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Isothiocyanate-Functionalized Bifunctional Chelates and *fac*-[M^I(CO)₃]⁺ (M = Re, ^{99m}Tc) Complexes for Targeting uPAR in Prostate Cancer

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Abstract

Developing new strategies to rapidly incorporate the *fac*-[M^I(CO)₃]⁺ (M = Re, ^{99m}Tc) core into biological targeting vectors in radiopharmaceuticals continues to expand as molecules become more complex and as efforts to minimize nonspecific binding increase. This work examines a novel isothiocyanate-functionalized bifunctional chelate based on 2,2'-dipicolylamine (DPA) specifically designed for complexing the *fac*-[M^I(CO)₃]⁺ core. Two strategies (postlabeling and prelabeling) were explored using the isothiocyanate-functionalized DPA to determine the effectiveness of assembly on the overall yield and purity of the complex with amine containing biomolecules. A model amino acid (lysine) examined (1) amine conjugation of isothiocyanate-functionalized DPA followed by complexation with *fac*-[M^I(CO)₃]⁺ (postlabeling) and (2) complexation of *fac*-[M^I(CO)₃]⁺ with isothiocyanate-functionalized DPA followed by amine conjugation (prelabeling). Conducted with stable Re and radioactive ^{99m}Tc analogs, both strategies formed the product in good to excellent yields under macroscopic and radiotracer

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00531. RP-HPLC chromatogram of **7**. Radio-HPLC chromatograms of **8a** after purification and following incubation in mouse serum for 6 h (PDF)

Notes

The authors declare no competing financial interest.

concentrations. A synthetic peptide (AE105) which targets an emerging biomarker in CaP prognosis, urokinase-type plasminogen activator receptor (uPAR), was also explored using the isothiocyanate-functionalized DPA strategy. In vitro PC-3 (uPAR+) cell uptake assays with the $^{99\text{m}}\text{Tc}$ -labeled peptide (**8a**) showed $4.2 \pm 0.5\%$ uptake at 4 h. In a murine model bearing PC-3 tumor xenografts, in vivo biodistribution of **8a** led to favorable tumor uptake ($3.7 \pm 0.7\%$ ID/g) at 4 h p.i. with relatively low accumulation ($<2\%$ ID/g) in normal organs not associated with normal peptide excretion. These results illustrate the promise of the isothiocyanate-functionalized approach for labeling amine containing biological targeting vectors with $\text{fac-}[\text{M}^{\text{I}}(\text{CO})_3]^+$.

Graphical abstract

INTRODUCTION

Molecular imaging techniques utilizing radioisotopes for single photon emission computed tomography (SPECT) and positron emission tomography (PET) are vital contributors to numerous diagnostic medical procedures. $^{99\text{m}}\text{Tc}$ [$t_{1/2} = 6.02$ h, 140 keV γ (89%)] remains the most widely used radionuclide in diagnostic radiopharmaceuticals due to its nearly ideal nuclear properties for SPECT and convenient on-site availability via ^{99}Mo - $^{99\text{m}}\text{Tc}$ generators. For targeted molecular imaging applications with $^{99\text{m}}\text{Tc}$, the $\text{fac-}[^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3]^+$ core is particularly attractive because of its ease of preparation via an aqueous-based kit, versatile coordination chemistry, and formation of inert complexes with a broad spectrum of ligands which can be conjugated to targeting biomolecules.^{1–4} Despite these advantages, labeling with the $\text{fac-}[^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3]^+$ core often requires high temperatures (>90 °C) for quantitative complexation in acceptable time frames (30–60 min) for radiopharmaceutical preparation. The *chelate, then click* method, wherein metal complexation with a chelator occurs prior to conjugating the resulting complex to a biomolecule targeting vector via a click reaction, allows radiolabeling with temperature-sensitive biomolecules (e.g., antibodies) which would normally be denatured under the complexation conditions.

Several reports have highlighted the potential benefits of the *chelate, then click* strategy for labeling targeting biomolecules with the $\text{fac-}[\text{M}^{\text{I}}(\text{CO})_3]^+$ ($\text{M} = \text{Re}, ^{99\text{m}}\text{Tc}$) core.^{5,6} While the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction has been heralded as the iconic click reaction,⁷ a growing body of literature has revealed the drawbacks of the copper catalyst in biological applications. The negative effects associated with the metal catalyst including Cu-mediated cleavage of peptides, coordination of Cu to targeting biomolecules, and biological toxicity remain considerable barriers to the use of the CuAAC reaction.

Therefore, alternative click reactions which do not require a metal catalyst are being investigated for generating targeted radiopharmaceuticals and other applications.^{8–13} Among these reactions, isoxazole formation,¹⁴ thioether formation,^{15,16} and hydrazone generation have been successfully utilized in proof-of-concept studies with the *fac*-[M^I(CO)₃]⁺ core.¹⁷

In addition to click reactions, amine-reactive functionalities have also been used in facile approaches for labeling biomolecules with Re and ^{99m}Tc complexes in prelabeling or *preconjugate* strategies, which are analogous to the *chelate, then click* strategy. Isothiocyanate moieties and several types of activated esters (e.g., *N*-hydroxy succinimide, tetrafluorophenyl) have been employed to conjugate an array of metal complexes in several oxidation states (M^{I–V}) to solvent-accessible amine groups in macromolecules, peptides, and small molecule targeting vectors.^{18–35} Conjugation reactions employing amine-reactive functionalities do not require a catalyst, and they proceed efficiently in mild conditions at physiological temperatures. While these coupling reactions lack the chemical selectivity of “ideal” click reactions, they are widely employed for conjugating amine-reactive bifunctional chelates with peptides and antibody targeting vectors to generate targeted imaging and therapeutic radiopharmaceuticals.^{36–38}

Widespread use of amine-conjugation strategies with targeting biomolecules prompted the present study’s development of a novel bifunctional chelate containing an isothiocyanate moiety for generating targeted radiopharmaceuticals with the *fac*-[M^I(CO)₃]⁺ core. Although divalent Re and ^{99m}Tc complexes containing isothiocyanates have been employed in prelabeling reactions with targeting vectors,^{20,21} the use of isothiocyanate-functionalized *fac*-[M^I(CO)₃]⁺ complexes in this labeling strategy has not been demonstrated in the literature. 2,2’-Dipicolylamine (DPA) was chosen as the chelate scaffold due to the favorable complexation and kinetic inertness of this chelate with the *fac*-[M^I(CO)₃]⁺ core.^{6,39–41} One of the main goals of the studies was to evaluate the use of the labeled complexes in the prelabeling strategy with compounds containing free amine groups. *N*-α-Boc-L-lysine-COOH (BocLys) was chosen as a model compound for analysis and was coupled with the bifunctional chelate and metal complexes via prelabeling and postlabeling strategies (Scheme 1).

The bifunctional chelate was also reacted with an established targeting peptide (AE105)^{42–46} which binds to the urokinase plasminogen activator receptor (uPAR) to generate a ^{99m}Tc-labeled analog for use in cancer applications. uPAR is an attractive biomarker due to its overexpression in a variety of malignancies including breast, pancreatic, colon, ovarian, and prostate cancer (CaP).^{47–49} Urokinase plasminogen activator (uPA) and uPAR are known components in numerous cell signaling pathways including cytoskeleton organization, vascularization, proteolysis of extracellular membrane components, cell growth, and motility.^{50–55} uPA/uPAR have also been implicated in cancer cell invasion, metastasis, and advanced disease.^{51,56–59} Various reports have targeted uPA/uPAR for preclinical imaging^{42–44,46,60,61} or therapeutic applications in cancer.^{48,62–66} Increased expression of uPAR has been associated with more aggressive and advanced CaP relative to indolent or localized disease.^{63,67–71} Developing uPAR-targeted analogs labeled with SPECT radionuclides would be desirable to aid CaP assessment in patients. In the present study,

the ^{99m}Tc -labeled AE015 peptide was utilized in binding assays in vitro with PC-3 cells and in biodistribution analyses in vivo with mice bearing PC-3 xenografts.

RESULTS AND DISCUSSION

The ligands and metal complexes were prepared as shown in Scheme 1. *tert*-Butyl (4-aminobutyl)carbamate was chosen as the linker for generating the DPA bifunctional chelate precursor. This six-atom spacer was envisioned to separate the coordination center from the isothiocyanate functionality for biomolecule attachment and to prevent interactions of nonchelate donors (amine, isothiocyanate, thiourea) with the metal center in subsequent steps.

Reductive amination reactions between the linker and 2-pyridinecarboxaldehyde using $\text{NaBH}(\text{OAc})_3$ successfully generated the Boc-protected DPA precursor ligand **1** in 90% yield after purification by column chromatography. Removal of the Boc group using $\text{TFA}/\text{CH}_2\text{Cl}_2$ followed by basic extraction yielded **2** in its neutral form in high yield (90%).

The primary amine group in the deprotected ligand was converted to the isothiocyanate functionality by adopting a generalized literature procedure from Munch et al.⁷² Reaction of **2** in EtOH with NEt_3 and excess CS_2 formed a dithiocarbamate intermediate which upon addition of di-*tert*-butyl-dicarbonate and catalytic DMAP decomposed to the desired isothiocyanate, **3**. The crude reaction was purified by column chromatography on basic alumina to give **3** in 70% isolated yield. All spectroscopic characterization results were consistent with the desired compound. Notably, strong bands at 2095 cm^{-1} in the IR spectra indicated the presence of the isothiocyanate group.

Ligand **3** was subsequently reacted with a protected amino acid to characterize the coupling reaction of this novel ligand with a small molecule model system prior to its use with more complicated targeting molecules. BocLys was chosen as the model amino acid since the unprotected ϵ -amine group would mimic the reactivity of solvent-exposed lysine side chains in macromolecules. Retention of the Boc protecting group was intended to prevent the α -amine group from reacting with a second ligand during conjugation. In a similar approach to the SAAC strategy,⁷³ the lysine conjugate could also be directly utilized in solid phase peptide synthesis for future applications if desired.

The conjugation conditions avoided inorganic bases and minimized aqueous solvent to prevent hydrolysis of the isothiocyanate functional group. A mixture of MeCN and minimal H_2O (~10%) with NEt_3 as the base was found suitable to maintain solubility of all components and promote smooth conjugation to the intended thiourea product. RP-HPLC analysis during the reaction with **3** indicated formation of a single new product under these conditions. The BocLys conjugate, **4**, was obtained in good yield (71%) following preparatory HPLC purification of the reaction mixture. All spectroscopic characterization data were as anticipated for the desired product.

Two separate routes were utilized to generate the metal complex of the BocLys conjugated chelate. In the prelabeling route, the isothiocyanate ligand **3** was complexed with *fac*- $[\text{Re}^{\text{I}}(\text{OH})_2(\text{CO})_3]^+$ to examine the effect of metal complexation prior to coupling with

BocLys. The main purpose of generating the rhenium complexes was to have characterized reference materials prior to performing tracer-scale reactions with ^{99m}Tc ; therefore, macroscale reaction conditions were not optimized to improve the isolated yields of these species. Heating **3** with $\text{fac-}[\text{Re}^{\text{I}}(\text{OH}_2)_3(\text{CO})_3]^+$ at 40–50 °C in MeOH was found suitable for complexation without significant hydrolysis of the isothiocyanate to the amine. These conditions produced the desired product, **5**, which was isolated as a pure species by preparatory RP-HPLC in a moderate yield of 34%. IR analysis showed the expected νCO stretching bands for the $\text{fac-}[\text{Re}^{\text{I}}(\text{CO})_3]^+$ core (2028 and 1903 cm^{-1}) as well as the presence of the isothiocyanate group (2107 cm^{-1}), and ESI-MS analysis was consistent with the desired complex (583.1 m/z). While the NMR spectra of **5** showed singlets for the methylene protons of the DPA chelate as opposed to AB quartets seen with several other $\text{fac-}[\text{M}^{\text{I}}(\text{CO})_3(\text{DPA})]$ complexes,^{5,17} the downfield shifts of these protons in **5** (4.88 ppm) compared to the original ligand (3.79 ppm) indicated that metal complexation occurred at the DPA portion of the chelate as expected.

This rhenium complex was subsequently conjugated with BocLys using similar conditions as for the free ligand. The reaction proceeded smoothly to produce a single new species, **6**, which was purified by preparatory HPLC with a favorable yield (64%). ESI-MS analysis confirmed the product was the desired complex (829.2 m/z). IR analysis indicated loss of the isothiocyanate group from the starting complex while the νCO stretching bands for the $\text{fac-}[\text{Re}^{\text{I}}(\text{CO})_3]^+$ core were retained in **6** as anticipated. Surprisingly, ^1H NMR showed splitting for the methylene DPA protons as opposed to the singlets observed for these species in **5** prior to conjugation with BocLys. This suggests that these protons experienced slightly different environments upon conversion of the isothiocyanate group to the thiourea group despite the presence of the butyl linker between these functionalities and the DPA chelate area. Complex **6** was also generated via the postlabeling strategy by complexing the lysine-conjugated ligand, **4**, with $\text{fac-}[\text{Re}^{\text{I}}(\text{OH}_2)_3(\text{CO})_3]^+$ in MeOH at 60–65 °C. These reaction conditions formed **6** as the predominant species with the same RP-HPLC t_{R} as observed in the prelabeling pathway (11.0 min for both routes). The desired complex was isolated with a 32% yield following preparatory RP-HPLC purification of the postlabeling reaction. All spectroscopic data of **6** obtained by the prelabeling and postlabeling pathways were identical, confirming that both routes are viable for generating the desired metal complex on the macroscopic level with Re.

Analogous reactions as used with Re were subsequently explored on the tracer scale with ^{99m}Tc . In the prelabeling pathway, ligand **3** at 1×10^{-4} M was complexed with $\text{fac-}[\text{Re}^{\text{I}}(\text{OH}_2)_3(\text{CO})_3]^+$ in pH 7.4 sodium phosphate buffer at 70 °C for 1 h to produce the corresponding complex, **5a**, in 92% radiochemical purity. As shown in Figure 1, the HPLC t_{R} of this species (10.7 min) correlated with the rhenium complex, **5** (10.5 min), indicating the ^{99m}Tc complex was generated as anticipated. Under these conditions, ~5% hydrolysis of the isothiocyanate group was observed.

Crude reaction mixtures of **5a** were used in subsequent coupling reactions with BocLys. Several different reaction conditions were used to explore the efficiency of the isothiocyanate conjugations at the tracer level with ^{99m}Tc , although extensive investigations to probe the limits of product formation or maximize product yields were beyond the scope

of these proof-of-concept studies. Performing the reactions at 37 °C in a mixed MeCN/ aqueous solution with NEt₃ as the base was intended to minimize hydrolysis of the isothiocyanate group and to mimic the conditions used with the rhenium complex at the macroscopic level. Since these conditions would denature or cause precipitation of proteins and other macromolecules used as targeting moieties, parallel reactions employed an aqueous NaHCO₃ solution free of organic solvents to be compatible with these alternative targeting biomolecules. Both the aqueous and mixed organic/aqueous conditions were performed with BocLys at either 10 mM or 50 mM to probe the coupling efficiency at different nucleophilic amine concentrations.

As shown in Figure 2, the HPLC t_R of the ^{99m}Tc complex generated by coupling **5a** with BocLys correlated with the rhenium analog, **6** (11.0 min), thereby confirming the identity of the product as the desired complex, **6a** (11.2 min). Table 1 shows the radiochemical yields of **6a** from the various prelabeling reaction conditions explored. The yields of the final complex consistently increased by extending the reaction times from 30 to 60 min, although the rate of product production appeared to be slower during the 30–60 min period as would be expected from a bimolecular reaction. In general, utilizing the mixed organic/aqueous conditions with 50 mM BocLys led to the greatest yields, although the complex was obtained in higher yields at 10 mM BocLys than at 50 mM using the conditions stated above. This unexpected result was due to increased hydrolysis of **5a** with the higher percentage of water from the aqueous BocLys stock solution in the 50 mM reaction compared to the 10 mM reaction. Further attempts were not made to minimize hydrolysis of the starting complex in the mixed organic/aqueous reactions. Greater amounts of the hydrolyzed products were observed in the aqueous conditions compared to the mixed organic/aqueous reactions, although increasing the concentration of BocLys in the aqueous reactions led to lower formation of the hydrolyzed species (data not shown). This is likely due to the greater efficiency of thiourea formation relative to isothiocyanate hydrolysis at higher BocLys concentrations. Time points beyond 60 min were not explored due to the diminishing changes in product yields and the increasing amounts of hydrolyzed byproduct observed with time. A more detailed kinetic analysis was not pursued in these studies as coupling reactions between isothiocyanate ligands and amines have been thoroughly investigated elsewhere.⁷⁴

It is well-known that the pH of the reactions has a significant effect on the rate of both thiourea formation and isothiocyanate hydrolysis.^{20,21,74–76} The pH of the aqueous NaHCO₃ conditions in the above studies was held above 10 due to the p*K*_a of the ε-amine group of lysine. Preliminary studies in aqueous NaHCO₃ at lower pH (~9) showed decreased thiourea product formation due to greater levels of protonation and diminished nucleophilicity of BocLys; greater levels of hydrolysis were also observed in these conditions (data not shown). The relationship between solution pH and the p*K*_a of the reactive nucleophile should be considered in future applications of the prelabeling methodology with the complex explored here to optimize labeling yields of the intended amine-bearing moiety.

Complex **6a** was also formed via the postlabeling strategy by heating **4** with *fac*-[^{99m}Tc^I(OH₂)₃(CO)₃]⁺ in pH 7.4 buffer at 70 °C for 30–60 min. As was observed for the rhenium analog, the HPLC t_R of **6a** produced via the postlabeling pathway (11.2 min)

matched the product of the prelabeling pathway at the tracer level with ^{99m}Tc . Employing ligand concentrations of 1×10^{-4} M or 5×10^{-5} M led to quantitative conversion from the $\text{fac-}[^{99m}\text{TcI}(\text{OH}_2)_3(\text{CO})_3]^+$ precursor in 30–60 min with >85% radiochemical purities (Table 2) as anticipated from previous studies with analogous DPA chelates.⁵

The above proof-of-concept studies show that while complex **5a** can successfully be used to label molecules containing amine groups with the $\text{fac-}[^{99m}\text{TcI}(\text{CO})_3]^+$ core via the prelabeling route, overall radiochemical yields were superior with the postlabeling strategy in the model systems explored here. Hydrolysis and incomplete thiourea formation from the isothiocyanate precursor would not be optimal during biomolecule labeling as purification would be required and specific activities of the final radiopharmaceuticals would be lower compared to quantitative reactions with the metal complex. Utilizing high concentrations (>1 mM) of biomolecules in the labeling reaction would further decrease specific activities; if the unlabeled and labeled components are not separated prior to in vivo administration, the unlabeled biomolecules may block target receptors during molecular imaging applications. Despite these shortcomings, the prelabeling route with this complex may be advantageous for labeling temperature-sensitive biomolecules with the $\text{fac-}[^{99m}\text{TcI}(\text{CO})_3]^+$ core since the coupling reactions can be accomplished at 37 °C instead of the significantly higher temperatures required for quantitative complexation of the ligand. Furthermore, the prelabeling strategy prevents the possibility of mixed or undefined coordination of the $\text{fac-}[^{99m}\text{TcI}(\text{CO})_3]^+$ core with nonspecific sites in macromolecules employed as targeting agents, thereby minimizing in vivo transchelation of the radiolabel from the targeting molecule. The collective results obtained with the BocLys model studies indicated the novel isothiocyanate chelate (**3**) was worthy of further use with a medically relevant biomolecule.

While most applications with isothiocyanate-functionalized ligands use macromolecules (e.g., antibodies) as the targeting molecule, the in vivo circulation times of these targeting agents (days) are generally longer than desirable for the relatively short half-life of ^{99m}Tc (6 h). The radionuclide typically decays appreciably before optimal uptake at the target and clearance from the blood pool and nontarget organs occur, thus leading to poor signal-to-noise ratios in the region of interest. Peptides, on the other hand, exhibit relatively fast pharmacokinetics and are better suited for use with ^{99m}Tc for imaging applications. AE105, a peptide which binds to uPAR and contains a single amine group, was selected as the targeting vector for assessment with the bifunctional chelate above.

AE105 has been conjugated with several chelators and successfully radiolabeled with ^{64}Cu ,^{42,45,77} ^{68}Ga ,⁴³ ^{18}F ,⁴⁶ ^{213}Bi ,⁶⁴ and ^{177}Lu .^{65,66} In vivo studies using xenograft tumor models have shown that uptake of the radiolabeled peptides within tumors correlates with uPAR expression.^{45,46} These constructs are thus potential candidates for noninvasive assessment of tumor status and disease progression over time. While Armstrong et al. previously generated a ^{99m}Tc -labeled peptide for assessment with uPAR, the analog was not used for in vivo analysis.⁷⁸ The current work presents a first-generation AE105 analog labeled with ^{99m}Tc for in vivo assessment with CaP.

The bifunctional isothiocyanate ligand, **3**, was used in slight excess compared to AE105 to ensure complete conjugation of the peptide (Scheme 2). Since the peptide contained a single

nucleophilic amine group, excess ligand was not anticipated to lead to multiple products during conjugation. Similar to reactions with the BocLys model systems explored earlier, a single major peak was observed during the reaction when NEt_3 and mixed $\text{H}_2\text{O}/\text{MeCN}$ conditions were employed overnight at room temperature. Purification of the reaction by RP-HPLC produced the desired peptide conjugate, **7**, in 40% yield with high purity (>98%) as indicated by RP-HPLC analysis of the isolated material (Supporting Information Figure S1). MALDITOF MS analysis ($1594.9\ m/z$) confirmed the identity of **7** as the desired product. The $\text{Re}(\text{CO})_3$ -complexed peptide conjugate, **8**, was prepared by reacting AE105 with complex **5** (Scheme 2). This reaction used the same conditions as employed for **7**, although low conjugation efficiency prompted heating at $37\ ^\circ\text{C}$ to help drive product formation. While partial hydrolysis of the isothiocyanate group of **5** was apparent in these conditions, further addition of the metal complex led to the peptide conjugate as the major species in the reaction. Purification of the mixture by RP-HPLC generated **8** in 43% yield with >97% purity as indicated by RP-HPLC analysis (Figure 3). MALDI-TOF MS analysis ($1864.8\ m/z$) confirmed the identity of **8** as desired.

AE105 conjugate **7** was subsequently radiolabeled with $^{99\text{m}}\text{Tc}$. Heating **7** at $5 \times 10^{-5}\ \text{M}$ with $\text{fac-}[^{99\text{m}}\text{Tc}^{\text{I}}(\text{OH}_2)_3(\text{CO})_3]^+$ in PBS/MeOH at $70\ ^\circ\text{C}$ for 60 min yielded a single species in >99% radiochemical purity by radio-HPLC analysis. Comparison of the t_{R} of this species (21.7 min) with that of the Re analog, **8** (21.5 min), confirmed the radiolabeled peptide as the desired product, **8a** (Figure 3). This species was isolated with decay-corrected yields of 59–60% following purification by HPLC method 3. **8a** was observed to be >96% stable in mouse serum up to 6 h at $37\ ^\circ\text{C}$ (Supporting Information Figure S3), indicating it would be a viable candidate for subsequent biological analyses.

In vitro cell uptake assays were performed to assess the uPAR-mediated uptake of **8a** with PC-3 cells. **8a** showed a time-dependent increase in cell uptake to yield $2.4 \pm 0.3\%$, $3.3 \pm 0.5\%$, and $4.2 \pm 0.5\%$ of the applied activity at 1, 2, and 4 h, respectively (Figure 4). The cell uptake of **8a** decreased to $1.1 \pm 0.9\%$, $1.3 \pm 0.4\%$, and $1.5 \pm 0.8\%$ of the applied activity at 1, 2, and 4 h, respectively, in the presence of unlabeled AE105 to block uPAR-mediated binding. These results established the uptake of **8a** with PC-3 cells was mediated by uPAR and indicated that the $^{99\text{m}}\text{Tc}$ labeled AE105 can specifically bind to its biological target.

The encouraging in vitro results with **8a** prompted its further investigation in vivo with mouse biodistribution studies. Figure 5 shows the uptake of **8a** in selected tissues at 1 and 4 h following tail vein injection of mice bearing PC-3 xenograft tumors. The radiolabeled peptide showed relatively low uptake (<2% ID/g) at 1 h p.i. in all normal tissues except those involved with excretion (liver, intestines, and kidneys with $12.0 \pm 1.3\%$, $4.2 \pm 0.8\%$, and $6.6 \pm 0.7\%$ ID/g, respectively) and the lungs ($3.2 \pm 0.4\%$ ID/g). The high uptake of **8a** in the liver follows the pattern observed with other small molecules and peptides labeled with the lipophilic $\text{fac-}[^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3(\text{DPA})]^+$ moiety. Relative to the 1 h time point, the uptake values decreased by 4 h p.i. for all normal organs as would be expected from typical peptide pharmacokinetics. Favorable uptake and retention of **8a** in the tumor was observed at both 1 and 4 h p.i., yielding $3.6 \pm 0.6\%$ and $3.7 \pm 0.7\%$ ID/g, respectively. Since the tumor uptake was higher than all other tissues except the liver, intestine, and kidneys at both time points,

8a may be an encouraging lead compound for future work as a SPECT imaging peptide for PC-3 tumors in vivo.

While radiolabeled uPAR analogs have been assessed with several tumor types in vivo, few reports utilizing CaP models have been published. An ^{18}F -AIF-NOTA conjugate of AE105 employed by Persson et al.⁴⁶ demonstrated an uptake of $4.2 \pm 0.1\%$ ID/g in PC-3 xenograft tumors at 1 h p.i. by quantitative PET imaging in mice. Additional PET and biodistribution analysis indicated the uptake of the radiolabeled peptide had declined to $\sim 1\%$ ID/g after 2 h p.i. The comparable uptake of **8a** within PC-3 tumors at both 1 and 4 h p.i. in the present study suggests the $^{99\text{m}}\text{Tc}$ analog may be more amenable for imaging uPAR-expressing tumors at later time points compared to the ^{18}F analog. Future analysis with **8a** is required to determine whether tumor uptake correlates with uPAR expression, as has been observed with related AE105 analogs labeled with other radionuclides.

In a subsequent study utilizing a transgenic PC-3M-LUC2 CaP model,⁶⁶ Persson et al. demonstrated a DOTA-AE105 analog labeled with ^{177}Lu (^{177}Lu -DOTA-AE105) bound to cells in vitro to a significantly greater level than a nonspecific AE105 mutant analog. Furthermore, ^{177}Lu -DOTA-AE105 inhibited metastatic spread of PC-3M-LUC2 in vivo, and PET imaging with ^{64}Cu -DOTA-AE105 was able to detect metastatic lesions which were not detectable with bioluminescent imaging. Considering the widespread availability and clinical utilization of $^{99\text{m}}\text{Tc}$ in diagnostic imaging, these pioneering studies with radiolabeled AE105 analogs in CaP models supported the development of the $^{99\text{m}}\text{Tc}$ -labeled construct shown here. The collective results from the current study and previous publications suggest AE105 analogs labeled with a variety of radionuclides show promise for continued preclinical development and potential translation to clinical CaP assessment in the future.

CONCLUSIONS

This report presents the first example of an isothiocyanate-functionalized DPA ligand for complexation with the $\text{fac-}[\text{M}^{\text{I}}(\text{CO})_3]^+$ core. The bifunctional ligand was synthesized and readily complexed with $\text{fac-}[\text{M}^{\text{I}}(\text{CO})_3]^+$ at both macroscopic and tracer levels. The ligand and metal complexes were successfully reacted with BocLys via prelabeling and postlabeling strategies to generate the intended final products. Exploration of the prelabeling pathway showed the potential of the novel isothiocyanate complexes for reacting with solvent-accessible amine groups at ambient temperatures in relatively short (~ 30 min) time periods as would be desirable for developing a wide variety of targeted radiopharmaceuticals with the $\text{fac-}[\text{M}^{\text{I}}(\text{CO})_3]^+$ core. Application of the bifunctional ligand with AE105 successfully generated a novel $\text{fac-}[\text{M}^{\text{I}}(\text{CO})_3]^+$ -labeled peptide for targeting uPAR. The radiolabeled analog (**8a**) showed uPAR-mediated uptake in PC-3 cells in vitro and favorable uptake and retention in PC-3 xenograft tumors in vivo. These results indicate that the novel chelate and uPAR-targeting construct shown here is worthy of future investigations for CaP assessment.

EXPERIMENTAL PROCEDURES

All reagents and solvents were of reagent grade or higher and obtained from commercial suppliers (Aldrich, Fluka, Acros, Fisher) and used as received unless noted otherwise. *N*- α -Boc-L-Lysine-COOH (BocLys) was from Chem-Impex International (Wood Dale, IL). AE105 (NH₂-Gly-Asp-Cha-Phe-D-Ser-D-Arg-Tyr-Leu-Trp-Ser-NH₂) was synthesized as previously described.^{46,79,80} *fac*-[Re^I(OH)₂]₃(CO)₃(SO₃CF₃),⁸¹ and *tert*-butyl (4-aminobutyl)carbamate⁸² were prepared from literature procedures. [^{99m}TcO₄]⁻ was obtained from Cardinal Health (Spokane, WA) or from Stanford Nuclear Medicine Clinic (Stanford, CA) and was used to prepare *fac*-[^{99m}Tc^I(OH)₂]₃(CO)₃⁺ via commercially available Isolink kits (Tyco, Inc.) as previously described.⁸³ Nuclear magnetic resonance (NMR) spectra were recorded at 293 K on a 300 MHz Varian Mercury Vx spectrometer using 5 mm NMR tubes. ¹H and ¹³C NMR spectra peak positions were referenced using residual solvent signals, and spectra were processed using Varian VNWR 6.1 software. Electrospray ionization mass spectrometry (ESI-MS) was performed by direct infusion of sample solutions on a Thermo-Finnigan LCQ Advantage instrument or an Agilent 1100 Ion Trap LC/MS/MS (scanned from 50 to 2000 *m/z* with the drying gas at 12 mL min⁻¹ at 350 °C and nebulizer pressure set at 50 psig). Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was performed at the Washington State University Molecular Biology and Genomics Core (Pullman, WA). Elemental analyses were conducted by Atlantic Micro Laboratories in Norcross, GA. Infrared (IR) spectra were recorded on a Thermo Nicolett 6700 FTIR with an ATR cell and analyzed with OMNIC 7.1a software. UV/vis spectra were recorded on a Varian Cary 50 Bio spectrophotometer and analyzed with Cary WinUV 3.00 software. Solutions of the compounds were prepared in UV/vis quality methanol and measured in 1 cm path length quartz cuvettes. Analytical separation and identification of small molecules and peptides by reversed-phase high performance liquid chromatography (RP-HPLC) were conducted using an Agilent Zorbax SB-Aq column (3.5 μ m, 4.6 $\times$ 150 mm) or a Varian Pursuit XRs column (C18, 5 μ m, 4.6 $\times$ 250 mm) with a Phenomenex security guard cartridge (C18, 4.0 $\times$ 3.0 mm). A PerkinElmer Series 200 analytical chromatography system equipped with a PerkinElmer Radiomatic 610TR detector and a Hitachi D-7000 series analytical chromatography system (L-7100 pump, L-7400 UV detector) equipped with a Berthold FlowStar LB 513 radiodetector were used during analyses. Unless noted otherwise, elutions performed on these systems used the following gradient profiles (HPLC methods 1 and 2) with 0.05% TFA (trifluoroacetic acid) in H₂O (solvent A) and methanol (MeOH, solvent B) as solvents at 1 mL/min and UV detection at 254 nm. HPLC method 1 (employing the Zorbax SB-Aq column): 0–1.5 min 100% A, 1.5–5 min linear gradient to 35% A/65% B, 5–10.5 min linear gradient to 25% A/75% B, 10.5–12 min isocratic 25% A/75% B, 12–15 min 100% B, return to 100% A at 15 min and equilibrate; UV was monitored at 254 nm; HPLC method 2 (employing the Pursuit XRs column): 0–3 min 100% A, 3–9 min isocratic 75% A/25% B, 9–20 min linear gradient to 100% B, 20–25 min 100% B, return to 100% A at 25 min and equilibrate. Chromatograms were plotted using OriginPro 8.5.1 (OriginLab Corporation, MA). Preparatory RP-HPLC separations and purifications of small molecules used an Agilent Zorbax SB-C18 PrepHT column (C18, 7 μ m, 21.2 $\times$ 250 mm) fitted with a matching guard cartridge (5 μ m, 21.2) on a Hitachi D-7000 series semipreparative chromatography system (L-7150 pump, L-7400 UV

detector). Elutions were performed with solvents A and B; UV was monitored at 220 nm. The PC-3 prostate cancer cell line was obtained from American Type Culture Collection (Manassas, VA). Male athymic nude mice used for biodistribution analysis were purchased from Charles River Laboratory (Wilmington, MA).

Small Molecule Synthesis and Complexation Reactions

tert-Butyl (4-(bis(pyridin-2-ylmethyl)amino)butyl)-carbamate, 1—*tert*-Butyl (4-aminobutyl) carbamate (193 mg, 1.03×10^{-3} mol), dichloroethane (15 mL), and 2-pyridinecarboxyaldehyde (0.215 mL, 2.26×10^{-3} mol) were combined in a flask. The flask was purged with N_2 and placed in an ice bath for 5 min. Sodium triacetoxyborohydride ($NaBH-(OAc)_3$), 546 mg, 2.58×10^{-3} mol) was added and the reaction was stirred under N_2 in the ice bath for an additional 5 min and then at room temperature for 2.5 h. The reaction was quenched with 8 mL H_2O and stirred for 1 h. A 2.5 M NaOH solution was added to the reaction to reach pH ~ 10 and the reaction mixture was transferred to a separatory funnel. The organic layer was collected and the remaining aqueous layer was extracted with CH_2Cl_2 (3×15 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated to dryness under reduced pressure. The crude residue was purified by silica gel flash chromatography using 3–4% MeOH in CH_2Cl_2 . Collected fractions containing the product ($R_f = 0.06$ in 3% MeOH/ CH_2Cl_2 on silica gel) were combined and concentrated to dryness under vacuum to yield **1** as a dark yellow oil (343.5 mg, 90%).

Anal. Calcd for $C_{21}H_{30}N_4O_2 \cdot 0.5MeOH$: C, 66.81; H, 8.35; N, 14.50. Found: C, 66.30; H, 8.11; N, 14.51. 1H NMR [δ (ppm), 300 MHz, $CDCl_3$]: 8.49 (d, 2 H), 7.61 (dd, 2 H), 7.47 (d, 2 H), 7.10 (dd, 2 H), 4.83 (s, 1 H), 3.76 (s, 4 H), 3.02 (q, 2 H), 2.51 (t, 2 H), 1.53 (m, 2 H), 1.47–1.35 (m, 11 H). ^{13}C NMR [δ (ppm), 75 MHz, $CDCl_3$]: 159.1, 155.6, 148.2, 135.7, 122.3, 121.3, 77.8, 59.7, 53.3, 53.0, 27.9, 27.2, 23.7. MS (+ESI): 393.2 m/z ; calculated for $[M + Na]^+$: 393.2 m/z . IR (neat oil, cm^{-1}): 2931, 1709, 1692, 1590, 1433, 1364, 1249, 1168, 758. UV/vis ϵ_{max} (202 nm): $11,300 M^{-1} cm^{-1}$; $\epsilon_{261 nm}$: $5,600 M^{-1} cm^{-1}$.

N¹,N¹-Bis(pyridin-2-ylmethyl)butane-1,4-diamine, 2—Compound **1** (291.8 mg, 7.9×10^{-4} mol) was dissolved in a 1:4 TFA: CH_2Cl_2 (5 mL) mixture and the solution was stirred at room temperature for 90 min. The mixture was concentrated to dryness under vacuum to yield a red-brown oil. The residue was dissolved in CH_2Cl_2 (20 mL) and washed with H_2O (20 mL) which had been adjusted to pH 10 with 1 M NaOH. The organic layer was then collected and the aqueous layer was extracted with CH_2Cl_2 (2×20 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated to dryness under vacuum to yield **2** as an amber-colored oil (192.2 mg, 90%).

Anal. Calcd for $C_{16}H_{22}N_4$: C, 71.08; H, 8.20; N, 20.72. Found: C, 71.01; H, 8.14; N, 20.59. 1H NMR [δ (ppm), 300 MHz, $CDCl_3$]: 8.45 (d, 2 H), 7.58 (dd, 2 H), 7.46 (d, 2 H), 7.07 (dd, 2 H), 3.74 (s, 4 H), 2.56 (t, 2 H), 2.48 (t, 2 H), 1.76 (br s, 2 H), 1.50 (m, 2 H), 1.35 (m, 2 H). ^{13}C NMR [δ (ppm), 75 MHz, $CDCl_3$]: 159.2, 148.3, 135.8, 122.3, 121.3, 59.8, 53.5, 41.2, 30.4, 23.8. MS (+ESI): 271.1 m/z ; calculated for $[M + H]^+$: 271.2 m/z . IR (neat oil, cm^{-1}): 3051, 3010, 2929, 2815, 1691, 1589, 1432, 1200, 1125, 760. UV/vis ϵ_{max} (204 nm): $12,500 M^{-1} cm^{-1}$; $\epsilon_{262 nm}$: $6,400 M^{-1} cm^{-1}$.

4-Isothiocyanato-N,N-bis(pyridin-2-ylmethyl)butan-1-amine, 3—Compound **2** (73 mg, 2.7×10^{-4} mol) was dissolved in absolute ethanol (EtOH, 1.5 mL), after which CS₂ (162 μ L, 2.7×10^{-3} mol) and triethylamine (NEt₃, 37.6 μ L, 2.7×10^{-4} mol) were added to the solution. The reaction was stirred at room temperature for 1.5 h and was then placed in an ice bath for 10 min. Di-*tert*-butyl-dicarbonate (57 mg, 2.6×10^{-4} mol) was added, followed immediately by 4-dimethylaminopyridine (DMAP, 1 mg, 8×10^{-6} mol) dissolved in absolute EtOH (0.25 mL). After 10 min, the reaction was allowed to come to room temperature and was stirred for 1 h. The mixture was then concentrated to dryness under vacuum. The crude residue was purified by flash chromatography on basic alumina using 1% MeOH/CH₂Cl₂. Collected fractions containing the product ($R_f = 0.38$ in 2% MeOH/CH₂Cl₂ on basic alumina) were combined and concentrated to dryness under vacuum to yield **3** as an amber-colored oil (59 mg, 70%).

Anal. Calcd for C₁₇H₂₀N₄S: C, 65.35; H, 6.45; N, 17.93. Found: C, 64.45; H, 6.51; N, 17.13. ¹H NMR [δ (ppm), 300 MHz, CDCl₃]: 8.51 (d, 2 H), 7.65 (dd, 2 H), 7.48 (d, 2 H), 7.14 (dd, 2 H), 3.79 (s, 4 H), 3.40 (t, 2 H), 2.55 (t, 2 H), 1.63 (m, 4 H). ¹³C NMR [δ (ppm), 75 MHz, CDCl₃]: 159.6, 149.0, 136.5, 123.0, 122.1, 60.5, 53.1, 44.8, 27.7, 24.2. MS (+ESI): 313.3 m/z ; calculated for [M + H]⁺: 313.2 m/z . IR (neat oil, cm⁻¹): 2932, 2814, 2176, 2095, 1588, 1432, 751. UV/vis $\epsilon_{\max}$ (203 nm): 19,200 M⁻¹ cm⁻¹; $\epsilon_{262 \text{ nm}}$: 8,600 M⁻¹ cm⁻¹.

(S)-6-(3-(4-(Bis(pyridin-2-ylmethyl)amino)butyl)thioureido)-2-((tert-butoxycarbonyl)amino) hexanoic acid, 4—Compound **3** (40 mg, 1.28×10^{-4} mol), BocLys (47.3 mg, 1.92×10^{-4} mol), and NEt₃ (52.3 μ L, 3.75×10^{-4} mol) were dissolved in a 9:1 acetonitrile (MeCN):H₂O mixture (5 mL), and the solution was stirred at room temperature overnight. The reaction was concentrated under reduced pressure and purified by preparatory HPLC using the following gradient method (prepHPLC method 1) with a flow rate of 12 mL/min: 0–3 min 100% A, 3–9 min isocratic 75% A/25% B, 9–25 min linear gradient to 100% B, 25–32 min 100% B, return to 100% A at 32 min and equilibrate. Fractions containing pure product were combined and concentrated to dryness under vacuum to yield **4** as an off-white solid (72 mg, 71%).

Anal. Calcd for C₂₈H₄₂N₆O₄S: C, 60.19; H, 7.58; N, 15.04. Found: C, 60.11; H, 7.51; N, 15.12. ¹H NMR [δ (ppm), 300 MHz, CD₃OD]: 8.66 (d, 2 H), 7.89 (dd, 2 H), 7.51 (d, 2 H), 7.45 (dd, 2 H), 4.62 (s, 4 H), 4.06 (dd, 1 H), 3.50 (m, 2 H), 3.45–3.32 (m, 4 H), 1.85 (m, 4 H), 1.74–1.53 (m, 6 H), 1.44 (s, 9 H). ¹³C NMR [δ (ppm), 75 MHz, CD₃OD]: 176.2, 163.1, 162.6, 158.1, 151.8, 150.3, 139.4, 125.5, 80.4, 58.5, 56.0, 54.8, 44.0, 32.5, 29.7, 28.7, 27.4, 24.2, 22.4. MS (+ESI): 559.3 m/z ; calculated for [M + H]⁺: 559.3 m/z . IR (solid, cm⁻¹): 3292, 2933, 1671, 1552, 1166, 1129, 761, 719. UV/vis: $\epsilon_{\max}$ (203 nm): 35,000 M⁻¹ cm⁻¹; $\epsilon_{242 \text{ nm}}$: 20,000 M⁻¹ cm⁻¹.

fac-[Re^I(CO)₃(3)](C₂F₃O₂), 5—Compound **3** (33 mg, 1.05×10^{-4} mol) was dissolved in 4 mL of MeOH in a 50 mL flask. A 0.1 M solution of *fac*-[Re^I(OH)₂(CO)₃](SO₃CF₃) (1.1 mL, 1.1×10^{-4} mol) was added and the reaction was stirred at 50 °C under a condenser for 1.75 h. The reaction was then concentrated under reduced pressure and purified by preparatory HPLC using the following gradient method (prepHPLC method 2) with a flow rate of 12.5 mL/min: 0–4 min 100% A, 4–12 min linear gradient to 40% A/60% B, 12–33

min linear gradient to 15% A/85% B, 33–36 min linear gradient to 100% B, 36–43 min 100% B, return to 100% A at 43 min and equilibrate. Fractions containing pure product were combined and concentrated to dryness under vacuum to yield **5** as an off-white crystalline residue (24.9 mg, 34%).

Anal. Calcd for $C_{22}H_{20}F_3N_4O_5ReS$: C, 37.98; H, 2.90; N, 8.05. Found: C, 37.86; H, 2.77; N, 7.87. 1H NMR [δ (ppm), 300 MHz, CD_3OD]: 8.87 (d, 2 H), 7.94 (dd, 2 H), 7.55 (d, 2 H), 7.38 (dd, 2 H), 4.88 (s, 4 H), 3.88 (m, 2 H), 3.75 (t, 2 H), 2.08 (m, 2 H), 1.83 (m, 2 H). ^{13}C NMR [δ (ppm), 75 MHz, CD_3OD]: 197.1, 162.1, 153.2, 141.6, 126.9, 124.5, 71.0, 68.6, 45.8, 28.1, 23.5. MS (+ESI): 583.1 m/z ; calculated for $[M]^+$: 583.1 m/z . IR (solid, cm^{-1}): 2951, 2107, 2028, 1903, 1734, 1683, 1135, 763, 705. UV/vis ϵ_{max} (235 nm): 9,900 $M^{-1} cm^{-1}$; $\epsilon_{261 nm}$: 7,800 $M^{-1} cm^{-1}$.

fac-[Re^I(CO)₃(4)](C₂F₃O₂), **6. Postlabeling Pathway from **4****—Compound **4** (36.4 mg, 4.62×10^{-5} mol) was dissolved in 0.5 mL of MeOH and 3 mL of H₂O in a 25 mL flask. A 0.1 M solution of *fac*-[Re^I(OH)₂(CO)₃](SO₃CF₃) (0.53 mL, 5.3×10^{-5} mol) was added and the reaction was stirred at 60 °C under a condenser for 3.5 h. The reaction was then diluted in MeOH to dissolve the creamy precipitate, filtered, and purified by preparatory HPLC using the following gradient method (prepHPLC method 3) with a flow rate of 12.5 mL/min: 0–3 min 100% A, 3–10 min isocratic 75% A/25% B, 10–32 min linear gradient to 100% B, 32–40 min 100% B, return to 100% A at 40 min and equilibrate. Fractions containing pure product were combined and concentrated to dryness under vacuum to yield **6** as a whitish semisolid residue (14.1 mg, 32%).

Prelabeling Pathway from **5**—Complex **5** (22.5 mg, 3.2×10^{-5} mol), BocLys (15.9 mg, 6.4×10^{-5} mol), and NEt₃ (18.5 μ L, 1.3×10^{-4} mol) were combined in a flask containing 4 mL of MeCN and 15 drops H₂O, and the reaction was stirred at room temperature overnight. The solution was then concentrated under reduced pressure and purified by preparatory HPLC using prepHPLC method 3 with a flow rate of 12.5 mL/min. Fractions containing pure material were combined and concentrated to dryness under vacuum to yield **6** as a white residue (19.5 mg, 64%).

Anal. Calcd for $C_{33}H_{42}F_3N_6O_9ReS$: C, 42.08; H, 4.49; N, 8.92. Found: C, 41.98; H, 4.45; N, 8.85. 1H NMR [δ (ppm), 300 MHz, CD_3OD]: 8.87 (d, 2 H), 7.93 (dd, 2 H), 7.54 (d, 2 H), 7.37 (dd, 2 H), 4.99–4.79 (m, 4 H), 4.07 (dd, 1 H), 3.95 (m, 2 H), 3.68 (br s, 2 H), 3.44 (br s, 2 H), 1.99 (m, 2 H), 1.92–1.51 (m, 8 H), 1.44 (s, 9 H). ^{13}C NMR [δ (ppm), 75 MHz, CD_3OD]: 197.2, 176.2, 162.2, 158.2, 153.1, 141.6, 126.9, 124.6, 80.5, 71.6, 68.7, 54.8, 44.1, 32.5, 29.7, 28.7, 27.8, 24.3, 22.7. MS (+ESI): 829.2 m/z ; calculated for $[M]^+$: 829.2 m/z . IR (solid, cm^{-1}): 3284, 2936, 2029, 1909, 1689, 1163, 765. UV/vis ϵ_{max} (203 nm): 37,000 $M^{-1} cm^{-1}$; $\epsilon_{240 nm}$: 22,200 $M^{-1} cm^{-1}$.

AE105 Peptide Conjugation and Complexation Reactions

AE105 Conjugate of Ligand **3, **7****—0.8 mg AE105 (5.3×10^{-7} mol) was dissolved in 100 μ L of H₂O. A solution of 0.4 mg **3** (1.28×10^{-6} mol) dissolved in 40 μ L of MeCN and 5 μ L of NEt₃ (3.59×10^{-5} mol) were added, and the reaction was stirred at room temperature overnight. The following day, 7.5 μ L of 10% TFA in H₂O was added and the reaction was

purified by analytical RP-HPLC using the Varian Pursuit XRs column with solvents A and B according to the following gradient method with a flow rate of 1 mL/min (HPLC method 3): 0–3 min 95% A/5% B, 3–9.5 min linear gradient to 55% A/45% B, 9.5–24.5 min linear gradient to 10% A/90% B, 24.5–29 min 100% B, return to 95% A/5% B at 29 min and equilibrate; UV was monitored at 238 nm. Fractions containing pure product were combined, concentrated by rotary evaporation, frozen, and lyophilized to yield 0.4 mg (40%) of the desired product. MALDI-TOF MS: 1594.9 *m/z*; calcd for [M + H]⁺: 1594.8 *m/z*.

AE105 Conjugate of Complex 5, 8—0.5 mg AE105 (3.31×10^{-7} mol) was dissolved in 60 μL of H₂O. A solution of 0.35 mg **5** (4.99×10^{-7} mol) dissolved in 26 μL of MeCN and 5 μL of NEt₃ (3.59×10^{-5} mol) were added, and the reaction was stirred at room temperature overnight. The following day, additional **5** (1.04×10^{-7} mol) dissolved in 8 μL of MeCN and 2 μL of NEt₃ (1.5×10^{-5} mol) was added, and the reaction was stirred at 37 °C for 4.5 h. 7 μL 10% TFA in H₂O was then added and the reaction was purified by analytical RP-HPLC by HPLC method 3. Fractions containing pure product were combined, concentrated by rotary evaporation, frozen, and lyophilized to yield 0.3 mg (43%) of the desired product. MALDI-TOF MS: 1864.8 *m/z*; calcd for [M]⁺: 1864.7 *m/z*.

General Procedures for ^{99m}Tc Reactions

Complexation of Isothiocyanate Ligand 3 and Postlabeling Pathway with 4—

Solutions of **3** or **4** in 10 mM pH 7.4 sodium phosphate buffer (250–450 μL) were sealed in 5 mL labeling vials and the vials were purged with N₂ for 5–10 min. Solutions of *fac*-[^{99m}Tc^I(OH₂)₃(CO)₃]⁺ (50–250 μL , 0.13–1.6 mCi) were added to bring the final ligand concentrations to 1×10^{-4} or 5×10^{-5} M in final volumes of 500 μL , and the vials were heated at 70 °C for 30–60 min. The vials were cooled prior to analysis and purification by radio-HPLC using HPLC method 1 or 2.

Prelabeling Pathway with Complex 5a. Mixed Organic/Aqueous Conditions—

Crude reaction mixtures of **5a** (50 μL , 6–150 μCi) were added to 5 mL labeling vials containing MeCN (375 or 415 μL), NEt₃ (25 μL), and 0.5 M BocLys in H₂O (50 or 10 μL) to bring the final BocLys concentrations to 50 mM or 10 mM in final volumes of 500 μL . The vials were sealed, purged with N₂ for 2–3 min, and heated at 37 °C for 30–60 min. The vials were analyzed by radio-HPLC using HPLC method 1 and purified by HPLC method 1 or 2.

Aqueous NaHCO₃ Conditions—Crude reaction mixtures of **5a** (50 μL , 6–10 μCi) were added to 5 mL labeling vials containing 0.1 M NaHCO₃ pH 10.2 buffer (400 or 440 μL) and 0.5 M BocLys in H₂O (50 or 10 μL) to bring the final BocLys concentrations to 50 mM or 10 mM in final volumes of 500 μL . The vials were sealed, purged with N₂ for 2–3 min, and heated at 37 °C for 30–60 min. The vials were analyzed by radio-HPLC using HPLC method 1 and purified by HPLC method 1 or 2.

Peptide Radiolabeling—A solution of 48 μg **7** (2.5×10^{-8} mol) dissolved in 30 μL of H₂O/MeOH, 220 μL of pH 7.4 PBS, and 50 μL of MeOH were combined in a 5 mL sealable labeling vial. The vial was sealed and sparged with N₂ for 5 min, and 200 μL of *fac*-

[^{99m}TcI(OH₂)₃(CO)₃]⁺ solution (79 μ Ci) was added to yield a final peptide concentration of 5×10^{-5} M in a final volume of 500 μ L. The reaction was then heated at 70–80 °C with periodic analysis by radio-HPLC using HPLC method 3. For the in vitro and in vivo biological analyses, the above radiolabeling procedure was followed utilizing 10 μ g **7** and 10 mCi *fac*-[^{99m}TcI(OH₂)₃(CO)₃]⁺ to yield the product peptide **8a**. The reaction solution was purified by radio-HPLC with a C18 column at a flow rate of 1 mL/min using HPLC method 4: 0–3 min 95% A/5% C (MeCN with 0.1% TFA), 3–18 min linear gradient to 5% A/95% C, 18–20 min hold 5% A/95% C, 20–23 return to 95% A/5% C and equilibrate. The collected product fraction was diluted to ten times its volume, passed through a C18 cartridge, and eluted with EtOH (1 mL). The radioactive fractions (2×100 μ L) were combined, adjusted to pH 7.4 with PBS (100 μ L), diluted with saline (2 mL) to give a solution of **8a** with <10% EtOH, and passed through a 0.22 μ M filter for subsequent use. The purity of this solution was verified by radio-HPLC using HPLC method 4 (Supporting Information Figure S2).

Peptide Stability Analysis—The serum stability of **8a** was determined as previously described.⁵ Briefly, HPLC-purified **8a** (100 μ Ci) in 50 μ L of PBS was incubated with mouse serum (500 μ L) at 37 °C for a total of 6 h. At periodic time points, the solution was filtered through a centrifugal filter (10 K; Millipore Corp.) and the filtrate was analyzed by radio-HPLC using HPLC method 4.

In Vitro and In Vivo Biological Evaluations

Cell Culture and Animal Model—Cell growth and tumor implantation procedures were performed as previously described.⁸⁴ Briefly, PC-3 cells were cultured in Ham's F-12K (Kaighn's) Medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator containing 5% CO₂ at 37 °C. A 70–80% confluent monolayer was detached with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and dissociated into a single-cell suspension for further cell culture and assays. The animal procedures were performed according to a protocol approved by the Stanford University Institutional Animal Care and Use Committee. Approximately 2×10^6 cultured PC-3 cells were suspended in 100 μ L of PBS and subcutaneously implanted in the left shoulders of male athymic nude mice. Tumors were grown to a size of 0.5–1 cm in diameter (5–6 weeks) prior to biodistribution studies.

In Vitro PC-3 Cell Uptake Assays—The uPAR-mediated binding of the radiolabeled peptide with PC-3 cells was performed as previously described.⁸⁴ Briefly, PC-3 cells suspended in Ham's F-12K (Kaighn's) Medium containing 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), 0.2% bovine serum albumin, and 0.3 mM 1,10-phenanthroline were seeded at a density of 1×10^5 per well in 24-well plates and allowed to attach overnight. The cells were then incubated at 37 °C for 1, 2, or 4 h with purified **8a** (1 μ Ci) alone or with 2 μ g AE105 to block uPAR-mediated binding. The cells were then washed 3 times with ice-cold PBS, lysed with 1 mL of 0.1 M NaOH containing 0.1% sodium dodecyl sulfate, and transferred to γ -counter tubes. Radioactivity was measured by a γ -counter (PerkinElmer model 1470), and cell uptake was expressed as the percentage of added radioactivity. The experiment was performed in quadruplicate wells.

In Vivo PC-3 Xenograft Mouse Biodistribution Analysis—For biodistribution studies, male athymic nude mice bearing PC-3 xenografts ($n = 4$ per group) were injected via the tail vein with approximately 100 μCi of **8a** and were euthanized at 1 or 4 h post injection (p.i.). Tumor and normal tissues of interest were removed and weighed, and their radioactivity was measured using a γ -counter. Radioactivity uptake was expressed as a percentage of the injected radioactive dose per gram of tissue (% ID/g).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BocLys	N- α -Boc-L-Lysine-COOH
CaP	prostate cancer
Cha	β -cyclohexyl-L-alanine
cpm	counts per minute
DMAP	4-dimethylaminopyridine
DPA	2,2'-dipicolylamine
ESI-MS	electrospray ionization mass spectrometry
EtOH	ethanol
HPLC	high performance liquid chromatography
IR	infrared
MALDI-TOF	matrix assisted laser desorption/ionization time-of-flight
MeCN	acetonitrile
MeOH	methanol
MS	mass spectrometry
NaBH(OAc)₃	sodium triacetoxyborohydride
NEt₃	triethylamine
NMR	nuclear magnetic resonance

PBS	phosphate buffered saline
RP-HPLC	reversed-phase HPLC
SAAC	single amino acid chelate
SPECT	single photon emission computed tomography
TFA	trifluoroacetic acid
t_R	retention time
uPAR	urokinase plasminogen activator receptor
UV/vis	ultraviolet/visible spectroscopy

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Figure 1. Normalized and offset RP-HPLC chromatograms of **5** (UV absorbance, 254 nm; earlier blue trace) at t_R 10.5 min and **5a** (radiodetector, counts per minute (cpm); later red trace) at t_R 10.7 min.

Figure 2. Normalized and offset RP-HPLC chromatograms of **6** (UV absorbance, 254 nm; earlier blue trace) at t_R 11.0 min and **6a** (radiodetector, counts per minute (cpm); later red trace) at t_R 11.2 min.

Figure 3. Normalized and offset RP-HPLC chromatograms of **8** (UV absorbance, 254 nm; earlier blue trace) at t_R 21.5 min and **8a** (radiodetector, counts per minute (cpm); later red trace) at t_R 21.7 min.

Figure 4.

PC-3 in vitro cell uptake assay. PC-3 cells (1×10^5 per well in 24-well plates) were incubated at 37 °C for 1, 2, or 4 h with purified **8a** (1 μ Ci) alone (blue diamonds) or with 2 μ g AE105 (red squares) to block uPAR-mediated binding. Data represent the amount of activity associated with the cells as a percentage of the total activity applied $\pm$ standard deviation of quadruplicate wells.

Figure 5. Biodistribution of **8a** in male athymic nude mice bearing PC-3 tumors 1 and 4 h after injection of approximately 100 μ Ci of **8a** via the tail vein. Data are expressed as % ID/g $\pm$ standard deviation ($n = 4$ per group).

Scheme 1.

Reaction Scheme for Generating the Isothiocyanate-Functionalized Ligand, Metal Complexes, and BocLys Conjugates via prelabeling and postlabeling Strategies

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Table 1Radiochemical Yields of 6a via the prelabeling Pathway from 5a^a

		6a^b	
	concentration of BocLys	30 min	60 min
Organic conditions ^c	10 mM	90%	94%
	50 mM	79%	85%
Aqueous conditions ^d	10 mM	31%	46%
	50 mM	65%	68%

^aReactions were heated at 37 °C for the times indicated and then allowed to cool briefly before aliquots were removed for analysis by radio-HPLC using HPLC method 1.

^bThe radiochemical yields shown were calculated from radio-HPLC chromatograms and are corrected to account for the initial radiochemical purity of **5a** from the crude reaction mixture prior to conjugation with BocLys according to the following equation:

$$\% \text{yield} = \frac{\% \text{radiochemical purity of BocLys conjugated complex}}{\% \text{radiochemical purity of initial isothiocyanate complex}}$$

^cOrganic conditions: mixed MeCN/phosphate buffer solutions with NEt₃ as the base.

^dAqueous conditions: aqueous pH 10.2 NaHCO₃ solutions.

Table 2Radiochemical Purities of 6a via the postlabeling Pathway from Ligand 4^a

ligand concentration	6a	
	30 min	60 min
1 × 10 ⁻⁴ M	96%	–
5 × 10 ⁻⁵ M	87%	91%

^aReactions were heated at 70 °C in 10 mM sodium phosphate pH 7.4 buffer for the times indicated and then allowed to cool briefly before aliquots were removed for analysis by radio-HPLC using HPLC method 1 or 2.