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, $[^{99m}\text{Tc}]$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 nM and 1.5±0.20 nM, respectively, while the $K_d$ defined for $[^{99m}\text{Tc}/^{99}\text{gTc}]$Demobesin 1 was 0.67±0.10 nM. $[^{99m}\text{Tc}]$Demobesin 1 was rather stable in murine plasma, whereas it degraded rapidly in kidney and liver homogenates. After injection in healthy Swiss albino mice, $[^{99m}\text{Tc}]$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 $[^{99m}\text{Tc}]$Demobesin 1 after injection in PC-3 tumour-bearing athymic mice. The initial high radiogand uptake of $16.2±3.1\%\text{ID/g}$ in the PC-3 xenografts at 1 h p.i. remained at a similar level ($15.6±1.9\%\text{ID/g}$) at 4 h p.i. Even after 24 h p.i., when the radioactivity had cleared from all other tissues, a value of $5.24±0.67\%\text{ID/g}$ was still observed in the tumour. The high and prolonged localization of $[^{99m}\text{Tc}]$Demobesin 1 at the tumour site and its rapid background clearance are very promising qualities for GRP-R-targeted tumour imaging in man.

Keywords: Bombesin – Gastrin releasing peptide – Tumour imaging – $[^{99m}\text{Tc}]$ – Tetraamine chelator

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 neumedin 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 111In [39] and 90Tc 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 111In- [39] and 186Re-based radiotherapeutic agents have been reported [47]. Due to the dominance of 99mTc in nuclear medicine diagnosis, most attempts have been focussed on 99mTc-based GRP-R-seeking radiotracers involving a wide range of chelators, such as the N₅S₂ [40, 41, 42, 43], the P₅S₂ [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 99mTc with formation of a single radiopeptide species, [99mTc]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)O₂⁺ 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 [99mTc]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 [99mTc]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-butyloxycarbonyl)-6-{[carboxymethoxy] acetyl}amino-benzyl]-1,4,8,11-tetraazaundecane will be reported elsewhere. [Tyr⁴]BN (Pyr-Gln-Arg-Tyr-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂) and the potent bombesin antagonist [(D)Phe₆,Leu-NH₂]¹³des-Met⁹[¹⁴]BN(6–14) were purchased from Bachem (Bubendorf, Switzerland). Iodine-125 was provided by MDS Nordion, SA (Fleurus, Belgium). Radiodiodination of [Tyr⁴]BN was performed according to a published protocol [53]. Technetium-99m in the form of [⁹⁹ᵐTc]NaTcO₄ in physiological saline was eluted from a commercial ⁹⁹ᵐTc²⁰¹Tc generator (Cis International, France). Technetium-99m was purchased from Oak Ridge National Laboratories, USA, as NH₂⁻¹⁰⁹TcO₄⁻. The impure black solid was purified prior to use by overnight treatment with H₂O₂ and NH₃·H₂O in MeOH. Evaporation of the solvent afforded NH₂⁻¹⁰⁹TcO₄ 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 ¹²⁵I or ⁹⁹ᵐTc 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 pixellized 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 (AQAD Navigator, Finnigan) using a Ha-