Toward Stable N_4-Modified Neurotensins for NTS1-Receptor-Targeted Tumor Imaging with ^{99m}Tc

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A series of Gly-neurotensin(8−13) analogues modified at the N-terminus by acyclic tetraamines (Demotensin 1−4) were obtained by solid-phase peptide synthesis techniques. Strategic replacement of amino acids and/or reduction of sensitive peptide bonds were performed to enhance conjugate resistance against proteolytic enzymes. During ^{99m}Tc-labeling, single species radiopeptides, [^{99m}Tc]Demotensin 1−4, were easily obtained in high yields and typical specific activities of 1 Ci/μmol. Peptide conjugates displayed a high affinity binding to the human neurotensin subtype 1 receptor (NTS1-R) expressed in colon adenocarcinoma HT-29 or WiDr cells and/or in human tumor sections. [^{99m}Tc]Demotensin 1−4 internalized very rapidly in HT-29 or WiDr cells by a NTS1-R-mediated process. [^{99m}Tc]Demotensin 3 and 4, which remained stable during 1 h incubation in murine plasma, were selectively studied in nude mice bearing human HT-29 and WiDr xenografts. After injection, [^{99m}Tc]Demotensin 3 and 4 effectively and specifically localized in the experimental tumors and were rapidly excreted via the kidneys into the urine, exhibiting overall biodistribution patterns favorable for NTS1-R-targeted tumor imaging in man.

Introduction

Neurotensin (NT) is a brain-gut neuropeptide consisting of 13 amino acids that was first isolated from bovine hypothalamus[1] and soon thereafter from bovine intestinal tissue.[2] NT exerts a spectrum of physiological functions both in the central nervous system and in the periphery. These actions are mediated by interaction with specific receptors found at the cell membrane of target cells. Three neurotensin receptor subtypes have been cloned so far, two of which (NTS1-R and nts2-R) belong to the superfamily of G-protein-coupled receptors having the typical seven-transmembrane domain configuration while the third one (nts3-R) is an entirely new type of neuropeptide receptor identical to gp95/sortilin with a single transmembrane domain.[3−5] The NTS1-R binds NT with a high affinity (K_d = 0.2 nM) and is insensitive to the potent H1 receptor antagonist levocabastine. Conversely, the nts2-R binds both NT and levocabastine with similar affinities (K_d = 2−10 nM).

The expression of NTS1-Rs was recently manifested in several human cancers, such as in 75% of exocrine ductal pancreatic adenocarcinomas,[6−8] Ewing's sarcomas, astrocytomas, and meningiomas.[9,10] Although NTS1-Rs have been detected in ample numbers in several human colon carcinoma cell lines,[11] their expression in human colonic cancers is still a matter of controversy.[11−13] The NTS1-R presence in human tumors versus their absence or minimal expression in normal surrounding tissue provide the molecular basis for developing NT-based radiopeptides for application in targeted diagnostic imaging and/or radionuclide therapy. This approach has been successfully pursued in the management of somatostatin subtype 2 receptor(sst_2)-positive tumors.[10,14] Taking into account that sst_2 expression is practically confined in the narrow group of neuroendocrine tumors, alternative peptide carriers, such as neurotensin, are soon expected to play an increasingly prominent role in the diagnosis and treatment of tumors devoid of sst_2.[10,14] Consequently, NTS1-R-rich tumors are emerging as putative targets for diagnostic and/or therapeutic neurotensin-based radiopharmaceuticals. Among them, exocrine ductal pancreatic adenocarcinoma is of particular clinical relevance due to its high prevalence and poor prognosis as well as its lack of sst_2-expression.[15,16] This devastating pancreatic malignancy is the fourth and fifth cause of cancer death in men and women, respectively, in western countries.[17,18] Due to its extremely rapid progression, exocrine ductal pancreatic adenocarcinoma is rarely curable by surgical intervention at the time of diagnosis. Therefore, novel clinical tools are most urgently needed for its early diagnosis and therapy.

Aiming toward this goal, several neurotensin analogues have been recently introduced carrying metal-chelates at the N-terminus, such as ^{111}In-DTPA/DOTA[19,20] or organometallic-containing ^{99m}Tc^{I}-*fac*-$(CO)_3$ fragments.[21−25] A common pursuit in these studies has been the design of radioactivity vehicles of sufficient biological stability to ensure the effective delivery of radionuclides at the tumor site(s) after in vivo administration. The short half-lives of either NT (1.5 min) or the truncated NT-(8−13) analogue (10 min) in human plasma do not allow for sufficient accumulation in the tumor, thereby precluding their clinical application.[26−29] Especially susceptible to enzymatic cleavage are the Arg^8-Arg^9, the Tyr^{11}-Ile^{12}, and the Pro^{10}-Tyr^{11} peptide bonds in the original NT(8−13) motif, the minimum C-terminal fragment of NT preserving full capacity for interaction with the receptor. Attempts have been directed toward stabilizing these rapidly biodegradable sites by reduction or methylation of peptide bonds in combination with strategic amino acid replacement(s) using unnatural sterically restricting residues. It should be noted that progress achieved so far is reflected by a recent clinical study reporting the first application

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Table 1. Analytical Data for Demotensin 1−4

compound	% purity	% yield	MW calcd, *m/z*	MW ES-MS,[a] [M + H$^+$]	HPLC t_R (min), UV trace system 1[b]	HPLC t_R (min), UV trace system 2[c]
Demotensin 1, N_4-Gly-Arg-Arg-Pro-Tyr-Ile-Leu-OH	>97	16	1059.67 [M + 2H$^+$]/2 = 530.84	1060.6 531.2	12.5	17.8
Demotensin 2, N_4-Gly-LysΨ[CH_2NH]Arg-Pro-Tyr-Ile-Leu-OH	>98	33	1017.68 [M + 2H$^+$]/2 = 509.84	1018.9 510.3	12.5	15.6
Demotensin 3, N_4-Gly-LysΨ[CH_2NH]Arg-Pro-Tyr-Tle-Leu-OH	>99	25	1017.68 [M + 2H$^+$]/2 = 509.84	1018.9 510.3	12.1	15.4
Demotensin 4, N_4-Gly-ArgΨ[CH_2NH]Arg-Pro-Tyr-Tle-Leu-OH	>98	21	1045.69 [M + 2H$^+$]/2 = 524.4	1046.8 524.4	12.1	14.5

[a] Peaks observed in the ES-MS spectra. [b] RP-HPLC with UV-detection (215 nm), Column: Vydac 218TP54 (C18, 5 μm, 250 mm × 4.6 mm), solvent A = 0.1% TFA (v/v) and solvent B = MeCN containing 0.1% TFA (v/v); linear gradient, 3% B to 100% B in 30 min; flow rate, 1 mL/min. [c] RP-HPLC with UV-detection (215 nm), Column: Vydac 218TP54 (C18, 5 μm, 250 mm × 4.6 mm), solvent A = 0.1% TFA (v/v) and solvent B = MeOH containing 0.1% TFA (v/v); linear gradient, 3% B to 100% B in 30 min; flow rate, 1 mL/min.

of a ^{99m}Tc-labeled NT radiotracer for targeted tumor scintigraphy in a small group of pancreatic cancer patients.[30]

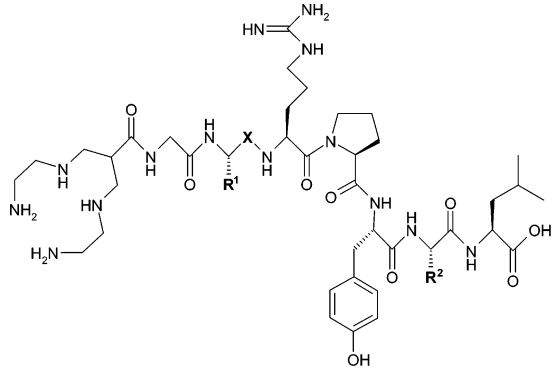
We have been recently involved in the development of ^{99m}Tc-based peptide radiotracers for application in targeted diagnostic imaging of cancer. For effective labeling with ^{99m}Tc, peptide carriers have been functionalized by acyclic tetraamines covalently coupled at the N-terminus either directly or via suitable linkers. This method has proven to be very successful in providing high purity and high specific activity radiopeptides that retain full capacity for receptor interaction both in vitro and in animal models.[31,32] First studies in neuroendocrine cancer patients have further established the applicability of this strategy in clinical oncology.[33,34] In this work, we have coupled an acyclic tetraamine chelator to the N-terminal Gly of the original Gly-NT(8−13) motif (Demotensin 1) and to single (Demotensin 2, Lys$^8\Psi$[CH_2NH]Arg^9) and doubly stabilized (Demotensin 3, Lys$^8\Psi$[CH_2NH]Arg^9/Tle;[12] Demotensin 4, Arg$^8\Psi$[CH_2NH]-Arg^9/Tle^{12}) derivatives thereof. The effects of these modifications on end-peptide stability and ability to interact with the human NTS1-R was investigated in vitro and in animal models carrying human colon adenocarcinoma xenografts.

Results

Synthesis of Conjugates. The peptide sequences corresponding to Demotensin 1−4 were assembled on solid support following Boc-protection strategies. Prior to addition of Gly^7 in the Demotensin 2−4 sequences, the peptide bond between residues 8 and 9 was reduced by reductive amination. Eventually, the Boc-protected tetraamine precursor was coupled to the terminal Gly^7, the conjugate was released from the resin with concomitant removal of lateral protecting groups by HF treatment and purified by chromatography. Isolated tetraamine−peptide conjugates, Demotensin 1−4 (Chart 1), were obtained as white solids after freeze-drying, in overall yields ranging from 16% to 33%. Yields of synthesis for individual products along with analytical data comprising high-performance liquid chromatography (HPLC) and electron spray mass spectroscopy (ES-MS) results are compiled in Table 1.

Radiopeptides. Incubation of Demotensin 1−4 with $^{99m}TcO_4^-$ generator eluate in an alkaline aqueous solution containing citrate anions and tin chloride led after 30 min to a nearly quantitative incorporation of the radiometal. Radiopeptides, [^{99m}Tc]Demotensin 1−4, were obtained in >98% yields as single radioactive species in typical specific activities of 1 Ci/μmol total peptide (Table 2). Formation of the [$^{99m}TcO_2$-N_4]$^+$−peptide conjugates was assumed on the basis of published reports demonstrating that acyclic tetraamines form monocationic octahedral metal dioxo complexes with both technetium and rhenium at the oxidation state of +5.[34−37] To confirm this hypothesis, the representative [$^{185/187}$Re(V)O_2-N_4]$^+$-complex of Demotensin 1, [Re]Demotensin 1, was synthesized from the [(n-C_4H_9)$_4$N][Re^VOCl_4] precursor, isolated in a pure form and identified by matrix-assisted laser desorption ionization mass spectroscopy time-of-flight (MALDI-TOF) method. Co-injection of [^{99m}Tc]Demotensin 1 and [Re]Demotensin 1 in an HPLC system coupled to twin radiometric (gamma trace for [^{99m}Tc]-Demotensin 1) and photometric (UV/Vis trace for [Re]Demotensin 1) detection modes resulted in identical elution profiles in two different chromatographic settings, suggesting the presence of isostructural metal-chelate species (Table 2). As revealed by combined HPLC and ITLC analysis of the labeling reaction mixture, radiopeptide purity was >98% with traces of total radiochemical impurities, comprising $^{99m}TcO_4^-$, [^{99m}Tc]-citrate, and $^{99m}TcO_2$·xH$_2$O, not exceeding 2%.

Chart 1. Demotensin 1−4 Formulas



Peptide conjugate	R^1	R^2	X
Demotensin 1	CH_2-CH_2-CH_2-NH-(C=NH)-NH_2 (Arg)	$CH(CH_3)$-CH_2-CH_3 (Ile)	C=O
Demotensin 2	CH_2-CH_2-CH_2-CH_2-NH_2 (Lys)	$CH(CH_3)$-CH_2-CH_3 (Ile)	CH_2
Demotensin 3	CH_2-CH_2-CH_2-CH_2-NH_2 (Lys)	$C(CH_3)_3$ (*tert*-Leu)	CH_2
Demotensin 4	CH_2-CH_2-CH_2-NH-(C=NH)-NH_2 (Arg)	$C(CH_3)_3$ (*tert*-Leu)	CH_2

Affinity of Demotensin 1−4 for the Human NTS1-R. Demotensin 1−4 affinities for the human NTS1-R were determined by competition binding assays in human colon adenocarcinoma HT-29 and WiDr cell membranes both expressing the NTS1-R.[10] [^{125}I-Tyr^3]NT was used as the radioligand in all experiments and data expressed as IC_{50} are compiled in Table 3; values for NT are included as well for comparison purposes. All conjugates, such as native NT, were able to

Table 2. Analytical Data for (Radio)metalated Peptide Conjugates

compound	HPLC t_R (min), gamma trace	
	system 3[a]	system 4[b]
[^{99m}Tc]Demotensin 1, [$^{99m}TcO_2N_4$]$^+$-Gly-NT(8−13)	15.6	30.2
[$^{185/187}$Re]Demotensin 1, [$^{185/187}ReO_2N_4$]$^+$-Gly-NT(8−13)	15.1[c]	29.7[c]
[^{99m}Tc]Demotensin 2, [$^{99m}TcO_2N_4$]$^+$-Gly-LysΨ[CH_2NH]Arg-Pro-Tyr-Ile-Leu-OH	17.2	27.0
[^{99m}Tc]Demotensin 3, [$^{99m}TcO_2N_4$]$^+$-Gly-LysΨ[CH_2NH]Arg-Pro-Tyr-Tle-Leu-OH	16.4	26.2
[^{99m}Tc]Demotensin 4, [$^{99m}TcO_2N_4$]$^+$-Gly-ArgΨ[CH_2NH]Arg-Pro-Tyr-Tle-Leu-OH	19.0	24.7

[a] RP-HPLC with gamma-detection, Column: Xterra RP-18 (5 μm, 150 mm × 4.6 mm), solvent A = 20 mM Na_2HPO_4 pH 11.5 in 0.45% NaCl and solvent B = MeOH; linear gradient, 0% B to 70% B in 30 min; flow rate, 1 mL/min. [b] RP-HPLC with gamma-detection, Column: Symmetry Shield RP-18 (5 μm, 150 mm × 3.9 mm), solvent A = 0.1% TFA (v/v) and solvent B = MeOH; linear gradient, 0% B to 60% B in 60 min; flow rate, 1 mL/min. [c] With UV-detection (220 nm).

Table 3. Affinities of Demotensin 1−4 for the Human NTS1-R[a]

peptide conjugate	IC_{50} (nM)	
	HT-29	WiDr
NT	0.62 ± 0.04	0.46 ± 0.23
Demotensin 1	0.32 ± 0.02	0.14 ± 0.01
Demotensin 2	0.41 ± 0.02	0.18 ± 0.01
Demotensin 3	1.50 ± 0.01	0.66 ± 0.05
Demotensin 4	0.85 ± 0.07	0.51 ± 0.01

[a] Results represent the mean (±SEM) IC_{50} values in nM for the nonradioactive NT and for Demotensin 1−4 as determined during competition binding assays in HT-29 and WiDr cell membranes expressing the human NTS1-R; [^{125}I-Tyr^3]NT was used as the radioligand in all experiments.

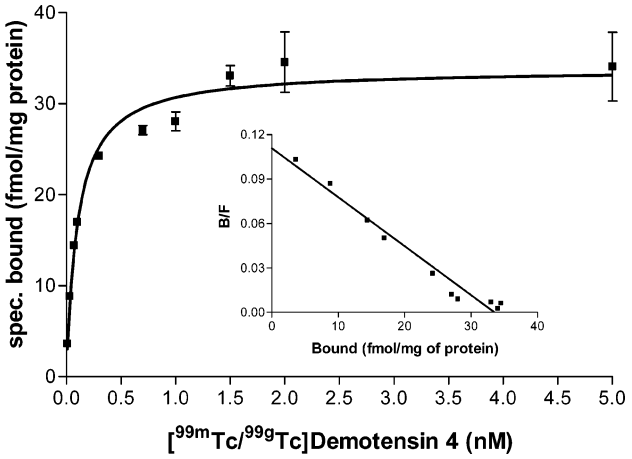


Figure 1. Saturation binding curve of [^{99m}Tc/^{99g}Tc]Demotensin 4 in human NTS1-R-positive WiDr cell membranes: Calculated equilibrium binding constant, K_d = 0.10 ± 0.015 nM, B_{max}= 34 ± 0.99 fmol/mg protein (Scatchard plot is shown in the inset).

displace the radioligand in a monophasic and dose-dependent fashion with respective IC_{50}s within the low or sub-nanomolar range.

Saturation Binding of [^{99m}Tc/^{99g}Tc]Demotensin 4. Saturation binding experiments were performed in NTS1-R-positive WiDr cell membranes selectively for [^{99m}Tc/^{99g}Tc]Demotensin 4 in order to determine if incorporation of the (radio)metal by the acyclic tetraamine framework and formation of the (radio)-metalated-peptide species is well tolerated by the receptor. Due to radiation safety issues related to the 520 Ci/μmol specific activity of ^{99m}Tc, the required amount of (radio)metalated peptide was obtained at "carrier-added" conditions. Thus, [^{99m}Tc/^{99g}Tc]Demotensin 4 was synthesized from a $^{99m}TcO_4^-$/$^{99g}TcO_4^-$ mixture and isolated in a chemically pure form by HPLC.[31,32,34] As revealed by the saturation curve in Figure 1, [^{99m}Tc/^{99g}Tc]-Demotensin 4 showed a strong and dose-dependent interaction with a single class of high affinity binding sites in WiDr cell membranes resulting in an equilibrium binding constant (K_d) of 0.10 ± 0.01 nM. This finding showed that, similarly to the unlabeled peptide conjugate, radiometalated Demotensin 1, used here as a representative example, retains a high affinity for the human NTS1-R.

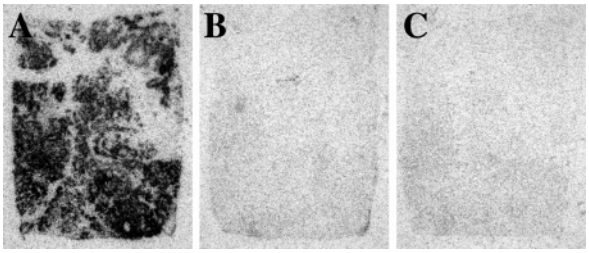


Figure 2. NTS1-R autoradiography in a human pancreatic carcinoma. A: Total binding of [^{125}I-Tyr^3]NT showing strong labeling of the whole tumor, B+C: Binding of [^{125}I-Tyr^3]NT in the presence of 10^{-6} M NT (B) or Demotensin 4 (C). Complete displacement is seen with NT or Demotensin 4.

Receptor Autoradiography of Demotensin 4 in Human Biopsy Samples. The IC_{50} value for Demotensin 4 determined by receptor autoradiography on cryostat sections of resected human exocrine ductal pancreatic carcinoma was 2.9 ± 0.4 nM (mean ± SEM, n = 4) while the respective value for native NT was 1.3 ± 0.27 nM (mean ± SEM, $n \geq 4$). Representative autoradiograms are included in Figure 2.

Internalization of [^{99m}Tc]Demotensin 1−4. Time-dependent internalization curves for [^{99m}Tc]Demotensin 3 and 4 in HT-29 and for [^{99m}Tc]Demotensin 1−4 in WiDr cells during incubation at 37 °C are presented in Figure 3, parts A and B, respectively. Radiopeptides were effectively and rapidly internalized in both cell lines reaching a 90−95% internalization plateau within 30 min. Internalized radioactivity remained high up to 2 h incubation, as reported also for other radiolabeled synthetic NT analogues.[21−25] As shown by the corresponding nonspecific internalization curves of Figure 3 (empty bullets), internalization was banned by incubation in the presence of 1 μM NT as expected for a receptor-mediated process.

Metabolism of [^{99m}Tc]Demotensin 1−4. As shown by the time-dependent plasma degradation curves of [^{99m}Tc]Demotensin 1−4 in Figure 4(A), the radiopeptide rank of stability in murine plasma is: [^{99m}Tc]Demotensin 4 = [^{99m}Tc]Demotensin 3 (stable up to 1 h incubation) ≫ [^{99m}Tc]Demotensin 2 ($t_{1/2}$ = 20−25 min) ≫ [^{99m}Tc]Demotensin 1 ($t_{1/2}$ = 3−5 min). It is evident that the presence of [$^{99m}TcO_2$-N_4]$^+$-chelate at the N-terminus of the Gly^7-NT(8−13) motif was not sufficient to inhibit the enzymatic degradation of [^{99m}Tc]Demotensin 1. Interestingly, replacement of Arg^8 by Lys^8 and concomitant reduction of the Lys^8-Arg^9 bond in [^{99m}Tc]Demotensin 2, although increasing plasma stability, did not effectively protected this radiopeptide from eventual degradation during longer exposure in plasma. Further replacement of Ile^{12} by Tle^{12} in [^{99m}Tc]Demotensin 3 efficiently protected this radiopeptide from plasma proteolytic enzymes up to 60 min incubation. Equally

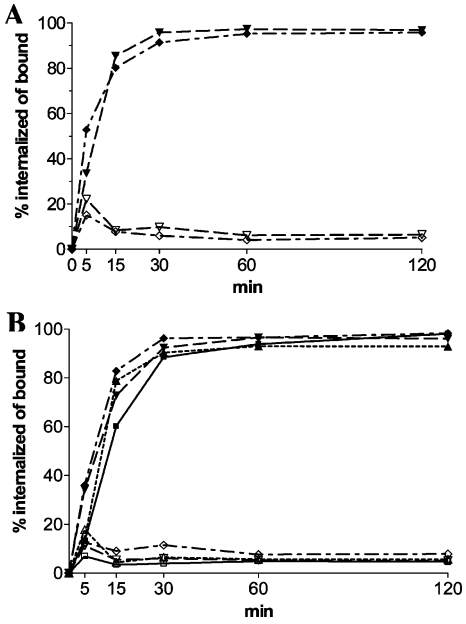


Figure 3. (A) Time-dependent internalization curves of [^{99m}Tc]-Demotensin 3 and 4 in HT-29 cells during incubation at 37 °C. (B) Time-dependent internalization curves of [^{99m}Tc]Demotensin 1−4 in WiDr cells during incubation at 37 °C. ■, [^{99m}Tc]Demotensin 1; ▲, [^{99m}Tc]Demotensin 2; ▼, [^{99m}Tc]Demotensin 3; and ◆, [^{99m}Tc]Demotensin 4; nonspecific internalization (in the presence of 1 μM NT) curves are indicated by the respective empty bullets.

high stability was attained by [^{99m}Tc]Demotensin 4, wherein the original peptide Arg^8-Arg^9 bond was reduced and Ile^{12} was replaced by Tle.[12] Representative radiochromatograms of [^{99m}Tc]-Demotensin 1 and 4 analysis of 15 min plasma incubates are shown in Figure 4, parts B and C, respectively.

During incubation in kidney homogenates [^{99m}Tc]Demotensin 1−4 were all degraded to hydrophilic products at different rates, as shown by the time-dependent degradation curves in Figure 5A. Degradation, although much faster in the kidney than in plasma, yielded a similar rank of radiopeptide stability: [^{99m}Tc]-Demotensin 3 ($t_{1/2}$ = 15−20 min) > [^{99m}Tc]Demotensin 4 ($t_{1/2}$ = 5−10 min) > [^{99m}Tc]Demotensin 2 ($t_{1/2}$ = 3−5 min) ≥ [^{99m}Tc]Demotensin 1 ($t_{1/2} \sim$ 2 min). Furthermore, urine analysis of radiopeptides 30 min after iv administration in mice revealed their eventual excretion totally (in the case of [^{99m}Tc]Demotensin 1 and 2) or predominantly (in the case of [^{99m}Tc]Demotensin 3 and 4) in the form of hydrophilic degradation species. As verified by parallel ITLC tests, the metabolites found in urine did not include free $^{99m}TcO_4^-$ released by metal-chelate breakdown in vivo, and further confirm previous reports on the high in vivo stability of $[^{99m}TcO_2\text{-}N_4]^+$-chelates.[33−35] A representative radiochromatogram of [^{99m}Tc]Demotensin 4 metabolites in urine is presented in Figure 5B, showing that, besides the hydrophilic products with t_R 1−5 min, there is still a significant amount of intact [^{99m}Tc]Demotensin 4 detected in the urine at the expected t_R of 19 min.

Biodistribution of [^{99m}Tc]Demotensin 3 and 4. Biodistribution data of [^{99m}Tc]Demotensin 3 and 4 in athymic mice bearing human HT-29 xenografts are summarized in Table 4 as %ID/g for the 1, 4, and 24 h time intervals after iv administration, whereas biodistribution data of the plasma degradable analogues, [^{99m}Tc]Demotensin 1 and 2, were not included in this study. Both radiopeptides showed a very rapid clearance from background tissues via the kidneys into the urine. Their in vivo profiles were generally very similar with the exception of renal uptake, which was significantly lower for

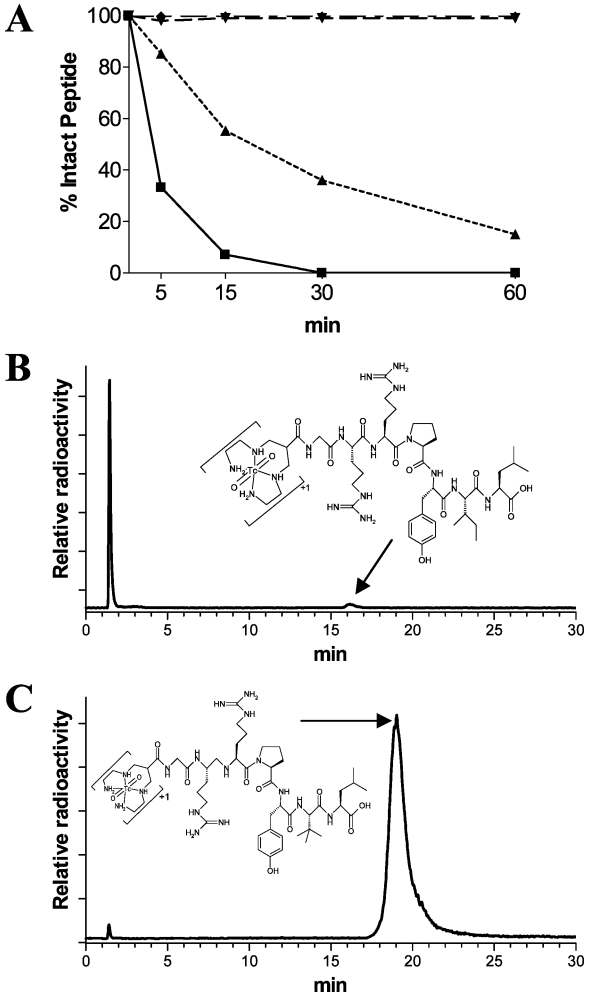


Figure 4. (A) Time-dependent enzymatic degradation of [^{99m}Tc]-Demotensin 1−4 by incubation at 37 °C in murine plasma; ■, [^{99m}Tc]-Demotensin 1; ▲, [^{99m}Tc]Demotensin 2; ▼, [^{99m}Tc]Demotensin 3; and ◆, [^{99m}Tc]Demotensin 4. (B) and (C) Representative HPLC gamma trace of 15 min murine plasma incubates of [^{99m}Tc]Demotensin 1 and [^{99m}Tc]Demotensin 4, respectively.

[^{99m}Tc]Demotensin 4 than for the Lys^8-substituted analogue, [^{99m}Tc]Demotensin 3, at all time intervals. Uptake by the human NTS1-R-expressing HT-29 tumor, although higher for [^{99m}Tc]-Demotensin 4 than for [^{99m}Tc]Demotensin 3, was not significantly different. Co-injection of excess unlabeled NT significantly reduced tumor uptake for both radiopeptides, suggesting a receptor-mediated accumulation. Likewise, intestinal uptake was found significantly reduced after treatment with excess NT.

Results derived from biodistribution experiments with [^{99m}Tc]-Demotensin 3 and 4 in athymic mice bearing human WiDr xenografts are listed in Table 5 as %ID/g for 1 h pi. WiDr tumor values were at similar levels to those found in the HT-29 tumor (Table 4) for both analogues and could be effectively reduced by treatment with "cold" NT. This finding is in accordance with in vitro data revealing comparable affinities and internalization capacities of tested NT analogues in both cell lines. As described above, intestinal uptake could be blocked by co-injection of cold NT. Kidney values for [^{99m}Tc]Demotensin 3, modified at position 8 by Lys, were found higher as compared to those of [^{99m}Tc]Demotensin 4, as was also observed in the kidneys of HT-29 tumor bearing mice.

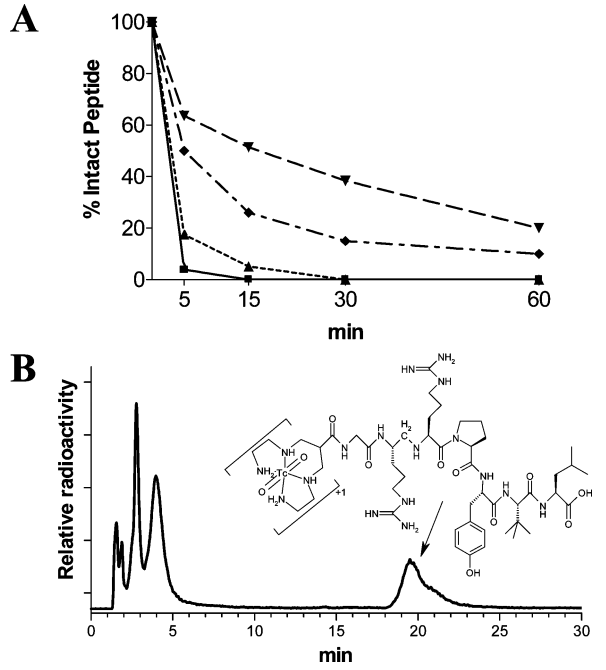


Figure 5. (A) Time-dependent enzymatic degradation of [^{99m}Tc]-Demotensin 1−4 by incubation at 37 °C in mouse kidney homogenates; ■, [^{99m}Tc]Demotensin 1; ▲, [^{99m}Tc]Demotensin 2; ▼, [^{99m}Tc]Demotensin 3; and ◆, [^{99m}Tc]Demotensin 4. (B) Radiochromatogram of HPLC analysis of mouse urine collected 30 min after injection of [^{99m}Tc]Demotensin 4.

Discussion

The overexpression of NTS1-Rs in human neoplasms,[6−14] like exocrine ductal pancreatic carcinomas or Ewing's sarcomas, has spurred research toward radiolabeled neurotensin analogues for application in targeted diagnostic imaging and radionuclide therapy of NTS1-R-positive tumors.[19−25] A major handicap of original NT or NT(8−13) motif-based analogues used for this purpose is their rapid degradation by plasma peptidases[26−29] eventually preventing the effective in vivo delivery of radioactivity at the target site(s). Attempts to stabilize NT analogues include N-terminal acetylation, reduction of biodegradable peptide bonds, amino acid replacement, and cyclization.

Joining this effort, we have developed four new Gly-NT(8−13) analogues suitable for labeling with the pre-eminent diagnostic radionuclide of nuclear medicine, ^{99m}Tc, by means of an acyclic tetraamine unit covalently tethered at the N-terminal Gly. Synthesis of peptide conjugates, Demotensin 1−4, was completed via typical SPPS and facile terminal coupling of a Boc-protected tetraamine bifunctional chelator precursor via its carboxy functionality.[31,32,34] To obtain stable radiopeptides, structural modifications comprised N_4-functionalization of the original Gly-NT(8−13) sequence (Demotensin 1), as well as reduction of the Arg/Lys^8-Arg^9 bond with or without parallel Ile^{12}/Tle^{12} replacement (Demotensin 2−4). These modifications were undertaken on the basis of previous studies reporting the Arg^8-Arg^9 bond as the first cleavage site of NT(8−13)-containing analogues by plasma peptidases followed by the Tyr^{11}-Ile^{12} bond.[21−25] Interestingly, the above modifications were not found to diminish the affinity of resulting Demotensin 1−4 bioconjugates for the human NTS1-R, as demonstrated by competition binding assays in human NTS1-R-positive colorectal adenocarcinoma HT-29 and WiDr cell preparations[10] and, selectively for Demotensin 4, by receptor autoradiography in resected human biopsy samples of exocrine ductal pancreatic carcinoma. Binding was neither affected by incorporation of the (radio)metal and formation of the corresponding monocationic [^{99m}TcO$_2^+$-N$_4$]-chelate, as representatively shown for [^{99m}Tc/^{99g}Tc]Demotensin 4 during saturation binding assays in WiDr cell membranes. Specific interaction of radiolabeled peptides, [^{99m}Tc]Demotensin 1−4, was further confirmed during incubation at 37 °C with HT-29 and WiDr cells which resulted in a very rapid and receptor-mediated internalization of radioactivity within the cell. Similar findings have been reported for other NT(8-13) analogues carrying different ^{99m}Tc-chelates at the N-terminus.[21−25]

Conversely, the effects of above-described modifications on individual end-radiopeptide stability were found to be diverse. More specifically, the presence of [^{99m}TcO$_2$−N$_4$]$^+$-chelate at the N-terminus alone was not sufficient to inhibit the proteolytic action of mouse plasma or kidney enzymes, as revealed by HPLC analysis of mouse plasma incubates and kidney homogenates in vitro. Accordingly, [^{99m}Tc]Demotensin 1 was degraded very rapidly in both assays similarly to previous reports.[21−25] Parallel ex vivo analysis of mouse urine revealed only the presence of hydrophilic metabolite(s) in the urine, but no detectable traces of the original radiopeptide, [^{99m}Tc]-Demotensin 1. It should be stressed that ^{99m}TcO$_4^-$ traces expected to be released from in vivo breakdown of the [^{99m}TcO$_2$−N$_4$]$^+$-chelate were not detected during any of the assays. This finding, supported by acetone/ITLC results, is in agreement with previous studies reporting on the high in vivo stability of [^{99m}TcO$_2$−N$_4$]$^+$-chelates.[31,33−35]

Replacement of Arg^8 by Lys^8 and reduction of the Lys^8-Arg^9 peptide bond moderately increased stability in [^{99m}Tc]Demotensin 2. Further replacement of Ile^{12} by Tle^{12} in reduced Lys^8/Arg^8-Arg^9 bond [^{99m}Tc]Demotensin 3/[^{99m}Tc]Demotensin 4 led to a high stability in mouse plasma without affecting the capacity of these radiopeptides to interact with the human NTS1-R. However, neither [^{99m}Tc]Demotensin 3 nor [^{99m}Tc]Demotensin 4 remained intact during in vitro incubation in kidney homogenates, implying a rapid renal degradation in vivo. In fact, such degradation was verified by ex vivo mouse urine analysis which revealed excretion of both radiopeptides in the urine predominantly in the form of catabolic products. However, renal degradation rates of doubly stabilized [^{99m}Tc]Demotensin 3 and [^{99m}Tc]Demotensin 4 were found to be substantially slower as compared to single stabilized [^{99m}Tc]Demotensin 2 and to nonstabilized [^{99m}Tc]Demotensin 1. It is characteristic that measurable amounts of original [^{99m}Tc]Demotensin 3 and [^{99m}Tc]Demotensin 4 were still detected in the urine as opposed to [^{99m}Tc]Demotensin 1 and [^{99m}Tc]Demotensin 2; the latter were excreted totally as degraded products. The eventual catabolism of doubly stabilized radiopeptides is suspected to have occurred by kidney proteases acting at a third cleavage site, most probably between Pro^{10} and Tyr^{11}, as previously described for similar analogues.

Due to their superior plasma stability, [^{99m}Tc]Demotensin 3 and [^{99m}Tc]Demotensin 4 were further investigated in vivo. Nude mice bearing either HT-29 or WiDr xenografts, both expressing the human NTS1-R, were employed as an in vivo model. Consistent with in vitro data, radiopeptides were capable to target both HT-29 and WiDr implants in vivo by a receptor-specific process resulting in comparable tumor radioactivity levels. Both analogues washed out very rapidly from non-target tissues via the kidneys. An especially excellent in vivo profile was exhibited by [^{99m}Tc]Demotensin 4. This radiopeptide consistently showed faster renal excretion and higher, although not statistically significant, tumor accumulation values as compared to [^{99m}Tc]Demotensin 3. Tumor values were found

Table 4. Biodistribution of [^{99m}Tc]Demotensin 3 and [^{99m}Tc]Demotensin 4 in Human HT-29 Xenograft-Bearing Nude Mice[a]

	[^{99m}Tc]Demotensin 3			[^{99m}Tc]Demotensin 4		
organ	1 h	4 h	24 h	1 h	4 h	24 h
blood	0.61 ± 0.09	0.15 ± 0.02	0.08 ± 0.03	0.73 ± 0.12	0.09 ± 0.00	0.02 ± 0.01
liver	0.89 ± 0.07	0.90 ± 0.08	0.47 ± 0.09	0.84 ± 0.11	0.76 ± 0.05	0.49 ± 0.03
heart	0.31 ± 0.03	0.12 ± 0.01	0.12 ± 0.01	0.50 ± 0.12	0.11 ± 0.03	0.03 ± 0.03
kidneys	18.17 ± 1.22	20.36 ± 2.64	7.96 ± 0.98	10.92 ± 1.94	7.10 ± 0.87	1.33 ± 0.52
stomach	1.14 ± 0.05	1.68 ± 0.11	1.01 ± 0.61	1.36 ± 0.20	0.92 ± 0.09	0.17 ± 0.10
intestines	1.31 ± 0.25	2.93 ± 0.13	2.84 ± 0.07	1.45 ± 0.28	1.20 ± 0.14	0.42 ± 0.10
blocked int.[b,c]	0.60 ± 0.03*	1.30 ± 0.40**		0.45 ± 0.04**	0.59 ± 0.09**	
spleen	0.45 ± 0.04	0.39 ± 0.03	0.34 ± 0.01	0.55 ± 0.01	0.30 ± 0.03	0.20 ± 0.02
muscle	0.15 ± 0.01	0.07 ± 0.01	0.10 ± 0.03	0.19 ± 0.03	0.06 ± 0.01	0.01 ± 0.01
lungs	1.23 ± 0.12	0.44 ± 0.02	0.35 ± 0.05	1.38 ± 0.14	0.34 ± 0.03	0.14 ± 0.03
HT-29 tumor	3.52 ± 0.66	2.53 ± 0.11	1.96 ± 0.38	4.94 ± 0.52	3.15 ± 0.66	1.21 ± 0.28
blocked tumor[b,c]	1.65 ± 0.22*	0.62 ± 0.10***		1.56 ± 0.02***	0.68 ± 0.22**	

[a] Data are expressed as ID%/g tissue and are presented as mean ± SD ($n = 4$) at 1, 4, and 24 h pi. [b] Blockade was achieved by co-injection of 350 μg native NT. [c] Statistical analysis was performed using the Student's *t*-test with *p* values indicating significant (*$p < 0.05$), very significant (**$p < 0.01$) and extremely significant (***$p < 0.001$) difference versus unblocked.

Table 5. Biodistribution of [^{99m}Tc]Demotensin 3 and [^{99m}Tc]Demotensin 4 in Human WiDr Xenograft-Bearing Nude Mice[a]

organ	[^{99m}Tc]Demotensin 3	[^{99m}Tc]Demotensin 4
blood	0.72 ± 0.04	0.64 ± 0.14
liver	0.82 ± 0.02	1.23 ± 0.07
heart	0.34 ± 0.03	0.45 ± 0.11
kidneys	24.14 ± 4.16	16.29 ± 3.22
stomach	0.83 ± 0.51	0.68 ± 0.10
intestine	1.53 ± 0.20	1.99 ± 0.23
blocked intestine[b,c]	0.75 ± 0.10**	0.64 ± 0.00**
spleen	0.49 ± 0.01	0.58 ± 0.09
muscle	0.16 ± 0.01	0.16 ± 0.02
lung	1.16 ± 0.06	1.16 ± 0.18
WiDr tumor	3.95 ± 0.64	4.72 ± 0.72
blocked tumor[b,c]	1.70 ± 0.14**	1.67 ± 0.28**

[a] Data are expressed as ID%/g tissue and are presented as mean ± SD ($n = 4$) at 1 h pi. [b] Blockade was achieved by co-injection of 350 μg native NT. [c] Statistical analysis was performed using the Student's *t*-test, and a value **$p < 0.01$ was considered as a very significant difference versus unblocked.

in good agreement with or superior to values previously reported for other ^{99m}Tc^{I}(CO)$_3$- or ^{111}In-DTPA/DOTA-based NTS1-R-avid radiotracers in the same or similar animal model(s).

Thus, by screening of DTPA-coupled stabilized NT analogues compound 2530 [DTPA-(Pip)Gly-Pro-(PipAm)Gly-Arg-Pro-Tyr-*t*BuGly-Leu-OH], showing high serum stability and high affinity binding to the NTS1-R, was selected for further evaluation in vivo.[19] When labeled with ^{111}In and injected in mice, it showed a 2.81 ± 0.75%ID/g at 1 h pi and 2.18 ± 0.12%ID/g at 4 h pi uptake in the HT-29 xenografts.[19] To enable stable binding of therapeutic radiometals, DTPA in 2530 was replaced by DOTA.[20] The resulting bioconjugate, 2656, when labeled with ^{111}In and injected in the same animal model, displayed lower tumor uptake (1.90 ± 0.31%ID/g at 1 h pi and 1.10 ± 0.64%ID/g at 4 h pi).[20] Apparently both analogues had a lower tumor uptake than [^{99m}Tc]Demotensin 4 in the same animal model (Table 4). Comparison of [^{99m}Tc]Demotensin 4 with another series of ^{99m}Tc^{I}(CO)$_3$-(N$_\alpha$His)Ac-carrying and stabilized NT(8−13) analogues revealed comparable values of HT-29 tumor uptake.[21−24] Although the time points tested are not identical, ^{99m}Tc-NT-XI (^{99m}Tc^{I}(CO)$_3$-(N$_\alpha$His)Ac-Lys-[ΨCH$_2$−NH]Arg-Pro-Tyr-Tle-Leu), the compound of choice for evaluation in ductal pancreatic adenocarcinoma patients,[30] displayed a 3.9 ± 0.7%ID/g uptake in the implant at 1.5 h pi while at 5 h pi tumor uptake declined to 1.7 ± 1.0%ID/g.[23] This value is in the same range as [^{99m}Tc]Demotensin 4 (Table 4).

In view of the above, [^{99m}Tc]Demotensin 4 undoubtedly warrants further evaluation as a candidate radiotracer for application in the targeted diagnostic imaging of NTS1-R-positive tumors in man.

Conclusion

A series of Gly-NT(8−13) analogues have been developed amenable to ^{99m}Tc labeling via N-terminal functionalization with acyclic tetraamines. Satisfactory stability in mouse plasma was achieved only by [^{99m}Tc]Demotensin 3 and [^{99m}Tc]Demotensin 4 which were doubly stabilized at the first (Lys^8/Arg^8-Arg^9 bond) and the second (Tyr^{11}-Ile^{12} bond) cleavage sites. Stabilization involved peptide bond reduction and concomitant Ile^{12} replacement by Tle.[12] Concordant to their capacity to specifically interact with the human NTS1-R in HT-29 and WiDr cell preparations in vitro, [^{99m}Tc]Demotensin 3 and [^{99m}Tc]Demotensin 4 were capable to specifically target this receptor in vivo. In nude mice bearing human HT-29 and WiDr xenografts, both radiopeptides showed high tumor uptake and rapid background clearance via the kidneys into the urine. [^{99m}Tc]Demotensin 4 showed a superior in vivo profile in comparison to [^{99m}Tc]-Demotensin 3 as a result of its faster renal washout and its higher tumor uptake. In view of these findings, [^{99m}Tc]Demotensin 4 seems to be a very promising candidate for application in the targeted diagnostic imaging of NTS1-R-positive tumors, such as exocrine ductal pancreatic carcinoma, in man.

Experimental Section

General. Unless otherwise reported reagent grade chemicals were used as such without further purification. Boc-protected amino acids Tyr[Bn(2,6diCl)]-OH, Pro-OH, Arg(Tos)-OH, Lys(Z)-OH, Gly, and the preloaded Leu-Merrifield resin used in solid-phase peptide synthesis (SPPS) were obtained from NovaBiochem (Läufelfingen, Switzerland). Boc-Tle-OH, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), trifluoroacetic acid (TFA), and dichloromethane were purchased from Fluka (Bornem, Belgium), while ethyl diisopropylamine (DIPEA), *N*-methylmorpholine (NMM), and 2-propanol were obtained from Acros (Geel, Belgium). SPPS was performed on a semiautomated Peptide Synthesizer SP640B (Labortec, Bubendorf, Switzerland). Purification of the peptide conjugates was performed on a semipreparative HPLC system (Gilson) using a RP C_{18} column (Vydac 218 TP 152022, 25 × 2.5 cm, 10−20 μm) eluted with a linear gradient (3% to 80% CH_3CN in H_2O, containing 0.1% TFA in 30 min) at a flow rate of 13 mL/min with UV detection at 215 nm. Peptide conjugates were analyzed on a Merck-Hitachi HPLC equipped with a L-3000 UV detector using a RP column and the conditions given in Table 1. Neurotensin (NT = Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) was purchased from Bachem (Bubendorf, Switzerland). Potassium perrhenate from Aldrich was used as the rhenium source.

ES-MS spectra were recorded on a VG Quatro II spectrometer using MassLynx 2.22 software for data analysis. MALDI-TOF of Re-metalated Demotensin 1 was carried out on a Voyager sSTR instrument (Applied Biosystems, Framingham, NY).

Technetium-99m was obtained in the form of [^{99m}Tc]$NaTcO_4$ by elution of a commercial ^{99}Mo/^{99m}Tc generator (Cis International, France), while ^{99g}Tc was provided by Oak Ridge National Laboratories in the form of $NH_4{}^{99g}TcO_4$ powder. Iodine-125 was obtained from MDS Nordion, SA (Fleurus, Belgium). All manipulations with gamma and beta emitting radionuclides and their solutions were performed behind suitable shielding in dedicated laboratories in compliance with national and international radiation safety guidelines. Radiochemical high performance liquid chromatography (HPLC) analyses were performed on a Waters Chromatograph (Waters, Vienna, Austria) with a 600 solvent delivery system 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 chromatographic control was conducted by the Millennium Software (Waters). Analyses were performed on RP-18 Symmetry Shield (5 μm, 3.9 mm $\times$ 150 mm) or XTerra RP-18 (5 μm, 4.6 mm $\times$ 150 mm) cartridge columns (Waters, Germany) applying the gradient systems given in the text. For instant thin-layer chromatography (ITLC) ITLC−SG strips from Gelman Sciences (Ann Arbor, MI) were used. All solvents used in analyses were HPLC grade and were previously filtered through 0.22 μm membrane filters (Millipore, Milford, MA) and degassed by helium flux. Radioactivity measurements were conducted in an automated well-type gamma counter [NaI(Tl) crystal, Canberra Packard Auto-Gamma 5000 series model] calibrated for ^{99m}Tc or ^{125}I.

The human colorectal adenocarcinoma cell line HT-29 was a kind gift from Dr. E. García-Garayoa (PSI, Villigen, Switzerland), while the human colorectal adenocarcinoma WiDr cell line was provided by LGC Promochem (Teddington, UK). All culture media were purchased from Gibco BRL, Life Technologies (Grand Island, NY) while supplements were provided by Biochrom KG Seromed (Berlin, Germany). For protein measurements the Bio-Rad protein assay kit (based on the Bradford procedure) by Bio-Rad Laboratories GmbH (Munich, Germany) was employed. The Brandel M-48 Cell Harvester (Adi Hassel Ingenieur Büro, Munich, Germany) was used for filtrations during binding assays. Animal experiments were carried out in compliance with European and national regulations. For metabolism experiments in-house male Swiss albino mice (30 $\pm$ 5 g) were used. For experimental tumor models female Swiss nu/nu mice of 7 weeks of age on the day of arrival (Charles Rivier Laboratories, France) were employed. The animals were kept under aseptic conditions until the day of biodistribution.

Synthesis of Conjugates. Demotensin 1. SPPS was performed on a Boc-Leu-substituted (1.04 mmol/g) Merrifield polystyrene resin. The Boc-deprotections were performed in a mixture of TFA/CH_2Cl_2/anisole (49/49/2) (5 min + 20 min). After filtration of the TFA mixture and neutralization by washing with 20% DIPEA/CH_2Cl_2 (3 times, 1 min), the couplings were performed by using 3 equiv of Boc-protected amino acid and DIC (3 equiv) in the presence of HOBt (3 equiv) in CH_2Cl_2. The completeness of couplings was monitored by the ninhydrin color test. After Boc deprotection of Gly and neutralization by washing with 20% DIPEA/CH_2Cl_2 (3 times 1 min), *N*,*N*′,*N*″,*N*‴-tetrakis(*tert*-butoxycarbonyl)-6-carboxy-1,4,8,11-tetraazaundecane (2 equiv) was coupled using TBTU (2 equiv) and NMM (12 equiv). The peptide conjugate was cleaved from the resin by treatment with HF(l) for 1.5 h at 0 °C, purified by HPLC, and lyophilized. Analytical data of the compound are presented in Table 1.

Demotensin 2. The NT(9−13) peptide sequence was assembled on the Merrifield resin as described above for Demotensin 1. After Boc-deprotection of Arg[9], the peptide resin trifluoroacetate was washed with DMF containing 1% AcOH (3 times, 1 min) and Boc-Lys(Z)-H[28] (2.5 equiv) in DMF was added, followed by the addition of $NaBH_4CN$ (2.5 equiv). The pH of the solution was regularly adjusted to 6 by addition of a 1% AcOH in DMF solution. After 2.5 h reaction, this reductive amination procedure was repeated, followed by washings with CH_2Cl_2 (3 times, 1 min), *i*-PrOH (3 times, 1 min) and CH_2Cl_2 (3 times, 1 min). The Kaiser ninhydrin test showed a reddish-brown color. After Boc-deprotection, the Gly residue and the chelator *N*,*N*′,*N*″,*N*‴-tetrakis(*tert*-butoxycarbonyl)-6-carboxy-1,4,8,11-tetraazaundecane were coupled and the peptide was cleaved from the resin and purified as described above.

Demotensin 3. The procedure described above was used, except that Boc-Tle was introduced in position 12 of the peptide sequence instead of Boc-Leu.

Demotensin 4. The peptide sequence was assembled on Merrifield resin as described above. After Boc-deprotection of Arg[8], a reductive amination was performed using Boc-Arg(Z_2)-H (2 times, 2.5 equiv)[29] and $NaBH_3CN$ (2 times, 2.5 equiv), followed by the procedure described above.

Radiolabeling. Labeling of Demotensins 1−4 with ^{99m}Tc was accomplished by following a published protocol.[31] Briefly, each lyophilized conjugate was dissolved in 50 mM acetic acid/EtOH 8/2 v/v to a final 1 mM peptide concentration. Each bulk solution was distributed in 50 μL aliquots in Eppendorf tubes and stored at −20 °C. Labeling was conducted in an Eppendorf vial, wherein the following solutions were consecutively added: (i) 0.5 M phosphate buffer pH 11.5 (50 μL), (ii) 0.1 M sodium citrate (5 μL), (iii) [^{99m}Tc]$NaTcO_4$ generator eluate (415 mL, 10−20 mCi), (iv) peptide conjugate stock solution (15 μL, 15 nmol), and (v) freshly made $SnCl_2$ solution in EtOH (30 μg, 15 μL). After reaction for 30 min at ambient temperature, the pH was brought to ~7 by adding 1 M HCl (10 μL).

A similar procedure was applied for the preparation of [^{99g}Tc/^{99m}Tc]Demotensin 4 at tracer added level.[31] [^{99g}Tc]NH_4TcO_4 was used as ^{99g}Tc-source (0.9 μg, 5 nmol) that was "spiked" with ^{99m}Tc found in the generator eluate. Complete reduction to Tc(V) was accomplished by addition of a higher amount of $SnCl_2$ (1 μmol/mL). The resulting [^{99g}Tc/^{99m}Tc]Demotensin 4 was isolated in a highly pure single species form by HPLC. For saturation binding experiments triplicates in the 10^{-12} to 10^{-16} M concentration range were prepared, as already described.[31,32,34]

Radioiodination of NT was performed by following the chloramine-T methodology, and [^{125}I-Tyr[3]]NT was obtained in a highly pure form after HPLC purification.[38]

Re-Metalation of Demotensin 1. The isostructural *trans*-[$Re^{V}(O)_2(N_4)]^+$ species of [^{99m}Tc]Demotensin 1 was prepared by reaction of Demotensin 1 with excess of the $[(n\text{-}C_4H_9)_4N][Re^{V}OCl_4]$ precursor in MeOH in the presence of Hünig's base (*N*-ethyldiisopropylamine).[34,35−37] All peptide present was transformed to a single species, [Re^{V}]Demotensin 1, isolated by HPLC, and identified by HPLC and MALDI-TOF. Retention times of the Re and ^{99m}Tc analogues were compared by HPLC coupled to parallel UV/Vis (for Re) and radiometric (for ^{99m}Tc) detection modes (Table 2). MW calculated for $[^{185/187}ReO_2N_4{}^0]^+$-Gly-NT(8−13): 1276.61/1278.61; MALDI-TOF (%, *m*/*z*): 1258.7 (58, $[M - H_2O]^+$)/1260.7 (100, $[M - H_2O]^+$).

Quality Control. For quality control of radiolabeled products, both RP-HPLC and ITLC were applied. In a typical HPLC experiment an aliquot (1 μL) of the test peptide labeling mixture was injected on the column. Elution at a 1.0 mL/min flow rate followed a linear gradient pattern with 20% solvent B at the start, reaching to 60% solvent B within the next 20 min (solvent A = 0.1% aqueous TFA and solvent B = acetonitrile). Under these conditions, [^{99m}Tc]citrate elutes at 2.0 min, $^{99m}TcO_4^-$ at 2.6 min, and the radiolabeled peptides after 12 min.[31] For detection of reduced hydrolyzed technetium traces ($^{99m}TcO_2{\cdot}xH_2O$), an aliquot of the test peptide labeling mixture was positioned on the ITLC strip. For development up to 10 cm from the origin, 1 M ammonium acetate/MeOH 1/1 (v/v) was used. Strips were left to dry in the open before they were cut into two sections: 1st = Start (origin + 0.5−1 cm): $^{99m}TcO_2{\cdot}xH_2O$ and 2nd = Front (the remaining part of the strip): $^{99m}TcO_4^-$, [^{99m}Tc]citrate, and [^{99m}Tc]peptide conjugate. Counts of each piece were measured in the gamma counter. For stability studies, tests were conducted at predetermined time points up to 6 h postlabeling.

Cell Culture. Human colorectal adenocarcinoma HT-29 and WiDr cells both expressing the human NTS1-R[10] were cultured in McCoy's GLUTAMAX-I supplemented by 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were kept in a controlled humidified atmosphere containing 5% CO_2 at 37 °C. Passages were performed every 4−5 days using a trypsin/EDTA (0.05%/0.02% w/v) solution.

Competition Binding Experiments for Demotensin 1−4. Competition binding assays were conducted both in HT-29 and in WiDr cell membranes harvested as previously described.[31,32] [^{125}I-Tyr^3]NT[38] was used as the radioligand and NT was used as reference compound. In brief, ∼40000 cpm radioligand were used per assay tube along with 50 μg of protein and tested peptide in increasing concentrations in 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 point were incubated for 30 min at 37 °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 Cell Harvester, 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 (Graph Pad Software, San Diego, CA). Nonspecific binding was defined as the amount of activity bound in the presence of 1 μM NT.

Saturation Binding Assays with [^{99m}Tc]Demotensin 4. Saturation binding assays were selectively conducted for [^{99g}Tc/^{99m}Tc]-Demotensin 4 in WiDr membranes following a previously described protocol.[31,32,34] Two sets of triplicates of the following concentrations were prepared: 0.06, 0.18, 0.42, 0.6, 1.8, 4.2, 6, 9, 12, 30, 60, and 300 nM (total [Tc]Demotensin 4 concentration = [^{99g}Tc]-Demotensin 4 + [^{99m}Tc]Demotensin 4 concentration) for total and nonspecific binding each. Each assay tube contained 50 μL of binding buffer (50 mM HEPES, pH 7.4, 1% BSA, 5 mM $MgCl_2$, 10 μM bacitracin), 50 μL of radioligand solution of the corresponding concentration, and 200 μL of WiDr membrane suspension containing 100 μg of protein. In a parallel series 50 μL of 0.6 μM NT solution was added instead of binding buffer to determine nonspecific binding. Tubes were incubated for 30 min at 37 °C in an Incubator-Orbital Shaker unit (MPM Instr. SrI, Italy). Incubation was terminated by addition of ice-cold washing buffer 10 mM HEPES (pH 7.4, 154 mM NaCl) followed by rapid filtration over glass fiber filters (Whatman GF/B, presoaked for at least 2 h in a 1% polyethyleneimine, PEI, aqueous solution) on a Brandel Cell Harvester. Filters were thoroughly rinsed with ice-cold washing buffer, and filter activity was counted in a gamma counter. The Scatchard plot was drawn and the K_d value was calculated employing the PRISM 2 program (GraphPad Software, San Diego, CA).

Receptor Autoradiography of Demotensin 4 on Human Tumors. The binding affinity of Demotensin 4 for the human NTS1-R expressed in human exocrine ductal pancreatic carcinoma was tested in cryostat sections of resected biopsy samples using [^{125}I-Tyr^3]NT (2000 Ci/mmol, Anawa, Wangen, Switzerland) as radioligand according to a published protocol.[6,9] Briefly, consecutive sample sections were incubated for 1 h at 4 °C with the radioligand in 50 mM Tris/HCl (pH 7.6, 0.2% BSA, 5 mM $MgCl_2$, 10 μM bacitracin) in the presence of increasing concentrations of either NT or Demotensin 4. Incubation was terminated by washing the sections in ice-cold buffer (50 mM Tris/HCl, pH 7.6). Sections were subsequently dried by a stream of cold air and placed over 3H hyperfilms (Amersham, Little Chalfont, Bucks, UK) for exposure in X-ray film cassettes. Autoradiograms were then quantified employing a computer-assisted image processing system using tissue standards for iodinated compounds (Amersham, Little Chalfont, Bucks, UK).

Internalization of [^{99m}Tc]Demotensin 1−4. Confluent cells, of either the HT-29 (for [^{99m}Tc]Demotensin 3 and 4) or the WiDr cell line (for [^{99m}Tc]Demotensin 1−4), were seeded in six-well plates ($1.0-1.5 \times 10^6$ cells per well) where they were incubated for 24 h. For the internalization experiment, cells were rinsed twice with ice-cold internalization buffer containing McCoy's GLUTAMAX-I supplemented by 1% (v/v) FBS, and fresh medium was added (1.2 mL). Approximately 300000 cpm test peptide (∼200 fmol total peptide in 150 μL of PBS/0.5%BSA buffer) was added and the experiment performed according to a published protocol.[31,32,34] Nonspecific internalization was determined by a parallel triplicate series containing 1 μM NT. Discrimination of the surface-bound radioactivity from that internalized was achieved by washing the cells at low pH. Considering that total activity comprises membrane-bound plus internalized activity, the percent internalized activity versus the selected time intervals could be calculated applying the Microsoft Excel program.

Metabolism of [^{99m}Tc]Demotensin 1−4. In Plasma. Blood (∼1 mL) was collected in heparinized polypropylene tubes from the heart of male Swiss albino mice while under ether anesthesia and centrifuged at 5000 rpm at 4 °C. The plasma was collected and incubated fresh with test radiopeptide at 37 °C. Fractions withdrawn at 5, 15, 30, 60, and 120 min were mixed with ethanol in a 2/1 EtOH/aliquot v/v ratio and centrifuged at 35000 rpm for 10 min. Supernatants were filtered through Millex GV filters (0.22 μm) and analyzed by HPLC (System 3). Furthermore, sample aliquots were positioned on ITLC−SG strips which were developed up to 10 cm from the origin with acetone to reveal any trace of $^{99m}TcO_4^-$ (R_f $^{99m}TcO_4^-$ = 1).

In Kidney Homogenates. Kidneys were excised from ether-anesthetized male Swiss albino mice and rapidly rinsed and immersed in ice-cold 50 mM TRIS/0.2 M sucrose buffer pH 7.4. They were subsequently homogenized in an Ultra-Turrax T25 homogenator for 1 min at 4 °C. Test radiopeptide was then incubated in fresh homogenate at 37 °C and the procedure described above ("In Plasma" section) was followed for detection of metabolites or released $^{99m}TcO_4^-$.

In the Urine. To detect metabolites excreted in the urine, each test peptide was injected as a 100 μL bolus (0.5−1 mCi) via the tail vein of male Swiss albino mice. Animals were kept in metabolic cages for 30 min with free access to water and food. They were then sacrificed by cardiac puncture under a mild ether anesthesia, and urine was immediately collected from their bladder with a syringe. This urine was mixed with that collected in the metabolic cages, filtered through a Millex GV filter (0.22 μm), and analyzed by HPLC (System 3) for detection of metabolites and ITLC ("In Plasma" section) for detection of $^{99m}TcO_4^-$ traces.

Biodistribution of [^{99m}Tc]Demotensin 3 and 4. In Human HT-29 Xenograft-Bearing Mice. A ∼150 μL bolus containing a suspension of $1.5-2 \times 10^7$ freshly harvested human HT-29 cells in sterile PBS was subcutaneously injected in the right flank of each female Swiss nu/nu mouse. The animals were kept under aseptic conditions and 2−3 weeks later developed well-palpable tumors at the inoculation site. For biodistribution, a 100 μL bolus containing 4−5 μCi test radiopeptide, [^{99m}Tc]Demotensin 1−4 (corresponding to ∼5 pmol total peptide) in PBS buffer pH 7.4 was injected in the tail vein of mice. Animals were sacrificed in groups of four to five by cardiac puncture under a mild ether anesthesia at 1, 4, and 24 h postinjection (pi). Two additional sets of four animals received intravenously (iv) 350 μg of NT along with tested radiopeptide (blocked animals). These were sacrificed at 1 and 4 h pi. Samples of blood and urine as well as organs of interest were collected, weighed, and measured for radioactivity in a gamma counter. Intestines were not emptied of their contents. Data were calculated as percent injected dose per gram tissue (%ID/g) using standard solutions and applying an in-house software program. Statistical analysis was performed using Student's *t* test.

In Human WiDr Xenograft-Bearing Mice. Female Swiss nu/nu mice were inoculated in the right flank with a ∼150 μL bolus containing a suspension of $1.5-2 \times 10^7$ freshly harvested human WiDr cells in sterile PBS. The animals were kept under aseptic conditions and 2−3 weeks later developed well-palpable tumors at the inoculation site. Biodistribution was conducted as described above selectively for [^{99m}Tc]Demotensin 3 and 4. Animals were sacrificed in groups of four to five by cardiac puncture under a

mild ether anesthesia at 1 h pi. For blockade, an additional set of four animals received 350 μg of NT iv along with test radiopeptide and were sacrificed at 1 h pi.

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