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[^{99m}Tc]Demomedin C, a Radioligand Based on Human Gastrin Releasing Peptide(18-27): Synthesis and Preclinical Evaluation in Gastrin Releasing Peptide Receptor-Expressing Models

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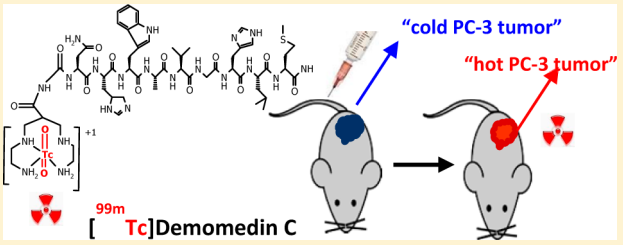
ABSTRACT: The synthesis and preclinical evaluation of [^{99m}Tc]Demomedin C in GRPR-expressing models are reported. Demomedin C resulted by coupling a Boc-protected N_4-chelator to neuromedin C (human GRP(18-27)), which, after ^{99m}Tc-labeling, afforded [^{99m}Tc]Demomedin C. Demomedin C showed high affinity and selectivity for the GRPR during receptor autoradiography on human cancer samples (IC_{50} in nM: GRPR, 1.4 ± 0.2; NMBR, 106 ± 18; and BB_3R, >1000). It triggered GRPR internalization in HEK-GRPR cells and Ca^{2+} release in PC-3 cells (EC_{50} = 1.3 nM). [^{99m}Tc]Demomedin C rapidly and specifically internalized at 37 °C in PC-3 cells and was stable in mouse plasma. [^{99m}Tc]Demomedin C efficiently and specifically localized in human PC-3 implants in mice (9.84 ± 0.81%ID/g at 1 h pi; 6.36 ± 0.85%ID/g at 4 h pi, and 0.41 ± 0.07%ID/g at 4 h pi block). Thus, human GRP-based radioligands, such as [^{99m}Tc]Demomedin C, can successfully target GRPR-expressing human tumors *in vivo* while displaying attractive biological features—e.g. higher GRPR-selectivity—vs their frog-homologues.



INTRODUCTION

The gastrin releasing peptide receptor (GRPR) has recently become a major molecular-target for radiolabeled peptide vectors owing to its abundant expression in many common human cancers, such as in mammary carcinoma, prostate cancer, or lung cancer.[1−7] Most of GRPR-directed radioligands developed and tested thus far are derivatives of the amphibian tetradecapeptide bombesin (BBN) (Figure 1) and its C-terminal octapeptide fragment BBN(7-14).[2,8,9] Coupling of a suitable chelator at the N-terminus has allowed binding of radiometals useful for SPECT/PET imaging and radionuclide therapy, while introduction of linkers between the peptide and the metal-chelate has been used to shape pharmacokinetics. The BBN-based radiopeptides massively internalize into target-cells, and often show ample and GRPR-mediated accumulation in human xenografts in mice.

We have previously reported on a group of such BBN and BBN(7-14) analogs modified with acyclic tetraamines for stable binding of ^{99m}Tc, [^{99m}Tc]Demobesin 3-6,[10] among which [^{99m}Tc]Demobesin 4 is currently undergoing clinical evaluation in a small group of prostate cancer patients. In contrast to other ^{99m}Tc-BBN analogs showing suboptimal tumor uptake and/or undesirably high levels of radioactivity in the abdomen,[11] [^{99m}Tc]Demobesin 4 shows an excellent pharmacokinetic profile.[10] In fact, the suitability of the open chain tetraamine framework to tag ^{99m}Tc to a variety of peptide-vectors has been documented in several preclinical and clinical studies.[11−15] The *in vivo* stability and hydrophilicity of the monocationic *trans*-[^{99m}TcO_2−N_4]$^+$-chelate seems to favor tumor targeting and pharmacokinetics both in mice and in man.

However, the application of BBN-agonists during pilot clinical studies has recently raised a few biosafety concerns. After intravenous (iv) injection of BBN-like agonists to patients, severe side-effects related to abdominal smooth muscle contraction and stimulation of gastrointestinal hormone secretion have been reported.[16−24] It should be stressed as well that BBN acts as a mitogen.[25−27] Frog BBN elicits such undesirable effects after binding to G-protein-coupled receptors

A: pGlu-Gln-Arg-Leu-**Gly-Asn**-Gln-**Trp-Ala-Val-Gly-His-Leu-Met**-NH_2

B: Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-**Gly-Asn**-His-**Trp-Ala-Val-Gly-His-Leu-Met**-NH_2

C: **Gly-Asn**-Leu-**Trp-Ala**-Thr-**Gly-His**-Phe-**Met**-NH_2

D: **Gly-Asn**-His-**Trp-Ala-Val-Gly-His-Leu-Met**-NH_2

Figure 1. (A) Amphibian tetradecapeptide BBN and its mammalian homologues, (B) GRP, (C) NMB, and (D) NMC; conserved amino acids are indicated in bold.

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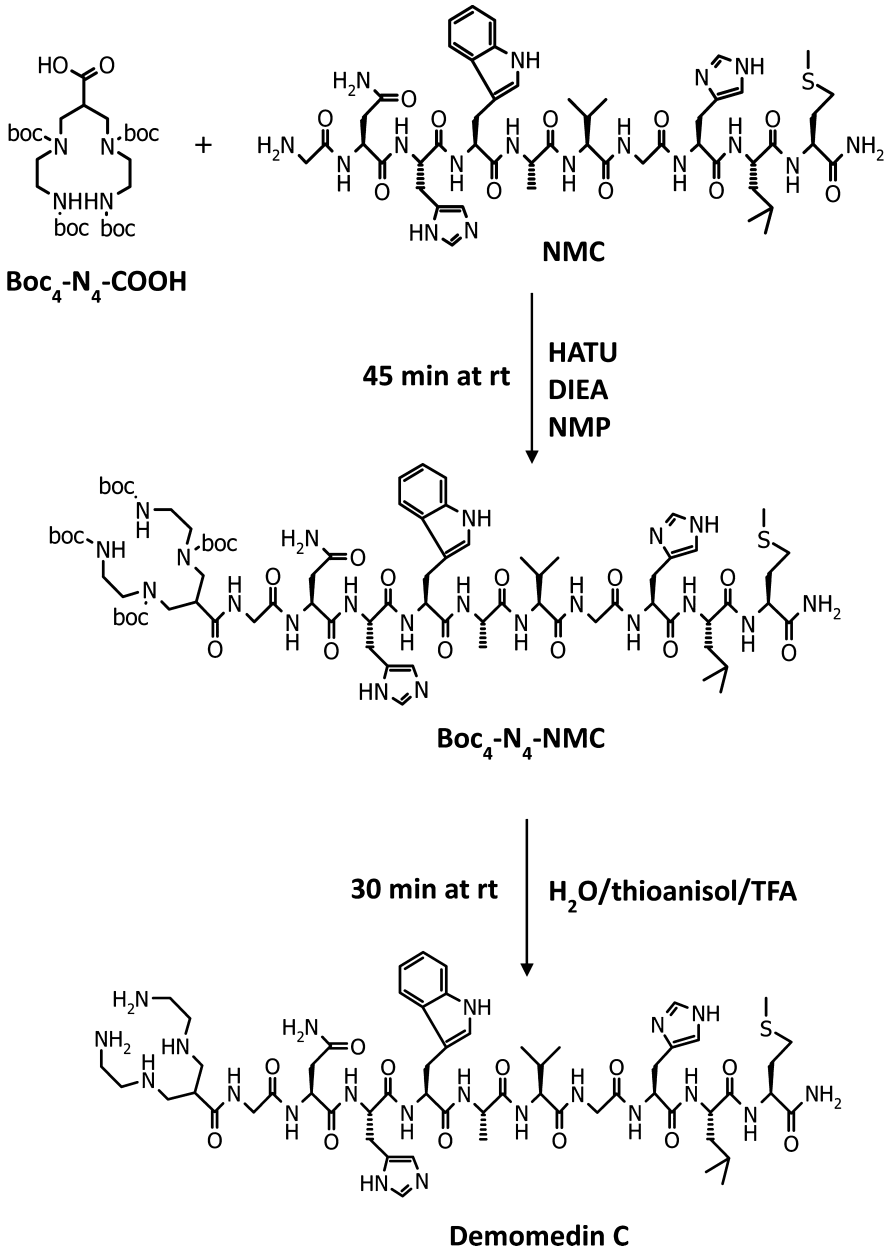

Figure 2. Two-step synthesis of Demomedin C in solution.

located on the surface of target-cells. These receptors comprise three members in humans pharmacologically distinguished by their selectivity toward native mammalian peptides with high homology to BBN (Figure 1).[16−28] Thus, the decapeptide neuromedin B (NMB) displays high affinity to NMBR (BB_1R), while the 27-mer GRP and its C-terminal decapeptide fragment neuromedin C (NMC, GRP(18-27)) show high affinity for the GRPR (BB_2R). Amphibian BBN binds with equal affinity to both GRPR and NMBR.[2] The BB_3R is an orphan receptor without a native ligand identified to date.

Radiolabeled BBN-agonists of poor GRPR-selectivity are expected to show high levels of background radioactivity by virtue of their binding to both GRPR and NMBR populations distributed in the body, especially in the abdomen, amplified by internalization. Furthermore, additive GRPR- and NMBR-mediated effects in the gastrointestinal tract will challenge peptide tolerability and biosafety after systemic administration of BBN-based radioagonists.[20−24] The impact of high GRPR-selectivity of agonist-based radioligands on background radioactivity levels as well as on the release of potent pharmacological responses warrants further investigation. On the other hand, the rarity of human tumors with bombesin receptors other than or in addition to the GRPR further compromises the benefits of using multibombesin receptor binding radioligands.[29,30]

In an effort to address some of these issues, we present [^{99m}Tc]Demomedin C, a radioligand based on the human decapeptide NMC. NMC (or GRP(18-27)) is GRPR-selective, and its His[20] corresponds to Gln[7] in the frog BBN tetradecapeptide sequence (Figure 1). In order to directly compare the effects of higher GRPR-selectivity on several biological parameters versus our previously reported BBN-based analogs, we have coupled the same acyclic tetraamine chelator to the primary amine of Gly[18] in NMC for stable binding of the diagnostic radionuclide ^{99m}Tc. It should be noted that [^{99m}Tc]Demobesin 3-6 shows indistinguishable affinity to

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Table 1. Analytical Data for Demomedin C Synthesis

			ESI-MS[a]		RP-HPLC t_R (min)	ITLC SiO_2; R_f
peptide	% purity	% yield	MW calcd m/z	found m/z (%)	sys1[b]	eluent[c]
Boc_4-N_4-NMC	>95		1709.06		45.2	0.07[d]
			$[M + 2H^+]^{2+}/2$, 855.54	854.6 (8)		
			$[(M-4Boc) + 2H^+]^{2+}/2$, 653.42	655.8 (42)		
			$[M-Boc +3H^+]^{3+}/3$, 537.01	533.7 (100)		
Demomedin C	>97	39	1306.57		21.8	
			$[M + H^+]^+$, 1307.58	1307.2 (5)		
			$[M + 2H^+]^{2+}/2$, 654.29	654.4 (100)		

[a]ESI-MS data for Boc_4-N_4-NMC and Demomedin C. [b]RP-HPLC on a RP18-Symmetry Shield column (5 μm, 150 mm × 3.9 mm) eluted at a flow rate of 1 mL/min with a linear gradient starting with 0%B and advancing to 100%B within 100 min for Boc_4-N_4-NMC and to 30%B within 30 min for Demomedin C; A = 0.1% aqueous TFA and B = pure MeCN; the corresponding sulfoxides eluted at 43.4 and at 19.5 min. [c]SiO_2 plates developed with $CHCl_3$/MeOH/NH_3 10/2/0.2. [d]Ninhydrin negative/UV positive; R_f Boc_4-N_4-COOH = 0.45, ninhydrin positive; R_f NMC = 0.04, ninhydrin positive.

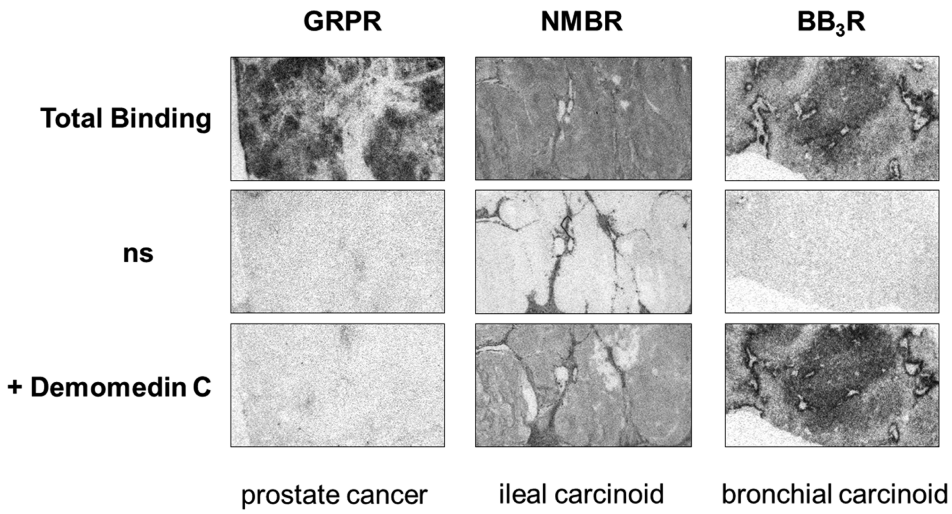

Figure 3. Receptor autoradiography showing the displacement of the universal radioligand by Demomedin C in GRPR-expressing human prostate cancer (1st column), while no displacement by Demomedin C was observed in NMBR-expressing ileal carcinoids (2nd column) and in BB_3R-expressing bronchial carcinoids (3rd column). 1st row: Autoradiograms showing total binding of [^{125}I-DTyr^6,βAla^{11},Phe^{13},Nle^{14}]BBN(6-14) with all three tumors labeled with this universal radioligand (bars = 1 mm). 2nd row: Autoradiograms showing nonspecific binding (in the presence of 10^{-7} M [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]BBN(6-14)). 3rd row: Autoradiograms showing displacement of [^{125}I-DTyr^6,βAla^{11},Phe^{13},Nle^{14}] BBN(6-14) with 10^{-7} M Demomedin C. Full displacement is seen in the GRPR-expressing tumor, almost no displacement in the NMBR-expressing tumor, and no displacement at all in the BB_3R-expressing tumor.

GRPR and NMBR.[10] Results will be further compared with data acquired by the GRPR-selective but noninternalizing antagonist [^{99m}Tc]Demobesin 1.[31,32]

The present study on the first GRPR-selective radioligand agonist may provide a supplementary platform for the development of future GRPR-directed radiopharmaceuticals combining lower background activity with prolonged retention in the tumor.

RESULTS

Synthesis of Demomedin C. The synthesis of Demomedin C was accomplished by coupling of the Boc-protected tetraamine precursor to the terminal Gly[1] of Neuromedin C in solution using HATU as the coupling agent in the presence of DIEA (1st step). The protecting Boc-groups were then removed by TFA treatment (2nd step), as depicted in Figure 2. The peptide conjugate was isolated in >97% purity by chromatographic methods and lyophilized, affording the product as a white solid. Synthesis yields along with analytical data including chromatographic and electron spray mass spectroscopy (ES-MS) results consistent with the expected formulas are presented in Table 1.

Radiotracer. Incorporation of ^{99m}Tc by Demomedin C was achieved in ≥98% yield and a typical specific activity of 0.5−1 Ci/μmol by brief incubation with $^{99m}TcO_4^-$ generator eluate, $SnCl_2$ as reducing agent, and citrate anions as transfer ligand in alkaline pH at ambient temperature. Quality control of the radiolabeled product combined HPLC and ITLC analysis. The total radiochemical impurities, comprising $^{99m}TcO_4^-$, [^{99m}Tc]-citrate, and $^{99m}TcO_2{\cdot}xH_2O$, did not exceed 2%, while a single radiopeptide species was only detected by RP-HPLC.

Receptor Autoradiography in Human Tumor Samples. The selective affinities of Demomedin C for each of the three bombesin receptor subtypes found in mammals were studied during *in vitro* receptor autoradiography competition binding assays against the universal radioligand ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]BBN(6-14)[30] in cryostat sections of well characterized human cancers preferentially expressing one of the subtypes,[32,33] as shown in the representative autoradiograms of excised human samples in Figure 3. Demomedin C showed high affinity for the GRPR expressed

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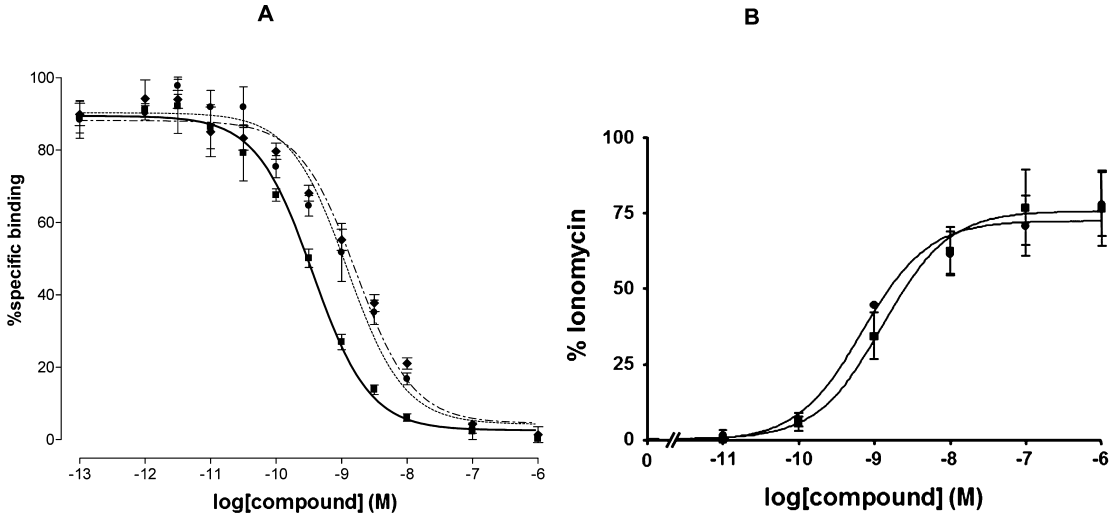

Figure 4. (A) Displacement of [^{125}I-Tyr^4]BBN from human GRPRs in PC-3 cell membrane homogenates by increasing amounts of Demomedin C (■, IC_{50}= 0.36 ± 0.01 nM), [Tyr^4]BBN (●, IC_{50} = 1.22 ± 0.18 nM), or NMC (♦, IC_{50} = 1.66 ± 0.22 nM); results are expressed as the mean IC_{50} ± SEM. (B) Dose−response curves of BBN and Demomedin C determined by the calcium release assay. PC-3 cells were treated either with BBN (●) or with Demomedin C (■) at concentrations ranging between 0.01 nM and 1 μM. Both compounds behave like agonists, exhibiting EC_{50} values of 0.71 nM (BBN) and 1.3 nM (Demomedin C). Data are expressed as percentage of maximum calcium response induced by ionomycin.

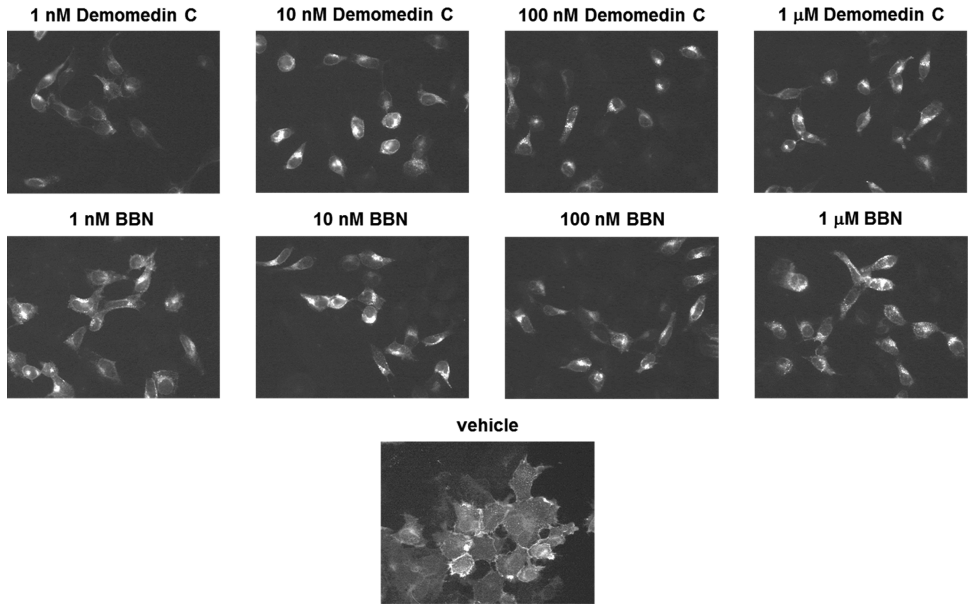

Figure 5. Demomedin C behaves like an agonist in the GRPR internalization assay. HEK-GRPR cells were treated for 30 min at 37 °C either with Demomedin C (upper row) or with BBN (middle row) or vehicle (last row) at concentrations ranging between 1 nM and 1 μM. Following incubation with the peptides, the cells were processed for immunofluorescence microscopy.

in resected prostate carcinoma specimens (IC_{50}= 2.4 ± 1.0 nM; n = 3), very low affinity for NMBR present in ileal carcinoid biopsy samples (IC_{50} = 106 ± 13 nM; n = 2), and no affinity for BB_3R expressed in bronchial carcinoid samples (IC_{50} > 1000 nM; n = 2). Thus, Demomedin C displayed good selectivity for the GRPR, in contrast to BBN-based radioligands such as Demobesin 3 (GRPR, IC_{50} = 0.47 nM; NMBR, IC_{50} = 1.6 nM; and BB_3R, IC_{50} > 100 nM) or [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]-BBN(6-14) (IC_{50} ≈ 1.0 nM for all subtypes).[10]

Affinity of Demomedin C for the Human GRPR in PC-3 Cell Membranes. As shown in Figure 4A, Demomedin C displaced [^{125}I-Tyr^4]BBN from GRPR-binding sites in PC-3 cell membrane homogenates[34] in a monophasic and dose-dependent fashion. When compared to nonmodified NMC (IC_{50} = 1.66 ± 0.22 nM) and [Tyr^4]BBN (IC_{50} = 1.22 ± 0.18 nM), Demomedin C showed higher affinity (IC_{50} = 0.36 ± 0.01 nM), revealing a favorable effect of the N-terminal coupled tetraamine framework on affinity.

Intracellular Calcium Release. Demomedin C was tested for its effect on signaling by using a calcium release assay in PC-3 cells and compared to BBN. As seen in Figure 4B, both Demomedin C and BBN behaved as agonists stimulating calcium mobilization with EC_{50} values of 1.3 nM (Demomedin C) and 0.71 nM (BBN).

Immunofluorescence Microscopy-Based Internalization Assay. Demomedin C demonstrated agonistic properties in immunofluorescence-based internalization assays[35] with HEK293 cells stably expressing the GRPR. Figure 5 illustrates

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that Demomedin C can trigger a massive GRPR internalization in HEK-GRPR cells already at a concentration of 1 nM, as compared to a condition without peptide. The agonistic effect of Demomedin C is comparable to the one of BBN in inducing GRPR internalization.

Internalization of [^{99m}Tc]Demomedin C in PC-3 Cells. Internalization curves of [^{99m}Tc]Demomedin C in PC-3 cells as a function of time are shown in Figure 6. Internalized fractions

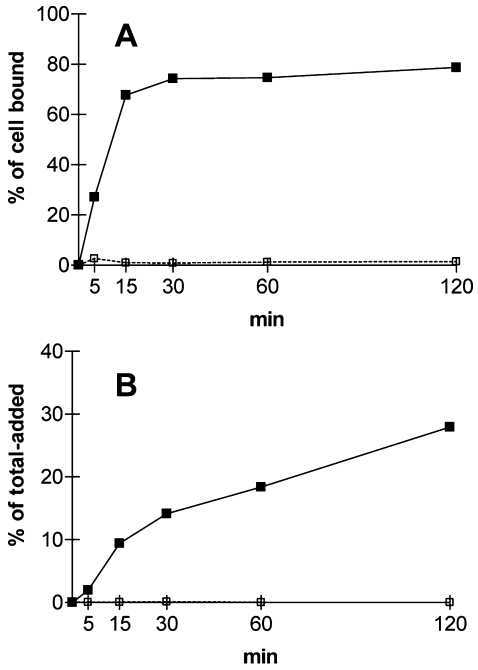

Figure 6. Time-dependent internalization curves of [^{99m}Tc]-Demomedin C in PC-3 cells during incubation at 37 °C without (■) or with addition of 1 μM [Tyr^4]BBN to indicate nonspecific internalization (□). Internalized fractions are presented (A) as percentages of cell-bound activity or (B) as percentages of total added activity per well; values are normalized for 10^6 cells per well and are the average of two experiments performed in triplicate.

of cell-associated activity reveal rapid intracellular migration of the radioligand, with the ≈80% internalization plateau reached within 30 min and maintained at this high level up to 2 h (Figure 6A). In the presence of 1 μM [Tyr^4]BBN, this value dropped to <1%, indicating a process mediated by the GRPR. Internalization is equally GRPR-specific when calculated as a fraction of total-added activity (Figure 6B), but intracellular accumulation progressively increases with time to exceed 30% at 2 h of incubation.

Metabolic Studies with [^{99m}Tc]Demomedin C. During incubation with mouse plasma at 37 °C, [^{99m}Tc]Demomedin C gradually degrades to more hydrophilic metabolic products. As shown in the radiochromatograms of Figure 7, two hydrophilic radiometabolites appear (A, t_R = 0.4 min; B, t_R = 15.2 min) as early as 15 min, with 80% [^{99m}Tc]Demomedin C (t_R = 22.6 min) remaining intact at this time point. [^{99m}Tc]Demomedin C slowly degrades further to 65% at 30 min, 45% at 60 min, and <20% at 120 min incubation. Degradation is much faster in incubates of mouse kidney-homogenates with [^{99m}Tc]-Demomedin C totally degraded within 5 min. Likewise, [^{99m}Tc]Demomedin C is found totally degraded in 30 min *ex-vivo* mouse urine (Figure 7). It should be stressed that the predominant radiometabolite (t_R = 0.4 min) is not $^{99m}TcO_4^-$, as evidenced by paper/acetone tests run in parallel (R_f $^{99m}TcO_4^-$ = 1; ≪1%), which is in agreement with metal-chelate stabilities reported for other ^{99m}Tc-tetraamine derivatized peptides.[10−15]

Biodistribution of [^{99m}Tc]Demomedin C in PC-3 Xenograft-Bearing SCID Mice. Tissue distribution data for [^{99m}Tc]Demomedin C are shown in Table 2 for the 1 h, 4 h, and 24 h time intervals. In a separate animal group, mice iv received excess [Tyr^4]BBN (40 nmol) together with the radioligand (10 pmol) to study the effects of *in vivo* GRPR-blockade on individual tissue uptake at 4 h pi and especially on GRPR-rich areas. Results are expressed as mean %ID/g values ± sd using at least four animals per group. The radiotracer shows high tumor uptake at 1 h pi (9.84 ± 0.81%ID/g) with ≈50% of this initial value still present at 24 h pi (4.71 ± 1.1% ID/g). Tumor uptake is GRPR-specific, as verified by the significant (P < 0.001) reduction observed in the *in vivo* GRPR-blockade animal group (6.36 ± 0.85%ID/g vs 0.41 ± 0.07%ID/g) at 4 h. Likewise, high and specific uptake was evident in the pancreas (34.38 ± 5.49%ID/g vs 0.59 ± 0.04%ID/g at 4 h pi) and the intestines (6.96 ± 0.42%ID/g vs 2.53 ± 0.66%ID/g at 4 h pi), as expected by the abundant GRPR-expression reported in the pancreas and the intestinal wall of mice.[16,18,28,36]

Blood clearance of [^{99m}Tc]Demomedin C was very rapid. Similarly fast was the overall clearance of radioactivity from the body of mice via the kidneys and into the urine, with the hepatobiliary system playing a less prominent role in the excretion of [^{99m}Tc]Demomedin C. It is interesting to note that radioactivity was not retained in the kidneys, as renal values declined with time (9.80 ± 0.81%ID/g at 1 h pi to 2.58 ± 0.3% ID/g at 24 h pi); recent kidney protection strategies may further accelerate passage of radioactivity from the kidneys into the urine.[37] The high and GRPR-specific tumor values attained by [^{99m}Tc]Demomedin C in combination with its rapid body clearance lead to an excellent pharmacokinetic profile, reflected in the high tumor/nontarget tissue ratios, which increase with time (Table 2).

■ DISCUSSION

The clinical relevance of GRPRs relies on their abundant expression in frequently occurring human tumors, such as prostate cancer, breast carcinoma, and lung cancer.[1−7] As a result, a great variety of radiolabeled analogs of the GRPR-avid amphibian tetradecapeptide BBN were developed and tested both in animals and in patients in recent years. Most of these are based on the C-terminal BBN(7-14) octapeptide fragment still capable of recognizing and interacting with the GRPR. Structural interventions have mostly involved attachment of different metal-chelators and/or diverse linkers as pharmacokinetic modifiers at the N-terminal part of the BBN(7-14) motif while data on radiolabeled human GRP sequences are lacking thus far.[2,8,9,11]

As a part of this research effort, we have previously developed analogs based both on the BBN(7-14) fragment as well as on the full BBN sequence at the N-terminus of which we have covalently coupled acyclic tetraamine chelators for stable binding of ^{99m}Tc.[10] [^{99m}Tc]Demobesin 3−6 are well internalizing agonists with equivalent affinity for the GRPR and NMBR while in mice they were able to specifically target GRPR-expressing PC-3 xenografts. We have recently expanded our search toward radioligands based on the mammalian homologue of BBN, namely the human 27-mer GRP and its C-terminal decapeptide fragment GRP(18-27) also known as

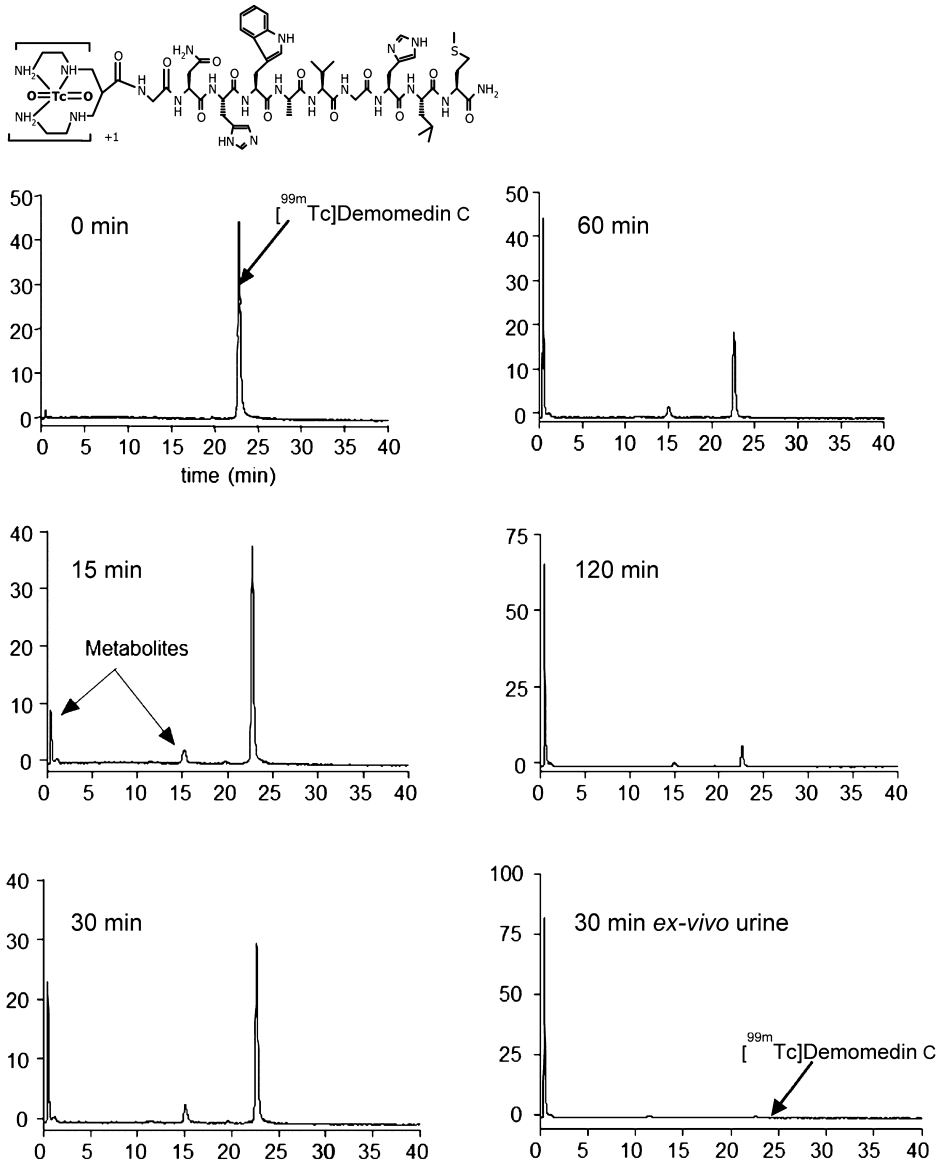

Figure 7. Degradation of [^{99m}Tc]Demomedin C in murine plasma incubates at 37 °C, as shown by the representative RP-HPLC radiochromatograms at 0 min, 15 min, 30 min, 60 min, and 120 min incubation; a RP18 Symmetry Shield column (5 μm, 3.9 mm × 150 mm) is eluted at 1 mL/min flow rate by applying a linear gradient system starting from 0% B and advancing to 30% B within 30 min (A = 0.1% aqueous TFA and B = MeCN). [^{99m}Tc]Demomedin C (t_R = 22.6 min) is gradually converted into two major hydrophilic radiometabolites (A, t_R = 0.4 min; B, t_R = 15.2 min) with time; in the last radiochromatogram, RP-HPLC analysis of 30 min *ex-vivo* mouse urine [^{99m}Tc]Demomedin C reveals the excretion of a totally degraded radiotracer into urine.

NMC, motivated by their reported selectivity for the GRPR.[2,17,18] GRPR-selectivity in combination with radioligand internalization has not been considered so far during the design of GRPR-seeking radiolabeled BBNs. It may nevertheless affect background clearance given that the radioligand will not be retained in NMBR-rich tissues in the body. Furthermore, less adverse reactions will be elicited by lack of interaction with the NMBR in the abdomen while GRPR-mediated internalization favoring longer retention in tumor-sites will be preserved. To enable labeling with the diagnostic radionuclide ^{99m}Tc, an acyclic tetraamine has been covalently coupled in solution to Gly[18] of GRP(18-27) to afford Demomedin C.

Demomedin C retained a very high affinity for the human GRPR (IC_{50} = 0.36 nM) as determined against [^{125}I-Tyr^4]BBN during competition binding assays which is in the same range reported for Demobesin 3−6. However, its affinity profile determined against the universal radioligand ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]BBN(6−14) during receptor autoradiography in human biopsy specimens expressing each one of the three mammalian bombesin receptors showed a much higher selectivity for the GRPR (IC_{50} in nM ± SEM: GRPR = 1.4 ± 0.2, NMBR = 106 ± 18 and BB_3R > 1000) as compared to Demobesin 3-6; the latter exhibited indistinguishable affinities for the GRPR and the NMBR and no affinity for the BB_3R.[10] It is interesting to note that high selectivity for the GRPR has been reported so far only for noninternalizing BBN antagonists,[31,32,38,39] such as Demobesin 1 (*vide infra*). Demomedin C behaved like a true GRPR-agonist in two functional assays. Thus, in a calcium release assay in PC-3 cells, Demomedin C triggered intracellular calcium release with comparable potency to BBN. Furthermore, Demomedin C triggered the massive internalization of the GRPR equally

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Table 2. Biodistribution of [^{99m}Tc]Demomedin C in PC-3 Xenograft-Bearing SCID Mice at 1 h, 4 h, and 24 h pi[a]

organs	1 h	4 h	4 h block[b]	24 h
blood	0.64 ± 0.37	0.13 ± 0.03	0.18 ± 0.09	0.04 ± 0.10
liver	1.24 ± 0.08	1.00 ± 0.14	1.64 ± 0.12	0.47 ± 0.15
heart	0.34 ± 0.04	0.09 ± 0.02	0.15 ± 0.02	0.20 ± 0.14
kidneys	9.80 ± 0.81	6.81 ± 0.89	7.22 ± 1.24	2.58 ± 0.38
stomach	1.24 ± 0.23	0.68 ± 0.10	0.70 ± 0.40	0.63 ± 0.18
intestines	6.87 ± 0.52	6.96 ± 0.42	2.53 ± 0.66[c]	1.35 ± 0.09
spleen	1.76 ± 0.60	1.12 ± 0.36	1.86 ± 1.26	0.82 ± 0.31
muscle	0.15 ± 0.02	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.02
lung	0.81 ± 0.04	0.26 ± 0.05	0.48 ± 0.12	0.24 ± 0.07
pancreas	40.95 ± 7.69	34.38 ± 5.49	0.59 ± 0.04[c]	15.65 ± 2.33
tumor	9.84 ± 0.81	6.36 ± 0.85	0.41 ± 0.07[c]	4.71 ± 1.09
tumor/blood	15.4	48.9	2.3	117.7
tumor/liver	7.9	6.4	0.2	10.0
tumor/kidneys	1.0	0.9	0.06	1.8
tumor/muscle	65.3	212	10.2	117.7

[a]Data are given as %ID/g tissue and are the average values ± sd ($n \geq 4$). [b]Co-injection of 40 nmol [Tyr^4]BBN for the *in vivo* GRPR-blockade. [c]Highly significant ($P < 0.001$) difference between blocked and unblocked animals (unpaired two-tailed Student's t test).

efficiently to BBN even at concentrations as low as 1 nM in HEK293 cells transfected to stably express the human GRPR. In accordance to this finding, the radiolabeled analog [^{99m}Tc]Demomedin C rapidly and massively internalized in PC-3 cells at 37 °C by a GRPR-mediated mechanism. This behavior resembles the fast, massive and GRPR-specific intracellular migration previously reported for [^{99m}Tc]-Demobesin 3−6.[10] In contrast, GRPR-antagonist radioligands, such as [^{99m}Tc]Demobesin 1, failed to internalize in GRPR-expressing cells.[31,32,38,39] The metabolism of [^{99m}Tc]-Demomedin C in mouse plasma is rather slow. A gradual conversion of the tracer to one predominant and very hydrophilic metabolite is observed, similarly to what was also reported for [^{99m}Tc]Demobesin 3 and 4.[10] This process was found to be extremely fast in mouse kidney homogenates where it is completed within 5 min. As a result, [^{99m}Tc]Demomedin C is excreted in the urine totally transformed to a major hydrophilic radiometabolite, as was verified by chromatographic analysis of *ex-vivo* mouse urine samples.

It is interesting to observe how the above qualities affect radioligand pharmacokinetics and body clearance as well as GRPR-targeting in physiological organs and in PC-3 xenografts in mice. As expected, the fast metabolism of [^{99m}Tc]-Demomedin C in the kidneys facilitates clearance of radioactivity from the body of mice via the kidneys rapidly into the urine. On the other hand, the slow plasma degradation of the radiopeptide allows sufficient delivery at the target and in combination with the high internalization rate documented *in vitro* lead to high uptake and retention in the tumor and in the GRPR-rich mouse pancreas. Compared to [^{99m}Tc]Demobesin 3−4, tumor values are in the same level except for the 24-h time point where [^{99m}Tc]Demomedin C displayed somewhat superior values (4.7 ± 1.1%ID/g vs. ≈2%ID/g for the BBN-based analogs).[10] Renal uptake was found much lower, especially in the 1-h and 4-h time intervals (9.8 ± 0.8%ID/g vs. ≈20%ID/g for [^{99m}Tc]Demobesin 3−4 at 1 h pi). In the intestinal tract expressing high numbers of GRPRs and NMBRs, [^{99m}Tc]Demomedin C showed lower uptake at 1 and 4 h pi as compared to the BBN-derived radiotracers in agreement with their respective GRPR-selectivities.[10]

However, when compared to the radioantagonist [^{99m}Tc]-Demobesin 1 the following conclusions can be drawn.[31] First, [^{99m}Tc]Demomedin C shows better clearance from background tissues, especially from major excretory organs like liver, intestines and kidneys. This could be attributed, at least in part, to the higher hydrophilicity of both [^{99m}Tc]Demomedin C and its radiometabolite(s) as opposed to more lipophilic [^{99m}Tc]-Demobesin 1 and its two major lipophilic radiometabolites. Second, pancreatic uptake of the GRPR-selective antagonist, although extremely high in the initial time points, very rapidly declines. In contrast, [^{99m}Tc]Demomedin C showed a more prolonged pancreatic retention, consistent with its rapid internalization capacity. Selectivity for the GRPR seems to be of little consequence for radioligand uptake and retention in the pancreas of mice. Studies in man are warranted to establish if and to what extent this observation applies in the human pancreatic uptake as well, since interspecies differences in the affinity and density of bombesin receptor subtype expression in several mouse and human tissues have been reported.[31,40,41] Finally, tumor uptake of [^{99m}Tc]Demomedin C is somewhat lower at 1 and 4 h pi. However, at 24 h the initial difference in tumor values recedes to equal levels (≈5%ID/g). Apparently, internalization slows down radioactivity washout from the tumor in the case of the radioagonist [^{99m}Tc]Demomedin C, as opposed to the radioantagonist [^{99m}Tc]Demobesin 1. This finding may be of significance for the successful application of GRPR-antagonists for radionuclide therapy. The real therapeutic efficacy of noninternalizing GRPR-antagonists needs to be established, given that shorter range particle emitters will not be able to access the cell nucleus while longer half-life radionuclides will run the risk of rapid washout from tumor sites at later time points.

CONCLUSIONS

[^{99m}Tc]Demomedin C is the first reported radioligand based on a human homologue of amphibian BBN. It is generated by covalent coupling of an open-chain tetraamine chelator to the primary amine of Gly^{18} of GRP(18−27) and labeling with ^{99m}Tc. Demomedin C shows high affinity and selectivity for the human GRPR. It is a potent agonist inducing intracellular calcium release and triggering GRPR-internalization in GRPR-expressing cells. After injection in mouse models [^{99m}Tc]-Demomedin C shows rapid clearance from nontarget tissues via the kidneys into the urine while targeting the GRPR-expressing

implants with high efficiency and specificity. This preclinical study using GRPR-positive *in vitro* and *in vivo* models has shown that analogs based on GRPR-preferring and well-internalizing human GRP motifs, such as on NMC, can be at least as effective for GRPR-tumor targeting as their amphibian BBN counterparts providing a supplementary platform for innovative structural interventions. Potential benefits inherent to GRPR-selective and well internalizing radioligands, such as better tolerability, lower background radioactivity levels and prolonged retention in tumor lesions, warrant further validation by future studies.

EXPERIMENTAL SECTION

Synthesis of Demomedin C. Synthesis of Demomedin C was conducted in solution in two successive steps, first step: coupling of the tetraamine chelator, second step: deprotection of the peptide conjugate.

1st Step − Coupling. Neuromedin C (6.8 mg, 6.1 μmol; NMC, H-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 − provided as an acetate salt by PeptaNova, Sandhausen, Germany) was dissolved in 0.4% (v/v) TFA (1 mL) previously degassed with He-flux and the solution was lyophilized to remove traces of acetic acid. The coupling agent HATU (19.2 mg, 50.5 μmol; O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, Fluka, Buchs, Switzerland) was dissolved in MeCN (200 μL) and then mixed with a solution of the Boc-protected tetraamine chelator in CH_2Cl_2 (300 μL, 11.7 mg, 16.3 μmol; (Boc-N)$_4$-COOH, N,N′,N″,N‴-tetrakis-(*tert*-butoxycarbonyl)-6-(carboxy)-1,4,8,11-tetraazaundecane)) and DIEA (7.2 μL, 5.5 mg, 42.3 μmol; N,N-diisopropylethylamine, Fluka, Buchs, Switzerland) was added. This solution was brought to react with a solution of NMC in NMP (375 μL; N-methylpyrrolidone, Fluka, Buchs, Switzerland) at room temperature for 45 min. After addition of 0.1 M NaOH the desired product was extracted in CH_2Cl_2 and the solvent removed under vacuum. The residue was dissolved in 1 mL MeCN, diluted with 15 mL H_2O and then slowly passed through a preconditioned solid phase extraction cartridge (Oasis HLB 200 mg, 6 mL; Waters, Vienna, Austria; conditioning with 12 mL MeCN and 12 mL 1 mM ammonium acetate). The cartridge was washed with 1 mM ammonium acetate (12 mL) and H_2O (12 mL) and finally, the Boc-protected N_4−NMC-conjugate was collected in MeCN (12 mL). The conjugate was obtained after evaporation of MeCN. Its formation was verified by ES-MS on an AQA Navigator electrospray phasmatometer (Finnigan, USA) using a Harvard syringe pump. Product purity was monitored by RP-HPLC and ITLC (Table 1).

2nd Step − Deprotection. The protected peptide conjugate was treated with a mixture of TFA (500 μL; trifluoroacetic acid, Fluka, Buchs, Switzerland), thioanisole (20 μL, Fluka, Buchs, Switzerland) and H_2O (25 μL) for 30 min at room temperature (rt). After addition of ice-chilled 0.5 M NaOH (12 mL) thioanisole was removed by extraction in CH_2Cl_2 (5 × 6 mL) and the aqueous phase volume reduced to 2 mL in vacuum. It was filtered through a 0.22 μm filter (Millex-GV; Millipore, Milford/USA) and purified by HPLC over a semipreparative RadialPak column (μBondapak, 10 μm, 8 mm ×100 mm; Waters, Vienna, Austria). The column was eluted at 1 mL/min flow rate by the following gradient system: 80% 0.1% TFA − 20% MeCN to 0% 0.1% TFA − 100% MeCN within 30 min. The product was isolated and the MeCN was removed under a N_2-stream; after lyophilization the white solid was stored at −20 °C. Overall yield= 39%; 2.3 μmol. Formation of Demomedin C was verified by ES-MS; a >95% purity was confirmed by RP-HPLC on a RP18-Symmetry Shield column (5 μm, 150 mm × 3.9 mm) eluted at a flow rate of 1 mL/min with a linear gradient starting with 0%B and advancing to 30%B within 30 min (A= 0.1% aqueous TFA and B= pure MeCN), as summarized in Table 1.

Radiolabeling. Lyophilized Demomedin C was dissolved in 50 mM acetic acid/EtOH 8/2 v/v to a final 1 mM concentration and bulk solutions were distributed in 50 μL aliquots in Eppendorf vials and stored at −20 °C. For ^{99m}Tc labeling[10,31] the following solutions were added into an Eppendorf tube containing 0.5 M phosphate buffer pH 11.5 (25 μL): 0.1 M sodium citrate (3 μL), [^{99m}Tc]$NaTcO_4$ (210 μL, 3.7 − 7.5 mCi) eluted from a commercial ^{99}Mo/^{99m}Tc generator (Cis International, Larmor Baden, France), Demomedin C stock solution (7.5 μL, 7.5 nmol) and finally fresh $SnCl_2$ solution in EtOH (5 μg, 5 μL). After 30 min incubation at ambient temperature the reaction mixture was neutralized by addition of 1 M HCl (4 μL) and EtOH was added (25 μL).

Radioiodination of [Tyr^4]BBN ([Tyr^4]Bombesin = Pyr-Gln-Arg-Tyr-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2; Bachem, Bubendorf, Switzerland) was performed by the chloramine-T methodology. The forming sulfoxide (Met^{14}=O) was reduced by dithiothreitol and [^{125}I-Tyr^4]BBN was isolated in a highly pure form by HPLC. Methionine was added to the purified radioligand solution to prevent oxidation of Met^{14} to the corresponding sulfoxide and the resulting stock solution in 0.1% BSA-PBS was kept at −20 °C; aliquots thereof were used for competition binding assays (specific activity of 2 Ci/μmol).

ATTENTION!!! All manipulations with radioactive solutions were performed by authorized and trained personnel behind sufficient lead shielding in supervised and licensed laboratories following national and international rules.

Quality Control. Labeling yield and radiochemical purity of [^{99m}Tc]Demomedin C were assessed by HPLC and ITLC. HPLC analyses were conducted on a Waters Chromatograph (Waters, Vienna, Austria) efficient with a 600 solvent delivery system; the chromatograph was coupled to twin detection instrumentation, comprising a Waters 996 photodiode array UV detector and a Gabi gamma detector from Raytest (RSM Analytische Instrumente GmbH Germany). Data processing and chromatography were controlled via the Millennium Software (Waters, USA). A RP18 Symmetry Shield column (5 μm, 3.9 mm × 150 mm; Waters Vienna, Austria) was eluted at 1 mL/min flow rate with a linear gradient system starting from 0% B and advancing to 30% B within 30 min, with solvent A = 0.1% aqueous TFA and B = MeCN.

For ITLC tests, samples (5 μL) were applied on ITLC-SG strips (Pall Corporation, NY/USA), developed up to 10 cm from the origin with 1 M ammonium acetate/MeOH 1/1 (v/v). Strips were left to dry in the open and then were cut into two pieces: first = Start (origin +1 cm): $^{99m}TcO_2$ and second = Front (the rest of the strip): $^{99m}TcO_4^-$, [^{99m}Tc]citrate and [^{99m}Tc]Demomedin C. For detection of free $^{99m}TcO_4^-$, Whatman 3MM paper strips (1 cm ×12 cm) were developed with acetone, left to dry and cut into 2 pieces: first = Start (origin +7 cm): all other forms of ^{99m}Tc, and second = Front (the rest of the strip): $^{99m}TcO_4^-$. Pieces were measured for their radioactivity content in an automated well-type gamma counter [NaI(Tl) crystal, Canberra Packard Auto-Gamma 5000 series model]. Samples from both radiopeptide labeling mixtures were analyzed up to 24 h post labeling.

Cell Culture. Human androgen-independent prostate adenocarcinoma PC-3 cells (LGC Promochem, Teddington, UK) endogenously expressing the human gastrin releasing peptide receptor (GRPR)[34] were cultured in Dulbecco's Modified Eagle Medium with GlutaMAX™-I (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco BRL, Life Technologies, Grand Island, NY/USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Biochrom KG Seromed, Berlin, Germany). Cells were kept in a controlled humidified atmosphere containing 5% CO_2 at 37 °C. Cells were inspected for confluency and 1:3 or 1:4 passages were performed (usually every 4−5 days) using a trypsin/EDTA (0.05%/0.02% w/v) solution. Alternatively, PC-3 cells were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH; DSMZ No: ACC465) and cultured at 37 °C and 5% CO_2 in Ham's F12K containing 2 mM L-glutamine and supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Human embryonic kidney 293 (HEK293) cells stably expressing the HA-epitope tagged human GRP receptor (HEK-GRPR), were generated and cultured at 37 °C and 5% CO_2 in DMEM containing 10% (v/v) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 750 μg/mL G418 as previously described.[32,38] All culture reagents

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were provided by Gibco BRL (Grand Island, NY) if not stated otherwise.

Receptor Autoradiography. Binding affinities of Demomedin C were determined by *in vitro* receptor autoradiography performed on cryostat sections of well characterized human tumor tissues, prostate carcinomas for GRP receptors, ileal carcinoids for NMB receptor and bronchial carcinoids for BB_3 receptor as previously described.[10,30,32] The universal radioligand ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]BBN(6−14) (2 Ci/μmol; ANAWA, Wangen Switzerland) was used as tracer known to identify all three bombesin receptor subtypes.[30,33] IC_{50} values are given in nM ± SEM for GRP receptors and in nM ± SD for NMB receptors.

Competition Binding in PC-3 Cell Membranes. Competition binding assays of Demomedin C against [^{125}I-Tyr^4]BBN were conducted in PC-3 cell membranes harvested as previously described;[10,31] [Tyr^4]BBN and NMC served as reference hormones. In brief, 50 pM radioligand (corresponding to ≈40,000 cpm) per assay tube were mixed with 25 μg protein and with tested substance of increasing concentrations to a total volume of 300 μL 50 mM HEPES (pH 7.4, 0.3% BSA, 5 mM $MgCl_2$, 10 μM bacitracin). Triplicates of each concentration were incubated for 45 min at 22 °C in an Incubator-Orbital Shaker unit, (MPM Instr. SrI, Italy). Incubation was interrupted by addition of ice-cold buffer 10 mM HEPES (pH 7.4, 154 mM NaCl) followed by rapid filtration over glass fiber filters (Whatman GF/B, presoaked in binding buffer) on a Brandel M-48 Cell Harvester (Adi Hassel Ingenieur Büro, Munich, Germany) and filters were thoroughly rinsed with chilled buffer. Filter activity was measured in a gamma counter and IC_{50} values were extracted by nonlinear regression according to a one site model applying the PRISM™ 2 program (GraphPad Software, San Diego, CA). Nonspecific binding was defined as the amount of activity bound in the presence of 1 μM [Tyr^4]BBN.

Calcium Release Assay. Intracellular calcium release was measured in PC-3 cells using the Fluo-4NW Calcium Assay kit from Molecular Probes, Inc. (Eugene, OR), as described previously.[32,38] In brief, PC-3 cells were seeded (10,000 cells per well) in 96 well plates and cultured for 2 days at 37 °C and 5% CO_2 in culture medium. At the day of the experiment, the cells were washed with assay buffer (1 x HBSS, 20 mM HEPES) containing 2.5 mM probenecid, loaded with 100 μL/well Fluo-4NW dye in assay buffer containing 2.5 mM probenecid for 30 min at 37 °C and 5% CO_2 and then for further 30 min at room temperature. To measure the intracellular calcium level after stimulation with the BBN analogs, the dye-loaded cells were transferred to a SpectraMax M2^e microplate reader (Molecular Devices, Sunnyvale, CA). Intracellular calcium release was recorded for 60 s in a kinetic experiment at room temperature monitoring fluorescence emission at 520 nm (with λ_{ex} = 485 nm) in the presence of the analogs at the concentrations indicated. Maximum fluorescence (F-max) was measured after the addition of 5 μM ionomycin. Baseline (F-baseline) measurements were taken for dye-loaded, untreated cells. Data are shown as percentage of maximum calcium response (F-max − F-baseline =100% of maximum calcium response) as reported previously.[32,38] All experiments were repeated three times in triplicate.

Immunofluorescence Microscopy-Based Internalization Assay. Immunofluorescence microscopy-based internalization assays with HEK-GRPR cells were performed as previously described.[32,35,38] Briefly, HEK-GRPR cells were grown on poly-D-lysine (20 μg/mL) (Sigma-Aldrich, St. Louis, MO) coated 35 mm four-well plates (Cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany). For the experiment, cells were treated either with vehicle alone or with BBN (Bachem, Bubendorf, Switzerland) or Demomedin C at concentrations between 1 nM and 1 μM for 30 min at 37 °C and 5% CO_2 in growth medium, and then they were processed for immunofluorescence microscopy using a mouse monoclonal HA-epitope antibody (Covance; Berkeley, CA) at a dilution of 1:1000 as first antibody and Alexa Fluor 488 goat antimouse IgG (H+L) (Molecular Probes, Inc.; Eugene, OR) at a dilution of 1:600 as secondary antibody. The cells were imaged using a Leica DM RB immunofluorescence microscope and an Olympus DP10 camera.

Internalization of [^{99m}Tc]Demomedin C in PC-3 Cells. Confluent PC-3 cells were seeded in six-well plates ((1.0−1.5) × 10^6 cells per well) and were incubated for 24 h. Cells were rinsed twice with ice-cold internalization buffer containing DMEM GlutaMAX-I supplemented with 1% (v/v) FBS, and then fresh internalization medium (37 °C) was added (1.2 mL). Approximately 300,000 cpm of test peptide (~200 fmol total peptide in 150 μL of 0.5% BSA PBS) was added per well, and the experiment was performed as previously described.[10,31] Nonspecific internalization was determined by a parallel triplicate series containing 1 μM [Tyr^4]BBN. Surface bound radioligand was separated from the internalized radioactivity by treatment of the cells with acidic buffer (50 mM glycine buffer, pH 2.8, 0.1 M NaCl). The percent internalized radioactivity at each time point could be calculated with the Microsoft Excel program.

Metabolic Studies with [^{99m}Tc]Demomedin C. *In Mouse Plasma.* Blood (~1 mL) was collected from the heart of male Swiss albino mice (NCSR "Demokritos" Animal House) while under ether anesthesia in prechilled heparinized polypropylene tubes on ice. Samples were centrifuged at 4 °C for 10 min at 2000*g*, and the plasma was isolated. [^{99m}Tc]Demomedin C (12.5 μL, 350 pmol corresponding to 150−350 μCi) was added to each polypropylene tube containing plasma (37.5 μL), and tubes were incubated for 0, 15, 30, 60, and 120 min at 37 °C. At the desired time points, EtOH (100 μL) was added and tubes were centrifuged at 4 °C for 10 min at 2000*g*. Supernatants were filtered through 0.22 μm Millex GV filters (Millipore, Milford, CT), and the EtOH was removed under a gentle stream of N_2. HPLC analyses were performed on a RP18 Symmetry Shield column (5 μm, 3.9 mm × 150 mm; Waters Vienna, Austria) eluted at 1 mL/min flow rate with a linear gradient system starting from 0% B and advancing to 30% B within 30 min, with solvent A = 0.1% aqueous TFA and B = MeCN. In addition, incubate aliquots were positioned on Whatman 3MM paper strips developed with acetone to rule out the formation of $^{99m}TcO_4^-$ (R_f $^{99m}TcO_4^-$ = 1).

In Mouse Kidney Homogenates. Kidneys were excised from euthanized male Swiss albino mice, rapidly rinsed, and immersed in ice-cold 50 mM TRIS/0.2 M sucrose buffer pH 7.4 in a 1/2 v/v ratio. They were minced on ice and homogenized using an Ultra-Turrax T25 homogenator for 1 min at 4 °C. [^{99m}Tc]Demomedin C (12.5 μL, 350 pmol corresponding to 150−350 μCi) was added to each polypropylene tube containing kidney homogenate (37.5 μL), and tubes were incubated for 0, 5, and 15 min at 37 °C. At the desired time points, EtOH (100 μL) was added and the procedure described above was followed.

In Ex Vivo Mouse Urine. [^{99m}Tc]Demomedin C was injected as a 100 μL bolus (≈2 nmol corresponding to 1−2 mCi) in the tail vein of male Swiss albino mice. Animals were kept in metabolic cages for 30 min with free access to water. They were then sacrificed by cardiac puncture under a mild ether anesthesia, and urine was immediately collected from their bladder with a syringe and placed on ice. After addition of EtOH in 1/2 v/v, the above-described procedure was followed.

Biodistribution of [^{99m}Tc]Demomedin C in PC-3 Xenograft-Bearing SCID Mice. Animal experiments were carried out in compliance with European and national regulations and after approval of protocols by local authorities. Female SCID mice of 6 weeks of age on the day of arrival (NCSR "Demokritos" Animal House) were inoculated in the right flank with a ~150 μL bolus containing a suspension of (1.5−2) × 10^7 freshly harvested human PC-3 cells in sterile normal saline. The animals were kept under aseptic conditions and 2−3 weeks later developed well-palpable tumors at the inoculation site. For biodistribution, a bolus containing [^{99m}Tc]Demomedin C (100 μL, 5 μCi, 10 pmol total peptide) in normal saline/EtOH 9/1 v/v was injected in the tail vein of mice. Animals were sacrificed in groups of 4 by cardiac puncture under a mild ether anesthesia at 1, 4, and 24 h postinjection (pi). An additional 4-h group of animals intravenously (iv) received a high excess [Tyr^4]BBN (100 μL, 100 μg, ≈40 nmol) in normal saline/EtOH 9/1 v/v along with the radioligand. Samples of blood and urine as well as organs of interest were collected, weighed, and measured for radioactivity in a γ counter. Intestines and stomach were not emptied of their contents. Data were calculated as

percent injected dose per gram tissue (%ID/g) with the aid of standard solutions and applying the Microsoft Excel program. Results represent mean values ± standard deviation as calculated by the PRISM 2.01 (GraphPad, San Diego, CA) software program. Statistical analysis using the unpaired two-tailed Student's *t* test was performed for the *in vivo* GRPR-blockade group; values $P < 0.05$ were considered as statistically significant.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

BBN, bombesin; NMB, neuromedin B; NMC, neuromedin C; GRP, gastrin releasing peptide; NMBR, NMB receptor; hGRPR, human GRP receptor; BB_3R, bombesin subtype 3 receptor; RP-HPLC, reverse phase high performance liquid chromatography; TLC, thin layer chromatography; t_R, retention time; ES-MS, electrospray mass spectrometry; PC-3 cells, androgen insensitive human prostate adenocarcinoma cells

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