Monooxorhenium(V) complexes with 222-N₂S₂ MAMA ligands for bifunctional chelator agents: Syntheses and preliminary in vivo evaluation

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Abstract

Introduction: Targeted radiotherapy using the bifunctional chelate approach with ^{186/188}Re(V) is challenging because of the susceptibility of monooxorhenium(V)-based complexes to oxidize in vivo at high dilution. A monoamine-monoamide dithiol (MAMA)-based bifunctional chelating agent was evaluated with both rhenium and technetium to determine its utility for in vivo applications.

Methods: A 222-MAMA chelator, 222-MAMA(*N*-6-Ahx-OEt) bifunctional chelator, and 222-MAMA(*N*-6-Ahx-BBN(7-14)NH₂) were synthesized, complexed with rhenium, radiolabeled with ^{99m}Tc and ¹⁸⁶Re (carrier added and no carrier added), and evaluated in initial biological distribution studies.

Results: An IC₅₀ value of 2.0 ± 0.7 nM for ^{nat}ReO-222-MAMA(*N*-6-Ahx-BBN(7-14)NH₂) compared to [¹²⁵I]-Tyr⁴-BBN(NH₂) was determined through competitive cell binding assays with PC-3 tumor cells. In vivo evaluation of the no-carrier added ^{99m}Tc-222-N₂S₂(*N*-6-Ahx-BBN(7-14)NH₂) complex showed little gastric uptake and blockable pancreatic uptake in normal mice.

Conclusions: The ¹⁸⁶ReO-222-N₂S₂(*N*-6-Ahx-BBN(7-14)NH₂) complex showed stability in biological media, which indicates that the 222-N₂S₂ chelator is appropriate for chelating ^{186/188}Re

in radiopharmaceuticals involving peptides. Additionally, the in vitro cell studies showed that the $\text{ReO-}^{222}\text{-N}_2\text{S}_2(\text{N-6-Ahx-BBN}(7\text{-}14)\text{NH}_2)$ complex (macroscopically) bound to PC3-tumor cell surface receptors with high affinity. The $^{99\text{m}}\text{Tc}$ analog was stable in vivo and exhibited pancreatic uptake in mice that was blockable, indicating BB2r targeting.

Abbreviated Title: Monooxorhenium(V) $^{222}\text{-N}_2\text{S}_2$ MAMA complexes

Key Words: Rhenium-186, rhenium(V), MAMA ligands, quantum chemical studies, bombesin, radiotherapy

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1. Introduction

Two medically useful rhenium isotopes (^{186}Re and ^{188}Re) are beta emitters with different half-lives, nuclear production routes [1-3], particle energies, and thus tissue ranges (**Table 1**). The nuclear properties [4] of these two Re radioisotopes, both available at no carrier added (*nca*) concentrations, makes them suitable for various radiotherapeutic applications (i.e., ^{186}Re for antibody labeling and smaller tumors and ^{188}Re for peptide labeling and larger tumors) for personalized tumor treatment. Rhenium is the third row transition-metal congener of technetium, which is widely used in $^{99\text{m}}\text{Tc}$ -based diagnostic agents for SPECT (single-photon emission computed tomography) imaging [5-7]. A bifunctional chelating agent conjugated to a targeting vector for in vivo localization that delivers Tc and Re to the tumor site could provide complementary diagnostic and therapeutic agents.

Table 1

A number of N_xS_{4-x} ligand systems have been reported for Re(V) [5,6,8,9], with the N_2S_2 -based monoamine-monoamide dithiol (MAMA) chelators of particular interest since the amine allows for conjugation of a single targeting vector. Kung *et al.* compared the 222-MAMA ligands for chelating oxotechnetium(V) with the bis(aminoethanethiol) (BAT) ligands and showed that the 222-MAMA chelators were more stable over time [10]. The MAMA-based complexes were more hydrophilic than their BAT analogues based on shorter HPLC retention times and lower rat brain uptake [10]. Subsequently, *N*-alkylated 222-MAMA derivatives were complexed with monooxotechnetium(V) [and to a lesser extent with monooxorhenium(V)] and shown to be stable under biological conditions [11-18].

The 222-MAMA-based bifunctional chelating agents were evaluated for forming stable $^{186/188}\text{Re}$ complexes for targeted peptide-based radiotherapy agents, using the seventh through

fourteenth amino acids (BBN(7-14)NH₂) of the bombesin peptide sequence as a potential bombesin receptor (BB2r) targeting vector [19]. The BBN(7-14)NH₂ sequence coupled to a variety of bifunctional chelating agents has been shown to specifically target the BB2r both in vitro and in vivo [6,20-26].

The synthesis, characterization, and radiolabeling of a new ²²²-MAMA derivatized bifunctional chelate for complexing ^{186/188}/_{nat}Re(V) are reported with initial stability studies and in vivo biodistributions.

2. Methods

2.1. General methods

All chemicals, unless otherwise indicated, were commercially available and used without further purification. Technetium-99m pertechnetate was available from saline elution of a ⁹⁹Mo/^{99m}Tc generator (Mallinckrodt Medical, St. Louis, MO). Rhenium-186 was obtained using acid dissolved enriched Al(¹⁸⁵ReO₄)₃ targets (94.6%; Isotech) from ¹⁸⁵Re(n,γ)¹⁸⁶Re production reactions at the University of Missouri Research Reactor (MURR, Columbia, MO) with a specific activity of 3.6-6.4 GBq/mg (0.098-0.172 Ci/mg). No carrier added ¹⁸⁶Re was produced at the Los Alamos National Laboratory Isotope Production Facility (LANL-IPF) using an encapsulated target of enriched ¹⁸⁶WO₃ Powder (99.9%; Isoflex USA) in a 40 MeV incident proton beam. The ¹⁸⁶Re was recovered as previously reported [27,28] with a slight modification: the anion exchange column was eluted with 8 M HNO₃ (2 x 50 mL) for ReO₄⁻ desorption. The nitric acid was removed by evaporation and the residue reconstituted in 0.1 M HCl. The specific activity was determined to be 790 ± 90 GBq/mg (21 ± 2 Ci/mg) [27,28]. PC-3 tumor cells were obtained from ATTC (Manassas, VA) and cultured by the MU Cell and Immunobiology Core (CIC). NMR studies were performed with a Bruker DRX 300 or 500 MHz Spectrometer (as noted). ESI-MS was performed

using a Finnigan TSQ7000 triple-quadrupole mass spectrometer. HPLC analysis was performed with a Shimadzu HPLC system outfitted with both UV-vis (254 and 358 nm) and radioisotope detectors. HPLC columns included a Betabasic-18 column (Thermo Scientific, Waltham, MA, 150 mm $\times$ 4.6 mm, 5 μ m), a Jupiter C-18 column (Phenomenex, Torrance, CA, 250 mm $\times$ 4.60 mm, 5 μ m, 300 Å pore size), and a Nova-Pac semi-prep C-18 column (Waters, Milford, MA, 300 mm $\times$ 19 mm, 6 μ m, 60 Å). All HPLC mobile phases used acetonitrile (AcN) in water with 0.1% trifluoroacetic acid (TFA); the specific conditions (i.e., column, gradient, flow rate) are indicated for purifications and analyses. Sep-Pak C18 Plus Light cartridges were purchased from Waters (Milford, MA). Radio-TLC strips (Saturation pads, Analtech, Newark, DE) were counted using a Bioscan 200 Imaging Scanner (gas ionization detector), or by cutting the TLC strips and counting them in a NaI(Tl) well detector (Harshaw Chemical Company, Serial Number EX-53, with Ortec electronics and a Canberra high-voltage supply) or a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer, Waltham, MA). A Beckman Coulter HPLC system in series with an ion trap mass analyzer (a LCQ FLEET instrument, Thermo Fisher Scientific, with positive ion ionization) was used to obtain LC/ESI-MS results (Betabasic-18, 10-50% AcN over 30 min, 1 mL/min). MS analysis was performed using the XCalibur software (Thermo Fisher Scientific). A Thermo Nicolet AEM FT-IR instrument was used for IR analysis (KBr pellets). UV-Vis analysis was performed with an OceanOptics USB2000 USB-ISS-UV/VIS spectrometer. All elemental analyses were performed by Atlantic Microlabs, Inc (Norcross, GA).

2.2. *Synthesis of compounds and complexes*

Compounds **1** and **2** (**Fig. 1**) were synthesized with trityl protecting groups following a preparation similar to Ono *et al.* and verified by NMR [17].

Figure 1

Ethyl 6-((2-oxo-2-((2-(tritylthio)ethyl)amino)ethyl)(2-(tritylthio)ethyl)amino)hexanoate (3) [trityl protected 222-MAMA(N-6-Ahx-OEt)]. Method 1: In oven-dried glassware under an Ar (g) atmosphere, **2** (1.15 g, 1.7 mmol) was added in 2 mL of anhydrous dimethylformamide (DMF). Anhydrous DMF (13 mL), ethyl-6-bromohexanoate (0.4 mL, 2.2 mmol), and diisopropylethylamine (0.33 mL, 1.9 mmol) were added in series. The reaction mixture was stirred under Ar (g) at 90 °C for 3 h. The product mixture was dried *in vacuo*, dissolved in ethylacetate (EtOAc; 10 mL) and extracted with a saturated NaCl solution (10 mL). The organic layer was dried over anhydrous Na₂SO₄, gravity filtered, rotary evaporated to an oil and placed under vacuum overnight. The product was purified on a silica gel column (1 in. × 6 in.) conditioned with 15% EtOAc in hexanes and eluted with 15% EtOAc in hexanes (100 mL), 30 % EtOAc in hexanes (100 mL), and 50% EtOAc in hexanes (300 mL), sequentially. The product, collected in two brown-colored fractions (eluted during the 30% EtOAc fraction and later in the 50% EtOAc fraction), were individually collected and dried to yield oils, which contained **3** and residual DMF. The two product fractions were combined and subsequently heated to 90 °C under vacuum for 24 h to obtain 0.367 g (0.4 mmol, 26.3% yield) of **3**. ¹H-NMR (500 MHz, CDCl₃, δ (ppm), letters correspond to **Fig. 1**): 1.16-1.36 (m, 7H, *n, h, i*), 1.53 (quintet, *J* = 7.6, 2H, *j*), 2.18-2.28 (m, 6H, *f, g, k*), 2.33-2.41 (m, 4H, *a, e*), 2.83 (s, 2H, *d*), 2.96-3.05 (m, 2H, *b*), 4.07-4.15 (m, 2H, *m*), 7.13-7.32 (m, 18H, *q, s*), 7.34-7.50 (m, 12H, *r*). ¹³C-NMR (500 MHz, CDCl₃, δ (ppm), letters correspond to **Fig. 1**): 14.3 (*n*), 24.7 (*j*), 26.8 (*h & i*), 29.9 (*f*), 32.0 (*a*), 34.2 (*k*), 37.9 (*b*), 53.8 (*g*), 54.6 (*e*), 58.2 (*d*), 60.2 (*m*), 66.7 (*o*), 126.7 (*s*), 127.9 (*q*), 129.5 (*r*), 144.7 (*p*), 171.2 (*c*), 173.5 (*l*). ESI-MS (*m/z*): 821.10 (calc. 821.38 [C₅₂H₅₇N₂O₃S₂⁺][M+H⁺]). Anal. Calcd (Found) for C₅₂H₅₆N₂O₃S₂·2HCl: C, 69.86 (69.88); H, 6.54 (6.84); N, 3.13 (3.22); S, 7.17 (7.49).

Method 2: To a scintillation vial equipped with a stir bar, **2** (1.01 g, 1.49 mmol) was dissolved in dry AcN (2 mL; dried over molecular sieves). Diisopropylethylamine (0.25 g, 1.93 mmol) in AcN (3 mL) was added, followed by slow addition of ethyl-6-bromohexanoate (0.43 g, 4.67 mmol) in 3 mL of AcN. The reaction mixture was placed in an oil bath and refluxed at 85 °C with under N₂ gas, and allowed to react for 24 h. The reaction vessel was brought to room temperature before removal of solvent *in vacuo*. EtOAc (5 mL) was added to the residue, and undissolved particulates were removed by filtration. The volume was reduced by half and 0.5 mL of hexanes was added. The crude product was purified on a silica gel column (10 g, 1.3 cm diameter) that had been pre-conditioned with 15% EtOAc in hexanes. The column was eluted with 30% EtOAc in hexanes (100 mL) followed by 50% EtOAc in hexanes (200 mL). Fractions (5 mL) were collected and analyzed by TLC analysis (silica gel; 50% EtOAc in hexanes. Fractions showing a single species with R_f = 0.4 were combined, and dried under vacuum overnight to obtain 1.08 g (1.32 mmol, 88% yield) of **3**.

6-((2-oxo-2-((2-(tritylthio)ethyl)amino)ethyl)(2-(tritylthio)ethyl)amino)hexanoic acid (4) [trityl protected 222-MAMA(N-6-Ahx-OH)]. **3** (0.14 g, 0.17 mmol) was dissolved in dry tetrahydrofuran (THF; 3 mL) and transferred to the reaction flask. Ethanol (EtOH; 3 mL) and aqueous 3 M NaOH (2 mL) were added. The mixture was refluxed for 9 h. The solvent was removed by rotary evaporation and the product left under vacuum overnight. **4** was dissolved in CHCl₃ and loaded onto a silica gel column (1 in. × 6 in.), eluted with CHCl₃ (200 mL) and, subsequently, 20% methanol (MeOH) in CHCl₃ (100 mL). The brown-colored fraction (obtained from the 20% MeOH eluent) contained 0.10 g (0.13 mmol, 76.5% yield) of **4**. ¹H-NMR (500 MHz, CDCl₃, δ (ppm), letters correspond to **Fig. 1**): 1.18-1.27 (m, 2H, *i*), 1.32 (tt, *J* = 7.4, 7.4 Hz, 2H, *h*), 1.54 (q, *J* = 7.5 Hz, 2H, *j*), 2.20-2.29 (m, 6H, *f*, *g*, *k*), 2.32-2.42 (m, 4H, *e*, *a*), 2.83 (s, 2H, *d*),

3.00 (quartet, $J = 6.3$ Hz, 2H, *b*), 7.14-7.30 (m, 18H, *o*, *q*), 7.35-7.42 (m, 12H, *p*), 7.44 (t, $J = 5.8$ Hz, 1H, *r*). ^{13}C -NMR (500 MHz, CDCl_3 , δ (ppm), letters correspond to **Fig. 1**): 24.5 (*j*), 26.7 (*i*), 26.7(*h*), 30.0 (*f*), 32.0 (*a*), 33.6 (*k*), 37.9 (*b*), 53.8 (*e*), 54.5 (*g*), 58.2 (*d*), 66.7 (*m*), 126.7 (*q*), 127.9 (*o*), 129.5 (*p*), 144.7 (*n*), 171.3 (*c*), 177.5 (*l*). LC-ESI-MS (m/z , 10-50 % AcN over 30 min, 1 mL/min, $R_t = 34.4$ min): 793.8 (calc. 793.35 $[\text{C}_{50}\text{H}_{53}\text{N}_2\text{O}_3\text{S}_2^+]$); ESI-MS (m/z): 791.38 (calc. 791.33 $[\text{C}_{50}\text{H}_{51}\text{N}_2\text{O}_3\text{S}_2^-][\text{M-H}^-]$). Anal. Calcd (Found) for $\text{C}_{50}\text{H}_{52}\text{N}_2\text{O}_3\text{S}_2 \cdot \text{HCl} \cdot \text{H}_2\text{O}$: C, 70.85 (70.86); H, 6.54 (6.30); N, 3.31 (3.34); S, 7.57 (7.53).

*N*¹-((5*S*,8*S*)-11-((1*H*-imidazol-4-yl)methyl)-5-carbamoyl-24-(1*H*-indol-3-yl)-8-isobutyl-20-isopropyl-17-methyl-7,10,13,16,19,22-hexaoxo-2-thia-6,9,12,15,18,21-hexaazatetracosan-23-yl)-2-(6-((2-mercaptoethyl)(2-((2-mercaptoethyl)amino)-2-oxoethyl)amino)hexanamido)pentanediamide (**5**) [222-MAMA(*N*-6-Ahx-BBN(7-14)NH₂)]. The peptide was synthesized in a model 396 multiple peptide synthesizer (AAPTEC, Louisville, KY) on a Sieber resin using standard, solid-phase, Fmoc protection strategy for linear elongation. Fmoc protected amino acids with protected side-chains (where appropriate) were used to synthesize the amino acid sequence -Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂ [BBN(7-14)]. Coupling was achieved by *in situ* activation with HBTU and DIPEA at every elongation step. **4** was added as the final amino acid using these same conditions. The product was cleaved and side chains deprotected with 85% trifluoroacetic acid (TFA) and non-reducing scavengers (phenol/water/triisopropylsilane, 5% each). The synthesis yielded 300 mg of impure **5** (85% pure), which was HPLC purified (20-40% AcN (0.1% TFA) in H₂O (0.1% TFA) over 40 min) resulting in 80 mg of **5** in >98% purity. LC/ESI-MS (m/z , 10-50 % AcN over 30 min, 1 mL/min, $R_t = 15.6$ min): 1230.3 (calc. 1230.59 $[\text{C}_{55}\text{H}_{88}\text{N}_{15}\text{O}_{11}\text{S}_3^+][\text{M}+\text{H}^+]$), 615.6 (calc. 615.80 $[\text{C}_{55}\text{H}_{89}\text{N}_{15}\text{O}_{11}\text{S}_3^{2+}][\text{M}+2\text{H}^{2+}]$).

N-(2-mercaptoethyl)-2-((2-mercaptoethyl)amino)acetamide (**6**) [*deprotected* 222-*MAMA*]. **2** (0.1 g, 0.1 mmol) was deprotected with 0.4 mL of a TFA solution (95% TFA:5% triethylsilane [TES]) at room temperature for 2 h with constant shaking. The solvent was evaporated under N₂ (g), and the resulting solids were extracted with hexanes (6 x 10 mL) to yield an oil (0.03 g, 0.1 mmol). ¹H-NMR (500 MHz, *d*₆-DMSO, δ(ppm), letters correspond to **Fig. 1**): 2.56 (dt, *J* = 7.2, 7.1 Hz, 2H, *a*), 2.73 (dt, *J* = 6.5, 6.5 Hz, 2H, *f*), 2.78-2.88 (m, 1H, *g*), 3.11 (t, *J* = 7.5 Hz, 2H, *e*), 3.30 (dt, *J* = 6.3, 6.4 Hz, 2H, *b*), 3.76 (s, 2H, *d*), 8.61 (s, 1H, *h*). ¹³C-NMR (500 MHz, *d*₆-DMSO, δ (ppm), letters correspond to **Fig. 1**): 19.5 (*f*), 23.3 (*a*), 42.1 (*b*), 47.4 (*d*), 49.4 (*e*), 165.1 (*c*).

ReO(222-*MAMA*(*N*-6-*Ahx-OEt*)) [*ReO*-3]. *Method 1*: **3** (0.9277 g, 1.1 mmol) was deprotected in a TFA solution (95% TFA:5% TES), washed with hexanes (6 × 10 mL), dissolved in MeOH (18 mL), and pH adjusted to 4.5-5 with 1 M NH₄OAc in MeOH using pH strips. ReOCl₃(PPh₃)₂ (0.98 g, 1.2 mmol) was added as a solid and the reaction mixture refluxed at 80 °C for 3.5 h. The product mixture was rotary evaporated to dryness, and dissolved in EtOAc (20 mL) and water (20 mL). The organic layer was extracted with saturated NaCl solution (20 mL), filtered to remove green solids, dried over anhydrous sodium sulfate, and taken to dryness *in vacuo*. The residue dissolved in CH₂Cl₂ was purified on a silica gel column (1 in. × 6 in.), eluted with 100% CH₂Cl₂ (250 mL), 1% MeOH in CH₂Cl₂ (200 mL), 2% MeOH in CH₂Cl₂ (300 mL), 4% MeOH in CH₂Cl₂ (200 mL), 7% MeOH in CH₂Cl₂ (200 mL), 10% MeOH in CH₂Cl₂ (200 mL), and 15% MeOH in CH₂Cl₂ (100 mL), sequentially. Colored bands were collected as separate fractions (i.e., a brown band, a red band, and a second brown band). Each fraction was rotary evaporated to dryness. The first (0.3735 g impure product) and second (0.0680 g impure product) brown bands were each HPLC purified (Betabasic-18, 10-40% AcN over 10 min, followed by 40-43.25% AcN

over 13 min, and followed by 43.25-50% AcN over 2 min, 1 mL/min) in portions to collect the HPLC peaks at 18.5 min and 19.2 min together. The collected peaks were rotary evaporated to dryness, dissolved in CHCl₃, dried over anhydrous Na₂SO₄, and rotary evaporated to dryness. Approximately 25% yield (0.15 g, 0.28 mmol) of solid ReO-**3** was obtained. IR (KBr Pellet): 1653.0 cm⁻¹ (C=O); 969.90 cm⁻¹ (Re=O). ¹H-NMR (500 MHz, CDCl₃, δ (ppm), letters correspond to **Fig. 1**): 1.27 (t, J = 7.0 Hz, 3H, *n*), 1.38-1.48 (quint, J = 7.5 Hz, 2H, *i*), 1.64 (dd, J = 4.5, 12.8 Hz, 1H, *e*), 1.72 (quint, J = 7.6 Hz, 2H, *j*), 1.77-1.92 (m, 2H, *h*), 2.36 (t, J = 7.3 Hz, 2H, *k*), 2.90 (dd, J = 4.5, 13.5 Hz, 1H, *f*), 3.10-3.32 (m, 3H, *a*, *b*, *e*), 3.38 (dt, J = 3.2, 13.3 Hz, 1H, *f*), 3.53 (td, J = 3.7, 12.9 Hz, 1H, *g*), 3.95 (dt, J = 5.9, 12.8 Hz, 1H, *g*), 4.10 (dt, J = 3.3, 13.5 Hz, *a*), 4.15 (q, J = 7.2 Hz, 2H, *m*), 4.19 (d, J = 16.5 Hz, 1H, *d*), 4.54 (dd, J = 5.7, 11.8 Hz, 1H, *b*), 4.70 (d, J = 16.5 Hz, 1H, *d*). ¹³C-NMR (500 MHz, CDCl₃, δ (ppm), letters correspond to **Fig. 1**): 14.2 (*n*), 23.5 (*h*), 24.4 (*j*), 26.3 (*i*), 33.8 (*k*), 39.1 (*f*), 47.7 (*a*), 59.6 (*b*), 60.7 (*m*), 63.6 (*g*), 64.2 (*e*), 67.0 (*d*), 173.5 (*l*), 188.1 (*c*). UV-Vis (ethyl acetate): 358 nm. LC/ESI-MS (m/z ; Betabasic-18, 10-50% AcN over 30 min, 1 mL/min; R_t = 30.1 min): 537.1 (calc. 537.09, [C₁₄H₂₆N₂O₄ReS₂⁺][M+H⁺]). Anal. Calcd (Found) for C₁₄H₂₅N₂O₄ReS₂·0.36CH₂Cl₂: C, 30.46 (30.82); H, 4.58 (4.42); N, 4.95 (4.88); S, 11.32 (10.98).

Method 2: Ammonium perrhenate (0.044 g, 0.16 mmol) and sodium citrate dihydrate (0.060 g, 0.20 mmol) were dissolved in 5 mL of DI water. Stannous tartrate (0.10 g, 0.37 mmol) was added and the reaction mixture was stirred at room temperature for 10 min to yield a blue solution. In a separate vial, **3** (0.050 g, 0.061 mmol) was deprotected in a TFA solution (95% TFA:5% TES), washed with hexanes (6 $\times$ 10 mL), and rotary evaporated to dryness to yield **6**. The rhenium-citrate solution was added to the vial containing **6** and the reaction was refluxed at 85 °C for 4 h. Sodium phosphate (pH 5) was added and the reaction was refluxed for another 4 hr. The

reaction mixture was cooled to room temperature, extracted with EtOAc (2×5 mL), the organic extracts were combined, dried over anhydrous Na_2SO_4 and rotary evaporated to dryness. Approximately 60% yield (0.2 g, 0.37 mmol) of solid ReO-**3** was obtained. HPLC: (Betabasic-18, 10-50 % AcN over 30 min, 1 mL/min) $R_t = 30.2$ min. LC/ESI-MS (m/z; Betabasic-18, 10-50% AcN over 30 min, 1 mL/min; $R_t = 29.4$ min): 537.3 (calc. 537.09, $[\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_4\text{ReS}_2^+][\text{M}+\text{H}^+]$).

ReO(222-MAMA(N-6-Ahx-BBN(7-14)NH₂)) [ReO-5]. Method 1: **5** (14.1 mg, 11.4 μmol) and $\text{ReOCl}_3(\text{PPh}_3)_2$ (12.5 mg, 15.0 μmol) were combined in a 1.5 mL snap cap centrifuge tube, followed by MeOH (400 μL) and 1 M NH_4OAc in MeOH (100 μL). The centrifuge tube was capped and the reaction mixture was heated at 80 °C for 2 h with stirring. The product mixture was centrifuged and the supernatant removed (~ 300 μL). A portion of the sample (50 μL) was removed for LC-ESI-MS. The solids were washed with 200 μL of MeOH, centrifuged, and the supernatant removed four times. The combined supernatants were rotary evaporated to yield 20.7 mg of impure product. The residue was dissolved in 1 mL of MeOH and HPLC purified (Nova-Pac semi-prep, 10-50% AcN over 60 min, 10 mL/min). The peaks from 47 to 50 min (38.5-40% AcN in water with 0.1% TFA) were collected. The product was further HPLC purified (Betabasic-18, 37-50 % AcN over 20 min, 1 mL/min; collected the 5.3 min peak) and the solvents removed *in vacuo* to obtain 2.0 mg of pure ReO-**5** (12 % isolated yield). LC-ESI-MS (m/z; Betabasic-18, 10-50 % AcN over 30 min, 1 mL/min; $R_t = 25.5$ min): 1430.4 (calc. 1430.52 $[\text{C}_{55}\text{H}_{85}\text{N}_{15}\text{O}_{12}\text{ReS}_3^+][\text{M}+\text{H}^+]$); 1452.5 (calc. 1452.5 $[\text{C}_{55}\text{H}_{84}\text{N}_{15}\text{O}_{12}\text{ReS}_3\text{Na}^+]$); 715.8 (calc. 715.8 $[\text{C}_{55}\text{H}_{86}\text{N}_{15}\text{O}_{12}\text{ReS}_3^{2+}][\text{M}+2\text{H}^{2+}]$).

Method 2: Ammonium perrhenate (2.69 mg, 10.04 μmol), sodium citrate dihydrate (5.91 mg, 20.1 μmol) and tin(II) tartrate (8.0 mg, 30.0 μmol) were added to 200 μL of DI water and sonicated for 15 min, or until the light blue color of Re-citrate had formed. In a separate vial, **5**

(9.27 mg, 0.648 μ mol), the Re-citrate solution, and 100 μ L AcN were combined in a 1.5 mL snap cap centrifuge tube, and heated at 70°C for 1 h. The reaction mixture was centrifuged and the supernatant collected. The solids were washed with 50:50 AcN:H₂O (500 μ L), centrifuged, and the second supernatant combined with the first. The combined supernatants were filtered through a 0.45 μ M filter, purified by HPLC (Betabasic-18, 10-50% AcN over 30 min at 1 mL/min), and the 25 min product peak was collected. The product was rotary evaporated to dryness to yield **ReO-5** (10 mg; 90%). LC-ESI-MS (m/z; Betabasic-18, 10-50 % AcN over 30 min, 1 mL/min; R_t = 25.4 min): 1430.4 (calc. 1430.52 [C₅₅H₈₅N₁₅O₁₂ReS₃⁺][M+H⁺]); 1452.5 (calc. 1452.5 [C₅₅H₈₄N₁₅O₁₂ReS₃Na⁺]).

^{99m}TcO(222-MAMA(N-6-Ahx-OEt)) [^{99m}TcO-3]. A GAS solution (56.4 mM sodium glucoheptonate, 0.25 M HCl, 3.7 mM SnCl₂ solution) was prepared. A mixture of **3** in EtOH (0.006 M, 0.3 mL), generator eluent (0.3 mL; 300 MBq (10 mCi)), and GAS (0.3 mL) was vortexed and allowed to react at 70 °C for 40 min. Phosphate buffer (0.9 mL of a 20 mM solution, pH 8-9) was added to the reaction mixture, which was then vortexed, and allowed to react at 70 °C for 30 min. The EtOH was removed from the reaction mixture under a stream of N₂ and the resultant mixture was extracted with EtOAc (3 × 2.0 mL). The organic layer was analyzed by radio-HPLC (Betabasic-18, 10-50% AcN over 30 min, 1 mL/min; R_t = 30.4 min). Radiochemical purity: 82 ± 7%.

^{99m}TcO(222-MAMA(N-6-Ahx-OH)) [^{99m}TcO-4]. The ^{99m}TcO-4 was prepared using the same procedure described for ^{99m}TcO-3, except with 0.3 mL of an EtOAc solution of **4** (4.97 mg in 0.32 mL EtOAc), with 23.2% isolated radiochemical yield. The organic layer was analyzed by radio-HPLC (Betabasic-18, 10-50% AcN over 30 min, 1 mL/min; R_t = 20.2 min).

^{99m}TcO(222-MAMA(N-6-Ahx-BBN(7-14)NH₂)) [^{99m}TcO-5]. A GAS solution (56.4 mM sodium glucoheptonate, 0.25 M HCl, 3.7 mM SnCl₂ solution) was prepared. A mixture of **5** in a 50:50 EtOH:H₂O solution (0.81 mM, 0.1 mL), 0.3 mL of generator [^{99m}Tc]-TcO₄¹⁻ eluent (~0.91 GBq (25 mCi)), and 0.3 mL of the GAS solution was vortexed, reacted in a 70 °C water bath for 40 min. Approximately, 0.3 mL of 20 mM phosphate buffer was added, and the resulting solution was pH 2. The product mixture was purified via a Waters Sep-Pak C18 Plus Light cartridge, which had been pretreated with 10 mL of EtOH followed by 10 ml of H₂O. Three fractions were collected, the load volume, a 1 ml H₂O wash, and a 1 ml EtOH elution. The EtOH fraction, contained ~0.21 GBq (5.7 mCi; 65.1% of the activity loaded). An aliquot of the concentrated ^{99m}TcO-**5** sample (7-8 µCi) was added to a sterile saline and checked for purity (Betabasic-18, 10-50% AcN over 30 min, 1 mL/min, R_t = 21.6 and 24.3 min in a 1:4 ratio). Radiochemical yield: 53 ± 6%, pre-purification; radiochemical purity: 88 ± 3%, post purification

Initial cysteine challenge experiment. The concentrated ^{99m}TcO-**5** product (50 µL, ~ 30 MBq (0.8 mCi) per vial) was added to approximately 450 µL of 1.1 mM cysteine in phosphate buffered saline (PBS) and incubated in a 37 °C water bath. The challenge experiment was performed twice (n = 3 each time), as was the experiment with pertechnetate blanks (using ^{99m}TcO₄¹⁻ generator eluent instead of the ^{99m}TcO-**5** solution). RadioTLC analysis was accomplished using saturation pads that were developed in saline and 50% AcN in water. Pertechnetate moved with the solvent front in both cases, while ^{99m}TcO-**5** remained at the origin in saline and moved with the solvent front in 50% AcN in water. The strips were cut in half and counted using a NaI(Tl) well detector at 0, 1, 4, and 24 h for both ^{99m}TcO₄¹⁻ and ^{99m}TcO-**5**.

Carrier Added ¹⁸⁶ReO(222-MAMA(N-6-Ahx-OEt)) [ca ¹⁸⁶ReO-3]. A GA10S solution (55 mg sodium glucoheptonate, 50 µL HCl, 35 mg SnCl₂·2H₂O, and 3.5 mL H₂O) was prepared. A

mixture of **3** in EtOAc (4.5 mM, 0.3 mL), 10 μ L of a [^{186}Re]- ReO_4^{1-} solution (8.25 MBq (223 μ Ci) of ^{186}Re ; 6.6 GBq/mg Re (0.179 Ci/mg)), and 0.3 mL of GA10S were combined, vortexed, and reacted in a 70 °C water bath for 40 min. Phosphate buffer (0.3 mL of a 20 mM solution, pH 8-9) was added, the solution was vortexed, and the reaction was continued in a 70 °C water bath for 30 min. The product mixture was extracted with EtOAc (2 $\times$ 2 mL) and the layers were counted using the dose calibrator (64.6% extracted yield). The first EtOAc collection vial contained 1.65 mg of gentisic acid (2,5-dihydroxybenzoic acid) to prevent radiolysis of the complex.

*Carrier Added $^{186}\text{ReO}(222\text{-MAMA}(N\text{-6-Ahx-BBN}(7\text{-14})\text{NH}_2))$ [*ca* $^{186}\text{ReO-5}$]. **5*** (0.8 mg, 0.7 μ mol) in 0.3 mL of EtOH and 0.15 mL of a carrier added [^{186}Re]- $\text{Al}(\text{ReO}_4)_3$ solution in water (32.9 MBq (889 μ Ci), 3.6 GBq/mg (0.098 Ci/mg), 0.0091 mg, 0.048 μ mol) were combined with 0.15 mL of deionized water and 0.3 mL of freshly prepared GA10S. The reaction mixture was vortexed, reacted at 75-80 °C in a dry bath for 40 min, and pH adjusted with pH 8-9 phosphate buffer (0.3 mL of a 20 mM phosphate buffer) to approximately 6.5. The product mixture was purified via a pretreated C-18 column (BondElut, Agilent Technologies, 1 in. $\times$ 2 in.) eluted with 15 mL of 50:50 AcN in H_2O . Three fractions were collected with volumes of 4 mL, 6 mL, and 5 mL, respectively. The 6 mL fraction (45% isolated radiochemical yield) was collected with gentisic acid (8 mg), concentrated in a 70 °C water bath with a stream of N_2 (g), and analyzed by radioTLC (0 and 1 h).

*No Carrier Added $^{186}\text{ReO}(222\text{-MAMA}(N\text{-6-Ahx-BBN}(7\text{-14})\text{NH}_2))$ [*nca* $^{186}\text{ReO-5}$]. **5*** (0.2 mg, 0.1 μ mol) in 100 μ L of absolute ethanol, 7.4 MBq (0.2 mCi) of *nca* $^{186}\text{ReO}_4^{1-}$ and 0.1 mL of GAS solution (56.4 mM sodium glucoheptonate, 0.25 M HCl, 3.7 mM SnCl_2 solution) were combined, vortexed and heated in a sand bath at 80°C for 1 h. After removing from heat, 0.1 mL of 20 mM phosphate buffer was added. The reaction mixture was purified on a C-18 column

(BondElut, Agilent Technologies, 0.5 in. $\times$ 0.5 in.) that had been pretreated with 5 mL of 50:50 AcN:H₂O. The reaction mixture was loaded, washed with 3 mL of H₂O, and eluted with 5 mL of 50:50 AcN:H₂O. HPLC (Jupiter C-18 column, at 25 °C with a gradient of 10-70% AcN with 0.1% TFA in water with 0.1% TFA over 15 min) retention time: 13.1 min, analogous to the macroscopic ReO-5 standard, indicated formation of *nca* ¹⁸⁶ReO-5.

Initial stability studies in 0.88 mM cysteine: The *ca* ¹⁸⁶ReO-5 was prepared as described above, evaporated to near dryness and reconstituted in EtOH. An aliquot of the ethanolic solution (40 μ L) was added to each of three tubes containing 160 μ L of 1.1 mM cysteine, which were placed in a 37 °C water bath. Samples were analyzed by HPLC (Betabasic-18, 10-30% AcN over 15 min, followed by 30-50% AcN over 15 min, 1 mL/min) at 0, 1 and 17.4 h. The 17.4 h sample was also analyzed by TLC (paper chromatography, saline and 50% AcN in water eluents) and LSC. A sample prepared without cysteine was used as the standard for comparison.

In vitro cell binding studies with ReO-5 and PC-3 tumor cells. The protocol used was previously reported [24]. Specifically, [¹²⁵I]-Tyr⁴-BBN(NH₂) (PerkinElmer, Waltham, MA) was used as the competitor; cell media [500 mL RPMI 1640 cell media (Invitrogen, Carlsbad, CA) was supplemented with approximately 30 μ M bovine serum albumin (BSA, 1 g, 15 μ mol) and 20.6 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES, 2.46 g, 10.3 mmol), filtered through a 0.22 μ m sterile filter, and pH adjusted to 7.4] was prepared for the study. Approximately 30,000 PC-3 cells were incubated at 37 °C, 95% humidity, and 5% CO₂ for 45 min, with approximately 30,000 cpm [¹²⁵I]-Tyr⁴-BBN(NH₂) and varying concentrations of ReO-5 (prepared by Method 1) in the modified cell growth media. The cells were washed with cold modified media to remove unbound activity. An LTI Multi-Wiper gamma counter (LTI Laboratory Technologies; Elburn, IL) was used to count the activity bound to the cells. The entire process was

performed three times in duplicate. The IC₅₀ value was calculated using GraFit (Erithacus Software, West Sussex, UK).

Pharmacokinetic studies in CF-1 mice. All animal experiments were conducted in accordance with protocols approved by the Harry S. Truman Memorial Veteran's Administration Hospital Subcommittee for Animal Studies. Normal mice (CF-1; 8 weeks old; Charles Rivers Laboratories; Wilmington, MA) were injected via the tail vein with approximately 0.37 MBq (10 μ Ci) of the radiolabeled material in approximately 100 μ L of sterile PBS. The mice were sacrificed at 1 h (eight mice, four with $^{99m}\text{TcO}_4^{1-}$ and four with $^{99m}\text{TcO-5}$) post injection. The organs and tissues were isolated, weighed, counted using a NaI(Tl) well counter, and the percent injected dose (% ID) and percent injected dose per gram (% ID/g) of each were calculated. The entire blood mass was estimated to be 6.5% of the total body weight [26]. All urinary bladder contents excreted from the time of injection to the time of sacrifice were collected, counted, and reported as urine (% ID).

2.3. Computational Methods

All calculations were performed using the Gaussian09 program package [29,30]. ReO-2 and ReO-222-MAMA(*N*-methyl) were fully optimized in the gas and solution phases (SMD chloroform [31]) using the PBE0/6-31G**,LANL2TZ method and basis set as validated in our previous work [32]. (In this notation, the first basis set (6-31G**) was used for the non-metal atoms; the second basis set (LANL2TZ) was used for the metal atom.) All equilibrium structures were verified as minima (no imaginary frequencies). The NMR calculations discussed herein were performed using the GIAO method [29] on the lowest-energy PBE0/6-31G**,LANL2TZ gas- or solution-phase equilibrium structure. The calculated NMR shifts are referenced to the ^{13}C - and ^1H -NMR shifts for tetramethylsilane computed at the same level and in the same phase.

3. Results and Discussion

The 222-MAMA ligand conjugated to BBN(7-14)NH₂ via a six carbon spacer was investigated as a potential bifunctional chelate for ^{186/188}Re(V), the most readily accessible oxidation state of Re from the available ^{186/188}ReO₄¹⁻ [6]. Although the 222-MAMA ligands have been evaluated with ^{99m}Tc, none conjugated to a peptide for chelating ^{186/188}Re have been reported. The overall scheme for preparing the oxorhenium(V) or oxotechnetium(V) complexes with the various 222-MAMA ligands is shown in **Fig.**

Figure 2

3.1. Ligand and bifunctional chelator synthesis

2 was coupled to BBN(7-14)NH₂ through a 6-carbon linker, which was complexed with rhenium for in vitro and technetium for in vivo studies. The target 222-MAMA(N-6-Ahx-BBN(7-14)NH₂) (**5**) was prepared in three steps as shown in **Schemes 1-3** using TFA in the final step to cleave **5** from the resin bead [10,33]. The trityl groups for the ligands (**2** and **3**) were removed using TFA and TES prior to reacting with ReOCl₃(PPh₃)₂ (Method 1) or rhenium-citrate (Method 2), or *in situ* with ^{99m}Tc or ¹⁸⁶Re on the radiotracer scale in the presence of Sn²⁺ and low pH conditions.

The bifunctional chelator (**4**), synthesized from **3**, was conjugated to the BBN(7-14) peptide via traditional solid-phase peptide synthesis utilizing the carboxylic acid derivatized product as the final amino acid-like reactant. **5** was cleaved from the Sieber resin bead. The crude product was 85% pure, and further purified by semi-preparative HPLC to obtain pure **5** (>98% pure).

Scheme 1

Scheme 2

Scheme 3

3.2. Rhenium Complexes

The oxorhenium(V) 222-MAMA complexes with **3** and **5** were synthesized from either $\text{ReOCl}_3(\text{PPh}_3)_2$ or $\text{ReO}(\text{citrate})_2^{1-}$, and exhibit absorbance around 358 nm (λ_{max}). The ^1H -NMR spectra exhibit complex splitting patterns indicating that the backbone protons became unique on coordination to the monooxorhenium(V) core. All chemical shifts are observed downfield on complexation with the exception of the methylene protons on the carbon alpha to the amine (*e* in the free ligands and the complexes, **Fig. 1**), with one of the protons shifted upfield and the other downfield, and identified via 2-D NMR experiments. Gas-phase NMR computations of the *syn* and *anti* isomers of ReO -222-MAMA(*N*-methyl) at the PBE0/6-31G**,LANL2TZ level using the GIAO method with chloroform solvent effects (SMD method) were used to validate the assignment of the upfield shifted proton. The assignment of protons in ReO -**3** is consistent with the ^1H -NMR results published by Katzenellenbogen and co-workers for *syn*- ReO -222-MAMA(*N*-benzyl) [11] and Jones and coworkers for the ReO -222-MAMA(*N*- β -Ala- OCH_2CH_3) complex [12].

The dramatic splitting of the protons on carbon *e* [2.33-2.41 ppm in **3** to 1.64 and 3.10-3.32 ppm in ReO -**3**] is due to the proximity to the $\text{Re}\equiv\text{O}$ bond. The quantum-mechanical NMR computations indicate that the complex isolated from our synthesis is the *syn* isomer. The 1.24 ppm difference in chemical shifts between the *e* protons determined computationally for the *syn* isomer is similar to the 1.6 ppm shift difference observed for ReO -**3** experimentally. Additionally, the downfield shift of peak *g* (**3**) from 2.18-2.28 multiplet to 3.53 ppm triplet of doublets and 3.95 ppm triplet of doublets indicates that the *syn* isomer was isolated [12,13]. The *e* proton shifts from our quantum-mechanical NMR computations for the *anti* isomer show a difference of 0.5-0.7 ppm.

According to our calculations, the *syn* and *anti* isomers are equally thermodynamically favorable, but the transition between the two requires inversion of the rhenium center (through an associative mechanism using water) or deprotonation (basic conditions) of the amine.

All of the ^{13}C -NMR carbon signals are observed downfield on complexation to the monooxorhenium(V) center. Again, the ^{13}C -NMR peak shifts closely match those reported for $\text{ReO-222-MAMA}(N\text{-}\beta\text{-Ala-OCH}_2\text{CH}_3)$ [12].

All Re complexes were characterized by reversed phase HPLC, as standards for the $^{99\text{m}}\text{Tc}$ and *nca* ^{186}Re analogues prepared at the radiotracer level using several methods. **Table 2** lists the retention times of the complexes by the two most commonly used methods.

Table 2

3.3. Radiolabeling of **3**

The radiolabeling of **3** was optimized using $^{99\text{m}}\text{TcO}_4^{1-}$ under a variety of reaction conditions (i.e., deprotected ligand or protected ligand, biphasic or aqueous reaction mixtures, reaction pH (1-9), reaction time (20-40 min), reaction temperature (30-70 °C), and ligand concentration (0.0025-6.7 mM in the total reaction mixture). These studies were monitored by EtOAc extraction (following the extraction method used by Oya *et al.* [10]). The optimal radiolabeling yield was observed with the protected ligand dissolved in EtOH (0.006 M, 0.3 mL), a 40 min reaction time at 70 °C with pH of ~1 (from the GAS solution), a pH adjustment with 20 mM pH 8-9 phosphate buffer, a 30 min heating at 70 °C, and an EtOAc extraction. A radiochemical yield of $85\pm 4\%$ was observed. HPLC analysis of the product mixture (**Error! Reference source not found.**) indicates that the product from the $^{99\text{m}}\text{Tc}$ labeling reaction with **3** (222-MAMA(*N*-hexanoate)) ($R_t = 30.4$ min) is analogous to the non-radioactive ReO-3 HPLC standard ($R_t = 30.2$ min).

Figure 3

The $^{186}\text{ReO-3}$ complex, using *ca* ^{186}Re , was prepared similarly to the $^{99\text{m}}\text{TcO-3}$ complex (by Method 1), utilizing a GAS solution with ten times the $[\text{Sn}^{2+}]$ (GA10S) and gave an extracted yield >60%. The increased Sn^{2+} concentration was required because rhenium is more difficult to reduce than technetium [34] and the $[\text{ReO}_4^{1-}]$ solution was carrier added. EtOAc extractions of $[\text{ReO}_4^{1-}]$ from saline contained 6.24% of the radioactivity. The EtOAc layer from the *ca* $^{186}\text{ReO-3}$ labeling-reaction product mixture extraction contained approximately 5 % $^{186}\text{ReO}_2$ (from TLC analysis). The amount of carrier added $^{186}\text{ReO-3}$ produced was insufficient for HPLC analysis.

3.4. Radiolabeling of **5**

The $^{99\text{m}}\text{Tc}$ labeled 222-MAMA(*N*-6-Ahx-BBN(7-14) NH_2) complex, $^{99\text{m}}\text{TcO-5}$, was prepared as described above with $53 \pm 6\%$ yield. The $^{99\text{m}}\text{TcO-5}$ was purified by Sep-Pak to remove $^{99\text{m}}\text{TcO}_4^{1-}$, $^{99\text{m}}\text{TcO}_2$, and excess **5**. Unreacted ligand **5** was still present according to the HPLC analysis; however, $^{99\text{m}}\text{TcO-5}$ was not further purified. Approximately 30% of the unlabeled **5** (average 0.03 mg) remained in solution following Sep-Pak purification. Radio-HPLC analysis of the purified $^{99\text{m}}\text{TcO-5}$ product mixture showed $88 \pm 3\%$ purity in the combined 21.6 and 24.3 min peaks, with the remainder present as pertechnetate.

When $^{99\text{m}}\text{TcO-5}$ was purified by Sep-Pak, two species were observed at 21.6 min and 24.3 min, presumably the *syn* and *anti* isomers. This isomerization was confirmed by collecting each individual product (peak) separately, reducing the volume, and reinjecting a sample (**Fig. 4**). The species at 21.6 min showed the growth of a peak at 24.3 min, which over time equilibrated to a 1:2 ratio favoring the later eluting species. The 24.3 min species changed very little over time, with <5% in-growth of the 21.6 min species. To verify this isomerization reaction, an aliquot of ReO-5

was dissolved in EtOH and analyzed by HPLC over time. Two species were observed at 22.1 and 24.8 min, with the 24.78 min species dominating (89%). In AcN only the later eluting species is observed. The $^{99m}\text{TcO-5}$ was relatively stable in sterile saline (without added gentisic acid) dropping from $\geq 90\%$ initially to $\geq 75\%$ remaining at 6 h, as confirmed by HPLC.

Figure 4

The *ca* $^{186}\text{ReO-5}$ complex was prepared similarly to *ca* $^{186}\text{ReO-3}$ and isolated by C18 column chromatography, and collected into a vial containing gentisic acid. The isolated fraction (0.988 mCi/mL) contained the majority of the desired product (approximately 66% of the total radioactivity). The isolated *ca* $^{186}\text{ReO-5}$ co-eluted with the *ReO-5* standard at 10.1 min (Jupiter C-18, 30-60% AcN in water without TFA over 15 min) or 20.5 min (Betabasic, 10-30% AcN in water with 0.1% TFA over 15 min and 30-50% AcN in water with 0.1% TFA over 5 min) and was unstable in the absence of gentisic acid. Previous results have shown that complexes containing beta emitters are less prone to radiolysis with the addition of a small amount of gentisic acid [35-38]. To assess the stability of *ca* $^{186}\text{ReO-5}$ in biological media, the complex was incubated with 0.88 mM cysteine at 37 °C in PBS. HPLC analysis showed only $^{186}\text{ReO-5}$ until 17.4 h, at which time TLC analysis indicated about 10-12% $^{186}\text{ReO}_4^{1-}$ and at most 1% $^{186}\text{ReO}_2$ had formed. Thus, after 17.4 h approximately 86-89% of the original $^{186}\text{ReO-5}$ complex remained intact. This amount is approximately the same as the amount of the $^{99m}\text{TcO-5}$ complex intact after 1 h. The difference in stability could be due to the addition of gentisic acid in the purified *ca* $^{186}\text{ReO-5}$ sample or the higher concentration of the *ca* $^{186}\text{ReO-5}$ synthesis. These results are consistent with the stability of $^{186}\text{ReO-MAMA}(N\text{-bisphosphonate})$ derivatives reported by Ogawa et al. [39].

The *nca* $^{186}\text{ReO-5}$ was synthesized similarly to $^{99m}\text{TcO-5}$ and was analyzed by HPLC (Jupiter C-18 column, at 25°C with a gradient of 10-70% AcN in water with 0.1% TFA over 15

min) (**Fig. 5**). Initially, only one peak at 13.1 min was observed, which over time separated into two peaks (likely the *syn* and *anti* isomers) observed at 12.5 and 13.5 min. These results are consistent with those observed for $^{99m}\text{TcO-5}$ and indicate formation of the desired *nca* $^{186}\text{ReO-5}$ product. Two isomers are observed for ReO-5 only when EtOH is present.

Figure 5

3.5. *In vitro* Stability Studies

Initial stability studies of HPLC purified $^{99m}\text{TcO-5}$ complex (without gentisic acid) in 1 mM cysteine at 37 °C showed that approximately 90% remained intact at 1 h and 48% at 24 h (**Fig.**). Oya et al. reported that 70% of the $^{99m}\text{TcO-2}$ complex was intact after incubation in rat serum at 37 °C for 30 min [10]; Yamamura et al. reported that 87.1% of $^{99m}\text{TcO-4}$ remained intact after 1 h in 10 μM cysteine in 0.05 M phosphate buffer (pH 7.0) at 37 °C [40].

Figure 6

3.5. *In vitro* competitive cell binding studies

In vitro competitive cell binding studies in PC-3 prostate cancer cells, known to express GRP (BB2r) receptors, of ReO-5 (prepared by Method 1) against [^{125}I]-Tyr⁴-BBN(NH_2) gave a calculated IC_{50} value of 2.0 ± 0.7 nM ($n = 6$). This value is similar to previous results with other metalated and non-metalated BB2r targeting compounds [21,22]. Additionally, this value is nearly identical to that reported for In-DOTA-5-Ava-BBN(7-14) NH_2 (1.7 ± 0.4 nM) [22] and is similar to the value reported for $\text{ReO-N}_3\text{S-5-Ava-BBN(7-14)NH}_2$ (1.0 ± 0.2 nM) [20].

3.6. *In vivo* biodistribution studies

Biodistribution studies with the $^{99m}\text{TcO-5}$ complex (prepared by Method 2, >95% pure) were performed in normal CF-1 mice, with four mice injected with TcO-5 (0.26-0.40 MBq; 7-8

μCi) and four mice injected with BBN[1-14] as a blocking agent 5 min prior to injection of $^{99\text{m}}\text{TcO-5}$ (0.26-0.40 MBq; 7-8 μCi). The biodistribution data obtained from these studies is tabulated in **Table 3** and shown graphically in **Figs. 7** and **8**. Approximately 2 % ID/g uptake was observed in the pancreas; this lower pancreatic uptake may be due to the presence of unlabeled **5** competing for receptor sites. Thus, HPLC purification may be necessary to increase the specific activity of the tracer, which may improve tumoral uptake in future in vivo studies. The pancreatic uptake does suggest BB2r targeting because the uptake was blocked by injection of BBN[1-14] prior to injection of $^{99\text{m}}\text{TcO-5}$. Additionally, the complex is very hydrophobic, as seen with its long HPLC retention time and its high liver and intestinal uptake.

Table 3

Figure 7

Figure 8

Conclusions and future directions

The $^{222}\text{MAMA}$ -based chelators were synthesized for potential use in radiopharmaceuticals that incorporate $^{186/188}\text{Re}$. The $^{222}\text{MAMA}$ chelator was conjugated to BBN(7-14) NH_2 and analyzed via $^{99\text{m}}\text{Tc}$, non-radioactive Re, and carrier-added and no carrier added ^{186}Re studies. Cell surface receptor binding studies with the ReO-5 complex and in vivo studies with $^{99\text{m}}\text{TcO-5}$ indicate BB2r targeting by these constructs. Thus, these studies indicate that $^{222}\text{MAMA}(N\text{-}6\text{-Ahx-BBN}(7\text{-}14)\text{NH}_2)$ chelates oxorhenium(V) and oxotechnetium(V) for delivery in vivo to BB2r-positive tissues. The bifunctional chelator (**3**) may be useful for conjugation to other biological targeting vectors and may have utility for delivering therapeutic doses of $^{186/188}\text{Re}$ to tumor tissues using peptides and antibodies. The availability of *nca* ^{186}Re gives impetus to develop $^{99\text{m}}\text{Tc}/^{186}\text{Re}$ “matched pair” agents for imaging and treating cancer.

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Figure Captions

Figure 1. **1-6**, and **ReO-3** with lettering scheme used for ^1H - and ^{13}C -NMR.

Figure 2. Overall schematic for rhenium and technetium complex formation (R is H, $(\text{CH}_2)_5\text{COOEt}$, $(\text{CH}_2)_5\text{COOH}$, or $(\text{CH}_2)_5\text{CO-QWAVGHLM-NH}_2$).

Figure 3. HPLC chromatograms (Betabasic, 10-50% AcN over 30 min, 1 mL/min) of A) $^{99\text{m}}\text{TcO-3}$ (radio-HPLC), and B) **ReO-3** (UV-vis, 358 nm). The 21.9 min peak is presumably $^{99\text{m}}\text{TcO-4}$ (from ester hydrolysis during ligand deprotection).

Figure 4. HPLC chromatograms (Betabasic, 10-50% AcN over 30 min, 1 mL/min) of A) $^{99\text{m}}\text{TcO-5}$, B) isolated 21.6 min peak re-injected (shows in-growth of 24.3 min species), and C) isolated 24.3 min peak (shows very little in-growth of 21.3 min species).

Figure 5. HPLC chromatograms (Jupiter C-18, 10-70% AcN over 15 min, 1 mL/min) of A) **ReO-5** and B) *nca* $^{186}\text{ReO-5}$.

Figure 6. $^{99\text{m}}\text{TcO-5}$ stability over 24 h in 1 mM cysteine at 37 °C.

Fig. 7. Biodistributions in percent injected dose (%ID) for $^{99\text{m}}\text{TcO-5}$ (at 1 h) and $^{99\text{m}}\text{TcO-5}$ (at 1 h) with blocking agent administered 5 min prior.

Fig. 8. Biodistributions in percent injected dose per gram (%ID/g) for $^{99\text{m}}\text{TcO-5}$ (at 1 h) and $^{99\text{m}}\text{TcO-5}$ (at 1 h) with blocking agent administered 5 min prior.

Fig. 1. 1-6, and ReO-3 with lettering scheme used for ^1H - and ^{13}C -NMR.

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Fig. 5. HPLC chromatograms (Jupiter C-18, 10-70% AcN over 15 min, 1 mL/min) of A) ReO-5 and B) *nca* ¹⁸⁶ReO-5.

Fig. 6. $^{99m}\text{TcO-5}$ stability over 24 h in 1 mM cysteine at 37 °C.

Fig. 7. Biodistributions in percent injected dose (%ID) for $^{99m}\text{TcO-5}$ (at 1 h) and $^{99m}\text{TcO-5}$ (at 1 h) with blocking agent administered 5 min prior.

Fig. 8. Biodistributions in percent injected dose per gram (%ID/g) for $^{99m}\text{TcO-5}$ (at 1 h) and $^{99m}\text{TcO-5}$ (at 1 h) with blocking agent administered 5 min prior.

Schemes

Scheme 1. Two-step preparation of target trityl-protected MAMA ligands.¹³

Scheme 2. Attaching linking group to chelator and subsequent saponification to obtain the bifunctional chelator.

Scheme 3. Solid-phase peptide synthesis (SPPS) and cleavage to give the bifunctional chelator conjugated to the targeting peptide.

Tables

Table 1. Nuclear properties of $^{186/188}\text{Re}$ [4].

Nuclide	$t_{1/2}$	$E_{\beta^- \text{ max}}$	γ decay energy (% abundance)	Range $\beta^- \text{ max}$ (H_2O)	Production routes [1-3]
^{186}Re	3.7 d	1.1 MeV	137 keV (8%)	4.8 mm	$^{185}\text{Re}(n,\gamma)^{186}\text{Re}$ $^{186}\text{W}(p,n)^{186}\text{Re}$ $^{186}\text{W}(d,2n)^{186}\text{Re}$
^{188}Re	17 h	2.1 MeV	155 keV (15%)	10.4 mm	$^{186}\text{W}(2n,\gamma)^{188}\text{W} \rightarrow \beta^- + ^{188}\text{Re} + \bar{\nu}$ $^{187}\text{Re}(n,\gamma)^{188}\text{Re}$

Table 2. HPLC retention times of compounds.

Compound	Betabasic-18, 10-50% AcN with 0.1% TFA over 30 min, 1 mL/min	Jupitor-18, 10-70% AcN with 0.1% TFA over 15 min, 1 mL/min
ReO-3 (method 1)	30.1 min	
ReO-3 (method 2)	30.2 min	
$^{99\text{m}}\text{TcO-3}$	30.4 min	
$^{99\text{m}}\text{TcO-4}$	20.2 min	
5	15.6 min	
ReO-5 (method 1)	25.5 min	
ReO-5 (method 2)	25.4 min	13.2 min
$^{99\text{m}}\text{TcO-5}$ (method 2)	24.3 min	
<i>nca</i> $^{186}\text{ReO-5}$		13.1 min

Table 3. Biodistribution in CF-1 normal mice for $^{99m}\text{TcO-5}$ and blocking study at 1 h post injection.

	%ID (n=4)		%ID/g (n=4)	
	$^{99m}\text{TcO-5}$	Blocked	$^{99m}\text{TcO-5}$	Blocked
Heart	0.02±0.02	0.05±0.07	0.11±0.16	0.40±0.51
Lung	0.13±0.16	0.15±0.07	0.55±0.69	0.80±0.41
Liver	20.61±4.47	12.47±4.27	10.38±1.88	6.51±1.98
Kidneys	2.00±0.23	1.60±0.39	4.24±0.60	3.57±0.89
Spleen	0.15±0.20	0.13±0.15	1.07±1.48	0.86±1.10
Stomach	1.64±0.71	1.44±0.33	3.60±2.15	1.87±0.29
S. Intestine	27.40±9.51	59.19±3.14	15.46±6.08	29.72±2.22
L. Intestine	23.20±10.47	0.39±0.05	18.04±8.25	0.37±0.11
Muscle	0.02±0.03	0.10±0.08	0.06±0.12	0.54±0.40
Bone	0.02±0.04	0.11±0.15	0.32±0.64	1.52±2.17
Brain	0.01±0.02	0.03±0.03	0.02±0.04	0.06±0.06
Pancreas	0.72±0.03	0.09±0.06	2.03±0.42	0.24±0.17
Bladder	2.46±1.12	1.40±0.16	0.05±0.02	0.3±0.06
Blood	0.83±0.23	1.09±2.80	0.38±0.09	0.49±0.52
Carcass	5.50±0.53	4.57±0.49	0.24±0.04	0.21±0.03
Urine	-	-	12.95±8.19	18.34±2.22
Cage Paper	-	-	4.76±9.25	0.23±0.42