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Radiochemical Investigations of Gastrin-releasing Peptide Receptor-specific [^{99m}Tc(X)(CO)$_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH$_2$)] in PC-3, Tumor-bearing, Rodent Models: Syntheses, Radiolabeling, and *in Vitro/in Vivo* Studies where Dpr = 2,3-Diaminopropionic acid and X = H_2O or $P(CH_2OH)_3$[1]

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ABSTRACT

Bombesin (BBN), a 14 amino acid peptide, is an analogue of human gastrin-releasing peptide (GRP) that binds to GRP receptors (GRPrs) with high affinity and specificity. The GRPr is overexpressed on a variety of human cancer cells, including prostate, breast, lung, and pancreatic cancers. The specific aim of this study was to develop ^{99m}Tc(I)-radiolabled BBN analogues that maintain high specificity for the GRPr *in vivo*. A preselected synthetic sequence via solid phase peptide synthesis was designed to produce 2,3-diaminopropionic acid (Dpr)-BBN conjugates with the following general structure: Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH$_2$). The new BBN constructs were purified by reversed phase high-performance liquid chromatography. Electrospray mass spectrometry was used to characterize the nonmetallated BBN conjugates. Re(I)-BBN conjugates were prepared by the reaction of [Re(Br)$_3$(CO)$_3$]$^{2-}$ and Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH$_2$) with gentle heating. Electrospray mass spectrometry was used to determine the molecular constitution of the new Re(I) conjugates. The ^{99m}Tc conjugates were prepared at the tracer level by preconjugation, postlabeling approach from the reaction of [^{99m}Tc(H$_2$O)$_3$(CO)$_3$]$^+$ and corresponding ligand. The ^{99m}Tc and Re(I) conjugates behaved similarly under identical reversed phase high-performance liquid chromatography conditions. Results from *in vitro* and *in vivo* models demonstrated the ability of these derivatives to specifically target GRPrs on human, prostate, cancerous PC-3 cells.

INTRODUCTION

Because of its wide range availability (^{99}Mo/^{99m}Tc generator system), ideal nuclear characteristics [$t_{1/2}$ = 6.04 h, Eγ = 140 keV (89%)], and well-established labeling chemistries, ^{99m}Tc continues to be the most versatile radioisotope in nuclear medicinal applications today. In fact, ^{99m}Tc accounts for >85% of all diagnostic applications performed in medical facilities each year (1). Aside from the traditional approach [*i.e.,* ^{99m}Tc(V) or ^{188}Re(V) labeling via N or S chelating donors] of radiolabeling small molecules and biologically active targets with technetium, a more recently developed "Organometallic" labeling strategy has been investigated (2–22). This effort was pioneered by Jaouen *et al.* (2); however, recent investigations by Alberto *et al.* have led to the development of some remarkable Tc(I) and Re(I) chemistry (3–6). Alberto's group has established the organometallic chemistry of Tc(I) and Re(I) tricarbonyl complexes containing the *fac*-M(CO)$_3$ moiety (3–6). They showed that the *fac*-M(CO)$_3$ moiety can be obtained by direct carbonylation of the permetallate salt by the action of borohydride under atmospheric carbon monoxide pressure (3–6). However, initial investigations during the development of a clinically useful ^{99m}Tc/^{188}Re tricarbonyl radiosynthon for the labeling of even the simplest biomolecules proved futile because of multistep, high-pressure synthetic protocols. With the advent of the new organometallic aquaion [^{99m}Tc(H$_2$O)$_3$(CO)$_3$]$^+$, a new avenue for the successful radiolabeling of bioactive molecules with low-valent ^{99m}Tc/^{188}Re has been developed (3–6). The new [^{99m}Tc(H$_2$O)$_3$(CO)$_3$]$^+$ aquaion has been found to be remarkably stable over a wide range of pH values, presumably because of the low-spin, d^6 electronic configuration of Tc(I). Furthermore, the lability of the three water molecules coordinated to the *fac*-M(CO)$_3$ moiety account for excellent labeling efficiencies with a number of donor groups, including amines, thioethers, phosphines, and thiols (3–6).

The feasibility of using the [^{99m}Tc(H$_2$O)$_3$(CO)$_3$]$^+$ aquaion as a radiosynthon for the successful labeling of bioactive molecules has been reported (6, 22). By simply functionalizing the NH$_2$ terminus of Neurotensin with histidine or (N_α-histidinyl)acetic acid, Alberto *et al.* were able to successfully radiolabel Neurotensin, achieving relatively high specific activity radiocomplexes. Furthermore, biological activity of the peptide was maintained (22).

In recent years, our laboratory has focused significant effort toward the successful radiolabeling of new BBN[3] analogues to be used as diagnostic and/or therapeutic radiopharmaceuticals in nuclear medicine (23–29). BBN is a 14 amino acid peptide with very high affinity for the GRPr. GRP function and *in vivo* distribution have been well established. Furthermore, the GRPr is expressed in the central nervous system and peripheral tissues, such as the pancreas or intestinal tract (30–35). A variety of tumors also expresses the BBN receptor/GRPr, including those of breast, prostate, gastric, colon, pancreatic, and small cell lung cancer (30–35). Therefore, radiolabeled BBN/GRP analogues hold potential to be used as site-directed diagnostic and/or therapeutic targeting motifs. We herein report a new method of radiolabeling the BBN analogue Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH$_2$) via the ^{99m}Tc(I)-precursor, [^{99m}Tc(H$_2$O)$_3$(CO)$_3$]$^+$. The *in vitro* and *in vivo* efficacy of targeting the GRPr on human, PC-3 cancer cells is reported.

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[3] The abbreviations used are: BBN, bombesin; GRP, gastrin-releasing peptide; GRPr, gastrin-releasing peptide receptor; SPPS, solid phase peptide synthetic; HPLC, high-performance liquid chromatographic; p.i., postinjection; ES-MS, electrospray ionization-mass spectrometry; %ID, percentage injected dose; RP-HPLC, reversed phase high-performance liquid chromatographic; SCID, severely compromised immunodeficient.

MATERIALS AND METHODS

^{99m}Tc, in the form of $^{99m}TcO_4^-$, was eluted from a $^{99}Mo/^{99m}Tc$ generator provided by Mallinckrodt Medical, Inc. (St. Louis, MO). SPPS techniques, using standard Fmoc chemistry, were used to make all BBN derivatives. SPPS was carried out using an Applied Biosystems 432A peptide synthesizer. Electrospray mass spectral analyses were performed by Synpep Corp. (Dublin, CA). HPLC analyses of radiolabeled and nonradiolabeled compounds were performed on a Waters 600E system equipped with a JASCO UV 975 tunable absorbance detector, an Eppendorf CH-30 column heater, an in-line EG&G ORTEC NaI solid scintillation detector, and a Hewlett Packard 3395 integrator. HPLC solvents were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification. $[^{99m}Tc(H_2O)_3(CO)_3]^+$ (1) was synthesized in a manner similar to that which is reported in the literature (3, 4). $[Re(Br)_3(CO)_3]^{2-}$ was synthesized in a manner reported previously and was used without further purification (16). All other chemicals were purchased from Aldrich Chemical Co. (St. Louis, MO) and used without further purification.

SPPS

Peptide synthesis was performed on a Perkin-Elmer–Applied Biosystems Model 432A automated peptide synthesizer using traditional Fmoc chemistry. The reaction of the HBTU-activated carboxyl group on the reactant with the NH_2-terminal amino group on the growing peptide, anchored via the COOH terminus to the resin, provided for stepwise amino acid addition. Rink amide MBHA resin (25 μmol) and Fmoc-protected amino acids, with appropriate side-chain protections, and Fmoc-Dpr(Fmoc)-OH were used for SPPS of the nonmetallated BBN conjugate. The preselected synthetic sequence was designed to produce the Dpr-(X)-BBN conjugate with the following general structure: Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2), 2 (Fig. 1). The final product was cleaved by a standard procedure using a cocktail containing thioanisol, water, ethanedithiol, and trifluoracetic acid in a ratio of 2:1:1:36 and precipitated into methyl-t-butyl ether. The crude peptide was purified by HPLC, and the solvents were removed on a SpeedVac concentrator. Typical yields of the crude peptide were 80–85%. ES-MS was used to determine the molecular constitution of the conjugate.

Synthesis of [$Re(H_2O)(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)], 3

To 10 mg of 2 was added excess $[Re(Br)_3(CO)_3]^{2-}$ in aqueous solution. The solution was allowed to heat for 1 h at 80°C with stirring. Quality control of the reaction mixture was determined by RP-HPLC. HPLC peak purification afforded the collection of the product, 3. Evaporation of solvent under reduced pressure afforded compound 3 as a pure white solid. ES-MS was used to determine the molecular constitution of the metallated Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) conjugate.

Radiolabeling of Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2), 4

To 100 μg (6×10^{-8} mol) of 2 was added 1 ml of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ (1). The solution was allowed to incubate at 75°C for 0.5 h. Quality control (radiochemical yield and purity determination) of the product was determined by RP-HPLC. Peak purification of the labeled species was performed by collecting the sample off of the chromatographic system into a solution of 1 mg/ml BSA/0.1 M Na_2HPO_4. All additional analyses were carried out using the HPLC-purified product.

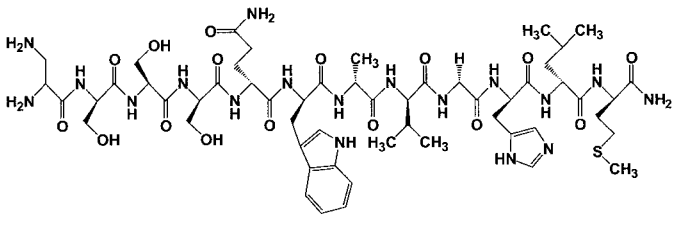

Fig. 1. Structure of Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2), 2.

Synthesis of [$^{99m}Tc(P(CH_2OH)_3)(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)], 5

To a peak collected sample of the radiolabeled conjugate 4 was added 100 μg of *tris*(hydroxymethyl)phosphine $[P(CH_2OH)_3]$ in 100 μl of deionized water. The solution was allowed to incubate at room temperature for 1 h. Quality control of the product was determined by RP-HPLC. Peak purification of the labeled species was performed by collecting the sample off of the chromatographic system into a solution of 1 mg/ml BSA/0.1 M Na_2HPO_4. All additional analyses were carried out using the HPLC-purified product.

HPLC Analysis of Conjugates 2–5

HPLC analysis of each new compound was performed using an analytical C-18 reversed phase column (Phenomenex, 50 × 4.6 mm, 5 μm). The mobile phase consisted of a linear gradient system, with solvent A corresponding to 100% water with 0.1% trifluoroacetic acid and solvent B corresponding to 100% acetonitrile with 0.1% trifluoroacetic acid. The mobile phase started with solvent compositions of 95% A:5% B. At 20 min, the solvent compositions were 20% A:80% B. Solvent compositions of 20% A:80% B were maintained for a period of 5 min, at which point the solvent compositions were changed to 95% A:5% B for column re-equilibration. The flow rate of the mobile phase was 1.5 ml/min. The chart speed of the integrator was 0.5 cm/min.

In Vitro Cell Binding Affinity Studies

***In Vitro* Receptor Binding.** The IC_{50} value of 3 was determined by a competitive displacement cell binding assay using ^{125}I-Tyr^4-BBN as the radiolabel. Briefly, ~3×10^6 PC-3 cells [suspended in D-MEM/F-12K media containing 0.01 M MEM and 2% BSA (pH 5.5)] were incubated at 37°C for 1 h in the presence of 20,000 cpm ^{125}I-Tyr^4-BBN and increasing concentrations of 3. On completion of the incubation, the reaction medium was aspirated, and the cells were washed four times with media. Cell-associated radioactivity was determined by counting in a Packard Riastar gamma counting system.

Internalization and Efflux Analysis. *In vitro* internalization analysis of 4 was carried out by incubation of ~3×10^6 PC-3 cells [in D-MEM/F-12K media containing 0.01 M MEM and 2% BSA (pH 5.5)] in the presence of 20,000 cpm of 4 at 37°C for selected time points of 10, 20, 30, 45, 60, 90, and 120 min. On completion of the incubation, the reaction medium was aspirated, and the cells were washed four times with media. Surface-bound radioactivity was removed by washing the cells with 0.2 N acetic acid/0.5 M NaCl (pH 2.5). The percentage of internalized, cell-associated radioactivity as a function of time was determined by counting in a Packard Riastar gamma counting system. Efflux evaluation was performed after a 40-min internalization period. The cellular medium was washed three times with buffer at room temperature and resuspended for further incubation. Selected sampling at 0-, 20-, 40-, 60-, 90-, 120-, and 150-min postinternalization was performed by an initial cold buffer wash of the cells, followed by washing with acetic acid/saline (pH 2.5 at 4°C).

Biodistribution Analyses of 4 and 5 in Normal, CF-1, Mouse Models

The biodistribution studies of 4 and 5 were determined in normal, CF-1 mice. The mice were injected with 5μCi (185kBq) of the complex in 50 μl of isotonic saline via the tail vein. The mice were euthanized, and the tissues and organs were excised from the animals after 1-, 4-, and 24-h p.i. Subsequently, the tissues and organs were weighed and counted in a NaI well counter, and the %ID and %ID/gram of each organ or tissue were calculated. The %ID in whole blood was estimated assuming a whole-blood volume of 6.5% the total body weight.

Biodistribution Analyses of 4 and 5 in PC-3 Tumor-bearing SCID Mice

The biodistribution studies of 4 and 5 were determined in SCID mice bearing human PC-3 tumors. Four- to 5-week-old female ICR SCID outbred mice were obtained from Taconic (Germantown, NY). The mice were housed five animals per cage in sterile microisolator cages in a temperature- and humidity-controlled room with a 12-h light/12-h dark schedule. The animals were fed autoclaved rodent chow (Rawlston Purina Company, St. Louis, MO) and water *ad libitum*. All animal studies were conducted in accordance with the highest standards of care as outlined in the NIH guide for Care and Use of

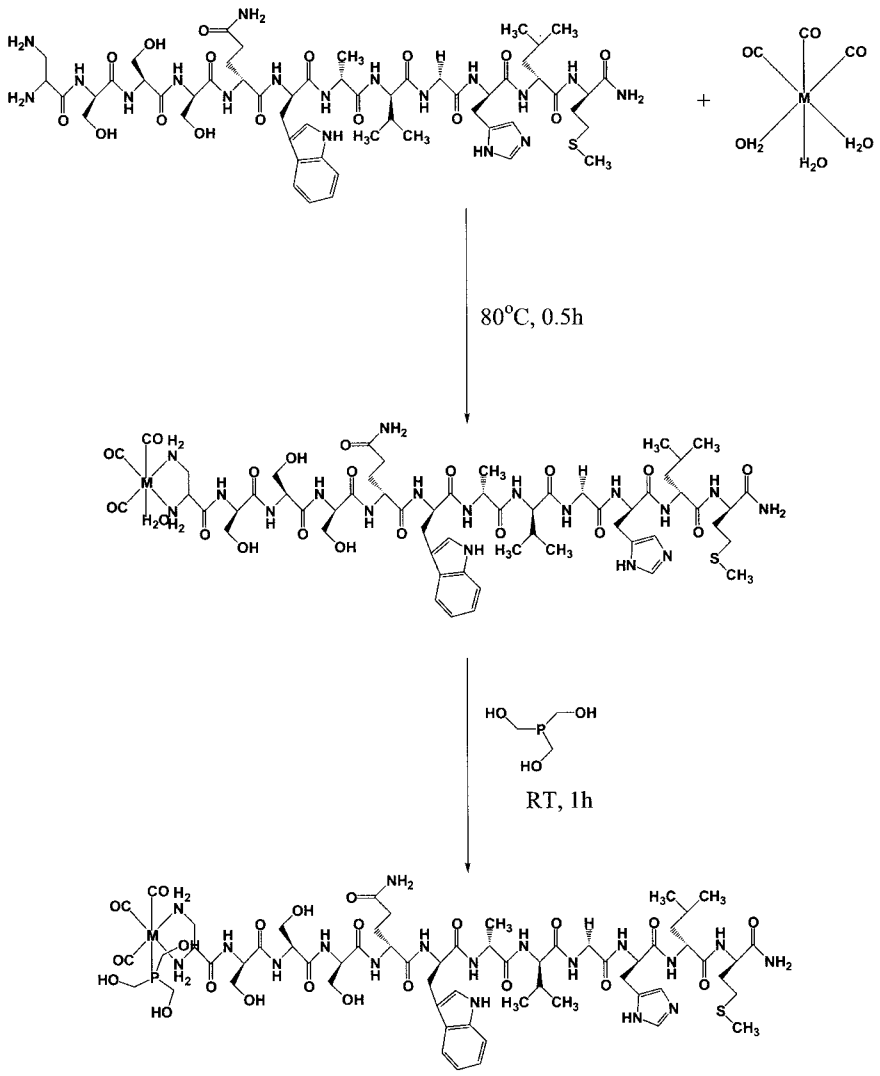

Fig. 2. Radiochemical syntheses of [^{99m}Tc(H_2O)$(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)], 4, and [^{99m}Tc($P(CH_2OH)_3$)$(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)], 5.

Laboratory Animals and the Policy and Procedures for Animal Research at the Harry S. Truman Memorial Veterans' Hospital. Animals were anesthetized for injections with isoflurane (Baxter Healthcare Corp., Deerfield, IL) at a rate of 2.5% with 0.4 liter of oxygen through a nonrebreathing anesthesia vaporizer.

Human prostate PC-3 cells were injected on the bilateral s.c. flank with $\sim 5 \times 10^6$ cells in a suspension of 100 μl of normal sterile saline per injection site. PC-3 cells were allowed to grow *in vivo* 2–3 weeks postinoculation, developing tumors ranging in sizes from 0.02 to 1.3 grams.

The mice were injected with 5 μCi of the ^{99m}Tc conjugates in 100 μl of isotonic saline via the tail vein. The mice were euthanized, and tissues and organs were excised from the animals at 1-, 4-, and 24-h p.i. Subsequently, the tissues and organs were weighed and counted in a NaI well counter, and the %ID and %ID/gram of each organ or tissue were calculated. The %ID in whole blood was estimated assuming a whole-blood volume of 6.5% the total body weight. Receptor-blocking studies were carried out by administration of 100 μg of commercially available BBN in conjunction with the conjugates. The animals were sacrificed at 1-h p.i. The tissues were removed, weighed, and counted as described previously.

RESULTS

The Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) peptide conjugate, 2 (Fig. 1), was conveniently synthesized by SPPS. The yield of the HPLC-purified conjugate was ~80%. ES-MS analysis of the conjugate was consistent with the calculated molecular weight (calculated, 1286.4; experimental, 1287.8).

The ^{99m}Tc(I)-synthon, 1, was prepared by methods similar to those reported previously (Refs. 3 and 4; Fig. 2). The radiosynthon was produced in high yields (≥95%, confirmed by RP-HPLC) on addition of $^{99m}TcO_4^-$ to a pressurized, 10-ml serum vial (1 atm of CO) containing $NaBH_4$ as the reducing agent. The pH of the reaction mixture during the formation of the ^{99m}Tc-precursor 1 was ~10. The radiometallated complex 1 was adjusted to a working pH of ~7.5 using 0.1 N HCl.

The new, metallated BBN conjugate, 3, was prepared by the addition of an aqueous solution of $[Re(Br)_3(CO)_3]^{2-}$ to the Dpr (SSS)-BBN Dpr.Ser-Ser-Ser-Glu-Trp-Ala-Ual-Gly-His-Leu-Met-(NH_2) (714)NH_2 peptide with heating. The conjugate was purified by RP-HPLC. Solvent removal under reduced pressure afforded 3 as a pale white solid. Electrospray mass spectrometry allowed for the determination of the molecular ion of the new nonradioactive Re(I) conjugate (calculated, 1557.8; experimental, 1557.8). No dissociation of the *fac*-Re(I)$(CO)_3$-moiety was observed from the Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) ligand framework, demonstrating the stability of the M(I)-N coordinate bond. The ancillary aquo (H_2O) ligand was not observed in any of the ES-MS analyses. This observation is consistent with coordinating bidentate ligands to low valent Tc(I)/Re(I) metal centers.[4]

[4] R. Schibli, personal communication.

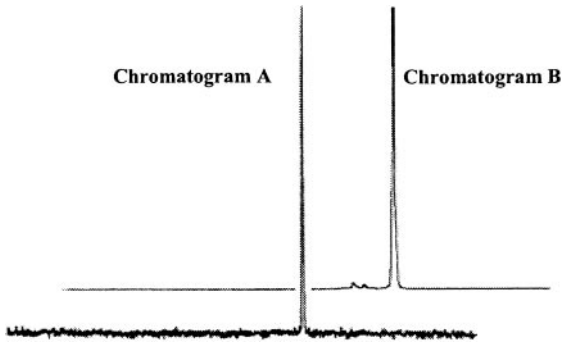

Fig. 3. HPLC elution profile of [^{99m}Tc(H_2O)$(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)], 4 (chromatogram A, t_R = 16.5 min), and [^{99m}Tc($P(CH_2OH)_3$)$(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)], 5 (chromatogram B, t_R = 15.7 min).

Aliphatic diamine ligands have been found to have relatively slow reaction rates with the [^{99m}Tc$(H_2O)_3(CO)_3]^+$ moiety as compared with those bidentate ligand frameworks containing an aromatic amine (7). The ^{99m}Tc-conjugate of the Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) peptide, on the other hand, was produced in high yield on addition of 1 to a vial containing 100 μg ($\sim 6 \times 10^{-8}$ mol) of 2 with heating (Fig. 2). The radiochemical yield of the new ^{99m}Tc conjugate was monitored by RP-HPLC. The HPLC chromatographic profile for the HPLC-purified ^{99m}Tc conjugate of Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) is shown in Fig. 3. The chromatogram shows a single peak (t_R = 16.5 min) corresponding to the new radiometallated conjugate. It can be concluded that the ^{99m}Tc-complex of Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) and nonradioactive Re-complex 3 are chemically similar based on the same respective HPLC retention times. Pertechnetate had a retention time of 3 min under identical HPLC conditions.

Over time, the *trans*-effect of the carbonyl ligand inherently labilizes the coordinating water molecule from the conjugate. In the presence of isotonic saline or dilute HCl, a mixed chlor-aquo species is observed by HPLC at 3-h postpurification (data not shown). The addition of $P(CH_2OH)_3$ to the radioconjugate served to displace either the labile H_2O or Cl^- ligands (Fig. 2), stabilizing the metal center while also increasing the hydrophilicity of the injected radiopharmaceutical. The radiolabeled conjugate, [^{99m}Tc($P(CH_2OH)_3$)$(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)], 5, was prepared by the addition of 100 μg of *tris*(hydroxymethyl)phosphine to an HPLC-purified solution of 4 at room temperature. The HPLC chromatographic profile of the new ^{99m}Tc conjugate is shown in Fig. 3. The chromatogram displays a single peak with a retention time of 15.7 min. This conjugate is stable in aqueous solution for time periods of $\geq$24 h.

The metallated Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) derivative exhibits high affinity binding to PC-3 cells, as demonstrated by competitive displacement assays. The IC_{50} for the metallated conjugate, [Re(H_2O)$(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)], was found to be 0.86 $\pm$ 0.22 nM.

Specific binding of the [^{99m}Tc(H_2O)$(CO)_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)] conjugate to GRPrs expressed on PC-3 cells was demonstrated after incubation (40 min) of 3×10^4 PC-3 cells with high specific activity ^{99m}Tc-analogue. In the absence of the corresponding nonmetallated analogue, $\sim$3–6% of the ^{99m}Tc activity was associated with the PC-3 cells. In contrast, if 10^{-5} M the corresponding unlabeled Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) conjugate or BBN (1–14) is present during the 30-min incubation, $<$0.5% of the ^{99m}Tc activity is cell associated. Fig. 4 summarizes the results of studies to assess the degree of uptake (internalization) of 4 in PC-3 cells. At 90-min postincubation, the amount of internalized activity is 80% of the total activity administered. Fig. 5 summarizes the results of studies to assess the degree of trapping (efflux) of 4 in PC-3 cells. The total ^{99m}Tc activity associated with the cells after the 40-min incubation was measured after washing the cells with the pH 7.4 incubation media. After washing these cells with the pH 2.5 buffer to remove surface bound ^{99m}Tc activity, $\sim$84% remained trapped by the cells (Fig. 5). Results of measurements at 20, 40, 60, 90, 120, and 150 min show that activity remains trapped by the PC-3 cells, with $\sim$46% of the ^{99m}Tc activity associated with the cells at t = 0 remaining residualized at 150 min. Thus, at 150 min, $\sim$55% of the activity remains residualized when normalized to the 84% trapped in the cells at t = 0. The specific trapping mechanism of ^{99m}Tc activity within the PC-3 cells is not fully understood. It is very likely that lysosomal proteases degrade the conjugate into peptide fragments. Those fragments to which ^{99m}Tc remains attached are residulaized within the cell, within the perinuclear space of the lysosome (36). Additional work is needed to identify the structures of these radiometallated fragments to elucidate the specific trapping mechanisms involved (29). The same studies, when performed with ^{125}I-Tyr^4-BBN, show that after a 40-min incubation of PC-3 cells with ^{125}I-Tyr^4-BBN, $\sim$100% of the cell-associated ^{125}I-activity is internalized (29). Furthermore, efflux of radioactivity of ^{125}I-Tyr^4-BBN is comparable with that of the ^{99m}Tc(I) conjugate. Therefore, incorporation of the ^{99m}Tc(I)-chelate onto Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) has little or no effect on the internalization properties of the ^{99m}Tc conjugate in GRPr-specific, PC-3 cells. The binding of these radioligands to PC-3 cells is receptor specific, because addition of 10^{-5} M corresponding unlabeled BBN analogues essentially eliminated the uptake of radioactivity by these cells.

Tables 1 and 2 summarize results of biodistribution studies in normal CF-1 mice at 1-h post-i.v. injection for the new conjugates 4 and 5. Incorporation of the serylserylserine tethering moiety into the

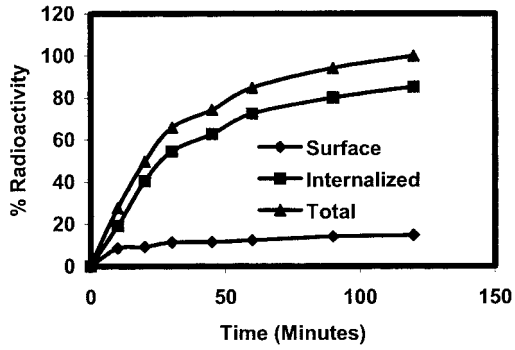

Fig. 4. Internalization of 4 in human prostate (PC-3) cancerous cells.

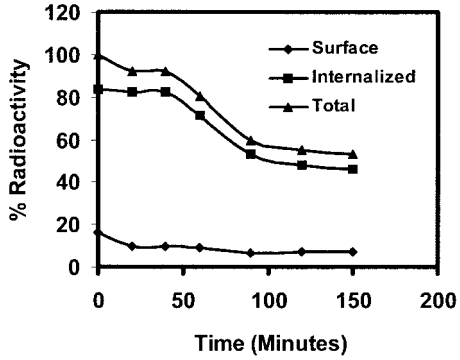

Fig. 5. Efflux of 4 in human prostate (PC-3) cancerous cells.

Table 1 *In vivo biodistribution analyses [%ID/gram (SD), n = 5] of [^{99m}Tc(H_2O)(CO)$_3$-Dpr-(SSS)-BBN[7–14]NH_2] in normal mice models (CF-1)*

Tissue/organ	1 h	4 h	24 h
Blood[a]	1.41 (0.22)	0.75 (0.06)	0.13 (0.08)
Heart	0.57 (0.07)	0.29 (0.04)	0.38 (0.74)
Lung	0.77 (0.14)	0.41 (0.11)	0.16 (0.10)
Liver	2.71 (0.71)	1.34 (0.20)	0.28 (0.02)
Spleen	1.48 (0.38)	0.60 (0.05)	0.04 (0.06)
Stomach	1.28 (0.47)	0.63 (0.10)	0.15 (0.03)
L. intestine	2.79 (0.49)	12.7 (2.14)	0.65 (0.18)
S. intestine	5.81 (2.61)	1.90 (0.26)	0.29 (0.03)
Kidney	6.02 (0.75)	3.32 (0.48)	0.73 (0.07)
Muscle	0.28 (0.14)	0.15 (0.02)	0.03 (0.04)
Pancreas	16.3 (1.38)	7.82 (0.87)	1.14 (0.13)
Urine (%ID)	67.4 (1.78)	72.7 (1.08)	82.2 (1.30)

[a] %ID in blood was estimated assuming the whole-blood volume to be 6.5% of the total body weight.

Table 2 *In vivo biodistribution analyses [%ID/gram (SD), n = 5] of [^{99m}Tc($P(CH_2OH)_3$)(CO)$_3$-Dpr-(SSS)-BBN[7–14]NH_2] in normal mice models (CF-1)*

Tissue/organ	1 h	4 h	24 h
Blood[a]	0.31 (0.10)	0.21 (0.10)	0.05 (0.04)
Heart	0.12 (0.04)	0.33 (0.17)	0.16 (0.18)
Lung	0.29 (0.08)	0.34 (0.13)	0.09 (0.08)
Liver	3.59 (0.69)	2.09 (0.43)	0.20 (0.03)
Spleen	1.26 (0.30)	1.73 (0.42)	0.44 (0.44)
Stomach	0.73 (0.14)	0.55 (0.33)	0.26 (0.17)
L. intestine	3.29 (0.58)	5.30 (1.12)	0.96 (0.22)
S. intestine	3.12 (0.50)	1.68 (0.27)	0.38 (0.04)
Kidney	4.17 (0.41)	1.34 (0.17)	0.26 (0.11)
Muscle	0.04 (0.03)	0.11 (0.13)	0.03 (0.04)
Pancreas	20.5 (4.12)	16.0 (1.61)	5.11 (0.80)
Urine (%ID)	76.2 (2.10)	81.4 (0.60)	85.7 (1.10)

[a] %ID in blood was estimated assuming the whole-blood volume to be 6.5% of the total body weight.

ligand framework (Table 1) and *tris*-hydroxymethylphosphine (Table 2) onto the metal center improved renal-urinary excretion as compared with BBN analogues investigated previously (29). There is no significant uptake or retention in the stomach, indicating that there is minimal, if any, *in vivo* dissociation of ^{99m}Tc from this ligand to produce $^{99m}TcO_4^-$. Pancreatic tissue expresses the GRPr in high density. Therefore, the accumulation of ^{99m}Tc activity in pancreatic tissue reflects the ability of these derivatives to target GRPr-expressing cells *in vivo*. Receptor-mediated uptake of 4 and 5 in normal pancreas was 16.3 ± 1.3 and 20.5 ± 4.12%ID/gram, respectively. Kidney retention for the ^{99m}Tc conjugates 4 and 5 was found to be consistent (*i.e.,* ~5%ID/gram). Blocking studies in which high levels of cold BBN (1–14) was administered 30 min before the ^{99m}Tc-ligands reduced the %ID/gram uptake/retention in the pancreas at 30-min p.i. by a factor of 8–10, demonstrating the *in vivo* specificity of these analogues for GRPr-expressing cells.

The pharmacokinetic properties of 4 and 5 (*i.e.,* blood clearance, excretability, and receptor-mediated pancreatic uptake) in CF-1 normal mice warranted biodistribution studies in tumor-bearing mouse models. Biodistribution studies of this conjugate in tumor-bearing (PC-3) SCID mice (Table 3) showed that it cleared efficiently from the bloodstream within 4 h p.i., *e.g.,* 1.58 ± 0.24%ID remained in whole blood at 4-h p.i. The majority of the radioactivity was excreted via the renal-urinary pathway (*i.e.,* 65.6 ± 4.64% was cleared via the urine at 1-h p.i.). The remainder of the radioactivity was excreted through the hepatobiliary pathway. The degree of receptor-mediated pancreatic uptake at 1-h p.i. was high. However, some efflux of activity was observed at 4- and 24-h p.i., respectively. Tumor uptake in human prostate (PC-3) cells for the new conjugate showed an average uptake of 3.68 ± 0.92%ID/gram at 1-h p.i. Tumor retention at 4- and 24-h p.i. demonstrates GRPr-mediated endocytosis of the agonist *in vivo* and complements *in vitro* analyses in human PC-3 cancerous cells. Biodistribution studies of 5 in tumor-bearing (PC-3) SCID mice showed average tumor uptakes of 2.68 ± 1.3%, 2.58 ± 1.41%, and 1.38 ± 1.05% at 1-, 4-, and 24-h p.i., respectively (Table 4).

DISCUSSION

This study describes an exciting new approach toward the radiolabeling of GRPr-specific bioconjugates via a "nontraditional" organometallic approach that has been recently described (2–22). ^{99m}Tc conjugates 4 and 5 can be prepared in high yield using the preconjugation, postlabeling approach by the reaction of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ with corresponding ligand (37). Recently, we have reported the design and development of ^{99m}Tc-labeled conjugates of BBN based on the structure N_3S-X-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2) [X = 0-Carbons, ω-$NH_2(CH_2)_2COOH$, ω-$NH_2(CH2)_4COOH$, ω-$NH_2(CH_2)_7COOH$, ω-$NH_2(CH_2)_{10}COOH$] (29). ^{99m}Tc conjugates of N_3S-X-BBN(7–14)NH_2 were produced in high yield via the prelabeling, postconjugation and postlabeling, preconjugation approaches using ^{99m}Tc(V)-gluconate as the synthon (29). The ^{99m}Tc-N_3S conjugates were shown to retain high *in vitro* and *in vivo* stability and specifically target GRPr-expressing cells *in vitro* and in CF-1 animal models. Results reported herein, however, suggest the new conjugates 4 and 5 to be superior to the ^{99m}Tc-N_3S conjugates in the same animal model.

The only accessible organ expressing GRPrs is the pancreas, and therefore, notably high pancreatic uptake is observed *versus* all other tissues. However, significant washout from normal pancreas is observed at 24-h p.i. for each of the two conjugates. Pancreatic uptake and retention for 5, however, is ~5%ID/gram even at 24-h p.i. for

Table 3 *($p(CH_2OH)_3$) biodistribution analyses [%ID/gram (SD), n = 5] of [^{99m}Tc(H_2O)(CO)$_3$-Dpr-(SSS)-BBN[7–14]NH_2] in PC-3 tumor-bearing mice models*

Tissue/organ	1 h	4 h	24 h
Blood[a]	2.75 (0.19)	1.58 (0.24)	0.23 (0.13)
Heart	1.00 (0.23)	0.62 (0.31)	0.26 (0.22)
Lung	1.66 (0.27)	0.72 (0.37)	0.12 (0.08)
Liver	3.97 (0.50)	2.03 (0.30)	0.33 (0.04)
Spleen	1.40 (0.39)	0.96 (0.55)	0.70 (0.66)
Stomach	1.91 (0.47)	0.84 (0.14)	0.41 (0.09)
L. intestine	3.42 (0.66)	10.8 (1.05)	0.89 (0.21)
S. intestine	10.2 (0.80)	2.56 (0.45)	0.48 (0.12)
Kidney	8.02 (1.04)	4.50 (0.49)	1.05 (0.28)
Muscle	0.36 (0.10)	0.08 (0.10)	0.13 (0.04)
Pancreas	23.3 (2.13)	12.3 (1.36)	1.56 (0.76)
Tumor	3.68 (0.92)	2.71 (0.78)	1.14 (1.07)
Urine (%ID)	65.6 (4.64)	75.7 (2.27)	84.5 (1.75)

[a] %ID in blood was estimated assuming the whole-blood volume to be 6.5% of the total body weight.

Table 4 *In vivo biodistribution analyses [%ID/gram (SD), n = 5] of [^{99m}Tc($P(CH_2OH)_3$)(CO)$_3$-Dpr-(SSS)-BBN[7–14]NH_2] in PC-3 tumor-bearing mice models*

Tissue/organ	1 h	4 h	24 h
Blood[a]	0.26 (0.32)	0.06 (0.04)	0.15 (0.22)
Heart	0.13 (0.20)	0.05 (0.17)	0.34 (0.33)
Lung	0.32 (0.21)	0.12 (0.15)	0.23 (0.26)
Liver	1.43 (0.18)	1.02 (0.09)	0.12 (0.06)
Spleen	0.66 (0.42)	0.40 (0.23)	0.36 (0.43)
Stomach	2.53 (4.26)	0.41 (0.19)	0.26 (0.16)
L. intestine	2.58 (0.60)	4.56 (0.77)	0.76 (0.17)
S. intestine	3.67 (1.78)	2.26 (1.78)	0.42 (0.23)
Kidney	4.23 (0.43)	1.53 (0.49)	0.37 (0.35)
Muscle	0.41 (0.48)	0.08 (0.03)	0.07 (0.16)
Pancreas	15.7 (2.73)	11.9 (6.70)	5.07 (0.73)
Tumor	2.68 (1.30)	2.58 (1.41)	1.38 (1.05)
Urine (%ID)	80.6 (5.41)	86.3 (1.55)	88.2 (3.61)

[a] %ID in blood was estimated assuming the whole-blood volume to be 6.5% of the total body weight.

reasons not fully understood. Tumor uptake and retention were apparent for each of the new ^{99m}Tc-Dpr conjugates 4 and 5, confirming the agonistic nature of the conjugates (Tables 3 and 4). However, uptake in normal pancreas *versus* tumor is evident and presumably caused by the ability of the conjugates to effectively target the well-vascularized pancreas and GRPrs thereon as compared with the inoculated tumor tissue. It is important to note that recent studies in our laboratory demonstrated successful control of tumors without significant radiotoxicity to the pancreas when targeted with ^{177}Lu/^{90}Y-labeled BBN conjugates (38). Furthermore, receptor density can vary greatly from rodent models to humans, potentially eliminating any radiotoxicity in human patients (39). Retention of ^{99m}Tc activity, even at 24-h p.i., complements *in vitro* studies in PC-3 cells (internalization and efflux) and is presumably caused by the presence of metabolized ^{99m}Tc-peptidic fragments within the lysosome (29, 36). The potential utility of a [^{99m}Tc(CO)$_3$-Nα-histidinyl acetate]-BBN (7–14) construct as a cancer-specific imaging agent was recently demonstrated by LaBella *et al.* (40) in PC-3 tumor-bearing mice. Their studies showed that [^{99m}Tc(CO)$_3$-Nα-histidinyl acetate]-BBN (7–14) localized minimally in tumors, presumably because of weak vascularization of the tumor model (40). These studies have shown that tumor uptake and retention of the new conjugates 4 and 5 are superior to [^{99m}Tc(CO)$_3$-Nα-histidinyl acetate]-BBN (7–14) in xenografted human prostate (PC3) cells in rodent models.

The *trans*-effect of the carbonyl ligand inherently labilizes the coordinating ancillary third ligand (*i.e.,* H_2O or Cl^-) from the bidentate conjugate. Although bidentate ligand frameworks generally are able to sterically protect the metal center from competitive displacement of the third ligand (41), the labile ligand position on the metal center could potentially result in nonspecific serum protein binding *in vivo* (*i.e.,* coordination to free thiols, histidine, or methionine residues). However, there is no evidence of serum-associated activity as indicated from biodistribution analyses of the conjugates in normal and tumor-bearing mice. Furthermore, the reaction with BBN(7–14)NH_2 showed little or no complexation with 1; thus, it may be ascertained that no nonspecific binding is occurring on the histidine or methionine residues of BBN. Biodistribution analyses show that these new, low-valent conjugates clear rapidly from the bloodstream, with little or no radioactivity present at 4-h p.i.

The *in vivo* stability and coordinating ability of the (hydroxymethyl)phosphine (-P($CH_2OH)_2$) functionality, a strong π-acid donor, to the *fac*-M$(CO)_3$ moiety have been well established (14). Therefore, we considered that the coordination of monodentate, *tris*(hydroxymethyl)phosphine (P($CH_2OH)_3$), as a third donor would eliminate potential dissociation or reactions of the metal center in competing environments and serve to increase the hydrophilicity of the conjugate, providing for more suitable pharmacokinetics of the radiopharmaceutical *in vivo*. The use of water-soluble phosphines as coligands at an ancillary position on the Tc/Re metal center has been well established. In fact, Liu and Edwards (42) have used trisulfonated triarylphosphines to stabilize the HYNIC ligand framework *in vitro*/*in vivo*. Introduction of P($CH_2OH)_3$ onto the metal center did not alter the degree of receptor-mediated pancreatic uptake (*i.e.,* pancreas = 20.5 ± 4.12%ID/gram at 1-h p.i., compared with 16.3 ± 1.38%ID/gram for X = H_2O), indicating retention of receptor specificity. Receptor-mediated tumor uptake for this conjugate was lower than that of the corresponding aquo derivative, however. A noticeable increase in the hydrophilicity of the radioconjugate was evident, which could provide an alternative method for tuning the *in vivo* pharmacokinetics of future radiolabeled conjugates.

The results of this study demonstrate that the [^{99m}Tc(X)(CO)$_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)] constructs discussed herein provide for ^{99m}Tc(I)-labeled conjugates that retain high *in vitro* and *in vivo* specificity targeting of GRPr-expressing cells. It was shown that the structures of these conjugates could be varied with little or no compromise of agonistic binding to GRPrs. The potential clinical utility of a [^{99m}Tc-N_3S-5-Ava-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)] construct, designed and developed in our laboratory, as a cancer-specific imaging agent was recently demonstrated by Van de Weile *et al.* (43, 44) in human patients with either prostate or breast cancer. Their studies showed that the N_3S conjugate localizes in tumors with high specificity producing good tumor:normal tissue uptake ratios and high-quality SPECT images (43, 44). Tumor uptake and retention in human prostate (PC-3) cells for the new conjugate [^{99m}Tc(H_2O)(CO)$_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)], 4, is superior to the ^{99m}Tc-N_3S conjugate in the same animal model (45). However, the clinical superiority of this compound over [^{99m}Tc-N_3S-5-Ava-Gln-Trp-Ala-Val-Gly-His-Leu-Met-(NH_2)] has yet to be established. These results further show the versatility of manipulating each the tethering moiety and ancillary third ligand, providing an effective strategy for optimizing pharmacokinetics of the radiolabeled BBN conjugates.

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