Published in final edited form as:

Bioconjug Chem. 2016 July 20; 27(7): 1762–1769. doi:10.1021/acs.bioconjchem.6b00271.

Evaluation of Nonpeptidic Ligand Conjugates for SPECT Imaging of Hypoxic and Carbonic Anhydrase IX-Expressing Cancers

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Abstract

As tumors grow, vasculature is often deficient or malformed, resulting in many localized areas of hypoxia. Cells located in these hypoxic regions exhibit an altered gene expression pattern that can significantly alter resistance to conventional anti-cancer treatments such as ionizing radiation and chemotherapeutic drugs. *A priori* knowledge of the level of hypoxia within a tumor may better guide clinical care. In an effort to create a hypoxia specific imaging agent, a ligand for the tissue hypoxia marker, carbonic anhydrase IX (CA IX), was synthesized and used as a targeting ligand to deliver an attached ^{99m}Tc-chelating agent. Binding of the resulting conjugates to hypoxic cancer cells was first characterized in vitro. Whole animal imaging and biodistribution studies then were performed to determine tumor specificity in vivo. Several conjugates were found to bind selectively to CA IX expressing tumors in a receptor-dependent manner. We suggest that such conjugates could prove useful in identifying hypoxic cancers and/or quantitating the level of hypoxia within a tumor.

Graphical abstract

Keywords

Hypoxia; carbonic anhydrase IX; hypoxia imaging; SPECT imaging

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Introduction

The tumor microenvironment can greatly affect the phenotype of cancer cells within a malignant mass. One of the most prominent microenvironmental effects is hypoxia that arises from the poorly formed vasculature common to most tumors^{1–4}. Since 1% to 1.5% of all genes are regulated by hypoxia⁵, hypoxic cancer cells can exhibit markedly different patterns of gene expression, including induction of an epithelial to mesenchymal transition and formation of cancer stem cells^{1,5}. These changes can lead to reduced sensitivity towards chemotherapeutic agents^{6–8}, increased aggressiveness, and eventual recurrence of the cancer^{5,6,9}. Since hypoxia plays such an important role in determining a cancer's phenotype, knowledge of each individual patient's level of tumor hypoxia could greatly inform the program for management of the disease.

Due to these hypoxic effects, efforts have been made to identify hypoxia markers to exploit for cancer imaging. One such marker, carbonic anhydrase IX (CA IX), is expressed via the activation of hypoxia-inducible factor-1 α (HIF-1 α) and has been found in cancers of the lung^{10–12}, colorectum^{13–15}, stomach¹⁶, pancreas¹⁷, breast^{18–22}, cervix^{23–26}, bladder^{27,28}, ovaries²⁹, brain^{30–32}, head & neck^{33–35} and oral cavity^{36–38}. Additionally, due to a mutation in the VHL gene that leads to constitutive HIF-1 α activation, cancers such as clear cell carcinoma of the kidney have been shown to upregulate CA IX up to 150-fold over basal levels^{39–41}. In normal cells, however, CA IX is only expressed in epithelial cells of the stomach and gallbladder where it appears to be catalytically inactive^{42,43}. Due to this highly restricted expression in normal tissues and presence on many hypoxic cancers, CA IX is an attractive target for specific delivery of imaging agents.

As CA IX has been touted as an excellent target for the specific delivery of imaging agents^{44–46}, both small molecule- and antibody-targeted conjugates have been prepared to image hypoxic tumors. For example, CA IX-specific peptide and small molecule ligands have been used to image mouse xenograft models of colon^{47–52}, renal⁵³ and cervical cancers⁵¹. CA IX-specific antibodies have also been exploited to image mouse cancer xenograft models of the kidney^{54–57}, head & neck⁵⁸, colon^{59,60} and cervix⁶¹ in addition to human patients with clear cell renal carcinomas^{62–66}. However, small molecule conjugates have primarily used only PET^{49,50} or fluorescence^{51–53} imaging modalities to visualize hypoxic tumors. To further develop CA IX-targeted conjugates that utilize the most widely used imaging agent in nuclear medicine, ^{99m}Tc⁶⁹ we created several ^{99m}Tc chelating probes linked to highly potent CA IX inhibitors^{67,68} via various linkers. Herein, we describe the synthesis, in vitro binding and selective in vivo SPECT imaging/biodistribution of CA IX-expressing xenograft tumors using these conjugates.

Results and Discussion

Synthesis and HT-29 binding of rhodamine conjugates

In an effort to identify a suitable CA IX targeting ligand, three potent CA IX inhibitors were identified^{67,68}. First, the inhibitors were coupled to rhodamine through an alkyl or PEG linker to test whether the attachment site would interfere with the inhibitor's ability to bind CA IX. In addition to steric hindrance, the attachment of the linker also adjusts the charge

state of the original inhibitors (+2 to +1 for L1, 0 to +1 for L2, and +2 to +1 for L3). Next, these three conjugates, shown in Figure 1, were incubated with HT-29 cells in the presence or absence of 100-fold excess unconjugated inhibitor and after washing were analyzed via confocal microscopy. As shown in Figure 2, the first conjugate did not appear to bind to the HT-29 cells, possibly due to interference caused by the attachment of the linker. The other two conjugates both bound the cells and were competed in the presence of excess unconjugated inhibitor, indicating a receptor-specific binding event. The two inhibitors that bound, L2 and L3, then were advanced for further conjugate construction and testing.

Synthesis and HT-29 binding of PEG₁₂-^{99m}Tc conjugates

To determine if the addition of a linker and the ^{99m}Tc binding moiety (EC20) would impact the binding of the two remaining CA IX inhibitors, a PEG₁₂ linker coupled to an EC20 moiety was attached to the inhibitors. PEG₁₂ was chosen as the linker due to its high flexibility, hydrophilicity and stability under physiological conditions. Additionally, the incorporation of a highly hydrophilic linker deters non-specific hydrophobic interactions and also ensures that these conjugates do not passively diffuse through cell membranes. ^{99m}Tc was chelated with these two conjugates, named L2-PEG₁₂-EC20 and L3-PEG₁₂-EC20 and were incubated with HT-29 cells in the presence or absence of unconjugated inhibitor. As shown in Figure 3, L2-PEG₁₂-EC20 and L3-PEG₁₂-EC20 exhibited binding affinities of 54 and 146 nM, respectively. The reported K_i of the original ligands L2 and L3 were 7.8 and 0.9 nM, respectively^{67,68}, meaning the addition of the PEG₁₂-EC20 decreased the binding by ~7- and 162-fold, respectively.

Synthesis and HT-29 binding of L2-PEG₃₆-^{99m}Tc and L2-Proline₃-PEG₁₂-^{99m}Tc conjugates

As the binding affinity for L2 was not as severely impacted by the addition of the linker, two additional conjugates using a PEG₃₆ and a Proline₃-PEG₁₂ linker were synthesized to explore the effect of different linkers on binding.

A longer PEG linker was chosen to explore the effect of greater distance between L2 and EC20. Since poly-prolines form a rigid helix with ~3 prolines per turn, a Proline₃ was incorporated into the PEG₁₂ linker determine the impact of a semi-rigid linker. As shown in Figure 3, the binding affinity for the two newly synthesized conjugates were 57 and 67 nM, essentially the same as the L2-PEG₁₂-EC20 conjugate.

Whole animal imaging and biodistribution timecourse

Prior to a larger animal study with all ^{99m}Tc-conjugates, a pilot study of the kinetics of a single conjugate, L2-PEG₃₆-EC20, was performed. Six mice bearing HT-29 xenografts were administered 10 nmol of conjugate via tail vein injection while three of these mice also received 100-fold excess unconjugated inhibitor. A single mouse from each of the two groups was imaged and then sacrificed for tissue/organ biodistribution analysis at 4, 9 and 24 hours post injection. As shown in Figure 4, when the kidneys were shielded, radioactive intensity was localized to the tumor with relative average intensities of ~1300, 750 and 350 at 4, 9 and 24 hours, respectively. Importantly, all mice concurrently injected with 100-fold excess unconjugated inhibitor did not exhibit any intensity in the tumor, indicative of receptor-dependent binding/uptake. Overall, the biodistribution (Figure 4) also exhibited a

similar pattern with a decrease in the amount of conjugate in all tissues over time. From these data, a 4 hour time point was chosen for a larger biodistribution study of all conjugates.

Biodistribution of ^{99m}Tc conjugates

To determine the uptake of the various ^{99m}Tc -bound conjugates in CA IX-expressing tumors, 10 nmol of each of the four conjugates shown in Figure 3 were injected into mice bearing HT-29 xenografts in the presence or absence 100-fold excess of the appropriate unconjugated CA IX inhibitor L2 or L3 (n=5 mice per group). As shown in Figure 5, the tumor was the only tissue that showed lower binding of all four conjugates when competing inhibitor was co-administered, indicating that no other tissue binding was CA IX receptor mediated. In fact, the difference between the tumor and tumor competition groups of all conjugates except L2-PEG₁₂-EC20 were significantly different with p-values < 0.01.

Overall, the four conjugates exhibited fairly similar biodistribution profiles. However, L2-PEG₁₂-EC20 did show a somewhat lower overall percentage of the injected dose being retained in the tissues. Otherwise, all conjugates appear to not be rapidly cleared from the bloodstream as measurable activity is still apparent in the blood after 4 hours. High activity located in the kidneys is most likely due to excretion, suggesting the renal system being the primary excretion route. However, the liver may also remove a portion of the L2 conjugates from the bloodstream as some activity was also present in the liver. The differences in kidney uptake were not statistically correlated to the conjugates solubility, calculated polar surface area, or cLogP. Additionally, no other tissues' uptake was statistically correlated to any of these chemical properties.

While the overall biodistribution and imaging of our conjugates are somewhat similar to that of other hydrophilic CA IX-targeted imaging agents previously reported^{47–52,69,70}, one major difference is that many other sulfonamide conjugates exhibit essentially no or barely detectable uptake in the tumor 4 hours post-injection^{47,48}. This absence of signal suggests these conjugates are rapidly cleared, even from CA IX-expressing tissues. In contrast, all four conjugates reported here still exhibit strong uptake in the tumor at 4 hours and even later time points. As the conjugate remains within the tumor, it is cleared from other CA IX negative tissues yielding high tumor-to-liver, tumor-to-blood, and tumor-to-muscle ratios as compared to other CA IX imaging agents. In fact, tumor-to-muscle ratios were >25 for all 4 conjugates at 4 hours whereas the ratio is generally only ~1 for other sulfonamide imaging agents 1 hour post-injection^{49,50}. The ability of these conjugates to remain within the tumor tissues for longer periods of time, even though the binding affinities are no better than that of other conjugates^{47–52,69,70} may be due to the fact that the ligands are dimeric in nature. Similarly, a trimeric sulfonamide derivative also exhibited enhanced retention in CA IX expressing tumors with tumor-to-muscle ratios of 9.5⁶⁹. While longer times post-injection may be detrimental to short-lived PET imaging agents, such as ^{18}F , the additional time is not impactful to longer-lived SPECT agents, such as ^{99m}Tc and is very beneficial in increasing the image contrast.

Conclusion

The imaging of cancer can be an important part of a patient's therapy, guiding which treatment modalities are employed. While many targeted imaging agents have been developed, very few of these agents are able to detect a vast array of different cancers. One receptor that is found on nearly every hypoxic cancer and not greatly expressed in any normal tissue is CA IX^{10–38}. Due to this preferential expression, many small molecule-^{47–53} and antibody-imaging conjugates^{54–66} have been used to image hypoxic cancers. To increase the repertoire of imaging agents, we endeavored to create CA IX conjugates using different CA IX inhibitors for imaging with ^{99m}Tc, the most widely used imaging agent in nuclear medicine⁷⁰.

To accomplish this, three potent CA IX inhibitors were initially identified and attached to imaging moieties. The best binding conjugate was identified and three different linkers, PEG₁₂, PEG₃₆ and Proline₃-PEG₁₂, were synthesized in an effort to test the impact of different linkers on the in vitro binding and in vivo biodistribution. While the different inhibitors appear to drive the binding affinity of CA IX conjugates in vitro, we show that the linker did not greatly impact the in vitro binding nor the biodistribution profile as the tumor was ~4% of the injected dose in all conjugates except L2-PEG₁₂-EC20. To increase the in vivo stability and/or contrast ratio between the tumor and other organs, especially the kidneys, alternative linker configurations could provide quicker renal clearance yet still retain higher levels of tumor accumulation. In conclusion, with selective SPECT probes developed, the imaging of nearly all solid tumors with regions of hypoxia can now be selectively imaged. This imaging may lead to better therapeutic regimens as chemotherapeutics that are more efficacious in hypoxic cancers now can be administered to verified hypoxic tumors. Therefore, additional testing of these imaging agents is warranted.

Materials and Methods

Materials

Protected amino acids were purchased from Chem-Impex International (Chicago, IL). H-Cys (Trt)-2-Cl-Trt resin was obtained from Novabiochem (San Diego, CA). 2-(1H-7-Azabenzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) was obtained from Genscript Inc. (Piscataway, NJ). Sulfuric acid, methanol, DMSO, DMF, TFA, isopropyl alcohol, NH₂-PEG₁₂-COOH-tBu, diisopropylethylamine (DIPEA), piperidine, CF₃COOH, CH₂Cl₂, K₂CO₃, tyramine and all other chemical reagents were purchased from Sigma Aldrich. Pure coat Amine 24-well microtiter plates were purchased from BD Biosciences (San Jose, CA). All other cell culture reagents, syringes and disposable items were purchased from VWR (Chicago, IL).

Synthesis of L1-Rhodamine, L2-Rhodamine and L3-Rhodamine

The CA IX inhibitors L1, L2 and L3 were prepared as previously described (67,68). The CA IX inhibitor was coupled with rhodamine derivative (5-((5-aminopentyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate hydrochloride) in the presence of EDC.HCl and HOBt in DMSO for 12 hours yielding the target conjugate. For

L1-Rhodamine, LRMS-LC/MS (m/z): $[M + H]^+$ calcd for $C_{48}H_{48}N_7O_9S$, 898.32; found, 898. For L2-Rhodamine, LRMS-LC/MS (m/z): $[M + H]^+$ calcd for $C_{52}H_{60}N_{10}O_{13}S_2$, 1097.39; found, 1097. For L3-Rhodamine, LRMS-LC/MS (m/z): $[M + H]^+$ calcd for $C_{49}H_{56}N_7O_{12}S$, 966.37; found, 966.1.

Synthesis of L2-PEG₁₂-EC20, L2-PEG₃₆-EC20, L2-Pro₃-PEG₁₂-EC20 and L3-PEG₁₂-EC20

All conjugates were synthesized by the following solid phase methodology. H-Cys(Trt)-2-chlorotrityl resin (100 mg, 0.56 mM) was swollen with 3 mL of dichloromethane (DCM) followed by 3 mL of dimethylformamide (DMF). For three times, a 3 mL solution of 20% piperidine in DMF was added to the resin with argon bubbled through for 5 min. The resin was washed three times with 3 mL of DMF and 3 times with 3 mL isopropyl alcohol (i-PrOH). After swelling the resin in DMF, a solution of Fmoc-Asp(tBu)-OH (2.5 equiv), PyBOP (2.5 equiv) and DIPEA (4.0 equiv) in DMF was added. Argon was bubbled for 2 h, and resin was washed three times with 3 mL of DMF and 3 times with 3 mL i-PrOH.

For L2-PEG₁₂-EC20, the above sequence was repeated for three more coupling steps for addition of Boc-DAP(Fmoc)-OH, Fmoc-NH-PEG₁₂-COOH, and CA IX inhibitor L2. For L2-PEG₃₆-EC20, the above sequence was repeated for three more coupling steps for addition of Boc-DAP(Fmoc)-OH, Fmoc-NH-PEG₃₆-COOH, and CA IX inhibitor L2. For L2-Pro₃-PEG₁₂-EC20, the above sequence was repeated for six more coupling steps for addition of Boc-DAP(Fmoc)-OH, Fmoc-NH-PEG₃₆-COOH, three times proline and CA IX inhibitor L2. For L3-PEG₁₂-EC20, the above sequence was repeated for three more coupling steps for addition of Boc-DAP(Fmoc)-OH, Fmoc-NH-PEG₁₂-COOH and CA IX inhibitor L3.

Final compounds were cleaved from the resin using a trifluoroacetic acid (TFA): H₂O: triisopropylsilane: cocktail (95:2.5:2.5) and concentrated under vacuum. The concentrated product was precipitated in diethyl ether and dried under vacuum. Crude conjugate was purified by preparative RP-HPLC [A = 2 mM ammonium acetate buffer (pH 7.0), B = CH₃CN, solvent gradient: 0% B to 100% B in 30 min] to yield the requisite product.

For L2-PEG₁₂-EC20 conjugate, LRMS-LC/MS (m/z): $[M + H]^+$ calcd for $C_{59}H_{97}N_{11}O_{28}S_3$, 1503.57; found, 752.8 (half mass). LogS, tPSA (polar surface area calculation) and cLogP were -2.025, 550.08, and -8.439, respectively. For the L2-PEG₃₆-EC20 conjugate, LRMS-LC/MS (m/z): $[M + H]^+$ calcd for $C_{108}H_{195}N_{11}O_{52}S_3$, 2574.21; found, 1281.6 (half mass). LogS, tPSA and cLogP were 0.3363, 771.6, and -11.797, respectively. For L2-Pro₃-PEG₁₂-EC20, LRMS-LC/MS (m/z): $[M + H]^+$ calcd for $C_{108}H_{195}N_{11}O_{52}S_3$, 1810.03; found, 1811.5. LogS, tPSA and cLogP were -3.835, 600.0, and -5.616, respectively. For L3-PEG₁₂-EC20, LRMS-LC/MS (m/z): $[M + H]^+$ calcd for $C_{54}H_{87}N_9O_{24}S_2$, 1310.44; found, 655.8 (half mass). LogS, tPSA and cLogP were -2.584, 458.17, and -6.33, respectively.

Fluorescent microscopy

HT-29 cells (10^5) were seeded into chambered coverglass plates and allowed to grow to confluence over 48–72 hr. Spent medium was replaced with 0.5 mL of fresh medium containing 0.5% bovine serum albumin and various concentrations of the dye conjugate alone or the dye conjugate plus 100-fold excess CA IX inhibitor L1, L2 or L3, where

appropriate. After incubation for 1 hour at 37 °C, cells were rinsed twice with 1 mL of incubation solution to remove unbound fluorescence and 0.5 mL of fresh incubation medium was added to the wells. Images were acquired using a confocal microscopy (FV 1000, Olympus).

^{99m}Tc conjugates binding to HT-29 cells

HT-29 cells (150,000 cells/well in 500 μ L) were seeded into 24-well plates and allowed to form monolayers over 48 h. Spent medium in each well was replaced with 0.5 mL fresh medium containing increasing concentrations (0.1 nM to 500 nM) of various conjugates bound with ^{99m}Tc in the presence or absence of 100-fold excess CA IX inhibitors L1, L2 or L3, where appropriate. After incubating for 1 h at 4 °C, cells were rinsed twice with 1 mL of medium and 1 mL of tris buffer. After dissolving in 0.5 mL of 0.25 M NaOH (aq), cells were transferred into individual γ -counter tubes and radioactivity was counted using a γ -counter (Packard, Packard Instrument Company). Apparent K_D was calculated by plotting bound radioactivity versus the concentration of radiotracer using GraphPad Prism 4.

Animal husbandry

Male athymic nu/nu mice were purchased from Harlan Laboratories, housed in a sterile environment on a standard 12 hour light–dark cycle and maintained on normal rodent chow. All animal procedures were approved by the Purdue Animal Care and Use Committee in accordance with National Institutes of Health guidelines.

^{99m}Tc conjugates in vivo imaging and biodistribution

Five-week-old male nu/nu mice were inoculated subcutaneously with HT-29 cells (5.0×10^6 /mouse) on their shoulders. Growth of the tumors was measured in two perpendicular directions every 2 days using a caliper (body weights were monitored on the same schedule), and the volumes of the tumors were calculated as $0.5 \times L \times W^2$ (L = longest axis and W = axis perpendicular to L in millimeters). Once tumors reached between 400 and 500 mm³ in volume, animals were administered ^{99m}Tc-bound conjugates (10 nmol, 150 μ Ci) in saline (100 μ L) via tail vein injection. At various times, animals were sacrificed by CO₂ asphyxiation. Images were acquired via a Kodak Imaging Station (In-Vivo FX, Eastman Kodak Company) in combination with CCD camera and Kodak molecular imaging software (version 4.0) with the kidneys shielded. Radioimages: illumination source = radio isotope, acquisition time = 3 min, f -stop = 4, focal plane = 5, FOV = 160, binning = 4. White light images: illumination source = white light transillumination, acquisition time = 0.05 s, f -stop = 16, focal plane = 5, FOV = 160 with no binning. Following imaging, animals were dissected and selected tissues were collected to preweighed γ -counter tubes. Radioactivity of preweighed tissues and ^{99m}Tc-bound conjugates (10 nmol, 150 μ Ci) in saline (100 μ L) was counted in a γ -counter. CPM values were decay corrected, and results were calculated as % injected dose (ID)/gram of wet tissue.

Acknowledgments

The authors gratefully acknowledge the campus-wide mass spectroscopy facility and support from the Purdue University Center for Cancer Research, P30CA023168.

Funding:

This work was supported by a grant from Endocyte Inc.

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L1-rhodamine **L2-rhodamine** **L3-rhodamine**

Figure 1.
Chemical structures of rhodamine conjugates.

Figure 2.

In vitro binding of rhodamine-labeled CA IX conjugates to HT-29 cells. Fluorescent conjugates were incubated with HT-29 cells in the presence or absence of 100-fold excess unlabeled ligand. After washing, white light and fluorescent microscopy were used to visualize binding.

Figure 3.

In vitro binding of EC20 conjugates to HT-29 cells. Various linkers and EC20 were conjugated to the L2 or L3 ligands. After chelation of ^{99m}Tc, all conjugates were incubated with HT-29 cells for 1 hour at 4 °C. Unbound conjugates were removed by washing and the remaining radioactivity was determined.

Figure 4.

In vivo imaging and biodistribution of L2-PEG36-EC20. Three mice bearing HT-29 xenograft tumors were administered 99mTc coordinated L2-PEG36-EC20 (10 nmol) via tail vein injection (left mouse in each panel). Three additional mice were simultaneously injected with 10 nmol L2-PEG36-EC20 and 1000 nmol unlabeled L2 ligand (right mouse in each panel). Whole animal images were taken 4, 9 and 24 hours post-injection. Organs then were excised, washed, weighed and the amount of radioactivity present was determined. The percentage of the injected dose per gram of tissue was determined and plotted.

Figure 5.

In vivo biodistribution of all CA IX conjugates. Mice bearing HT-29 xenograft tumors were administered 10 nmol of ^{99m}Tc coordinated conjugates via tail vein injection (n=5). Additional mice were simultaneously injected with 100 nmol of unlabeled L2 or L3 ligand (n=5). Four hours post injection, major tissues/organs were removed and the amount of radioactivity was determined. The percentage of injected dose/g of tissue was calculated and plotted. Error bars represent standard deviation. * denotes p-value < 0.01.