Two is better than one: difunctional high-affinity PSMA probes based on a [CpM(CO)3] (M = Re/99mTc) scaffold

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Abstract: More than 10% of all men will be given the diagnosis “prostate cancer” during their lifetime. Most of the current radio-diagnostic vehicles involve both expensive and localized production with cyclotrons as well as the use of bulky chelators for the radiometal. We report the use of a new multifunctional cyclopentadiene (Cp) platform to prepare difunctional and monofunctional, PSMA-targeting rhenium and technetium-99m complexes. The Cp-complexes and the free ligands are prepared by straightforward functionalization with either one or two Lys-urea-Glu (LuG) PSMA binding motifs. Cell binding assays revealed that the difunctional rhenium complex displays a dissociation constant (KD = 2.1 nM) that is an order of magnitude lower than the monofunctional compound (KD = 24.2 nM). The 99mTc complexes can be prepared in one step and 15 min in high yields. These difunctional Cp-Re(I)/99mTc(I) complexes represent a new class of imaging agents with binding affinities comparable to clinically evaluated compounds. Additionally, this study demonstrates that the Cp-platform can readily be derivatized with amine-containing biomolecules. Extending this work to incorporate both targeting and therapeutic moieties could lead to theranostic systems with Re/99mTc.

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Two is Better than One: Difunctional High-affinity PSMA Probes Based on a [CpM(CO)$_3$] (M = Re/^{99m}Tc) Scaffold

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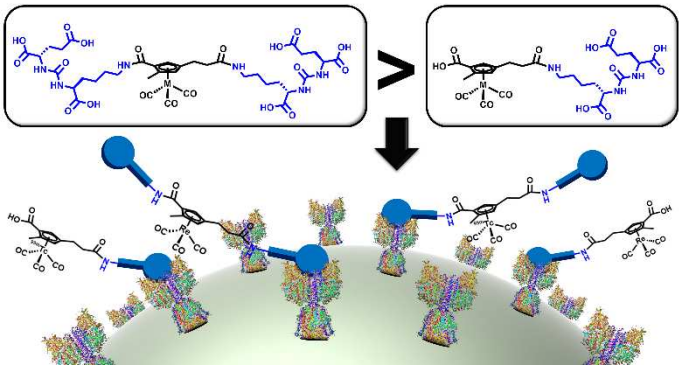


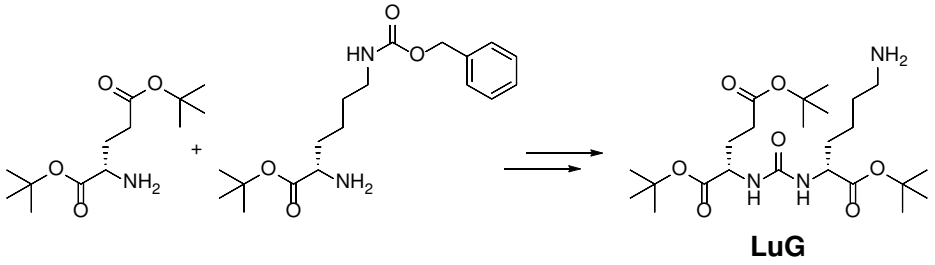
Abstract

More than 10% of all men will be given the diagnosis “prostate cancer” during their lifetime. Most of the current radio-diagnostic vehicles involve both expensive and localized production with cyclotrons as well as the use of bulky chelators for the radiometal. We report the use of a new multifunctional cyclopentadiene (Cp) platform to prepare difunctional and monofunctional, PSMA-targeting rhenium and technetium-99m complexes. The Cp-complexes and the free ligands are prepared by straightforward functionalization with either one or two Lys-urea-Glu (LuG) PSMA binding motifs. Cell binding assays revealed that the difunctional rhenium complex displays a dissociation constant (K_D = 2.1 nM) that is an order of magnitude lower than the monofunctional compound (K_D = 24.2 nM). The ^{99m}Tc complexes can be prepared in one step and ≤ 15 min in high yields. These difunctional Cp-Re(I)/^{99m}Tc(I) complexes represent a new class of imaging agents with binding affinities comparable to clinically evaluated compounds. Additionally, this study demonstrates that the Cp-platform can readily be derivatized with amine-containing biomolecules. Extending this work to incorporate both targeting and therapeutic moieties could lead to theranostic systems with Re/^{99m}Tc.

In the 21st century, one out of nine men will be diagnosed with prostate cancer during his lifetime. While current treatments have vastly increased the mean survival rate of patients, over 30'000 people are still expected to die from prostate cancer in 2019 in the US alone.[1] The most common cause of mortality in prostate cancer patients is the occurrence of metastases. A major factor in reducing the death toll of this disease is the improvement of early detection and monitoring techniques. The prostate specific membrane antigen (PSMA) is an extracellular receptor and a promising target for nuclear imaging as its overexpression is proportional to the stage and grade of tumor progression.[2] Many PSMA-binding imaging agents based on ^{11}C, ^{18}F, ^{64}Cu, ^{68}Ga, ^{86}Y, ^{89}Zr, ^{99m}Tc, ^{111}In, and various radionuclides of iodine have been reported.[3] The necessity and associated high cost of a cyclotron for the generation of most of these radionuclides limits their widespread availability. ^{68}Ga and ^{99m}Tc are exempt from this limitation as these two radionuclides can be delivered over large distances by means of relatively low-cost generators. New imaging agents based on both gallium and technetium chelators coupled to a high PSMA-affinity Lys-urea-Glu (LuG) motif are currently in advanced clinical trials.[4] Multifunctional radiotracers containing more than one binding unit have higher binding avidity to membrane-proteins. This has already been exploited with integrin $\alpha_v\beta_3$ protein-targeted radiotracers linked to cyclic arginine-glycine-aspartic (RGD) peptides.[5] Multiple studies found superior binding for difunctional RGD-chelates compared to their monofunctional counterparts.[6] Indeed, ^{99m}Tc-3PRGD$_2$ is a difunctional cyclic RGD-based imaging agent which is currently undergoing multiple phase I clinical trials.[7] In light of these promising results, it is surprising that little work has been done on multifunctional PSMA-targeting compounds. In 2012, Schäfer *et al.* reported a ^{68}Ga-based structure containing two LuG units that displayed improved PSMA affinity, higher cell uptake and prolonged cell surface retention time compared to the monofunctional analogue.[8] To the best of our knowledge, the only ^{99m}Tc-complexes conjugated to multiple PSMA-binding units were reported by Frangioni and coworkers in 2007. They prepared difunctional and trifunctional $^{99m}Tc(V)$ compounds based on a phosphinyl-containing PSMA-ligand. The bi- and trifunctional complexes showed significantly higher PSMA affinities than their

monofunctional counterpart.[9] These promising preliminary results illustrate that there is unexplored potential in multifunctional PSMA-targeted radiotracers.

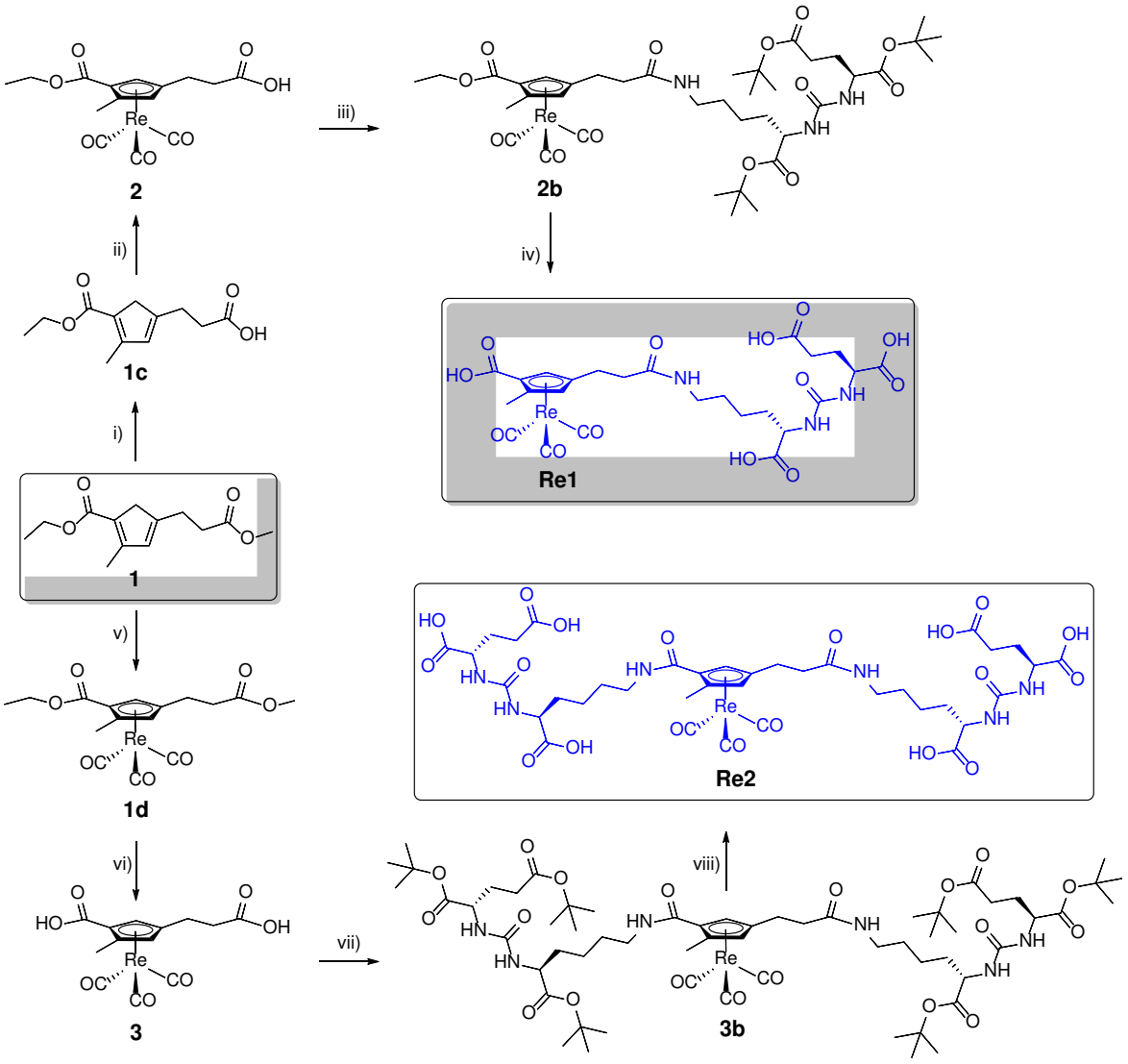
In this work, we describe the synthesis and evaluation of a compact Re/^{99m}Tc(I) system containing either one or two LuG units. In other reported PSMA-binding systems, the chelator is often as large or even larger as the LuG moiety. Cyclopentadiene (Cp) ligands are ideal chelators for the Re/^{99m}Tc(I) tricarbonyl system due to their small size, high stability of the formed complexes, and the possibility to functionalize them with one or more targeting units.[10] By using a multifunctional Cp as both the metal-coordinating moiety and as the linker to the two PSMA-ligands the usually large and bulky chelating part of the compound can be minimized to only a fraction of the resulting complex.



Scheme 1. Synthesis of **LuG**. Reaction conditions: i) triphosgene, triethylamine (NEt_3), dry CH_2Cl_2, 0 – 25 °C, 6 h, 65%. ii) H_2, Pd/C (10%), MeOH, 25 °C, 16 h, 96%.

In recent work, our group has prepared Cp-ligand **1** through a straightforward synthesis adapted from Hatanaka *et al.* and found that the respective Cp-piano stool type complexes of $[Re(CO)_3]^+$ and $[^{99m}Tc(CO)_3]^+$ could be prepared readily and in good yields. The two ester-groups of **1** are sufficiently different in reactivity on the free Cp to allow for step-wise hydrolysis and functionalization with amine-bearing functionalities.[11] The rhenium complexes **Re1** and **Re2** were prepared from compound **1** based on a synthetic strategy recently reported by our group. **LuG** was prepared according to a previously reported procedure and reacted with **2** and **3** using hydroxybenzotriazole (HOBt) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Scheme 1 and 2).[12] The resulting complexes (**2b** and **3b**, see Supporting Information) were purified by preparative HPLC. In the case of **3b**, the two diastereomers resulting from combining the planar chirality of the rhenium complex with the enantiomerically pure **LuG** could be separated and

characterized (**3b'** and **3b''**, Figure S9). In the case of **2b**, the diastereomers could not be separated but the ^{13}C NMR revealed two sets of peaks for all carbons, indicating their presence in a 1:1 ratio (Table S1). The *tert*-butyl protecting groups were cleaved with NaOH in MeOH under microwave (MW) conditions and further purification by preparative HPLC yielded pure **Re1** and **Re2**. Upon hydrolysis of **3b'** and **3b''**, the resulting compounds could not be differentiated anymore by either HPLC or NMR experiments (Figure S26 and S27). Hence, in a new batch, the diastereomers of **3b** were not separated and used as a mixture for the deprotection step. This yielded the final compound **Re2** as a mixture of diastereomers. The complexes were characterized by NMR spectroscopy and high-resolution mass spectrometry.



Scheme 2. Synthetic route to **Re1** and **Re2**. Reaction conditions: i) 0.5 eq NaOH, MeOH, MW, 120 °C, 15 min. ii) Re_2CO_{10}, N,N-dimethylformamide (DMF), MW, 220 °C, 15 min. iii) **LuG**, HOBt, EDC, N,N-diisopropylethylamine (DIPEA), DMF, 25 °C, 24 h, 30% (from **1**). iv) NaOH, MeOH, MW, 120 °C, 15 min, 76%. v) Re_2CO_{10}, *o*-Xylene, MW, 220 °C, 15 min, 76%. vi) NaOH, MeOH, MW, 120 °C, 15 min, 68%. vii) **LuG**, HOBt, EDC, DIPEA, DMF, 25 °C, 24 h, 31%. viii) NaOH, MeOH, MW, 120 °C, 15 min, 56%.

To determine the PSMA-binding affinity of **Re1** and **Re2**, LNCaP (PSMA-positive) and PC-3 (PSMA-negative) cells were incubated for 1 h with different concentrations of the rhenium complexes.[13] The cells were then washed, digested, and the total amount of Re was determined by inductively coupled plasma mass spectrometry (ICP-MS). Both compounds displayed binding affinities in the low nanomolar range for the LNCaP cells (Figure 1). With a K_D of 2.1 nM, the binding affinity of the difunctional complex **Re2** was 12 times higher than for the monofunctional **Re1** (24.2 nM) and on par with PSMA-binders currently in clinical trials.[14] In the PC-3 negative controls, no significant binding (< 0.05 ppb Re) was observed for both complexes at all tested concentrations. The tenfold higher non-specific binding (NS) observed for **Re1** as compared to **Re2** could stem from the difference in hydrophilicity of the two compounds. **Re2** comprises six carboxylate groups compared to only four on **Re1**. The difference in hydrophilicity is also evident from their different UPLC retention times (**Re1**: 2.35 min vs. **Re2**: 2.16 min). From the calculated B_{max} value and the known number of cells per sample an estimate of around 10^6 binding sites per LNCaP cell was obtained which is in good agreement with literature reports and supports the values obtained in our experiments.[15] Interestingly, the amount of rhenium detected at the highest incubation concentrations (B_{max}) for **Re1** was almost twice that of **Re2**. A possible explanation for this finding is that **Re2** is able to occupy two PSMA binding sites per rhenium atom, resulting in a lower amount of overall rhenium, while for **Re1**, only one rhenium atom is expected per one binding site.

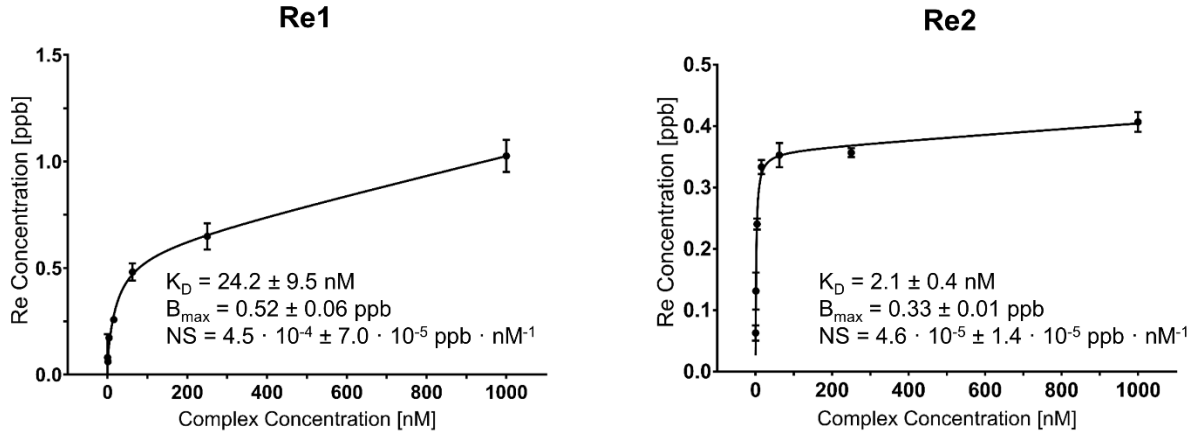
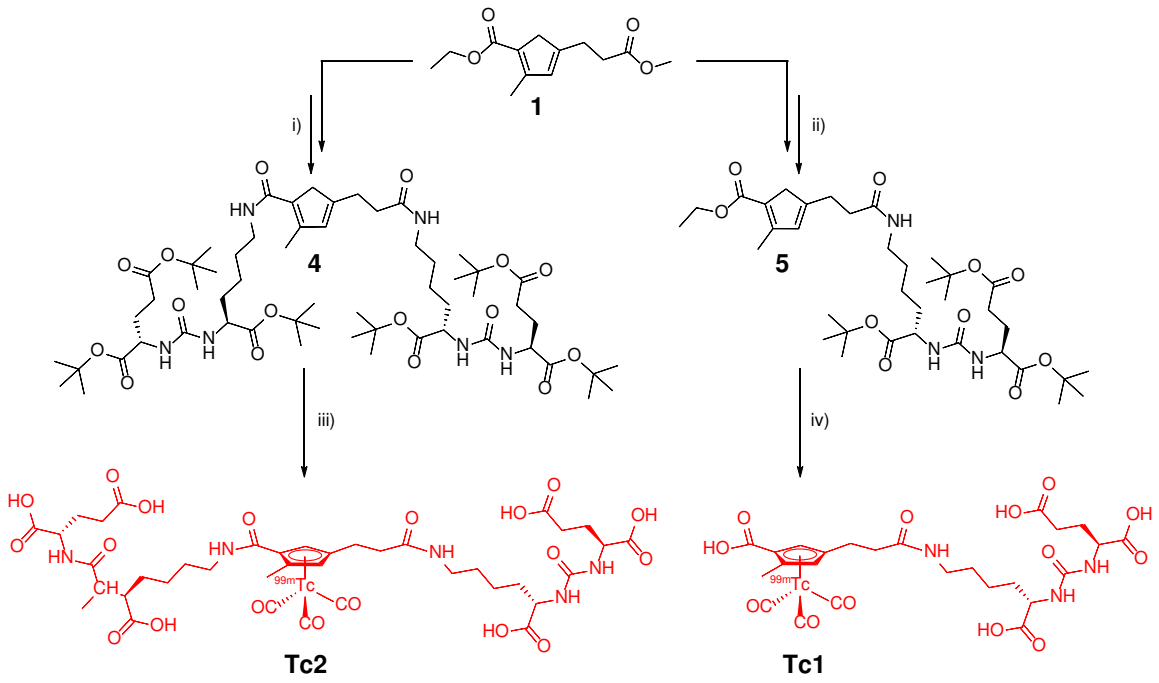


Figure 1. PSMA-binding affinity of complexes **Re1** and **Re2**. Binding affinities were obtained by fitting rhenium content of cell-samples incubated with different concentrations of complex. Each data point is the average of three different samples (error bars indicate standard deviation).

Having confirmed the ability of **Re1** and **Re2** to bind PSMA efficiently, the next step was to demonstrate that the corresponding ^{99m}Tc complexes can be prepared under radiolabeling conditions. Therefore, the free ligands **4** and **5** were prepared from **1** and **LuG** in two steps (Scheme 3). The pure ligands were then applied directly and without deprotection for radiolabeling with ^{99m}Tc. Reacting **4** and **5** (5 mM) with $[^{99m}Tc(OH_2)_3(CO)_3]^+$ at 140 °C in a microwave reactor in water at pH = 13 (adjusted with 1 M NaOH) led to formation of **Tc1** and **Tc2** presumably as diastereomeric mixtures (as with rhenium) within 15 min, as evidenced by HPLC co-injection with the respective rhenium complex (Figure 2, non-optimized radiochemical yields of crude reaction: 61% and 74% respectively). The main side-products were $[^{99m}TcO_4]^-$ and $[^{99m}Tc(OH_2)_3(CO)_3]^+$. The peak for **Tc2** was observed to be rather broad, which can partly be ascribed to the fact that the gamma detector will register radioactivity a little while before and after the compound enters the detector area. The shoulders of the peak could indicate that some tert-butyl protecting groups are still present in the crude, leading to peaks with slightly different retention times.



Scheme 3. Synthetic route to **Tc1** and **Tc2**. Reaction conditions: i) a) 0.93 eq. NaOH, MeOH, MW, 120 °C, 15 min. b) **LuG**, HOBt, EDC, DIPEA, DMF; 25 °C, 24 h, 17% (from **1**). ii) a) NaOH, MeOH, MW, 120 °C, 15 min. b) **LuG**, HOBt, EDC, DIPEA, DMF; 25 °C, 24 h, 15% (from **1**). iii) $[^{99m}Tc(OH_2)_3(CO)_3]^+$, H_2O (pH = 13), MW, 140 °C, 15 min. iv) $[^{99m}Tc(OH_2)_3(CO)_3]^+$, H_2O (pH = 13), MW, 140 °C, 10 min.

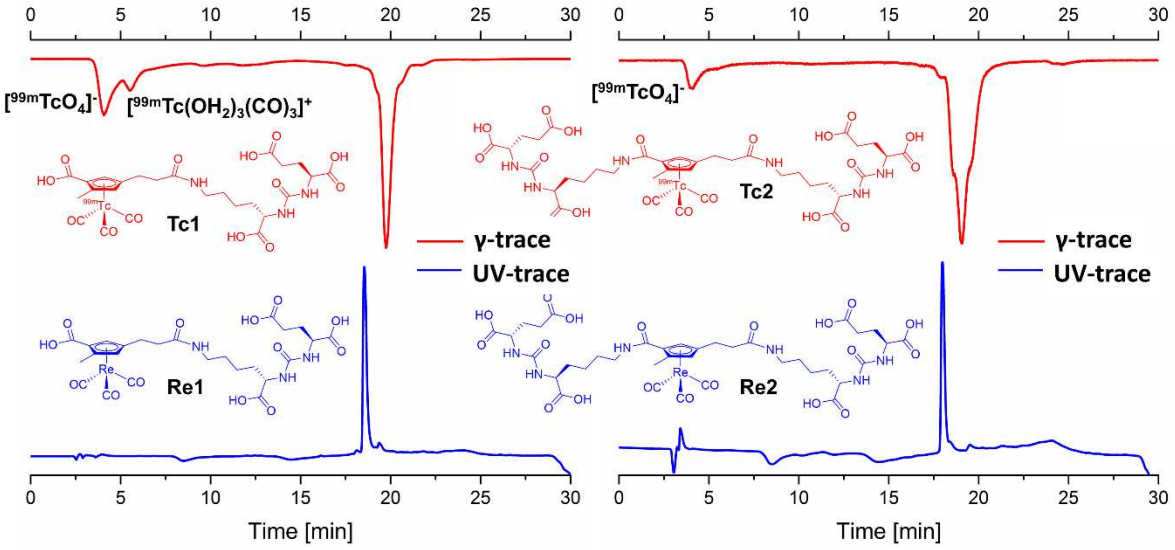


Figure 2. UV (254 nm) and γ-traces of the co-injection of the rhenium compounds **Re1** and **Re2** with the ^{99m}Tc compounds **Tc1** and **Tc2** respectively. Time differences are due to detector separation.

This work demonstrates that our previously developed cyclopentadiene-system lends itself to easy and straightforward functionalization with biomolecules. The coupling reactions proceed with good overall yields and the products are readily purified by preparative HPLC. Importantly, the synthesis of the respective ^{99m}Tc complexes is also possible from highly pre-functionalized Cp-ligands, in one step and in 15 min or less. The successful conjugation of two targeting units to our platform opens up the possibility to attach both a targeting and a cytotoxic moiety to the complex, which could lead to targeted chemotherapeutic compounds. In conclusion, this proof-of-concept illustrates the potential of multifunctional Cp-ligands in radiotracer design, and encourages their application with further targeting and bioactive modalities.

Acknowledgements

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Experimental Section

Materials. All chemicals were of reagent-grade quality or higher and were obtained from commercial suppliers. Solvents were used as received or dried over molecular sieves. Sodium boranocarbonate was a gift from Mallinckrodt Medical B.V. (The Netherlands). Na[$^{99m}TcO_4$] in 0.9% saline was eluted from a $^{99}Mo/^{99m}Tc$ UTK FM generator purchased from Mallinckrodt Medical B.V. (The Netherlands)

Instrumentation and methods. ^{1}H and ^{13}C NMR spectra were recorded in deuterated solvents on a Bruker DRX 400 (^{1}H: 400 MHz, ^{13}C: 100.6 MHz) or DRX 500 (^{1}H: 500 MHz ^{13}C: 125.8 MHz) MHz spectrometer at 300 K. The chemical shifts, δ, are reported in ppm (parts per million) relative to residual solvent peaks. The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), br (broad). Microwave reactions were performed using a Biotage Initiator+ Robot Eight or Anton Paar (AP) Monowave 200 instrument. UPLC-ESI-MS was performed on a Waters Acquity UPLC System coupled to a Bruker HCT™, using an Acquity UPLC BEH C18 1.7µm (2.1 x 50 mm) column. UPLC solvents were formic acid (0.1% in millipore water) (solvent A) and acetonitrile UPLC grade (solvent B). The temperature was regulated with a Peltier thermostatic system to the specified temperatures. High-resolution mass spectrometry (HR-MS) was performed on a Thermo DFS double-focusing system (ThermoFisher Scientific, Germany). Preparative HPLC was performed on a Varian ProStar 320 system, using a Dr. Maisch Reprosil C18 100-7 (40 x 250 mm) column (flow rate: 40.0 ml/min) or a LiChroCART RP-18e (10 x 250 mm) column. HPLC solvents were 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B), HPLC grade. Analytical HPLC was performed on a Merck Hitachi L7000 system, equipped with a L-7400 UV-detector and an in-line radio-detector Berthold FlowStar LB513, using an analytical Macherey-Nagel Nucleosil C18 5 μm (4.6 × 250 mm) column. Analytical UPLC was performed on a VWR Hitachi Chrommaster Ultra, using an Acquity UPLC BEH C18 1.7 µm (2.1 x 50 mm) column. UPLC solvents were trifluoroacetic acid (0.1% in Millipore water) (solvent A) and acetonitrile UPLC grade (solvent B).

HPLC/UPLC Methods

UPLC-MS. The UPLC-MS runs (flow rate 0.6 mL/min) were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC-grade)) and B (distilled water containing 0.1 % formic acid): *t* = 0–0.5 min, 5 % A; *t* = 4.0 min, 100 % A; *t* = 5.0 min, 100% A.

UPLC (U1). The UPLC runs (flow rate 0.5 mL/min) were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC-grade)) and B (distilled water containing 0.1% TFA): *t* = 0–0.5 min, 5% A; *t* = 4.0 min, 100% A; *t* = 5.0 min, 100% A.

HPLC. The HPLC runs (flow rate 0.5 mL/min) were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC-grade)) and B (distilled water containing 0.1 % TFA): *t* = 0–2.1 min, 10% A; *t* = 25 min, 100% A; *t* = 35 min, 100% A; *t* = 37 min, 10% A; *t* = 40 min, 10% A.

radioHPLC. The radioHPLC runs (flow rate 0.5 mL/min) using a gradient of A (MeOH (Sigma-Aldrich HPLC-grade)) and B (distilled water containing 0.1 % TFA t = 0 – 3 min: 10% A; 3 – 3.1 min: 0 – 25% A; 3.1 – 9 min: 25% A; 9 – 9.1 min: 25 – 34% A; 9.1 – 18 min: 34 - 100% A; 18 – 25 min: 100% A, 25 – 25.1 min: 100 - 10% A; 25.1 – 30 min: 10% MeOH. Comparison of the HPLC retention times for the ^{99m}Tc compounds with the corresponding ^{99}Tc and Re compound confirms identity.

Preparative HPLC (Method A). The preparative HPLC runs (flow rate 40 mL/min) were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC-grade)) and B (distilled water containing 0.1% TFA): *t* = 0–2.1 min, 10% A; *t* = 25 min, 100% A; *t* = 40 min, 100% A.

^{99m}Tc experiments. Caution! ^{99m}Tc is a *γ*-emitter (E= 140 keV, $t_{1/2}$= 6.02 h) which should only be handled by trained personnel in a licensed and appropriately shielded facility. Sodium boranocarbonate releases CO gas which is highly toxic, it is recommended to be handled only in ventilated hoods.

Cell culture

Cell lines were obtained from American Type Culture Collection (ATCC). LNCaP cells were maintained in RPMI 1640 medium without phenol red (Gibco) at 37 °C, 5% CO_2. PC-3 cells were maintained in DMEM/F12 medium (Gibco) at 37 °C, 5% CO_2. Cell culture

media were supplemented with 10% fetal bovine serum (Gibco) and penicillin-streptomycin (Invitrogen).

Cell binding assay

Cells were harvested at 70% confluence, washed once with PBS and resuspended in full medium. For cell binding assays, aliquots of $5 \cdot 10^6$ cells were incubated with the indicated concentrations of the complexes in 5 ml medium for 1 hour at 4 °C. Thereafter, cells were collected by centrifugation at 100 g and 4 °C for 10 min, and washed three times with ice-cold PBS.

ICP-MS sample preparation.

Cell pellets were digested in 150 µL ultrapure HNO_3 (60%) at 80 °C overnight, supplemented with 60 µLNH_4F (1.011 M) filtered and diluted with Millipore water to a final volume of 3 ml.

ICP-MS measurements

Rhenium was measured against a Re single element standard (Merck 170344.100) and verified by a control (Agilent5188-6524 PA Tuning 2). The rhenium content of the samples was determined by means of 6 calibration standards that were produced in the range between 0 and 100 ppb in Re ($R^2 > 0.99$) with a background equivalent concentration of BEC: 9.4 ppt and a detection limit of DL: 2.6 ppt. The isotope ^{185}Re (37.4% abundance) ^{187}Re (62.6% abundance) was evaluated in “no-gas” mode and He-gas mode. Spiking the samples with untreated negative controls (to account for potential carbon content from the biological samples) resulted in equivalent values within error ranges. A solution containing indium (100 ppb) was used as internal standard. The results are expressed as ppb Re as means ± standard deviations of three experiments.

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