the excellent in vivo profile of [^{99m}Tc]Demobesin 1 for GRP-R-targeted applications.

In conclusion, [^{99m}Tc]Demobesin 1 is a new potent GRP-R-specific radioligand that is accessible through a convenient labelling protocol and is easily applicable in a clinical environment. Due to its excellent in vivo performance (high and prolonged retention in human GRP-R-positive xenografts, fast background clearance), [^{99m}Tc]Demobesin 1 may provide a valuable tool in the early diagnosis and staging of GRP-R-positive tumours, such as primary and metastatic prostate and breast carcinomas. Moreover, [^{99m}Tc]Demobesin 1 may play an important role in monitoring response to treatment of GRP/GRP-R-associated malignant disease with bombesin antagonists. Therefore, first clinical studies have already been scheduled in a small number of prostate cancer patients in order to provide an initial rough estimate of the validity of the new radiotracer. The potential diagnostic value of [^{99m}Tc]Demobesin 1 becomes more apparent when one takes into account several logistical parameters operating in parallel, for example the cost-effectiveness, wide availability and superior nuclear properties of ^{99m}Tc in combination with the high frequency of GRP-R-positive neoplasms. Furthermore, due to the similarities of technetium and rhenium chemistries, a ^{188}Re-based analogue for internal radiotherapy of GRP-R-positive tumours will provide the radiotherapeutic counterpart of [^{99m}Tc]Demobesin 1. For this purpose, work aiming toward the development of a therapeutic ^{188}Re-based GRP-R-specific radiopharmaceutical is currently in progress.

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