^{99m}Tc-Labeled Vasoactive Intestinal Peptide Receptor Agonist: Functional Studies

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Vasoactive intestinal peptide (VIP) is a naturally occurring 28-amino acid peptide with a wide range of biological activities. Recent reports suggest that VIP receptors are expressed on a variety of malignant tumor cells and that the receptor density is higher than for somatostatin. Our aims were to label VIP with ^{99m}Tc—a generator-produced, inexpensive radionuclide that possesses ideal characteristics for scintigraphic imaging—and to evaluate ^{99m}Tc-VIP for bioactivity and its ability to detect experimental tumors. **Methods:** VIP_{28} was modified at the carboxy terminus by the addition of four amino acids that provided an N_4 configuration for a strong chelation of ^{99m}Tc. To eliminate steric hindrance, 4-aminobutyric acid (Aba) was used as a spacer. VIP_{28} was labeled with ^{125}I, which served as a control. Biological activity of the modified VIP_{28} agonist (TP3654) was examined in vitro using a cell-binding assay and an opossum internal anal sphincter (IAS) smooth muscle relaxivity assay. Tissue distribution studies were performed at 4 and 24 h after injection, and receptor-blocking assays were also performed in nude mice bearing human colorectal cancer LS174T. Blood clearance was examined in normal Sprague-Dawley rats. **Results:** The yield of ^{99m}Tc-TP3654 was quantitative, and the yields of ^{125}I-VIP and ^{125}I-TP3654 were >90%. All in vitro data strongly suggested that the biological activity of ^{99m}Tc-TP3654 agonist was equivalent to that of VIP_{28}. As the time after injection increased, radioactivity in all tissues decreased, except in the receptor-enriched tumor (P = 0.84) and in the lungs (P = 0.78). The tumor uptake (0.23 percentage injected dose per gram of tissue [%ID/g]) was several-fold higher than ^{125}I-VIP (0.06 %ID/g) at 24 h after injection in the similar system. In mice treated with unlabeled VIP or TP3654, the uptake of ^{99m}Tc-TP3654 decreased in all VIP receptor–rich tissues except the kidneys. The blood clearance was biphasic; the α half-time was 5 min and the β half-time was approximately 120 min. **Conclusion:** VIP_{28} was modified and successfully labeled with ^{99m}Tc. The results of all in vitro examinations indicated that the biological activity of TP3654 was equivalent to that of native VIP_{28} and tumor binding was receptor specific.

Key Words: vasoactive intestinal peptide; ^{99m}Tc; imaging; colorectal tumors; hybrid-peptide technique

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A large number of peptides are known to have a high affinity for glycoprotein receptor molecules expressed on malignant cells (*1–6*), stimulated neutrophils (*7–9*) and activated platelets (*10–12*). However, only one neuropeptide—^{111}In[diethylenetriamine pentaacetic acid (DTPA)-D-Phe^1]octreotide, a somatostatin analog—is available commercially. Although successful in diagnostic imaging of a variety of neuroendocrine tumors (*3*), its applications are restrictive, primarily because the tracer it uses is ^{111}In, which is cyclotron produced, expensive and not easily available for immediate use.

The physical characteristics (half-time [$t_{1/2}$] = 6 h, γ = 140 keV, 90%) and the generator availability have rendered ^{99m}Tc as one of the most attractive and indispensable radionuclides in nuclear medicine imaging. Octreotide and another analog of somatostatin, vapreotide, have been labeled with ^{99m}Tc in our laboratory (*13–17*) and evaluated in vitro and in vivo with experimental human tumors. However, Reubi (*4*) recently demonstrated that more human tumors express vasoactive intestinal peptide (VIP) receptors than somatostatin receptors and that, in many cases, VIP receptor density is much higher than somatostatin receptor density.

VIP is a 28-amino acid peptide that was initially isolated from porcine intestine some 25 years ago (*18*). VIP's structure is common in humans, pigs and rats. It is a hydrophobic, basic peptide containing three lysine (15, 20 and 21), two arginine (12 and 14) and two tyrosine (10 and 22) residues. VIP receptors have been identified on the cell membranes of intestinal endothelial cells (*19,20*), lungs (*21,22*) and various tumor cells, including colonic adenocarcinomas (*21–25*), carcinoids (*26*) and pancreatic carcinoma (*27*). It is for these reasons that Virgolini et al. (*26*) labeled VIP (Tyr^{10} and Tyr^{22}) with cyclotron-produced ^{123}I and imaged several human tumors. These data, supported by the work of Reubi and colleagues (*4,28,29*)—who observed that extensive quantity of VIP receptors were localized on 100% of the breast, ovarian, prostate and colon carcinomas, including their metastatic lesions—led us to hypothesize that ^{99m}Tc-labeled VIP may be a useful agent for imaging endocrine tumors in general and colorectal carcinoma in particular.

MATERIALS AND METHODS

In our initial attempts at ^{99m}Tc labeling, VIP was conjugated at the N terminal (His^1) with one of two well-known bifunctional chelating agents (BFCAs): CPTA [[4-(1,4,8,11-tetraazacyclotetra-

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dec-l-yl)methyl]benzoic acid] or MAG_3 [[*N*-[*N*[*N*-(benzylthio)acetyl]-glycyl]glycyl]glycin]. This work, published elsewhere, suffered primarily from two fundamental drawbacks: radiochemical impurities and a loss of biological activity (*30*). These data were consistent with a report that the histidine residue in the number-one position, at which the BFCA conjugation was performed, played an important role in the biological activity of VIP (*31*).

Experience generated from these attempts led us to further modify VIP, as explained later, thus forming the basis of this work.

Synthesis of VIP Receptor Agonist TP3654

In this approach, a new analog to modify VIP at the carboxy terminus of Asn^{28} was designed. We chose 4-aminobutyric acid (Aba) as a spacer and extended the molecule to include Gly-Gly-(D)-Ala-Gly. This tetrapeptide moiety provided an N_4 configuration for the chelation of ^{99m}Tc (*32*).

The peptide was custom synthesized by PeptidoGenic Research and Co., Inc. (Livermore, CA), using Wang resin and ABI 430 synthesizer. The peptide was cleaved from the resin with 90% trifluoroacetic acid (TFA) and was precipitated by the addition of diethylether at –20°C. The product was purified using preparative high-performance liquid chromatography (HPLC; Shimadzu LC-10 AD, Columbia, MD) and a 5-μ C_{18} HAIsil column. Fractions were collected and lyophilized, and the resultant compounds were characterized using a Perkin Sciex APZ ion-spray mass spectrometer (Norwalk, CT). VIP-Aba-Gly-Gly-(D)-Ala-Gly (Fig. 1) had the observed molecular weight of 3654.48 when the theoretically expected molecular weight was 3654.32. This analog is referred to as TP3654. The chemical aspects of this preparation have been described elsewhere (*33*).

Preparation of ^{125}I-VIP and ^{125}I-TP3654

Both VIP and TP3654 contain two tyrosine residues (positions 10 and 22) and facilitate radioiodination, as demonstrated previously (*26*). In brief, 100 μg iodogen in 10 μL $CHCl_3$ was placed in a clean, siliconized, conical glass vial, and chloroform was evaporated by a gentle stream of nitrogen. Then, 10 μg VIP or TP3654 in 10 μL 0.5 mol/L phosphate buffer, pH 7.5, was added, followed by approximately 1 mCi [^{125}I]NaI solution in approximately 100 μL 0.5 mol/L phosphate buffer, pH 7.5. The vial was sealed, and the solution was mixed in a vortex mixer and allowed to react for 30 min at 22°C. The reaction was terminated by adding 500 μg sodium metabisulfite. The product was purified using a Rainin 4.6 mm × 25 cm, C_{18} (5-μ) microbond reverse-phase column (Woburn, MA) connected to a Rainin HPLC equipped with NaI (Tl) radioactivity detector and an ultraviolet monitor. The eluting solvent consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) at a flow rate of 1 mL/min. The mono-iodinated VIP or TP3654 was collected and solvent evaporated, and then the ^{125}I-VIP or ^{125}I-TP3654 was taken up in a suitable volume of 0.1 mol/L tris(hydroxymethyl)aminomethane (Tris) buffer, pH 10, containing 0.1% human serum albumin (HSA) and stored at –80°C. On one occasion, ^{125}I-VIP was purchased from Amersham Life Sciences (Buckinghamshire, UK).

Preparation of ^{99m}Tc-TP3654

To a clean, nitrogen-flushed, 10-mL glass vial was added 50 μg TP3654 in acetate buffer (pH 4.6), 50 μg $SnCl_2 \times H_2O$ in 10 μL 0.005 mol/L HCl and 300 μL 0.1 mol/L trisodium phosphate (pH 12.0). The content was frozen immediately by placing the vial in an acetone dry-ice bath. The vial was then placed in a GeneVac lyophilizer (Sheffield, UK) and was lyophilized for 2 h. The vials were then filled with nitrogen, sealed and stored at –20°C.

To a vial at 22°C, 10–40 mCi ^{99m}Tc in 0.1–0.6 mL 0.9% NaCl was added and mixed using a vortex mixer. The mixture was incubated for 15 min. Then, the pH of the reaction mixture was raised to 6–6.5 by the addition of 1–1.5 mL 0.1 mol/L Na_2HPO_4 solution, pH 5.2. Ascorbic acid (500 μg) was then added as a stabilizing agent. HPLC analysis was performed using a Rainin HPLC with a reverse-phase C_{18} microbond column and with 0.1% TFA in H_2O (solvent A) and 0.1.% TFA in acetonitrile (solvent B). The gradient was such that solvent B was 10% at 0 min and 90% at 28 min. In instant thin-layer chromatography (ITLC-SG; Gelman Sciences, Ann Arbor, MI) using pyridine:acetic acid:water (3:5:1.5) as a mobile phase, colloid remains at Rf 0.0 and ^{99m}Tc-TP3654 migrates at Rf 1.0.

Stability of ^{99m}Tc-TP3654

To measure the in vitro stability of ^{99m}Tc-TP3654, a known quantity of the preparation was incubated at 37°C for 24 h, with 100 molar excess of DTPA, HSA or cysteine. At 0, 5 and 24 h, samples were withdrawn for HPLC analysis and the radioactivity that was associated with the challenging agent and that remained as ^{99m}Tc-TP3654 (Rt = 11.8 min) was measured.

Functional Assay Using Opossum Internal Anal Sphincter Smooth Muscle Tissues

This assay was performed by using the method of Rattan and colleagues (*31,34*) to examine the biological activity of TP3654 and CPTA-VIP. Native VIP_{28} was used as a control. Multiple lines of evidence suggest that VIP is one of the inhibitory neurotransmitters in the gut. The assay is based on the binding of VIP to specific receptors that cause a decrease in the resting tension of the internal anal sphincter (IAS) smooth muscle. This was determined in the presence of increasing concentrations of VIP until maximum fall was reached.

Preparation of Smooth Muscle Strips. Adult opossums (*Didelphis virginiana*) of either sex were anesthetized with pentobarbital (40 mg/kg intraperitoneally) and then were killed. The anal canal was removed and was transferred quickly to oxygenated Krebs' physiological solution of the following composition (in mmol/L): 118.07 NaCl, 4.69 KCl, 2.52 $CaCl_2$, 1.16 $MgSO_4$, 1.01 NaH_2PO_4, 25 $NaHCO_3$ and 11.10 glucose. The large blood vessels and extraneous tissues that contained the external anal sphincter were removed by sharp dissection, and the anal canal was opened and pinned flat with the mucosal side up on a dissecting tray containing oxygenated Krebs' solution. The mucosa was removed by using forceps and fine scissors, and the IAS circular smooth muscle strips (approximately 2 mm wide and 1 cm long) were cut from the lowermost part of the anal canal. Silk suture was tied to both ends of these muscle strips for isometric tension measurements.

Measurement of Isometric Tension. The IAS smooth muscle strips were transferred to temperature-controlled 2-mL muscle baths containing Krebs' solution bubbled continuously with a mixture of 95% O_2 and 5% CO_2.

The lower end of the muscle strip was tied to the bottom of the muscle bath with the tissue holder, and the other end was attached to an isometric force transducer (model FTO3; Grass Instruments Co., Quincy, MA). Isometric tensions of the smooth muscle strips were recorded on a Beckman Dynograph recorder (Beckman Instruments, Schiller Park, IL). Initially, 1 g of tension was applied to the muscle strips, which were then allowed to equilibrate for about 1 h with occasional washings. During this equilibration

period, strips developed steady tension. Only strips that developed steady tension and relaxed in response to electrical field stimulation were used. Both optimal length and base line of the muscle strips were determined, as described previously (*31,34*).

Drug Responses. TP3654 and CPTA-VIP were chosen as test agents and VIP_{28} as a control. The effect of different concentrations of these agents on resting IAS tension was examined using cumulative concentration responses. After a given concentration-response curve was derived, the muscle strips were washed continuously for 45–60 min before testing for the concentration-response curve of another agent. Maximal relaxation (100%) of the smooth muscle strips was determined after completely relaxing the muscle strips with 5 mmol/L ethylenediaminetetraacetic acid (EDTA).

^{125}I-VIP Receptor Displacement Assay with TP3654 and VIP

Colon adenocarcinoma cell line HT-29 was purchased from American Type Culture Collection (ATCC [Manassas, VA]) and maintained in McCoy's 5A medium containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% minimum essential medium (MEM) vitamins (Mediatech, Inc., Herndon, VA) at 5% CO_2 and 37°C.

Confluent cells were collected, washed with 50 mmol/L Tris × HCl buffer (pH 7.5) and then resuspended in 50 mmol/L Tris × HCl buffer (pH 7.5) that contained 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$ and 0.15 mol/L NaCl at 4°C. Approximately 2.5×10^6 viable cells were dispensed, in triplicate, in several siliconized borosilicate test tubes. Cells were then incubated at 4°C for 1 h with 40 pmol/L ^{125}I-VIP (2000 Ci/mmol) in the absence or presence of competitor TP3654 with a known quantity, ranging from 10^{-10} mol/L to 10^{-5} mol/L. Cells were then centrifuged and washed twice with 1 mL 50 mmol/L Tris × HCl buffer (pH 7.5) at 4°C; radioactivity bound to the cells was measured using a Packard 5000 series autogamma counter (Packard Instrument Corp., Meriden, CT). Similar assays were performed in triplicate in which VIP_{28}, instead of TP3654, was used as a competitor. Using the Munsen National Institutes of Health (NIH) ligand-binding program (Bethesda, MD), binding curves were plotted and half-maximal inhibitory concentrations (IC_{50}) were determined.

Tissue Distribution Studies

With ^{99m}Tc-TP3654. The ability of ^{99m}Tc-TP3654 to detect human colorectal carcinoma was examined in nude mice.

Human colorectal cancer cells LS174T (ATCC) were grown in culture and 5×10^6 viable cells were implanted intramuscularly in the right thighs of athymic NCr nude mice that weighed 20–25 g. Tumors were grown to no more than 1 cm in diameter. Each animal received approximately 700 μCi of ^{99m}Tc-TP3654 (1450 Ci/mmol) in 200 μL of saline through a lateral tail vein. Exact activity injected was determined by measuring the syringe, before and after injection, in a calibrated ionization chamber (Capintech, Ramsey, NJ). A suitable standard of ^{99m}Tc was prepared at the time of injection.

At 4 or 24 h later, animals were killed and imaged using a GE STARCAM gamma camera (Milwaukee, WI) equipped with a low-energy parallel-hole collimator and a dedicated computer. Tissues were dissected and weighed, and associated radioactivity was counted (Packard 5000 series gamma counter), along with a standard, in duplicate. Results were calculated as percentage injected dose per gram of tissue (%ID/g) and were analyzed by using Student *t* test.

With ^{125}I-VIP. Approximately 1 μCi ^{125}I-VIP (2000 Ci/mmol) was diluted to 200 μL in 0.05 mol/L phosphate buffer at pH 6.5; 2 μg VIP_{28} was added as a carrier so that the quantity of VIP injected per animal would be the same as for ^{99m}Tc-TP3654. A tissue distribution study was performed in a manner similar to that for ^{99m}Tc-TP3654. These data served as positive controls against which ^{99m}Tc-TP3654 data were compared.

With ^{99m}Tc-G(D)AGG Aba. G(D)AGG-Aba, the chelating moiety and the spacer, was labeled with ^{99m}Tc using the same procedure as for ^{99m}Tc-TP3654. The HPLC retention time for a single-peak ^{99m}Tc-G(D)AGG-Aba was 6.9 min, distinctly different from the 11.8 min required for ^{99m}Tc-TP3654. For animal tissue-distribution studies, a protocol similar to that for ^{99m}Tc-TP3654 was followed. Although G(D)AGG-Aba has considerably smaller amino acid residues than TP3654, these data were important because they helped determine whether any cleaving of ^{99m}Tc-G(D)AGG-Aba had occurred by metabolic interaction with ^{99m}Tc-TP3654.

Receptor Blocking

Unlabeled VIP-28 or TP3654 (50 μg intravenously) was injected into each of the five LS174T tumor-bearing nude mice; ^{99m}Tc-TP3654 (1450 Ci/mmol; 700 μCi intravenously) was administered 30 min later. The amount of VIP_{28} for 25-g mice (50 μg) was chosen randomly but with the knowledge that a larger amount would be toxic and the assumption that a smaller amount would block only a few receptors that would not be significantly effective in decreasing ^{99m}Tc-TP3654 uptake. Mice were killed 24 h later, and percentage injected dose per gram of tissues was determined. Data were compared to 24 h distribution in mice (n = 5) given 700 μCi ^{99m}Tc-TP3654 but not pretreated with unlabeled VIP or TP3654.

Blood Clearance in Rats

Three Sprague-Dawley rats, each weighing approximately 250 g, were injected through a lateral tail vein with 1 mCi ^{99m}Tc-TP3654 and serial blood samples were drawn, in triplicate, through the other lateral tail vein at 1, 5, 10, 15 and 30 min and then at 1, 2, 4, 6, 18 and 24 h. Blood samples were weighed, radioactivity was counted with a standard ^{99m}Tc solution prepared at the time of injection and the percentage injected dose per gram of blood was plotted as a function of time.

RESULTS

The theoretical molecular weight as computed for VIP-Aba-Gly-Gly-(D)-Ala-Gly was 3654.32, whereas the observed molecular weight was 3654.48. In this analog, now called TP3654, no impurities were detected by either HPLC or Sciex APZ ion-spray mass spectrometry. The preparation of ^{99m}Tc-TP3654 and its proposed structure are presented schematically in Figure 1. The yield of ^{99m}Tc-TP3654 was quantitative. The HPLC elution profile (Fig. 2) indicated that, unlike MAG_3-VIP or CPTA-VIP, the ^{99m}Tc-TP3654 preparations were eluted in a single peak (Rt = 11.8 min) and that the radiochemical yield was >99%. The HPLC analysis (free ^{99m}Tc Rt = 3.8 min), also indicated that the product was stable for up to 24 h when it was stored at 22°C and when challenged with 100 molar excess of DTPA, HSA

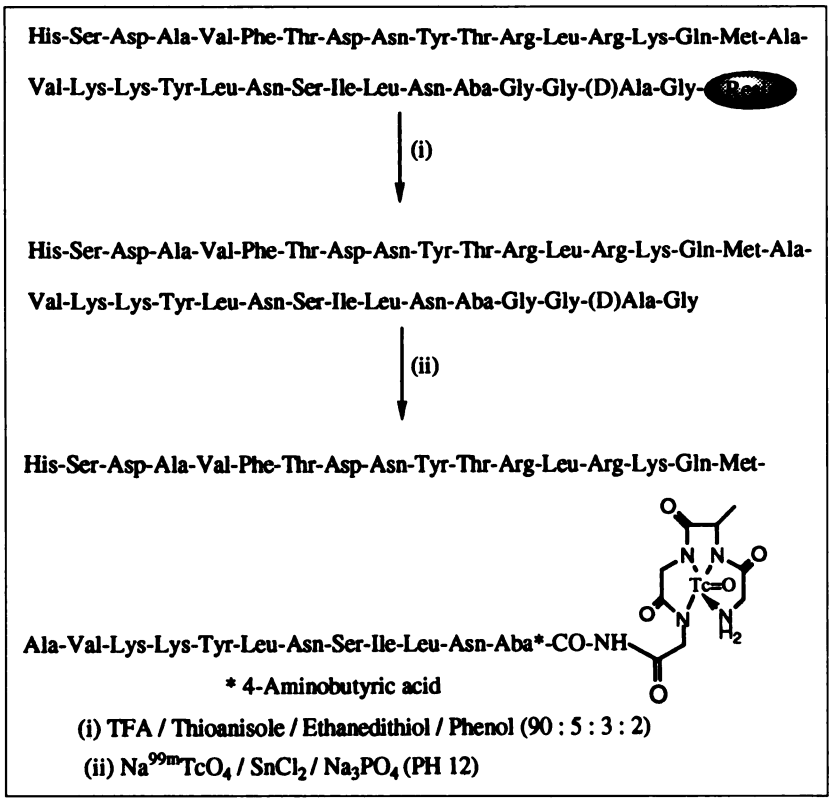

FIGURE 1. Amino acid sequence, preparation and proposed structure of ^{99m}Tc-TP3654.

or cysteine for 5 h at 37°C. However, at 24 h of incubation, approximately 11% of the radioactivity was displaced. Colloid content at all times was <5%.

The yields for ^{125}I-VIP and ^{125}I-TP3654 were approximately 90% and the retention times for mono-iodo and di-iodo products were 19.3 min and 20.1 min, respectively. The specific activity of the mono-iodo product, free of unlabeled peptide, was approximately 1000 Ci/mmol.

The results of IAS smooth muscle tissue assays, which indicated that TP3654 was able to exert 95% relaxivity at 10^{-6} mol/L, are shown in Figure 3. At this concentration, relaxivity by VIP_{28} was 80% and relaxivity by CPTA-VIP was 35% (*33*). These data confirm our previous findings (*30*) that the biological activity of CPTA-VIP was impaired and that the VIP analog TP3654 was as biologically active as VIP_{28}. These data corroborated with the results of our experiments in which TP3654 and VIP_{28} displaced the ^{125}I-VIP binding of receptors expressed on human colorectal carcinoma cells (HT29). These data are given in Figure 4. The IC_{50} values, as determined by the NIH ligand-binding

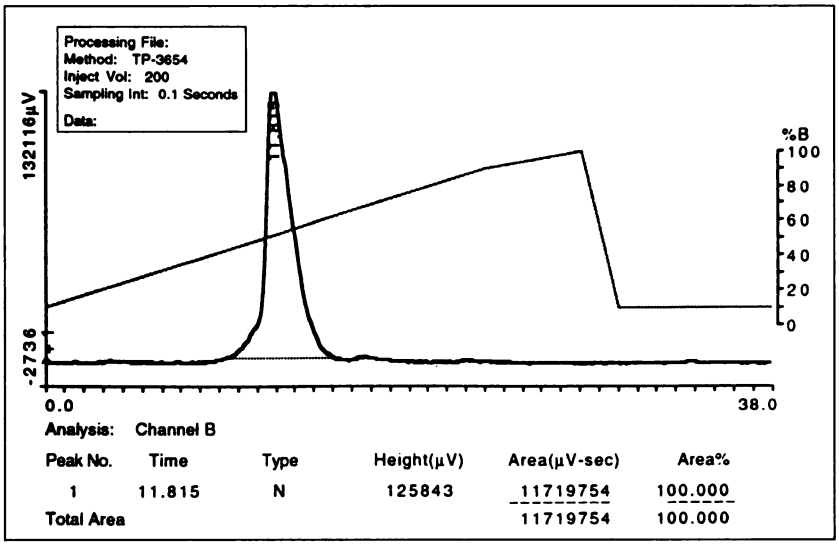

FIGURE 2. HPLC elution profile of ^{99m}Tc-TP3654. One hundred percent radioactivity is eluted in single peak at Rt 11.8 min. This is considerable improvement over multiple peaks with CPTA-VIP and MAG_3-VIP analogs. Diagonal line represents gradient composition.

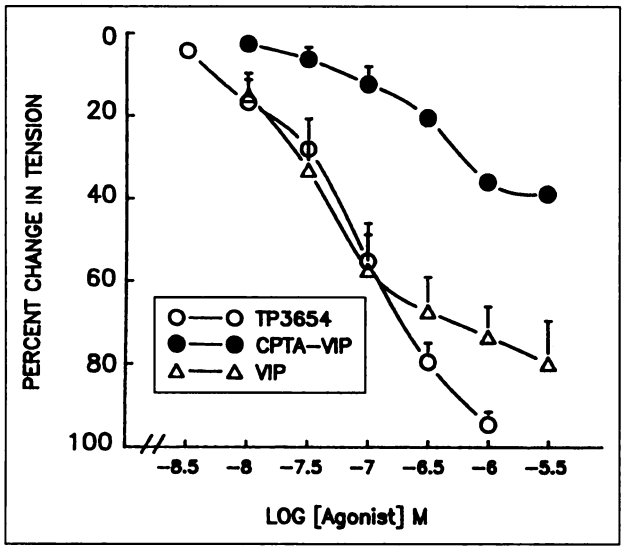

FIGURE 3. Effect of increasing concentration of VIP_{28}, unlabeled TP3654 and CPTA-VIP on resting IAS tension. At 10^{-6} mol/L TP3654, 95% muscle relaxivity was achieved, whereas relaxivity was approximately 75% with VIP_{28} and 35% with CPTA-VIP at same concentration.

program, for both TP3654 and VIP_{28} were approximately 15 nmol/L. These values were consistent with those previously reported for VIP_{28} (*5,31*).

In Vivo Evaluation

In Table 1, the tissue distribution data for 4 and 24 h of ^{99m}Tc-TP3654 and ^{125}I-VIP are given together with the 24 h tissue distribution of ^{99m}Tc-G(D)AGG Aba. The distribution of ^{99m}Tc-G(D)AGG Aba served as a reference and helped to ascertain that the distribution of ^{99m}Tc-TP3654 was distinctly different from that of the ^{99m}Tc-chelating moiety. Data indicate that ^{99m}Tc-TP3654 cleared by renal excretion with 18.99 ± 3.75 %ID/g at 4 h and 3.54 ± 0.4 %ID/g at 24 h. The liver uptake at these time points was 1.12 ± 0.08 %ID/g and 0.33 ± 0.04 %ID/g, respectively, followed by the tumor uptake of 0.24 ± 0.08 %ID/g and 0.23 ± 0.13 %ID/g, respectively. At 24 h after injection, ^{99m}Tc-TP3654 activity declined significantly in all organs ($P < 0.01$) except the VIP receptor–rich tumor and the lungs ($P = 0.84$ and $P = 0.78$, respectively).

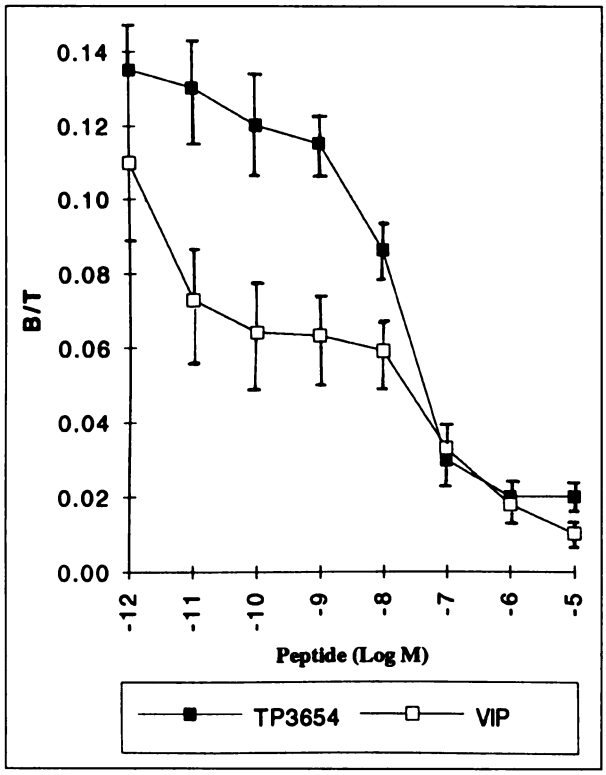

FIGURE 4. ^{125}I-VIP displacement curves with unlabeled TP3654 and VIP_{28}.

Although the tumor uptake was quantitatively low and remained unchanged, the tumor-to-blood and tumor-to-muscle ratios improved significantly ($P < 0.01$) at 24 h after injection. The tumor uptake was nearly four times higher than that for ^{125}I-VIP at 24 h after injection ($P < 0.01$). As the time after injection elapsed, ^{125}I-VIP radioactivity in all tissues, including the tumor and lungs, declined, indicating deiodination from VIP. At 24 h after injection, the ^{99m}Tc-G(D)AGG Aba was significantly lower in all tissues than that of ^{99m}Tc-TP3654 or ^{125}I-VIP.

A composite of posterior gamma-camera images of nude mice bearing human colorectal tumor (LS174T), obtained with ^{99m}Tc-TP3654 and ^{99m}Tc-G(D)AGG-Aba, are given in Figure 5. The image with ^{99m}Tc-TP3654 (left) is distinctly different from the one with ^{99m}Tc-G(D)AGG-Aba (right) and delineates the tumor and both kidneys.

Results given in Table 2 indicate that in mice treated with unlabeled TP3654 or VIP, the uptake of ^{99m}Tc-TP3654 decreased in all VIP receptor–rich tissues except the kidneys. The uptake of ^{99m}Tc-TP3654 in the spleen, liver, muscle and intestine in untreated mice was already low, and even though it was decreased, it was not statistically significantly different after VIP or TP3654 treatment ($P = 0.07$, $P = 0.07$, $P = 0.42$ and $P = 0.48$, respectively). Statistically significant decreases were observed in the heart, lungs, blood and tumor ($P < 0.01$, $P < 0.02$, $P < 0.01$ and $P < 0.01$, respectively). A similar trend was also apparent when mice were treated with unlabeled TP3654. Particularly encouraging was the decreased uptake in the tumor and lungs, which suggested a receptor-blocking phenomenon and indicated that the uptake of ^{99m}Tc-TP3654 was receptor specific. The increased kidney uptake may have been due to increased metabolites or to any pharmacological effects that may have been exerted by 50 μg VIP or TP3654. The blood clearance curve given in Figure 6 was biphasic; the α-$t_{1/2}$ was approximately 5 min and the β-$t_{1/2}$ was approximately 120 min. The α-$t_{1/2}$ of 5 min for ^{99m}Tc-TP3654 obtained in this study was similar to that reported by Bolin et al. (*35*). The results suggest that the TP3654, the VIP_{28} analog, has retained the receptor specificity of VIP_{28}, which does not change when TP3654 is labeled with ^{99m}Tc.

TABLE 1
Biodistribution (%ID/g ± SD) of ^{99m}Tc-TP3654, ^{125}I-VIP and ^{99m}Tc-G(D)AGG-Aba at 4 and 24 h Postinjection in Nude Mice Bearing LS174T Colorectal Tumors (n = 5)

Tissue	^{99m}Tc-TP3654		^{125}I-VIP		^{99m}Tc-G(D)AGG-Aba
	4 h*†	24 h*‡§	4 h†‡	24 h‡	24 h§
Muscle	0.09 ± 0.01	0.04 ± 0.01	3.32 ± 0.54	0.16 ± 0.03	0.01 ± <0.01
Small intestine	0.18 ± 0.05	0.05 ± 0.01	2.12 ± 0.16	0.09 ± 0.01	0.03 ± 0.01
Heart	0.10 ± 0.00	0.06 ± 0.01	1.65 ± 0.07	0.09 ± 0.03	0.01 ± <0.01
Lung	0.17 ± 0.01	0.16 ± 0.09	3.98 ± 2.17	0.19 ± 0.10	0.03 ± <0.01
Blood	0.21 ± 0.02	0.12 ± 0.02	3.40 ± 0.55	0.07 ± 0.01	0.03 ± <0.01
Spleen	0.19 ± 0.05	0.11 ± 0.02	2.13 ± 0.36	0.12 ± 0.05	0.06 ± 0.03
Kidney	18.99 ± 3.75	3.52 ± 0.40	2.46 ± 0.42	0.26 ± 0.03	0.20 ± 0.02
Liver	1.12 ± 0.08	0.33 ± 0.04	1.98 ± 0.29	0.16 ± 0.03	0.16 ± 0.02
Tumor	0.24 ± 0.08	0.23 ± 0.13	2.15 ± 0.36	0.06 ± 0.08	0.03 ± <0.01
T/M ratio	2.73 ± 1.09	6.28 ± 3.09	0.54 ± 0.39	0.38 ± 0.56	1.88 ± 0.36
T/B ratio	1.16 ± 0.29	1.98 ± 1.44	0.65 ± 0.16	0.88 ± 0.16	0.77 ± 0.03

*P values for 4 h and 24 h ^{99m}Tc-TP3654 are ≤0.01 for all tissues except for lung (P = 0.78), tumor (P = 0.84) and T/B ratio (P = 0.25).
†P values for 4 h ^{99m}Tc-TP3654 and 4 h ^{125}I-VIP are ≤0.01 for all tissues.
‡P values for 24 h ^{99m}Tc-TP3654 and 24 h ^{125}I-VIP are ≤0.01 for all tissues except for heart (P = 0.11), lung (P = 0.55), spleen (P = 0.60) and T/B ratio (P = 0.02).
§P values for 24 h ^{99m}Tc-TP3654 and ^{99m}Tc-G(D)AGG-Aba are ≤0.01 for all tissues except for T/B ratio (P = 0.02).
%ID/g = percentage injected dose per gram; T/M = tumor-to-muscle; T/B = tumor-to-blood.

DISCUSSION

^{111}In-[DTPA-D-Phe^1]octreotide has been identified as a useful agent in diagnosis, prognosis and treatment of cancers and has stimulated increasing interest in inherent capabilities of radiolabeled receptor–specific peptides for use in nuclear medicine. Through the original work of Virgolini et al. (*26*) and Reubi et al. (*4,28,29*), another neuropeptide (VIP_{28}) has emerged as a highly potent compound for use in diagnostic imaging. VIP1 and VIP2 receptor subtypes are expressed in higher densities and on more kinds of tumors than somatostatin. These data, together with the excellent physical characteristics of ^{99m}Tc, prompted us to undertake the task of preparing ^{99m}Tc-VIP and evaluating it for functional integrity.

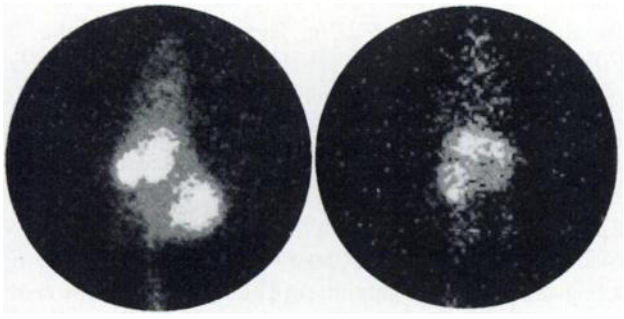

FIGURE 5. Posterior gamma-camera images of nude mice bearing LS174T human colorectal tumor in right thigh. Both images were obtained 24 h after injection of ^{99m}Tc-TP3654 (left) and ^{99m}Tc-G(D)AGG-Aba (right). Tissue distribution is distinctly different. Despite marginal uptake (0.2 %ID/g), tumor in right thigh is delineated with ^{99m}Tc-TP3654 because of low body background. This tumor is not delineated with ^{99m}Tc-G(D)AGG-Aba. Both tumors weighed approximately 1 g.

Our earlier work (30), in which we labeled VIP with ^{99m}Tc by conjugating with BFCAs to His^1 at the amino terminus of VIP, met with difficulty: multiple radioactive species were formed and a significant amount of biological activity was lost. The current approach has eliminated these drawbacks and offers several advantages.

VIP_{28}, modified at Asn^{28} by providing a tetrapeptide for strong chelation of ^{99m}Tc, results in a single radioactive compound with quantitative yield and without compromising on the biological characteristics of VIP_{28}. Using this configuration, we have labeled TP3654 with stable rhenium for nuclear magnetic resonance (NMR) and computer modeling studies. This hybrid peptide approach eliminates the lengthy and frequently inefficacious procedures for synthesizing and conjugating BFCAs, blocking and deblocking certain functional groups and the laborious purifying and characterizing of conjugated peptide. Using the same approach, we have labeled a straight-chain hexapeptide, a straight-chain heptapeptide and a cyclized octapeptide with ^{99m}Tc (*36*). The Aba spacer eliminates any steric hindrance from the chelating moiety and helps preserve the biological activity of the peptide. The chelating moiety can be added to either the carboxy or the amino terminus as needed. Other amino acid combinations to provide the N_4 configuration also can be chosen.

The IC_{50} values for VIP_{28} and TP3654 as determined by data shown in Figure 4 are 15 nmol/L and are consistent with those previously reported for VIP_{28} (*5,31*). Even more encouraging are the results of the functional IAS assay, shown in Figure 3. This assay was based on tissue relaxivity, a true functional characteristic of VIP_{28}, and provides confidence in the use of TP3654.

TABLE 2
Tissue Distribution (%ID/g ± SD) of ^{99m}Tc-TP3654 at 24 h in Nude Mice Bearing Human Colorectal Tumors LS174T and Given Intravenously 50 μg TP3654 or 50 μg VIP, 30 min Before the Administration of ^{99m}Tc-TP3654 (n = 5)

Tissue	^{99m}Tc-TP3654	TP3654	*P* values*	VIP	*P* values†
Muscle	0.04 ± 0.01	0.04 ± 0.02	0.28	0.03 ± <0.01	0.42
Small intestine	0.05 ± 0.01	0.07 ± 0.02	0.13	0.04 ± 0.01	0.48
Heart	0.06 ± 0.01	0.04 ± 0.00	<0.01	0.02 ± <0.01	<0.01
Lung	0.16 ± 0.09	0.09 ± 0.03	0.08	0.05 ± 0.01	0.02
Blood	0.12 ± 0.02	0.04 ± 0.01	<0.01	0.04 ± <0.00	<0.01
Spleen	0.11 ± 0.02	0.16 ± 0.01	<0.01	0.07 ± 0.01	0.07
Kidney	3.52 ± 0.40	5.45 ± 1.48	0.04	12.98 ± 2.11	<0.01
Liver	0.33 ± 0.04	0.52 ± 0.03	<0.01	0.25 ± 0.06	0.07
Tumor	0.23 ± 0.13	0.09 ± 0.02	<0.01	0.07 ± 0.03	<0.01
T/M ratio	6.28 ± 3.09	2.35 ± 1.01	0.02	2.45 ± 2.52	0.01
T/B ratio	1.98 ± 1.44	2.02 ± 0.52	0.05	1.54 ± 0.22	0.21

*P values for 24 h ^{99m}Tc-TP3654 in mice without and with treatment of 50 μg TP3654.
†P values for 24 h ^{99m}Tc-TP3654 in mice without and with treatment of 50 μg VIP.
%ID/g = percentage injected dose per gram; VIP = vasoactive intestinal peptide; T/M = tumor-to-muscle; T/B = tumor-to-blood.

The VIP receptor density for human colorectal cell line LS174T is not precisely known and may vary from tumor to tumor. Nonetheless, tumors were clearly delineated with ^{99m}Tc-TP3654 but not with ^{99m}Tc-G(D)AGG, the chelate. VIP receptors are ubiquitous on normal tissues, although their density may be lower than on the malignant cells. Consistently, a large proportion of ^{99m}Tc-TP3654 was observed in all tissues examined at 4 h after injection (Table 1).

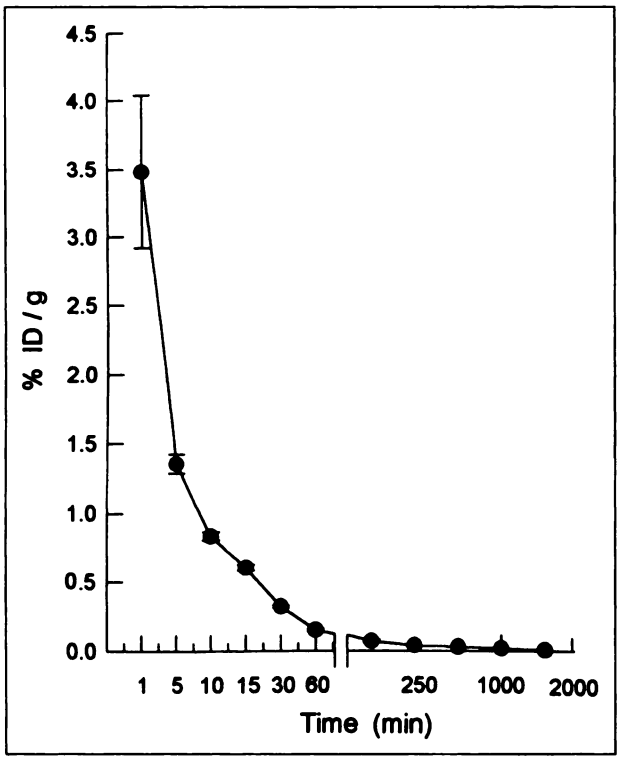

FIGURE 6. Blood clearance curve of ^{99m}Tc-TP3654 in healthy adult Sprague-Dawley rats. Data are presented as percentage injected dose per gram (%ID/g) circulating blood at specified time.

At 24 h after injection, this radioactivity decreased in all tissues except in the lungs (P = 0.78) and in the tumor (P = 0.84). Whether or not the radioactivity in the lungs and tumor was internalized is not yet known. As a result, tumor-to-muscle ratios increased significantly (P = 0.04) as the time after injection was prolonged, whereas tumor-to-blood ratios improved only marginally.

The absolute tumor uptake was 0.23 ± 0.13 %ID/g, which is considerably less than that with radiolabeled monoclonal antibodies. However, it was significantly greater than ^{125}I-VIP (0.06 ± 0.08 %ID/g; P = 0.01). Such low tumor uptakes of other radiolabeled receptor specific peptides have also been observed in mice (0.08 %ID/g) (*17*), in rats (0.04 %ID/g) (*37*) and in humans (0.05 %ID/g) (*38*). Whether or not low tumor uptake of ^{125}I-VIP at 24 h after injection was caused by dehalogenation is not yet clear. In the clinical images (*26*), the lung uptake, although not quantified, was very high. In 25-g mice (Table 1), the lung uptake of ^{99m}Tc-TP3654 at 4 h after injection was 0.17 ± 0.01 %ID/g, whereas in 250-g rats, the lung uptake at the same time averaged 0.73 ± 0.12 %ID/g. These data suggest that the VIP receptor density in lungs varies from species to species; therefore, the animal data may not be a true indication of results in humans. Nevertheless, a systematic evaluation in animals remains a vital stage in development of all radiopharmaceuticals.

Although the lung uptake was the same as the tumor in mice on a per unit weight basis (Table 1), lungs were not delineated by scintigraphic imaging (Fig. 5), probably because of their low weight and wide area spread. The maximum uptake of ^{99m}Tc-TP3654 was in the kidneys. However, this was not contributed by ^{99m}Tc-G(D)AGG-Aba (0.2 ± 0.02 %ID/g; P = 0.01) (Table 1), indicating that the chelating moiety was intact and that the renal uptake may be caused by the uptake of ^{99m}Tc-TP3654 or its metabolic products. The fact that the renal uptake significantly in-

creased ($P < 0.01$) in the receptor-blocking experiment (Table 2) indicates that this uptake may not be receptor specific and may represent nonspecific interaction with TP3654 or VIP metabolites. It may have been compounded by pharmacological effects exerted by the unlabeled VIP and TP3654 injected. The long-chain VIP_{28} may be more susceptible to proteolytic metabolism than smaller peptides. It is possible to block this nonspecific renal uptake by pre- or co-administration of D-lysine, as demonstrated by Lang et al. (*39*) and Kobayashi et al. (*40*). Additional evidence for receptor specificity of ^{99m}Tc-TP3654 was evident in the receptor-blocking experiment, because its uptake in VIP receptor–enriched lungs (*26*) and the tumor decreased significantly.

CONCLUSION

A new hybrid peptide technique has been developed for labeling peptides with ^{99m}Tc. It is applicable to labeling any peptide with ^{99m}Tc—short or long, cyclized or not—and also is applicable to peptide labeling with radionuclides of rhenium. This method is simple and efficient and has several advantages over conventional bifunctional chelating methods.

Using this method, we successfully labeled VIP_{28} with ^{99m}Tc and evaluated the agent in vitro and in nude mice bearing human colorectal carcinoma LS174T. In vitro data indicated that the biological activity of ^{99m}Tc-labeled VIP (^{99m}Tc-TP3654) was equivalent to that of VIP_{28}. The 24-h tumor uptake of ^{99m}Tc-TP3654 was higher than that of ^{125}I-VIP_{28} and could be blocked when mice were pretreated with unlabeled VIP_{28} or TP3654. The blood clearance of ^{99m}Tc-TP3654 was studied in rats and α-$t_{1/2}$ was equal to that of VIP_{28}.

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