Long-chain rhenium and technetium glucosamine conjugates†

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A series of five glucosamine-conjugated organometallic complexes of the tricarbonyl cores of technetium-99m and rhenium were made. Glucosamine was derivatized at the *C*-2 nitrogen with long chain alkyl spacers linked to either pyridyl-*tert*-nitrogen-phenol tridentate chelates or cyclopentadienyl ligating groups. The metal complexes of the tridentate ligands were formed by refluxing with $[Re(CO)_3(H_2O)_3]Br$, or with a base and $[^{99m}Tc(CO)_3(H_2O)_3]^+$. These ligands were found to be competent chelates in binding the $[^{99m}Tc(CO)_3]^+$ core as radiolabeling yields ranged from 87 to 93% and the resulting complexes are stable to cysteine and histidine challenges for 24 h. The cyclopentadienyl analogues were formed using a double ligand transfer reaction for the rhenium complexes and a single ligand transfer for the technetium-99m complexes. All five rhenium complexes were tested as substrates of hexokinase; two of these complexes were tested as hexokinase inhibitors and they were found to be competent inhibitors, suggesting that they may be able to interact with hexokinase. MTT cytotoxicity studies were performed and the complexes tested were found to be non-toxic to the concentrations tested (100 μM or 1 mM). GLUT-1 mediated cell uptake studies were performed on all five technetium-99m complexes, and their cell entry was found to parallel their lipophilicities, suggesting that cellular uptake is by passive diffusion and is not mediated by GLUT-1.

Introduction

In recent years, radiolabeled glucose analogues have attracted attention due to the successful clinical application of [^{18}F]-FDG (see Fig. 1). This has inspired research aimed at developing SPECT carbohydrate analogues to take advantage of the nuclide ^{99m}Tc.[1–6] ^{99m}Tc is the most commonly used isotope in nuclear medicine,[7] partly due to its near ideal physical properties; $t_{1/2}$ = 6 h, γ = 140 keV. These properties are complemented by its production in a ^{99}Mo generator, making it low cost, easily transportable, and normally available wherever there is a SPECT scanner. Technetium does not have any non-radioactive isotopes, so its third row congener, rhenium, is used to optimize cold chemistry and perform standard characterization techniques.

A kit preparation (Isolink™ by Covidien) of the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core pioneered by Alberto *et al.*[8] has sparked great interest in researching new SPECT radiotracers of this radionuclide core. The $[^{99m}Tc(CO)_3]^+$ core is attractive because it is small, kinetically inert, stable to oxidation, and amenable to chelation by several types of ligating atom. The tricarbonyl core has been used in many bioconjugates, including peptides,[9–11] nucleotides,[12] lipids,[13] and small biomolecules such as biotin.[14] There has also been interest in labeling carbohydrate complexes, though none of the ^{99m}Tc-carbohydrate complexes reported to date have been successfully developed into radiopharmaceuticals.

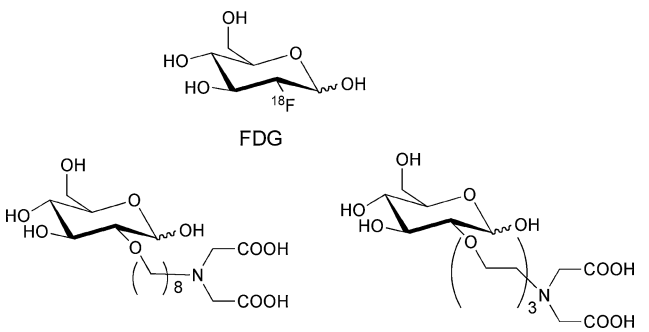

Fig. 1 FDG (above); long-chain glucose bioconjugates synthesized and coordinated as tridentate NO_2 ligands to the tricarbonyl core.[22]

Tracers of glucose metabolism need to have biological activity with key enzymes such as the GLUT family of glucose transporters, and hexokinase. The GLUTs are transmembrane proteins that facilitate transport of carbohydrates across lipid membranes.[15] Hexokinase catalyzes the transfer of a phosphate group from ATP to substrate, giving a negatively charged compound that cannot diffuse out of the cell. Tumor cells have increased energy requirements, and therefore higher amounts of proteins such as GLUT-1 and hexokinase compared to healthy tissue.[16] In order to image glucose metabolism, a compound should be recognized as a carbohydrate at least to the point where it gets into cells (GLUT)

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† Electronic supplementary information (ESI) available: Experimental details and characterisation of all organic compounds and precursors (**2–7** and **10–13**), and selected ^{1}H and ^{13}C NMR spectra. See DOI: 10.1039/b914309b

and gets trapped there (hexokinase), developing a concentration differential in different cell types.

Since the first organometallic carbohydrate containing complexes of group 7 metals were reported earlier this decade,[17] most work on $[^{99m}Tc(CO)_3]^+$ carbohydrate conjugates has focused on compounds with a short linker between the carbohydrate and the metal binding sphere.[18–21] Most of these studies have not reported on the interactions between the compounds synthesized and GLUT and/or hexokinase. Schibli and coworkers observed hexokinase inhibition when using glycoconjugates with a long alkyl linker between the carbohydrate and the $[M(CO)_3]^+$ core.[22] They found that the *C*-2 functionalized glucose derivatives with long alkyl and poly(ethylene glycol) (PEG) linkers (Fig. 1) exhibited hexokinase inhibition, $K_i = 0.25–5.8\ \mu M$.[22] None of the shorter chain compounds tested were found to exhibit significant inhibition of hexokinase.[22] *In silico* modeling showed that the linker length required to distance the bulky metal binding sphere such that it does not interfere with the hexokinase cleft closing was seven methylene units.[22]

We are interested in glucosamine-based carbohydrate conjugates due to the higher tolerances in structural variation of glucosamine analogues compared to glucose analogues exhibited by hexokinase and GLUTs.[23,24] The combination of this sugar with the benefits of a long linker chain may provide a viable carbohydrate-based imaging agent.

Previously, we have studied short chain glucosamine-conjugates of the $[M(CO)_3]^+$ core, combined with bidentate, tridentate and Cp-based ligands.[2,3,5] We have reported the successful incorporation of a phenolate as a monoanionic donor species into the stable N_2O binding sphere of tricarbonyl complexes,[25] and, recently, others have also used this group.[26] The use of a lipophilic oxygen donor such as a phenolate may impart beneficial properties in terms of overall lipophilicity or π-stacking ability to the chelating sphere that are not available with use of the more common acid group. Thus this work incorporates a phenolate chelating arm linked with a long linker to a glucosamine, probing for synergistic effects between three potentially beneficial functionalities.

Cyclopentadienyl (Cp^-) ligands bind well to the $[M(CO)_3]^+$ (M = ^{99m}Tc, Re) core *via* a η^5 coordination mode to give neutral, 18-electron organometallic complexes.[27] The $Cp^{99m}Tc$ core is attractive in its small size, stability and lipophilicity, and for these reasons has been used to label a variety of biomolecules.[28–32] We have previously reported the synthesis of two glucosamine appended Cp ligands and their Re and ^{99m}Tc tricarbonyl complexes (Fig. 2).[5] These compounds were found to inhibit the phosphorylation of glucose by hexokinase, with the most efficacious (Fig. 2, R = H, M = Re) having $K_i = 330 \pm 70\ \mu M$.[5] Extending the linker may improve the affinity of these compounds for both hexokinase and glucose transporters.

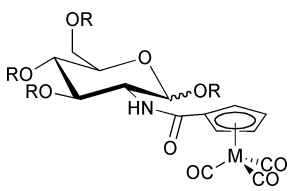


Fig. 2 Glucosamine appended Cp ligands for the tricarbonyl core (R = H, OAc; M = ^{99m}Tc, Re).[5]

Herein we present the synthesis and characterization of a series of organometallic technetium- and rhenium-tricarbonyl complexes of glucosamine, derivatized at *C*-2 with long alkyl spacers linked to either pyridyl-*tert*-nitrogen-phenol or cyclopentadienyl chelating systems. The rhenium(I) complexes were assessed as substrates for hexokinase, and select compounds were assayed for competitive inhibition of hexokinase and cell toxicity. The corresponding ^{99m}Tc complexes were assessed for GLUT-1 mediated cell uptake, and the tridentate compounds were tested for *in vitro* stability.

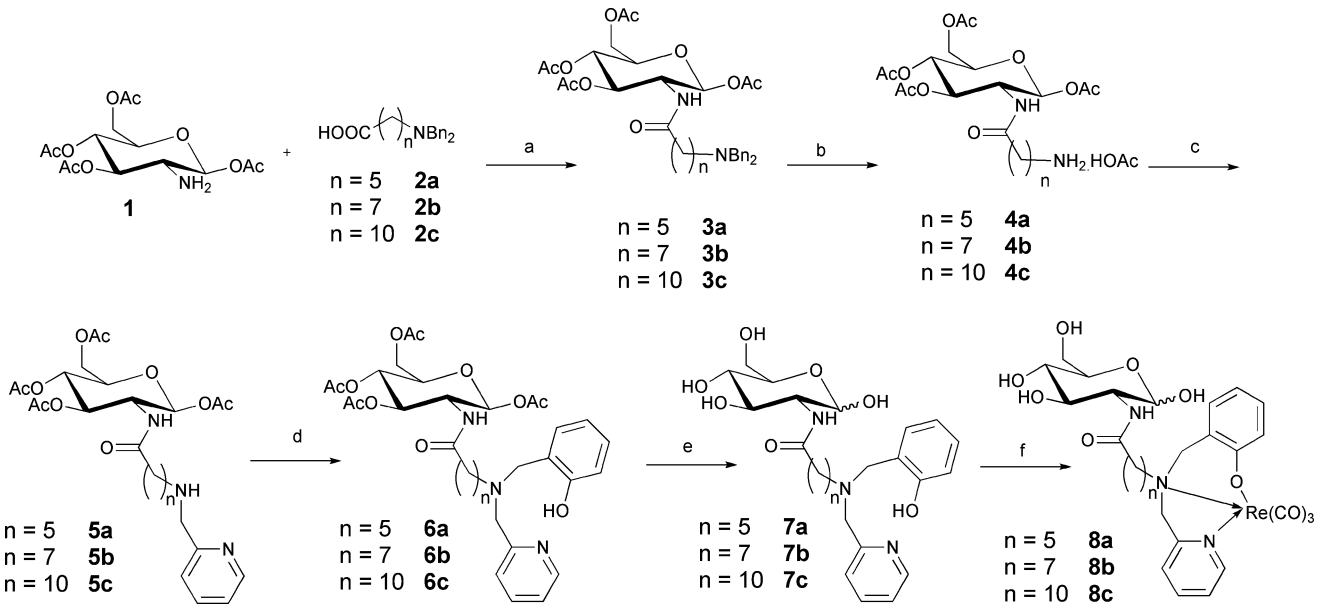
Results and discussion

Synthesis and characterization

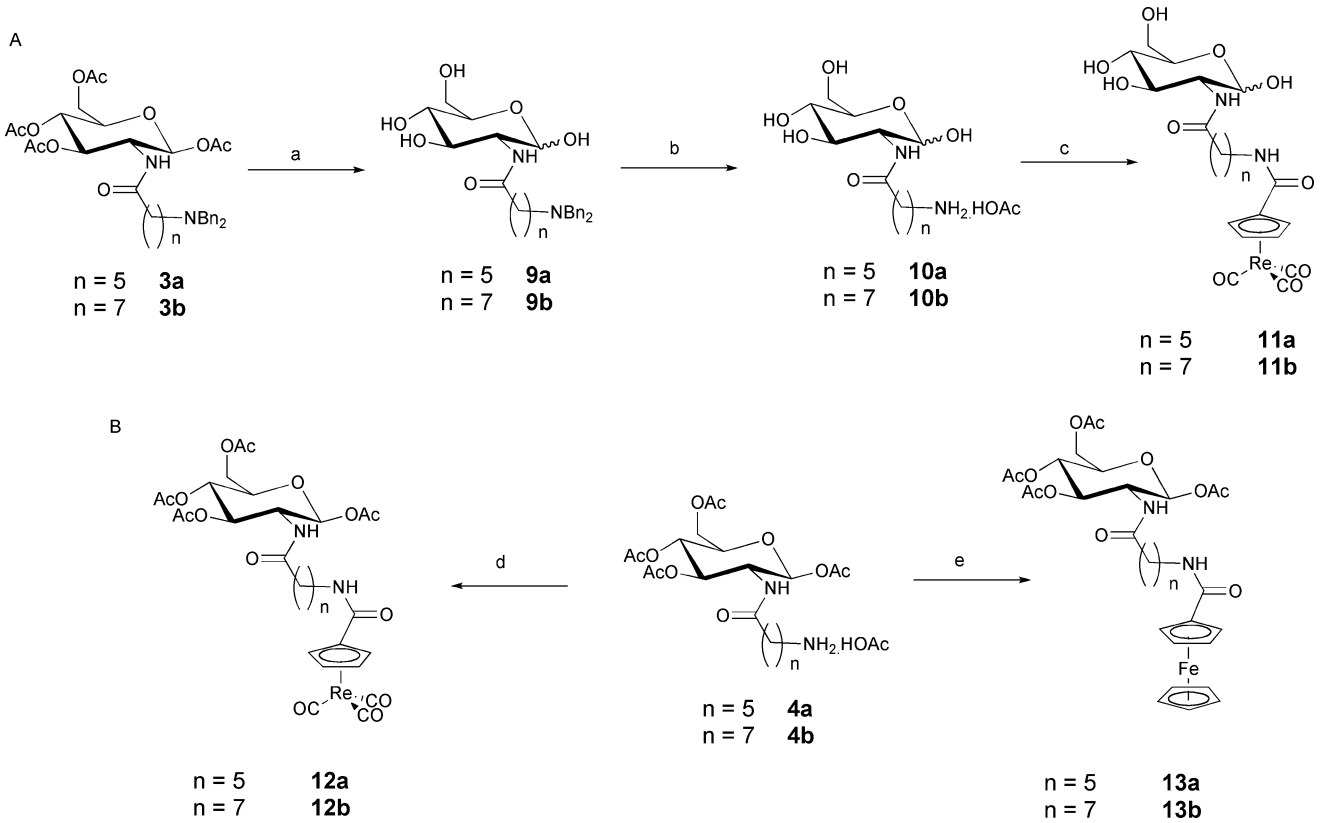
The synthetic sequence for making the ligands is shown in Scheme 1. The tetraacetylated glucosamine starting material **1** was made, as previously[33] reported, in four steps from commercially available glucosamine. N-Benzyl-protected amino-acids **2** were synthesized from the corresponding amino-acid under basic conditions in the presence of one equivalent of benzyl bromide, in a manner similar to that previously published.[34] The previous procedure produced a solid, whereas the compounds made here were oils, and thus were purified by column chromatography rather than precipitation. An EDC-mediated amide coupling reaction produced **3**, and removal of the benzyl protecting groups using $Pd(OH)_2$ on carbon with H_2 gas in glacial acetic acid gave **4**. The two chelating arms were then added sequentially, the first with a $NaBH_4$ mediated reductive amination to give **5**, then, following purification, a $NaBH(OAc)_3$ mediated reductive amination to yield **6**. The acetyl groups were removed with NaOMe in methanol to yield the deprotected proligands **7**. These reactions all occurred more slowly than the equivalent reactions in short-chain compounds described in the accompanying paper.[35] Proligands **7** were refluxed in methanol with equimolar amounts of $[Re(CO)_3(H_2O)_3]Br$, to give the corresponding tridentate rhenium complexes, **8**, in good yield after six hours. Proligands **7** could alternatively be stirred with NaOEt in ethanol before being refluxed with an aqueous solution of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ for 30 min to produce the corresponding ^{9m}Tc complexes **9** in good yields (Scheme 3A).

The rhenium-Cp compounds **11** and **12** were made indirectly, as shown in Scheme 2. A double ligand transfer (DLT) reaction was used to synthesize the rhenium starting material: $[HO_2CC_5H_4Re(CO)_3]$, following a literature procedure.[36] This was followed by an amide coupling to the appropriate sugar-linker compound (protected **4**, or deprotected **10**) to give the functionalized metal complexes **11** and **12**, respectively (Scheme 2). The carbohydrate-substituted ferrocenes **13**, were prepared as substrates for the single ligand transfer (SLT) reaction to access the $[Cp^{99m}Tc(CO)_3]$ complexes (see Scheme 3B). These ferrocene derivatives were made by an amide coupling of the sugar-linker compounds' N-terminus to the acid group of ferrocenecarboxylic acid (Scheme 2B).

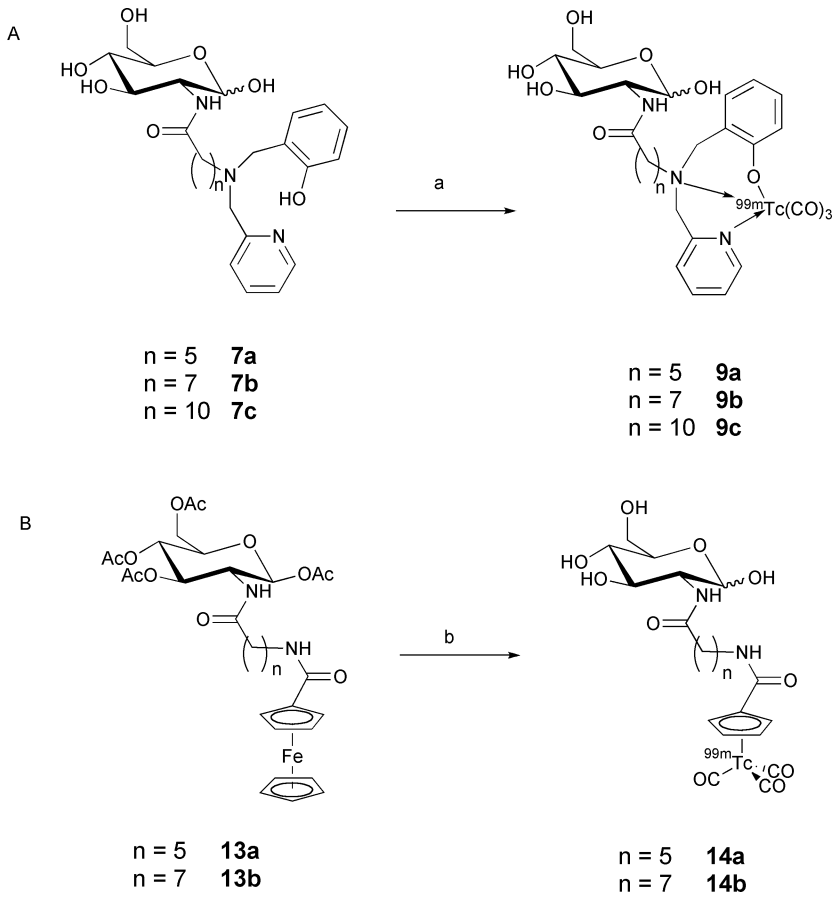
^{1}H and ^{13}C NMR spectroscopy, IR spectroscopy, mass spectrometry and elemental analysis were used to characterize the compounds as appropriate. As some of the compounds were not very stable, they were used directly in the next step of the reaction sequence following mass spectrometric and/or ^{1}H NMR spectroscopic analysis. The final ligands and rhenium complexes and most



Scheme 1 a) EDC·HCl, HOBt·H_2O, DMAP, DMF; b) $Pd(OH)_2/C$, H_2, glacial acetic acid; c) dichloroethane, 2-pyridinecarboxyldehyde, $NaBH_4$; d) salicylaldehyde, Na_2CO_3, CH_2Cl_2, then $NaBH(OAc)_3$; e) NaOMe, MeOH; f) $[Re(CO)_3(H_2O)_3]Br$, MeOH, reflux. Reaction times and yields vary with the length of the chain, n.



Scheme 2 a) NaOMe, methanol; b) $Pd(OH)_2/C$, H_2, glacial acetic acid; c) DCC, $[HO_2CC_5H_4Re(CO)_3]$, dry DCM, DMF; d) EDC, $[HO_2CC_5H_4Re(CO)_3]$, dry DCM; e) EDC, ferrocene carboxylic acid, dry DCM. Reaction times and yields vary with the length of the chain, n.



Scheme 3 a) NaOEt, EtOH:H_2O, $[^{99m}Tc(CO)_3(H_2O)_3]^+$, reflux, 40 min; b) H_2O:DMSO, $[^{99m}Tc(CO)_3(H_2O)_3]^+$, reflux, 2 h.

of the intermediate compounds were thoroughly characterized, and the spectra were all in accordance with the predicted structures (see ESI†).

Metal ion coordination of the tridentate ligands could be verified by shifts in the peaks in both the ^1H and ^{13}C NMR spectra. For example, the aromatic proton and carbon atoms of the tridentate ligand **7c** shifted considerably upon binding to the rhenium tricarbonyl core. The proton with the highest chemical shift moves 0.21 ppm downfield upon metal coordination, while one other aromatic proton is unchanged and the remaining six are shifted upfield by between 0.02 and 0.29 ppm. The carbohydrate protons shift very little, confirming the pendant nature of the carbohydrate moiety in solution. In the ^{13}C NMR spectra the appearance of two peaks around 197 ppm indicates the presence of the Re-CO group, and the largest shifts are again seen in the aromatic residues. The diagnostic isotope pattern of the rhenium in the mass spectrometer could also be used to confirm the presence of this metal, for example in the C_6 compound **8a** the ESI+ mass spectrum shows a peak with 100% intensity at 760 and a peak with ~60% intensity at 762, corresponding to the ^{185}Re and ^{187}Re isotopes, respectively.

Similar results were found for the Cp compounds. The ^1H and ^{13}C NMR spectra showed shifts in the Cp ring that was bound to the metal ion, with the shift being very different for the two metal ions Fe^{2+} and Re^+. As for the tridentate compounds, all metal complexes showed very small or no shifts in the carbohydrate residues, showing the lack of interaction between these portions of the molecules and the metal ions in solution. High resolution mass spectra pointed to the molecular composition being as expected, and the isotopic ratios of peaks in the metal complexes were diagnostic for either Re or Fe. Infrared spectroscopy was also used to confirm the presence of key functional groups.

^{99m}Tc Labeling

As the tridentate ligands, **7**, require deprotonation, a base was necessary for the radiolabeling to proceed on a radiochemically appropriate timeframe. The rhenium complexes could be formed without base by refluxing each ligand with the rhenium tricarbonyl precursor in methanol overnight, but this is not practical when dealing with technetium, its six hour half life, and the ensconced technology. The NaOEt/EtOH method of deprotonation outlined in the experimental section had worked for similar phenolate compounds,[25] was applied here, and gave good results. The proligand was dissolved in ethanol and three equivalents of NaOEt were added. This mixture was purged with nitrogen for 10 min before addition of $[^{99m}Tc(CO)_3(H_2O)_3]^+$. The reaction mixture was heated at 80 °C for 40 min (Scheme 3), giving consistently good radiochemical yields (87–93%). These three ligands all possessed the same tridentate binding sphere, and labeling yields were largely independent of the chain length (Table 1).

A C_{18} HPLC column was used to analyze the radiolabeling, and under these conditions the retention times (RTs) increased with the length of the linker between the glucosamine and the metal

Table 1 ^{99m}Tc Labeling and stability studies of tridentate glucosamine-based ligands

Ligand	RT (Re compound)[a]	RT (^{99m}Tc compound)[a]	Radiochemical yield	Stability in cysteine—24 h	Stability in histidine—24 h
7a	18.8 min	17.9 min	87%	99 ± 2%	95 ± 4%
7b	20.8 min	21.7 min	93%	100 ± 3%	99 ± 3%
7c	22.0 min	22.2 min	93%	100 ± 3%	93 ± 3%

[a] HPLC conditions—100% H_2O (with 0.1% TFA) linear gradient to 100% ACN at 30 min.

binding sphere (Table 1). There are small discrepancies between the retention times of analogous technetium and rhenium complexes, as the detectors for the two compounds (radiation and UV-vis, respectively) are connected in series.

The radiolabeling reaction to make the $[Cp^{99m}Tc(CO)_3]$ complexes, **14**, required slightly longer reaction times; however, they were still reasonable given the 6 h half life of ^{99m}Tc. The aqueous solution of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ was neutralized to pH 7 before addition to the ferrocene compound to begin the SLT reaction. HPLC analysis showed that the acetyl protecting groups on the glucosamine were removed during the reaction (Scheme 3B), as a major component of the reaction mixture had a RT coinciding with that of the deprotected rhenium standard. Further evidence of the *in situ* deacetylation was seen in that reaction with NaOMe in MeOH did not change the HPLC trace of either reaction mixture, as would be expected if there were acetyl groups present.

The radiation traces of these reaction mixtures show product, unreacted starting material and very small amounts of other compounds. Although there is still ^{99m}Tc starting material present after 2 h, longer reaction times led to the appearance of more peaks and decreased yields of the desired products. Others have also seen the benefit of protection of hydroxyl groups in reactions of this type, with hydroxyl acetylation increasing the yield from 12 to 41% in a similar SLT reaction.[32]

Stability

The stability of the three tridentate technetium complexes, **9a**,**b** and **c**, was challenged with 100 fold excess concentration of cysteine and histidine. As these compounds share the same tridentate binding moiety their stabilities were expected to be very similar. This was found to be true, with all three ligands binding tightly to ^{99m}Tc for 24 h in the presence of large excesses of competing amino acids (Table 1).

GLUT-1 Cell uptake assay

The technetium compounds **9** and **14** showed modest cellular uptakes, which did not change in the presence of glucose, suggesting that the cellular uptake observed is not due to the same facilitated mechanisms that are used to transport glucose. As can be seen in Fig. 3, the uptake of the compounds parallels their lipophilicity, with the C_{11} compound **9c** exhibiting the highest uptake and the C_6 compounds **9a** and **14a** the lowest. Fig. 3 also shows that addition of a large excess of glucose does not significantly affect the cellular uptake of any of the test compounds. These data together suggest that the observed uptake is due to passive diffusion through the lipid bilayer, where more lipophilic compounds can diffuse more easily.

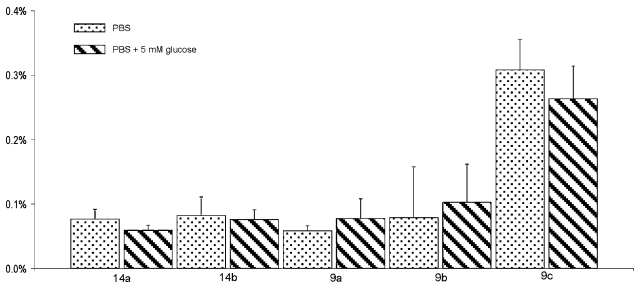


Fig. 3 Percentage of activity taken up into LCC6-HER2 cells during a 30 min incubation, each compound was tested in glucose free media (dotted bars) and in 5 mM glucose (striped bars) (n = 4, error bars show standard deviation).

Similar uptake tests were performed by Schibli and coworkers on the long-chain glucose based compounds shown in Fig. 1, in HT29 cells, a human colon cancer cell line also known to overexpress GLUT-1.[22] The uptakes they found for their long chain glucose compounds are similar to those found in our glucosamine compounds, both exhibiting cell uptakes of 0.1–0.2% of the total activity after a 30 min incubation.[22] It seems unlikely that there is a significant difference between the GLUT-1 interactions of glucose and glucosamine substituted bioconjugates of this type.

Hexokinase inhibition studies

Hexokinase is the first enzyme in the oxidative phosphorylation chain, and is responsible for transferring a phosphate group from ATP to the *C*-6 position of a hexose.[37] A convenient way to quantitate this process is to couple this phosphorylation to the next step in the oxidative phosphorylation chain where glucose-6-phosphate dehydrogenase dehydrates glucose-6-phosphate and, in the process, converts NAD^+ into NADH.[38] NADH absorbs strongly at 340 nm, and as the hexokinase catalyzed reaction is the rate determining step, this can be used to monitor the rate of substrate phosphorylation in the presence and absence of inhibitory compounds.

The C_6 and C_8 tridentate ligands and complexes, **7a**, **7b**, **8a**, and **8b** that were tested all showed interaction with hexokinase: **7a** K_i = 220 ± 30 μM, **8a** K_i = 500 ± 60 μM, **7b** K_i = 210 ± 40 μM, **8b** K_i = 70 ± 20 μM. The two proligands **7a** and **b** have similar K_i values, while **8b** has a stronger interaction and **8a** a weaker interaction with hexokinase. One possible reason **8b** exhibits a stronger interaction with hexokinase than **7b** is that by coordinating the metal ion the chelating portion of the ligand becomes more ordered. The chelating arms no longer have the ability to rotate freely as they do in the ligand, so the metal complex may end up taking up less space. The reverse is seen

for the C_6 compounds where the ligand **7a** displays a stronger interaction with the enzyme than does the metal complex **8a**. In the C_6 compounds the metal binding moiety is closer to the active site than in the equivalent C_8 compound, therefore making the complex bulkier would have a greater effect. It may also be that one of the compounds has a functional group in place to specifically interact with a residue on hexokinase, either in a favorable or an unfavorable manner. Despite some minor differences between the test compounds, they all displayed μM inhibition constants with hexokinase. For comparison, FDG has an inhibition constant for hexokinase of 1060 μM in this assay.[5] The K_i values of these compounds suggest that they may interact with hexokinase.

Schibli and coworkers investigated the hexokinase inhibition properties of their glucose-based long chain conjugates and found similar results.[22] They tested a range of compounds in their assay, and found that the two long chain analogues (Fig. 1) were the only ones that exhibited any appreciable inhibition of hexokinase. The PEGylated compound had $K_i = 250$ μM and the C_8 compound $K_i = 5800$ μM.[22] The addition of two oxygen atoms into the alkyl linker chain of the PEG changes the affinity for hexokinase by more than an order of magnitude. The affinities of the long chain glucose and glucosamine analogues for hexokinase are very similar, though the glucosamine compounds may interact more strongly. Comparing the K_i values of the non-PEGylated glucose rhenium complex with that of **8b**, the glucosamine analogue has an interaction with hexokinase that is nearly two orders of magnitude stronger than the glucose compound ($K_i = 70$ *vs.* 5800 μM), showing the potential utility of the glucosamine-based approach.

Hexokinase substrate studies

Four rhenium compounds were tested in this assay; **8a**, **8b**, **11a** and **11b**, as solubility constraints prevented the testing of the C_{11} compound **8c**. Unfortunately none of the compounds tested showed any significant phosphorylation by hexokinase, as measured by ADP production in an HPLC assay. As there was no significant ADP production above the hydrolysis levels of the control samples, these results suggest that no appreciable phosphorylation is occurring. For a compound to be a substrate of hexokinase, the cleft needs to close around the molecule in the active site so that the phosphate group can transfer, and it seems that this cannot occur with these compounds.

MTT Assay

The C_6 and C_8 tridentate ligands, **7a** and **7b**, and rhenium complexes, **8a** and **8b**, were tested in an MTT cell viability assay.[39] 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is metabolized by mitochondrial reductase in living cells to formazan, which gives a purple colour that indicates cell viability and can be quantified by measuring absorbance at 570 nm (A_{570}). None of the ligands or rhenium complexes tested were found to exhibit any toxicity up to the maximum concentrations tested (1 mM for **7a**, **7b** and **8a** and 100 μM for **8b** due to solubility constraints). The typical amount of a ^{99m}Tc complex injected into a patient is 800 MBq, ~45 pmol (usually with large excesses of ligand).[40] As this is distributed throughout the whole body, the concentration found in any one place, even sites of preferential accumulation are so low that chemical toxicity at these levels will not be of concern.

Conclusion

We have shown the synthesis, characterization and several *in vitro* assays of a series of long chain tridentate and Cp based ligands and their coordination to the $[M(CO)_3]^+$ cores (M = Re, ^{99m}Tc). The phenolate-based tridentate ligands were found to label quickly and in good yields, and to provide stable compounds. Unfortunately these compounds are not transported or phosphorylated as carbohydrate analogues. In synthesizing these metal chelates, the sugar has been modified to such an extent that it is no longer recognized as such by the key enzymes GLUT-1 and hexokinase. It is not known how much change is too much, but work like that shown here aims to determine, and take advantage of, these acceptable limits. As the long linker length is required for the hexokinase activity, but also reduces the water solubility of the complexes, alternatives such as a PEGylated linker are now under investigation in our lab.

Experimental

Materials and methods

Except where noted otherwise, all reactions and manipulations were carried out at room temperature. All chemicals were reagent grade as purchased from and were used as received unless stated otherwise. Reagents were purchased from Acros, Aldrich or Fluka unless otherwise stated. Solvents were HPLC grade, and were purchased from Fisher Scientific. Dichloromethane was dried in a solvent purification tower, and was used as dispensed. Hydrogen and argon were purchased from Praxair. Isolink™ kits were provided by Mallinckrodt Inc. (now Covidien). $Na^{99m}TcO_4$ was provided by the Nuclear Medicine Department at the University of British Columbia Hospital. 1,3,4,6-Tetra-*O*-acetyl-β-D-glucosamine **1** was prepared as previously described.[33] $[Re(CO)(H_2O)_3]Br$ was made from $Re(CO)_5Br$ (Strem) according to literature procedures.[41] Tricarbonyl(cyclopentadienylcarboxylic acid)rhenium was made in three steps from 1,1′-ferrocenedicarboxylic acid, according to literature procedures.[36,42]

The analytical TLC plates (aluminium backed ultra pure silica gel 60 Å, 250 μm) and the flash column silica gel (standard grade, 60 Å, 32-63 mm) were provided by Silicycle. TLC spots were visualized under a UVG-54 Mineralight® short-wave UV lamp (λ = 254 nm) and carbohydrate-containing compounds were visualized using TLC plates that were developed in a 5:95 sulfuric acid: ethanol mixture using a heat gun. All NMR spectra were acquired in deuterated solvents at ambient temperature in the UBC Department of Chemistry NMR facility on a Bruker Avance 300 spectrometer (300 MHz), a Bruker Avance 400 spectrometer (400 MHz), or a Bruker DRX400 (400 MHz) spectrometer. The NMR spectra and chemical shifts are expressed on the δ scale and referenced to the residual peaks of the deuterated solvent (purchased from Cambridge Isotope Labs). Infrared spectra were recorded on a Nicolet 6700 FT-IR spectrophotometer in transmission mode between 400 and 4000 cm^{-1} at a resolution of ± 0.09 cm^{-1}. ESI mass spectra were recorded on a Micromass LCT

instrument. High resolution mass spectra (Micromass LCT TOF-MS) were provided by the Analytical Services Facility, Department of Chemistry, University of British Columbia. HPLC analysis of non-radioactive compounds was done on a Phenomenex Synergi 4 μm Hydro-RP 80 Å column (250 × 4.6 mm) in a Waters WE 600 HPLC system equipped with a 2478 dual wavelength absorbance UV detector run controlled by Empower software package. HPLC analyses of radiolabeled complexes were performed on a Knauer Wellchrom K-1001 HPLC instrument equipped with a K-2501 absorption detector and a Capintec radiometric well counter using a Phenomenex Synergi 4 μm Hydro-RP 80 Å column (250 × 4.6 mm).

Synthesis and analytical data of all organic compounds and inorganic precursors are detailed in the ESI.†

Synthesis

(2-((6-*N*-(2-Hydroxybenzyl)-*N*-(pyridin-2-ylmethyl)amino)-hexylamido)-2-deoxy-*D*-glucopyranosyl) tricarbonylrhenium(I) (8a). **7a** (23.1 mg, 0.047 mmol) was dissolved in 2 mL MeOH. $[Re(CO)_3(H_2O)_3]Br$ (20.2 mg, 0.05 mmol) was added in one portion. Et_3N (10 μL) was added and the reaction mixture stirred for 5 h at reflux. Following consumption of the starting material by TLC (15% MeOH: 85% CH_2Cl_2) and mass spectrometry. The solvent was removed under reduced pressure leaving a pale yellow solid. The solid was purified *via* silica gel column chromatography (15% MeOH: 85% CH_2Cl_2) resulting in a pale white solid (23 mg, 64% yield). $R_f = 0.34$ (silica-15% MeOH: 85% CH_2Cl_2). ^{1}H NMR (MeOH-d_4, 300 MHz, δ): 8.72(m, 1H), 7.82(m, 1H), 7.33(m, 2H), 6.95(m, 2H), 6.46(m, 2H), 5.48(d, J = 1.95 Hz, 1H), 5.12(d, J = 7.98 Hz, 1H), 4.53(m, 2H), 3.78(m, 6H), 3.44(m, 2H), 2.20(m, 2H), 1.91-1.64(m, 4H), 1.43-1.13(m, 4H). ^{13}C NMR (MeOH-d_4, 75 MHz, δ): 176.70, 162.68, 158.56, 153.55, 141.06, 131.99, 126.34, 126.18, 124.42, 120.04, 118.27, 97.55, 92.97, 73.45, 72.66, 67.00, 63.18, 62.59, 56.21, 55.25, 37.10, 28.23, 27.01, 25.64. MS (ES+, 100% MeOH): $m/z = 760$ (M–H$^+$); HR-MS (ES+ of M–H$^+$) m/z calcd for $C_{28}H_{34}N_3O_{10}Re$: 760.1880, found: 760.1874. Elemental analysis calcd for $C_{28}H_{35}N_3O_{10}Re{\cdot}H_2O$: C, 43.29; H, 4.67; N, 5,54. found: C, 43.58; H, 5.07; N, 5.35.

(2-((8-*N*-(2-Hydroxybenzyl)-*N*-(pyridin-2-ylmethyl)amino)-octylamido)-2-deoxy-*D*-glucopyranosyl) tricarbonylrhenium(I) (8b). The experiment procedures of preparation of **8b** are similar to **8a**. **7b** (21.2 mg, 0.04 mmol), $[Re(CO)_3(H_2O)_3]Br$ (20.0 mg, 0.049 mmol) and 5μL Et_3N were used to give a yellow solid product. The solid was purified *via* silica gel column chromatography (12% MeOH: 88% CH_2Cl_2) resulting in a pale white solid (25 mg, 65% yield). $R_f = 0.45$ (silica-12% MeOH: 88% CH_2Cl_2). ^{1}H NMR (MeOH-d_4, 300 MHz, δ): 8.73(m, 1H), 7.86(m, 1H), 7.35(m, 3H), 6.98(m, 2H), 6.51(m, 1H), 5.50(d, J = 3.21, 1H-β), 5.12(m, 1H-α), 4.41(m, 2H), 4.34(m, 1H), 3.82(s, 2H), 3.79(s, 2H), 3.72(m, 1H), 3.48(m, 2H), 2.27(t, J = 12.57 Hz, 2H), 1.89(m, 2H), 1.66(m, 2H), 1.45(m, 8H).^{13}C NMR (MeOH-d_4, 75 MHz, δ): 162.28, 153.18, 140.70, 131.62, 131.24, 125.96, 119.66, 117.91, 97.20, 92.59, 76.02, 73.09, 72.58, 66.68, 62.82, 58.68, 55.82, 36.98, 30.12, 28.09, 26.83, 25.41. MS (ES+, 100% MeOH): m/z = 788 (M-H$^+$); HR-MS (ES$^+$ of M–H$^+$) m/z calcd for $C_{30}H_{39}N_3O_{10}Re$: 788.2193, found: 788.2220. Elemental analysis calcd for $C_{30}H_{39}N_3O_{10}$ $Re{\cdot}3H_2O$: C, 42.85; H, 5.27; N, 5.00. found: C, 42.71; H, 5.30; N, 5.10.

(2-((11-*N*-(2-Hydroxybenzyl)-*N*-(pyridin-2-ylmethyl)amino)-undecylamido)-2-deoxy-*D*-glucopyranosyl) tricarbonylrhenium(I) (8c). The experiment procedures of preparation of **8c** are similar to **8a**. **7c** (40.0 mg, 0.071 mmol), $[Re(CO)_3(H_2O)_3]Br$ (29.1 mg, 0.072 mmol) and 5μL Et_3N were used to give a yellow solid product. The solid was purified *via* silica gel column chromatography (12% MeOH: 88% CH_2Cl_2) resulting in a pale white solid (27 mg, 46% yield). $R_f = 0.25$ (silica-12% MeOH: 88% CH_2Cl_2). ^{1}H NMR (MeOH-d_4, 300 MHz, δ): 8.72(m, 1H), 7.80(m, 1H), 7.33(m, 2H), 6.93(m, 2H), 6.47(m, 2H), 5.10(s, 1H-β), 4.99(s, 1H-α), 4.57(m, 1H), 4.50(m, 2H), 3.81(s, 2H), 3.77(s, 2H), 3.71(m, 1H), 3.43(m, 2H), 2.24(t, J = 12.09 Hz, 2H), 1.87(m, 2H), 1.35(m, 16H). ^{13}C NMR (MeOH-d_4, 100 MHz, δ):197.00, 196.63, 176.31, 161.87, 152.80, 140.30, 132.10, 131.23, 130.81, 129.84, 129.12, 129.08, 129.02, 128.78, 128.06, 127.80, 125.57, 125.42, 123.66, 119.28, 117.49, 96.79, 94.61, 92.20, 77.61, 75.66, 73.03, 72.67, 72.19, 71.90, 70.23, 68.09, 66.34, 62.44, 61.84, 58.30, 56.21, 55.41, 54.63, 40.63, 37.04, 36.68, 36.59, 30.20, 30.09, 30.04, 29.99, 29.93. (ES+, 100% MeOH): $m/z = 852$ (M–Na$^+$); HR-MS (ES$^+$ of M–Na$^+$) m/z calcd for $C_{33}H_{44}N_3O_{10}NaRe$: 852.2482, found: 852.2468. Elemental analysis calcd. for $C_{33}H_{45}N_3O_{10}Re$: C, 47.82; H, 5.35; N, 5.07. found: C, 47.43; H, 5.64; N, 4.82.

^{99m}Tc Labelling—tridentate complexes (9a,b,c). $Na^{99m}TcO_4$ (200 MBq) was obtained as a saline solution from the University of British Columbia Hospital, Department of Nuclear Medicine. It was added to an Isolink™ kit (Covidien) and the volume made up to 1 mL with 0.9% saline solution. The resulting solution was heated at ~ 90 °C for 30 min. After cooling, the solution was neutralized with 1 M HCl (0.12 mL) to pH 7 as tested with pH paper. Meanwhile, 10^{-4} M ethanolic solutions of the ligands, **9**, were prepared, and 0.5 mL of each was transferred to a reaction vial with three equivalents of NaOEt (0.15 mL of a 10^{-3} M solution in ethanol). The vial was purged with nitrogen for 10 min to provide an inert atmosphere and to ensure time for deprotonation of the phenolates. Between 0.1 and 1 mL of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ was added to each vial, and the vial heated at 80 °C for 40 min. After cooling, the solutions were injected into the HPLC (with the analogous cold standard rhenium complex) for analysis.

^{99m}Tc Labelling—cyclopentadienyl complexes (14a,b). The appropriate ferrocene precursor (**13a**,**b**) was dissolved in dimethylsulfoxide to a final concentration of 1 mg/mL. This precursor solution (300 μL) was transferred to a 3 mL reaction vial which was sealed with a rubber septum and purged with nitrogen for 10 min. An aqueous solution of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ (made using an Isolink™ kit as detailed above) adjusted to pH 7 (300 μL) was added through the septum, and the reaction mixture stirred at 80 °C for 120 min. Upon cooling, a sample was injected into the radioHPLC for analysis. For cell uptake studies the reaction mixture was purified by HPLC; the appropriate peaks were collected and used in the studies.

Cysteine and histidine challenges

The ^{99m}Tc complexes were synthesized as outlined above. Cysteine and histidine solutions were freshly made at 10^{-3} M in aqueous PBS buffer (110 mM, pH 7.4, 0.9% saline). Cysteine or histidine solution (0.9 mL) was placed in a vial, and 0.1 mL of the technetium complex (in the reaction solution described above) was

added to give a final volume of 1 mL with the concentration of competing amino acid one hundred times greater than that of the ligand used for making the technetium complex. The solution was incubated at 37 °C and aliquots removed at 1 h, 4 h and 24 h for HPLC analysis. Each timepoint was done in triplicate from three separate incubation vials. The percentage of the initial complex remaining was examined, and the mean and standard deviation of three trials for each ligand with each of cysteine and histidine was calculated.

GLUT-1 Cell uptake studies

These experiments were conducted by using LCC6-HER2 cells—a human breast cancer cell line chosen for its overexpression of the glucose transporter GLUT-1.[43] Suspensions of cells with concentration 1×10^6 cells/mL were prepared in 1% PBS (pH 7.4). The suspension was aliquoted into 1.5 mL Eppendorf vials to get a final volume of 0.5 mL in each vial (5×10^5 cells). The compound to be tested was then added to the cells and the vial gently inverted. Each ^{99m}Tc complex (15 μCi) or ^{18}F-FDG (30 μCi) was used in 500 μL PBS solution. Each compound was added to the cells in glucose-free conditions, and in a final concentration of 5 mM D-glucose. The resulting suspensions were incubated at 37 °C for 30 min with shaking at 400 RPM. Following incubation, the vials were centrifuged at 1100 RPM for 5 min then 900 μL of the supernatant was removed. Cold PBS solution (900 μL) was added, and gentle mixing of the cells into the solution was achieved by gentle uptake and release of the solution from the pipette tip three times. This centrifuging and washing procedure was repeated four times in total to give the original supernatant and three washing supernatants. Finally the cells were vortexed in cold PBS (1 mL) to remove them from the vial wall and transferred to a tube for gamma counting. The activity in each cell sample was divided by the sum of all the supernatants for that sample to give a percentage of the original amount of activity that ended up associated with the cells. All experiments were carried out in quadruplicate. FDG was used as a positive control, and was repeated each day that these experiments were carried out to ensure experimental integrity.

Hexokinase inhibition assay

Hexokinase kits made up to contain 1 U/mL hexokinase, 1 U/mL glucose-6-phosphate dehydrogenase, 1 mM ATP and 1.5 mM NAD^+ were used in this assay. An aliquot (500 μL) of this solution was heated to 25 °C for 7–8 min and then transferred to a 1 cm pathlength cuvette for each trial. Rates were determined by adding one of four amounts of glucose: 10, 25, 50 or 75 μL of a standard solution (1.0 mg/mL glucose) to the cuvette, and the volume made up to 1250 μL with distilled water. The cuvette was gently inverted three times before being placed in a UV-vis spectrometer fitted with a water jacket equilibrated to 25 °C. The absorbance at 340 nm was recorded every 30 sec for at least five min. For each test compound five different concentrations were examined with each of the four glucose concentrations used above. The inhibitors were tested by adding either 25, 75, 100, 125 or 250 μL of a 5 mM stock solution of the compound to the assay mixture described above (final concentration of 0.1–0.5 mM) prior to addition of glucose. As all other factors were kept constant, changes in the rate of phosphorylation of glucose in the presence of a test compound were attributed to inhibition of the process by that test compound. The initial rates were taken from the linear portion of the A_{340} *vs.* time graphs (normally 210 sec), and these rates were manipulated through Lineweaver-Burke and inhibition constant plots using least squares fitting to determine the K_i values.

Hexokinase phosphorylation studies

A 5 mM aqueous solution of test compound (100 μL) was added to a reaction vial such that a final concentration of 0.5 mM test compound, 10 U/mL hexokinase, 1 mM ATP, 4 mM $MgCl_2$ in a 30 mM TEOA (triethanolamine, pH = 6) buffer was achieved. The resulting solution was mixed gently by inverting each vial three times, and then incubated for 16 h at 37 °C. Each solution (20 μL) was analysed by HPLC to determine whether any of the ATP had been converted to ADP, as would accompany the phosphorylation of a substrate. Using a Phenomenex Synergi 4 μm Hydro-RP 80 Å column (250 × 4.6 mm) and eluting with 30 mM KH_2PO_4, the retention time of ATP is 8.5 min, whereas that of ADP is 12.5 min.

MTT Cytotoxicity assay[39]

Human hepatocytes were used in this study, and the cells were cultured and maintained in the UBC Chemistry Department Biological Services facility. On day 1 of the study, the cells were diluted with media to a final concentration of 1×10^5 cells/mL, and 100 μL of this solution was added to each well of a 96 well plate which was incubated overnight. On day 2, test compound (100 μL of various concentration) was added to each well to bring the final well volume to 200 μL. The plates were incubated at 37 °C in 5% CO_2 for 72 h. On day 5, a 2.5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS was prepared and added to each well (50 μL), and the plates were then incubated for a further 3 h at 37 °C. The solution was removed, and dimethylsulfoxide (150 μL) was added to each well. The plates were gently shaken and the purple formazan was quantified using a plate reader to measure absorbance at 570 nm. At least four concentrations of each compound were tested, and all were done in quadruplicate. Cisplatin was used as a positive control for cytotoxicity, and was found to have an IC_{50} value of 2.6 ± 0.3 μM, which is consistent with the literature.[44]

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Notes and references

1 M. L. Bowen and C. Orvig, *Chem. Commun.*, 2008, 5077–5091.
2 T. Storr, C. L. Fisher, Y. Mikata, S. Yano, M. J. Adam and C. Orvig, *Dalton Trans.*, 2005, 654–655.
3 S. R. Bayly, C. L. Fisher, T. Storr, M. J. Adam and C. Orvig, *Bioconjugate Chem.*, 2004, **15**, 923–926.

4 T. Storr, M. Obata, C. L. Fisher, S. R. Bayly, D. E. Green, I. Brudzinska, Y. Mikata, B. O. Patrick, M. J. Adam, S. Yano and C. Orvig, *Chem.–Eur. J.*, 2005, **11**, 195–203.
5 C. L. Ferreira, C. B. Ewart, S. R. Bayly, B. O. Patrick, J. Steele, M. J. Adam and C. Orvig, *Inorg. Chem.*, 2006, **45**, 6979–6987.
6 C. L. Ferreira, S. R. Bayly, D. E. Green, T. Storr, C. A. Barta, J. Steele, M. J. Adam and C. Orvig, *Bioconjugate Chem.*, 2006, **17**, 1321–1329.
7 J. R. Dilworth and S. J. Parrott, *Chem. Soc. Rev.*, 1998, **27**, 43–55.
8 R. Alberto, R. Schibli, A. Elgi and P. A. Schubiger, *J. Am. Chem. Soc.*, 1998, **120**, 7987–7988.
9 S. Alves, A. Paulo, J. D. G. Correia, L. Gano, C. J. Smith, T. J. Hoffman and I. Santos, *Bioconjugate Chem.*, 2005, **16**, 438–449.
10 S. R. Banerjee, M. K. Levadala, N. Lazarova, L. Wei, J. F. Valliant, K. A. Stephenson, J. W. Babich, K. P. Maresca and J. Zubieta, *Inorg. Chem.*, 2002, **41**, 6417–6425.
11 Y. Liu, J. K. Pak, P. Schmutz, M. Bauwens, J. Mertens, H. Knight and R. Alberto, *J. Am. Chem. Soc.*, 2006, **128**, 15996–15997.
12 F. Zobi, B. Springler, T. Fox and R. Alberto, *Inorg. Chem.*, 2003, **42**, 2818–2820.
13 M. M. Saw, P. Kurz, N. Agorastos, T. S. A. Hor, F. X. Sundram, Y. K. Yan and R. Alberto, *Inorg. Chim. Acta*, 2006, **359**, 4087–4094.
14 S. James, K. P. Maresca, J. W. Babich, J. F. Valliant, L. Doering and J. Zubieta, *Bioconjugate Chem.*, 2006, **17**, 590–596.
15 M. S. McAllister, L. Krizanac-Bengez, F. Macchia, R. J. Naftalin, K. C. Pedley, M. R. Mayberg, M. Marroni, S. Leaman, K. A. Stanness and D. Janigro, *Brain Res.*, 2001, **904**, 20–30.
16 R. S. Brown, T. M. Goodman, K. R. Zasadny, J. K. Greenson and R. L. Wahl, *Nucl. Med. Biol.*, 2002, **29**, 443–453.
17 J. Petrig, R. Schibli, C. Dumas, R. Alberto and P. A. Schubiger, *Chem.–Eur. J.*, 2001, **7**, 1868–1873.
18 T. L. Mindt, H. Struthers, L. Brans, T. Anguelov, C. Schweinsberg, V. Maes, D. Tourwe and R. Schibli, *J. Am. Chem. Soc.*, 2006, **128**, 15096–15097.
19 D. J. Yang, C. G. Kim, N. R. Schechter, A. Azhdarinia, D. F. Yu, C. S. Oh, J. L. Bryant, J. J. Won, E. E. Kim and D. A. Podoloff, *Radiology*, 2003, **226**, 465–473.
20 S. R. Banerjee, J. W. Babich and J. Zubieta, *Inorg. Chim. Acta*, 2006, **359**, 1603–1612.
21 M. Gottschaldt, D. Koth, D. Muller, I. Klettte, S. Rau, H. Gorls, R. P. Baum and S. Yano, *Chem.–Eur. J.*, 2007, **13**, 10273–10280.
22 R. Schibli, C. Dumas, J. Petrig, L. Spadola, L. Scapozza, E. Garcia-Garayoa and P. A. Schubiger, *Bioconjugate Chem.*, 2005, **16**, 105–112.
23 L. Speizer, R. Haugland and H. Kutchai, *Biochim. Biophys. Acta, Biomembr.*, 1985, **815**, 75–84.
24 K. Yoshioka, M. Saito, K.-B. Oh, Y. Nemoto, H. Matsuoka, M. Natsume and H. Abe, *Biosci. Biotech. Biochem.*, 1996, **60**, 1899–1901.
25 N. C. Lim, C. B. Ewart, M. L. Bowen, C. L. Ferreira, C. A. Barta, M. J. Adam and C. Orvig, *Inorg. Chem.*, 2008, **47**, 1337–1345.
26 K. P. Maresca, S. M. Hilliier, F. J. Femia, C. N. Zimmerman, M. K. Levadala, S. R. Banerjee, J. Hicks, C. Sundararajan, J. F. Valliant, J. Zubieta, W. C. Eckelman, J. L. Joyal and J. W. Babich, *Bioconjugate Chem.*, 2009, **20**, 1625–1633.
27 R. Schibli and P. A. Schubiger, *Eur. J. Nucl. Med. Mol. Imaging*, 2002, **29**, 1529–1542.
28 S. Top, E. B. Kaloun, S. Toppi, A. Herrbach, M. J. McGlinchey and G. Jaouen, *Organometallics*, 2001, **20**, 4554–4561.
29 J. Bernard, K. Ortner, B. Spingler, H.-J. Pietzsch and R. Alberto, *Inorg. Chem.*, 2003, **42**, 1014–1022.
30 G. Tamagnan, R. M. Baldwin, N. S. Kula, R. J. Baldessarini and R. B. Innis, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 1113–1115.
31 M. B. Skaddan, F. Wust and J. A. Katzenellenbogen, *J. Org. Chem.*, 1999, **64**, 8108–8121.
32 S. Masi, S. Top, L. Boubekeur, G. Jaouen, S. Mundwiler, B. Spingler and R. Alberto, *Eur. J. Inorg. Chem.*, 2004, 2013–2017.
33 D. J. Silva, H. Wang, N. M. Allanson, R. K. Jain and M. J. Sofia, *J. Org. Chem.*, 1999, **64**, 5926–5929.
34 R. A. Breitenmoser and H. Heimgartner, *Helv. Chim. Acta*, 2001, **84**, 786–796.
35 M. L. Bowen, N. C. Lim, C. B. Ewart, R. Misri, C. L. Ferreira, U. Häfeli, M. J. Adam and C. Orvig, *Dalton Trans.*, 2009, DOI: 10.1039/b914310f.
36 T. W. Spradau and J. A. Katzenellenbogen, *Organometallics*, 1998, **17**, 2009–2017.
37 W. S. J. Bennett and T. A. Steitz, *Proc. Natl. Acad. Sci. U. S. A.*, 1978, **75**, 4848–4852.
38 R. J. L. Bondar and D. C. Mead, *Clin. Chem.*, 1974, **5**, 586–590.
39 T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55–63.
40 C. B. Sampson, *Text Book of Radiopharmacy: Theory and Practice*, Taylor and Francis, Oxford, 1994.
41 N. Lazarova, S. James, J. W. Babich and J. Zubieta, *Inorg. Chem. Commun.*, 2004, **7**, 1023–1026.
42 T. W. Spradau and J. A. Katzenellenbogen, *Bioconjugate Chem.*, 1998, **9**, 765–772.
43 W. H. Dragowska, T. J. Ruth, M. J. Adam, P. Kozlowski, K. Skov, M. B. Bally and D. T. T. Yapp, *American Association for Cancer Research*, 2005, 900.
44 A. Wu, D. C. Kennedy, B. O. Patrick and B. R. James, *Inorg. Chem.*, 2003, **42**, 7579–7586.