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Development of the ^{99m}Tc-Hydroxamamide Complex as a Probe Targeting Carbonic Anhydrase IX

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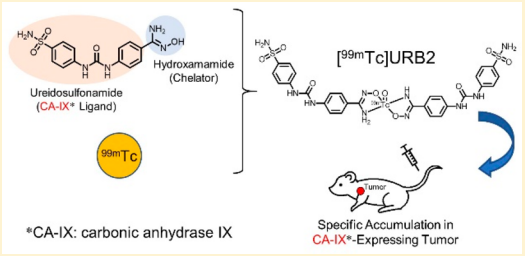
Supporting Information

ABSTRACT: Carbonic anhydrase IX (CA-IX) is regarded as a favorable target for in vivo imaging because of its specific expression in hypoxic regions of tumors. Hypoxia assists tumor propagation and growth and is resistant to chemotherapy and radiotherapy. Here, we designed and synthesized [^{99m}Tc]hydroxamamide ([^{99m}Tc]Ham) and [^{99m}Tc]methyl-substituted-hydroxamamide ([^{99m}Tc]MHam) complexes including a bivalent CA-IX ligand, sulfonamide (SA), and ureidosulfonamide (UR). In a cell binding assay, [^{99m}Tc]Ham complexes with bivalent SA ([^{99m}Tc]SAB2A and [^{99m}Tc]SAB2B) and UR ([^{99m}Tc]URB2A and [^{99m}Tc]URB2B) showed significantly greater uptake into CA-IX high-expressing (HT-29) cells than that into CA-IX low-expressing cells. Since the binding affinity of [^{99m}Tc]URB2A and [^{99m}Tc]URB2B for CA-IX was significantly higher than that of [^{99m}Tc]SAB2A and [^{99m}Tc]SAB2B, we additionally synthesized [^{99m}Tc]MURB2 (a [^{99m}Tc]MHam complex with bivalent UR) and evaluated the CA-IX-specific binding affinity of [^{99m}Tc]URB2A, [^{99m}Tc]URB2B, and [^{99m}Tc]MURB2. Their uptake into HT-29 cells was reduced by the addition of a CA inhibitor, acetazolamide, suggesting their CA-IX-specific binding affinity. A biodistribution study in HT-29 tumor-bearing mice was carried out using [^{99m}Tc]URB2A and [^{99m}Tc]MURB2 with the highest specificity for HT-29 cells. [^{99m}Tc]URB2A showed moderate tumor uptake and reduction by coinjection with acetazolamide; however, the tumor/blood ratio was insufficient for in vivo imaging. These results provided key information for the design of novel Ham-based imaging probes targeting CA-IX.



KEYWORDS: *carbonic anhydrase IX, technetium-99m, hydroxamamide, imaging*

INTRODUCTION

Carbonic anhydrase IX (CA-IX) is specifically expressed in hypoxic regions of tumors. Hypoxia assists tumor propagation and growth and is resistant to chemotherapy and radiotherapy.[1−10] CA-IX catalyzes the reversible reaction of carbon dioxide with water to bicarbonate ions and protons to maintain cellular acid−base homeostasis.[1,3,4,7−9] The CA-IX expression is controlled by hypoxia-inducible factor-1α (HIF-1α), which is reduced by von Hippel Lindau (VHL).[1,3−5,7,11,12] In hypoxic regions of tumors, the degradation of HIF-1α through VHL is suppressed, resulting in the overexpression of CA-IX,[1,3−5,7,11,12] which is observed in various tumors.[13−19] On the other hand, CA-IX expression is primarily limited to the gastrointestinal tract in normal tissue;[20] therefore, CA-IX is regarded as a favorable target for the in vivo imaging of tumors.

Several antibody-based nuclear medical imaging probes targeting CA-IX have been reported. Among them, iodine-124-labeled girentuximab showed a favorable property for the in vivo imaging of CA-IX, and clinical studies were performed to evaluate its efficacy and safety.[21,22] However, they showed pharmacokinetic limitations, such as slow blood clearance and nontargeted tissue and nonspecific normal organ uptake, raising issues of a low signal-to-background ratio and high radiation toxicity. Small-molecule probes have also been developed for the in vivo imaging of CA-IX. Fluorine-18 and gallium-68-labeled probes could be used to successfully visualize a CA-IX high-expressing tumor in a mouse model.[23−25] However, these probes are solely used for positron emission tomography (PET), which is only available at limited facilities. Therefore, it is necessary to develop nuclear medical imaging probes for single photon emission computed tomography (SPECT), which is more versatile than PET. We previously reported an indium-111-labeled probe for SPECT imaging of CA-IX, which successfully visualized CA-IX high-expressing tumors in mice.[26] Here, we developed a SPECT imaging probe including a more useful radiometal, technetium-99m (^{99m}Tc).

^{99m}Tc is the mainly used radioisotope in clinical practice. It is available from a commercial molybdenum-99/technetium-99m generator column, and its 141-keV γ ray is nearly optimal for imaging with SPECT. The half-life of ^{99m}Tc is 6 h, which is moderate for in vivo imaging. Therefore, ^{99m}Tc has played an important part in nuclear medicine. In order to apply ^{99m}Tc to

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molecular imaging, it is necessary to incorporate a chelating moiety into an imaging probe. Many effective coordination molecules for ^{99m}Tc have been developed, such as type N_2S_2 diamidedithiols, tricarbonyl, hydrazinonicotinic acid (HYNIC), and diethylenetriaminepentaacetic acid (DTPA).[27] Among them, we have previously reported several imaging probes based on hydroxamamide (Ham).[28]

Ham is a chelating agent including no thiol, which can form the ^{99m}Tc complex with high stability and a high radiochemical yield.[29,30] It can be synthesized easily and simply from nitrile in a one-step reaction. Moreover, Ham provides a ^{99m}Tc complex consisting of two Ham ligands, indicating that Ham would be helpful in the preparation of a ^{99m}Tc complex with a bivalent targeting vector.[30] Because enhancement in the binding of a multivalent ligand compared with its monovalent ligand was reported,[31,32] the [^{99m}Tc]Ham complex with a bivalent compound may be a candidate for in vivo imaging.

In this study, we designed [^{99m}Tc]Ham complexes with the aim of selective and specific in vivo imaging of CA-IX. We selected two types of structures as CA-IX ligands, sulfonamide (SA) and ureidosulfonamide (UR), and synthesized [^{99m}Tc]-SAB2 and [^{99m}Tc]URB2. SA is a common structure incorporated in many types of public CA inhibitors, such as acetazolamide. UR has been reported as a highly effective inhibitor of CA-IX.[33] The bulky structures of these [^{99m}Tc]-Ham complexes may contribute to cell membrane impermeability according to Lipinski's rule of five (molecular weight ≥500),[34] preventing them from binding to cytosolic CA isozymes. We also synthesized [^{99m}Tc]BHam, the [^{99m}Tc]Ham complex without CA-IX ligand, as a negative control. We evaluated their binding affinity using CA-IX high-expressing cells, HT-29, and RCC4 plus vector alone (RCC4-VA). After the most effective compound ([^{99m}Tc]URB2) was identified, we designed a UR derivative-incorporated *N*-methyl-substituted hydroxamamide (MHam), which provides a [^{99m}Tc]-MHam complex constituting two *N*-methyl-substituted Ham ligands with high stability,[35] and synthesized [^{99m}Tc]MURB2. After confirming its cellular binding affinity, we evaluated the biological properties of [^{99m}Tc]URB2 and [^{99m}Tc]MURB2 in HT-29 tumor-bearing mice.

EXPERIMENTAL SECTION

General. All reagents were purchased and used without further purification unless otherwise indicated. Na^{99m}TcO$_4$ was purchased from Nihon Medi-Physics Co., Ltd. (Tokyo, Japan). Silica gel column chromatography was performed with W-Prep 2XY (Yamazen, Osaka, Japan) on a Hi Flash silica gel column (40 μm, 60 Å; Yamazen). ^{1}H NMR and ^{13}C NMR spectra were recorded using a JNM-ECS400 (JEOL, Tokyo, Japan) with tetramethylsilane as an internal standard. Coupling constants are reported in Hertz. Multiplicity was defined as singlet (s), doublet (d), triplet (t), or multiplet (m). Mass spectra were obtained using a Shimadzu LCMS-2020 (an LC-20AT pump with an SPD-20A UV detector, λ = 254 nm; Shimadzu) with a Cosmosil C_{18} column ($5C_{18}$-AR-II, 4.6 mm × 150 mm; Nacalai Tesque, Kyoto, Japan) was used for high-performance liquid chromatography (HPLC).

Chemistry. *Synthesis of N′-Hydroxy-4-sulfamoylbenzene-1-carboximidamide (**1**).* To a solution of 4-cyanobenzene-1-sulfonamide (182 mg, 1 mmol) in EtOH (10 mL) were added hydroxylamine hydrochloride (208 mg, 3 mmol) and triethylamine (416 μL, 3 mmol). The reaction mixture was heated to reflux overnight. After being cooled to room temperature, water (50 mL) was added. The mixture was extracted with ethyl acetate (30 mL × 2). The organic phases were combined, dried over $MgSO_4$, and filtered. After evaporation of the filtrate, the residue was purified by silica gel chromatography (ethyl acetate/hexane = 9/1) to give 72 mg of **1** (34% yield). ^{1}H NMR (400 MHz, dimethyl sulfoxide (DMSO)-d_6, δ): 5.96 (s, 2H), 7.39 (s, 2H), 7.81 (m, 4H), 9.89 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 125.5 (2C), 125.8 (2C), 136.5 (1C), 144.1 (1C), 149.9 (1C). MS (ESI) m/z: 216.1 $[M + H]^+$.

*Synthesis of 4-{[(4′-Cyanophenyl)carbamoyl]amino}-benzene-1-sulfonamide (**2**).* To a solution of 4-amino-benzene-1-sulfonamide (172 mg, 1 mmol) in DMSO (5 mL) was added 1,1-carbonyldiimidazole (195 mg, 1.2 mmol). The reaction mixture was stirred at room temperature overnight. 4-Aminobenzonitrile (118 mg, 1 mmol) was added, and the reaction mixture was stirred at room temperature for 6 h. After lyophilization of the reaction mixture, the residue was purified by silica gel chromatography (chloroform/methanol = 9/1) to give 41 mg of **2** (13% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.24 (s, 2H), 7.64 (m, 4H), 7.75 (m, 4H), 9.29 (s, 1H), 9.36 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 112.4 (1C), 117.9 (2C), 118.3 (2C), 119.3(1C), 126.9 (2C), 133.4 (2C), 137.4 (1C), 142.3 (1C), 143.9 (1C), 151.9 (1C). MS (ESI) m/z: 317.1 $[M + H]^+$.

*Synthesis of N′-Hydroxy-4-{[(4-sulfamoylphenyl)-carbamoyl]amino}benzene-1-carboximidamide (**3**).* To a solution of **2** (20 mg, 0.06 mmol) in EtOH (5 mL) were added hydroxylamine hydrochloride (13 mg, 0.2 mmol) and triethylamine (26 μL, 0.2 mmol). The reaction mixture was stirred and heated to reflux overnight. After being cooled to room temperature, water (50 mL) was added. The mixture was extracted with ethyl acetate (30 mL × 2). The organic phases were combined, dried over $MgSO_4$, and filtered. After evaporation of the filtrate, the residue was purified by silica gel chromatography (chloroform/methanol = 9/1) to give 18 mg of **3** (84% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 6.69 (s, 2H), 7.23 (s, 2H), 7.53 (d, J = 9.2 Hz, 2H), 7.64 (t, J = 8.4 Hz, 4H), 7.75 (d, J = 8.4 Hz, 2H), 10.19 (s, 1H), 10.32 (s, 1H), 10.66 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 117.1 (3C), 117.3 (3C), 126.9 (3C), 127.5 (2C), 136.8 (1C), 142.9 (1C), 152.4 (1C). MS (ESI) m/z: 350.1 $[M + H]^+$.

*Synthesis of 4-({[4-(5-oxo-4,5-Dihydro-1,2,4-oxadiazol-3-yl)phenyl]carbamoyl}amino)benzene-1-sulfonamide (**4**).* To a solution of **3** (104 mg, 0.30 mmol) in DMSO (10 mL) was added 1,1-carbonyldiimidazole (58 mg, 0.36 mmol). The reaction mixture was stirred and heated gradually from room temperature to 95 °C for 6 h. The reaction mixture was lyophilized and purified by silica gel chromatography (chloroform/methanol = 9/1) to give 46 mg of **4** (42% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.08 (s, 1H), 7.23 (s, 2H), 7.62 (m, 4H), 7.73 (m, 4H), 9.40 (s, 1H), 9.44 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 117.8 (2C), 118.2 (2C), 118.3 (1C), 126.9 (2C), 127.2 (2C), 137.1 (1C), 142.5 (1C), 142.7 (1C), 152.2 (1C), 159.3 (1C), 163.1 (1C). MS (ESI) m/z: 376.0 $[M + H]^+$.

*Synthesis of 4-({[4-(4-Methyl-5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)phenyl]carbamoyl}amino)benzene-1-sulfonamide (**5**).* Iodomethane (55 μL, 0.88 mmol) and potassium carbonate (55 mg, 0.44 mmol) were added to a solution of **4** (55 mg, 0.15 mmol) in *N*,*N*-dimethylformamide (DMF) (5 mL). The reaction mixture was stirred at room temperature for 3 h. After the addition of water (50 mL), the mixture was

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extracted with ethyl acetate (30 mL × 2). The organic phases were combined, dried over $MgSO_4$, and filtered. After evaporation of the filtrate, the residue was purified by silica gel chromatography (chloroform/methanol = 9/1) to give 19 mg of **5** (34% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 3.24 (s, 3H), 7.24 (s, 2H), 7.64 (m, 2H), 7.69 (m, 4H), 7.75 (m, 2H), 9.32 (s, 1H), 9.34 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 29.7 (1C), 116.3 (2C), 117.8 (2C), 118.3 (1C), 126.9 (2C), 129.3 (2C), 137.2 (1C), 142.5 (1C), 142.9 (1C), 158.9 (1C), 159.5 (1C). MS (ESI) m/z: 390.2 $[M + H]^+$.

*Synthesis of N′-Hydroxy-N-methyl-4-{[(4-sulfamoylphenyl)carbamoyl]amino}benzene-1-carboximidamide (**6**).* Compound **5** (19 mg, 0.05 mmol) was dissolved in 1 M NaOH (aq)/DMF (1/1, 10 mL). The reaction mixture was stirred at 95 °C for 14 h. After neutralization with 1 M HCl (aq) in an ice bath, the mixture was extracted with ethyl acetate (30 mL × 2). The organic phases were combined, dried over $MgSO_4$, and filtered. After evaporation of the filtrate, the residue was purified by silica gel chromatography (chloroform/methanol = 9/1) to give 7 mg of **6** (38% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 3.51 (s, 3H), 5.68 (d, J = 5.2 Hz, 1H), 7.33 (d, J = 8.0 Hz, 4H), 7.50 (d, J = 8.0 Hz, 2H), 7.62 (d, J = 8.0 Hz, 2H), 7.73 (d, J = 8.0 Hz, 2H), 9.51 (s, 1H), 9.79 (s, 1H), 10.00 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 29.7 (1C), 117.4 (2C), 117.8 (2C), 126.9 (2C), 128.8 (2C), 136.9 (1C), 139.9 (1C), 142.8 (1C), 152.3 (1C), 155.2 (1C). MS (ESI) m/z: 364.1 $[M + H]^+$.

*Synthesis of N′-Hydroxybenzenecarboximidamide (**7**).* To a solution of benzonitrile (102 μL, 1 mmol) in EtOH (10 mL) were added hydroxylamine hydrochloride (208 mg, 3 mmol) and triethylamine (416 μL, 3 mmol). The reaction mixture was heated to reflux for 2 h. After being cooled to room temperature, water (50 mL) was added. The mixture was extracted with ethyl acetate (30 mL × 2). The organic phases were combined, dried over $MgSO_4$, and filtered. After evaporation of the filtrate, the residue was purified by silica gel chromatography (ethyl acetate/hexane = 3/2) to give 22 mg of **7** (16% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 5.85 (s, 2H), 7.37 (m, 3H), 7.67 (m, 2H), 9.63 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 125.4 (2C), 128.1 (2C), 128.9 (1C), 133.4 (1C), 150.9 (1C). MS (ESI) m/z: 137.1 $[M + H]^+$.

*Synthesis of 3-Phenyl-1,2,4-oxadiazol-5(4H)-one (**8**).* To a solution of **7** (1013 mg, 7.4 mmol) in DMSO (20 mL) was added 1,1-carbonyldiimidazole (1446 mg, 8.9 mmol). The reaction mixture was stirred and heated gradually from room temperature to 95 °C for 6 h. The reaction mixture was lyophilized and purified by silica gel chromatography (chloroform/methanol = 9/1) to give 360 mg of **8** (30% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.60 (m, 3H), 7.82 (d, J = 7.2 Hz, 2H), 12.98 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 123.7 (1C), 126.4 (2C), 129.7 (2C), 132.6 (1C), 157.7 (1C), 160.4 (1C). MS (ESI) m/z: 163.0 $[M + H]^+$.

*Synthesis of 4-Methyl-3-phenyl-1,2,4-oxadiazol-5(4H)-one (**9**).* Iodomethane (232 μL, 3.72 mmol) and potassium carbonate (257 mg, 1.86 mmol) were added to a solution of **8** (100 mg, 0.62 mmol) in DMF (5 mL). The reaction mixture was stirred at room temperature for 3 h. After the addition of water (50 mL), the mixture was extracted with ethyl acetate (30 mL × 2). The organic phases were combined, dried over $MgSO_4$, and filtered. After evaporation of the filtrate, the residue was purified by silica gel chromatography (chloroform/methanol = 9/1) to give 61 mg of **9** (55% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 3.37 (s, 3H), 7.62 (m, 2H), 7.68 (m, 1H), 7.75 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 29.5 (1C), 123.3 (1C), 128.4 (2C), 129.2 (2C), 132.0 (1C), 159.1 (1C), 159.4 (1C). MS (ESI) m/z: 177.0 $[M + H]^+$.

*Synthesis of N′-Hydroxy-N-methylbenzenecarboximidamide (**10**).* Compound **9** (115 mg, 0.65 mmol) was dissolved in 1 M NaOH (aq)/DMF (1/1, 10 mL). The reaction mixture was stirred at 95 °C for 14 h. After neutralization with 1 M HCl (aq) in an ice bath, the mixture was extracted with ethyl acetate (30 mL × 2). The organic phases were combined, dried over $MgSO_4$, and filtered. After evaporation of the filtrate, the residue was purified by silica gel chromatography (chloroform/methanol = 9/1) to give 76 mg of **10** (78% yield). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 3.34 (s, 3H), 5.76 (d, J = 5.2 Hz, 2H), 7.40 (s, 5H), 9.59 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 30.4 (1C), 128.2 (2C), 128.8 (2C), 132.6 (1C), 155.4 (1C). MS (ESI) m/z: 151.1 $[M + H]^+$.

Radiolabeling. $Na^{99m}TcO_4$ solution (100 μL) and tin(II) tartrate hydrate solution (3.0 mM in water, 30 μL) were added to a solution of **1**, **3**, **6**, **7**, or **10** (1 mg) in acetic acid/EtOH (1/4, 100 μL). After a 10 min incubation at room temperature, the mixture was purified by reversed-phase HPLC (RP-HPLC) on a Cosmosil C_{18} column using a mobile phase (10 mM phosphate buffer (pH 7.4)/acetonitrile = 9/1 (0 min) to 1/1 (30 min) ([^{99m}Tc]SAB2) or 4/1 (0 min) to 3/2 (30 min) ([^{99m}Tc]URB2, [^{99m}Tc]BHam, [^{99m}Tc]MURB2, and [^{99m}Tc]-MBHam)) at a flow rate of 1.0 mL/min. After the HPLC purification, the elusion buffer containing the ^{99m}Tc-labeled compound was subject to a stream of argon gas to remove acetonitrile. Subsequently, the ^{99m}Tc-labeled compound was extracted with ethyl acetate, and the extract was concentrated through a stream of argon gas. The residue was used after being resolved with a solvent for each experiment.

Cell Culture. HT-29 human colorectal carcinoma cells and RCC4-VA renal cell carcinoma cells, which are VHL-deficient, were used as CA-IX high-expressing cells, and MDA-MB-231 human breast adenocarcinoma cells and RCC4 plus VHL (RCC4-VHL) renal cell carcinoma (cell line RCC4 stably transfected with pcDNA3-VHL) cells were used as CA-IX low-expressing cells. HT-29 and MDA-MB-231 cells were purchased from Sumitomo Dainippon Pharma (Osaka, Japan). RCC4-VA and RCC4-VHL cells were purchased from DS Pharma Biomedical (Osaka, Japan). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (4000 mg/L of glucose; Nacalai Tesque), including 10% fetal bovine serum and a 100 U/mL concentration of penicillin and streptomycin at 37 °C in an atmosphere containing 5% CO_2.

Cell Binding Assay. Cells (HT-29, MDA-MB-231, RCC4-VHL, and RCC4-VA) were incubated in 12-well plates (4×10^5 cells/well) at 37 °C in 95% air/5% CO_2 for 24 h. After removing the medium, the ^{99m}Tc-labeled compound (37 kBq) in DMEM (1 mL) was added to each well, and the plates were incubated at 37 °C in 95% air/5% CO_2 for 2 h. Nonspecific binding of [^{99m}Tc]MURB2, [^{99m}Tc]MBHam, and [^{99m}Tc]-URB2 was evaluated in the presence of acetazolamide (2.0 μM) using HT-29 and MDA-MB-231 cells. The half-maximal inhibitory concentration (IC_{50}) was calculated by the addition of acetazolamide with increasing concentrations (2000, 400, 80, 16, 3.2, 0.64, 0.128, 0.0256, and 0.00512 nM) using HT-29 cells. After incubation, the wells were washed with 1 mL of phosphate-buffered saline (pH 7.4) (Thermo Fisher Scientific, Massachusetts, U.S.A.), and the cells were lysed with 1 M NaOH (aq) (0.2 mL × 2). Radioactivity in the cell solution

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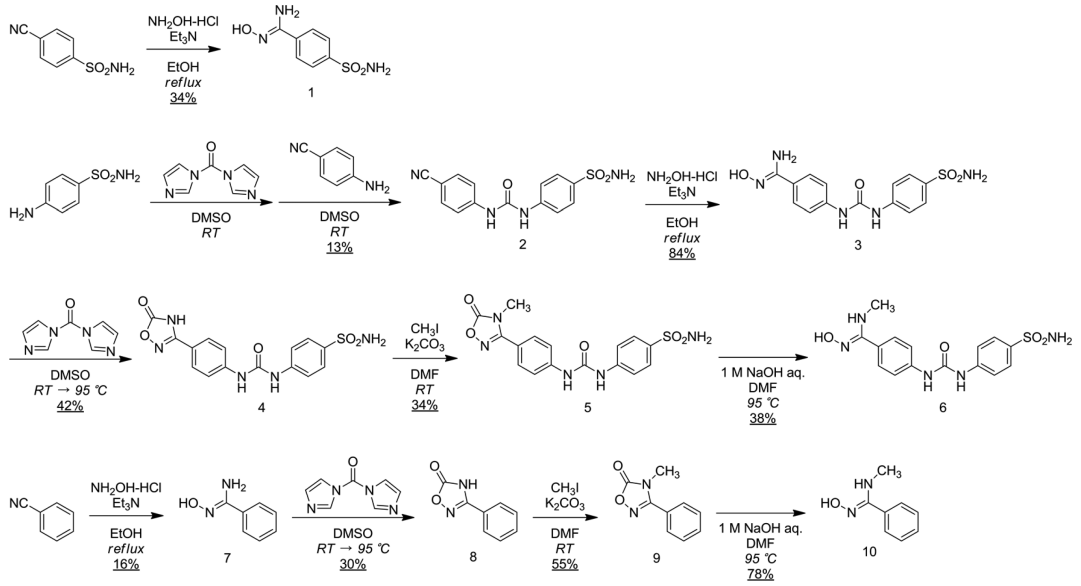
Scheme 1. Synthetic Route for Ham and MHam Compounds



was measured with a γ counter (Wallac 2470 Wizard; PerkinElmer, Massachusetts, U.S.A.). The protein concentration in the cell solution was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific).

Stability in Murine Plasma Assay. The experiments with animals were conducted according to our institutional guidelines and approved by Kyoto University Animal Care Committee. ddY mice (male, 5 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). After collection of plasma (200 μL) from ddY mice, [^{99m}Tc]MURB2 or [^{99m}Tc]URB2 (740 kBq) was added, and the solution was incubated at 37 °C for 1, 4, and 7 h. After the addition of acetonitrile (200 μL), they were centrifuged (4000g, 5 min). Subsequently, the supernatant was analyzed by RP-HPLC. The analytical method of RP-HPLC was the same as that written in the Radiolabeling section.

Biodistribution Assay. Nude mice (BALB/c *nu*/*nu*; male, 5 weeks old) were purchased from Japan SLC, Inc. HT-29 or MDA-MB-231 cells (1×10^7) were injected subcutaneously into the left flank of mice. After 4 weeks, [^{99m}Tc]URB2A or [^{99m}Tc]MURB2 (480 kBq, 150 μL in saline supplemented with 0.1% Tween 80) was intravenously injected, and the mice were sacrificed at 1, 3, 6, or 24 h post-injection (p.i.) (n = 5 per time-point). In the blocking experiment, acetazolamide (10 mg/kg mouse) was added to the injection. After collection of the blood, organs of interest (spleen, pancreas, stomach, intestines, kidneys, liver, heart, lungs, brain, muscle, and tumor) were removed and weighed. The radioactivity in each sample was measured with a γ counter (PerkinElmer). Moreover, the thyroid of MDA-MB-231 tumor-bearing mice was collected at 24 h p.i., and radioactivity was measured. The results are expressed as a percentage of the injected dose per gram (% ID/g) for selected organs, except the stomach and thyroid, whose results are expressed as a percentage of the injected dose.

Statistical Analysis. The significance of differences was assessed by a Student's t-test. Differences at the 95% confidence level ($P < 0.05$) were regarded as significant.

RESULTS AND DISCUSSION

Chemistry. Synthesis of the Ham and MHam compounds is shown in Scheme 1. The UR derivative **2** was synthesized by reacting 4-aminobenzonitrile and 4-aminobenzene-1-sulfonamide with 1,1-carbonyldiimidazole. The reaction of 4-cyanobenzene-1-sulfonamide, **2**, or benzonitrile with hydroxylamine gave Ham compounds according to a previously reported method,[30] which afforded **1**, **3**, and **7** in yields of 34, 84, and 16%, respectively. The precursors for [^{99m}Tc]-MURB2 (**6**) and [^{99m}Tc]MBHam (**10**) were prepared in three steps from the precursors for [^{99m}Tc]URB2 (**3**) and [^{99m}Tc]-BHam (**7**), respectively. The reaction of **3** or **7** with 1,1-carbonyldiimidazole provided compound **4** or **8** in DMSO. Methylation of compounds **4** and **8** with iodomethane provided compounds **5** and **9**, which were decyclized by heating in alkaline solution to compounds **6** and **10**, respectively (Scheme 1).

Radiolabeling. ^{99m}Tc labeling was performed using ^{99m}Tc pertechnetate, the Ham or MHam precursor, and tin(II) tartrate hydrate (Scheme 2). [^{99m}Tc]SAB2, [^{99m}Tc]URB2, and [^{99m}Tc]BHam were synthesized from the precursors **1**, **3**, and **7**, respectively. The RP-HPLC analysis of the reaction mixture indicated that two kinds of ^{99m}Tc-labeled compounds were generated as reported in previous reports on [^{99m}Tc]Ham complexes, suggesting that two specific isomers of them were given by the ^{99m}Tc labeling reaction using Ham (Figure 1A).[30,35] Hereinafter, the isomers observed at shorter retention times on RP-HPLC are referred to as the A-form ([^{99m}Tc]-SAB2A, [^{99m}Tc]URB2A, and [^{99m}Tc]BHamA), and the others as the B-form ([^{99m}Tc]SAB2B, [^{99m}Tc]URB2B, and [^{99m}Tc]-BHamB). The ^{99m}Tc labeling reaction with **6** and **10** provided a single radioactive peak at a retention time of 12.2 ([^{99m}Tc]MURB2) and 13.5 min ([^{99m}Tc]MBHam) on RP-HPLC, respectively (Figure 1B). In contrast, the reaction with **3** or **7** rendered two peaks at retention times of 9.7 and 12.7 min ([^{99m}Tc]URB2) or 7.5 and 15.5 min ([^{99m}Tc]BHam) (Figure 1A). This suggests that introduction of the methyl group into Ham would be useful to obtain a single [^{99m}Tc]MHam complex.[35] All ^{99m}Tc-labeled compounds were obtained in a 14−61% radiochemical yield and over

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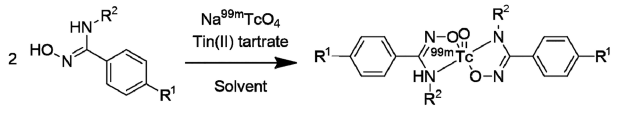
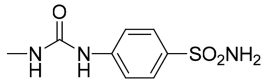
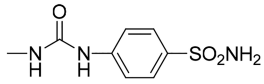
Scheme 2. ^{99m}Tc Labeling Route and Structure of [^{99m}Tc]Ham and [^{99m}Tc]MHam Complexes



compound	R^1	R^2
[^{99m}Tc]SAB2	—SO_2NH_2	—H
[^{99m}Tc]URB2		—H
[^{99m}Tc]BHam	—H	—H
[^{99m}Tc]MURB2		—CH_3
[^{99m}Tc]MBHam	—H	—CH_3

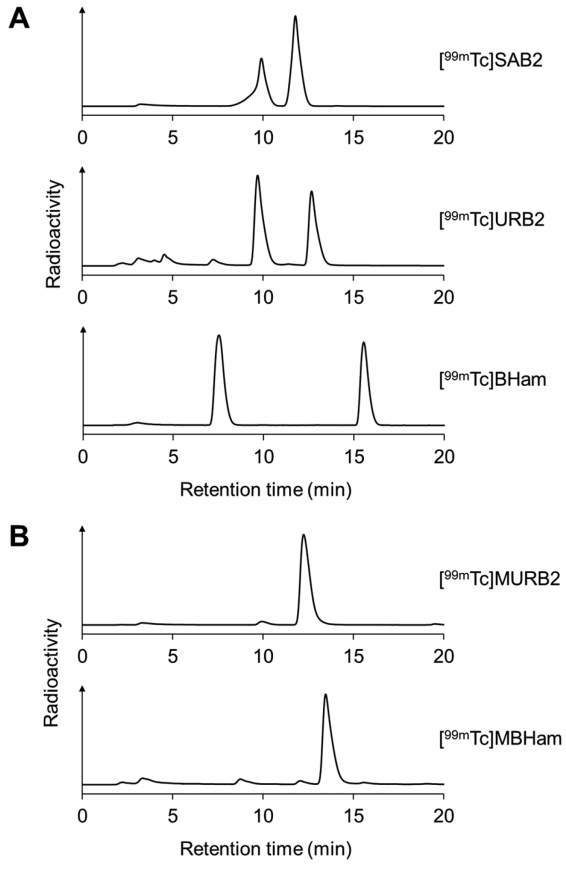


Figure 1. Radiochromatograms of [^{99m}Tc]Ham (A) and [^{99m}Tc]-MHam (B) complexes.

95% radiochemical purity without contamination by the precursors, which were observed at 0−5 min of the retention times on RP-HPLC (Table S1).

Cell Binding Assay. We previously evaluated in vitro expression of CA-IX in HT-29, MDA-MB-231, RCC4-VHL, and RCC4-VA cells by Western blotting. A high CA-IX expression was observed in HT-29 and RCC4-VA, and almost no expression was present in the others.[26] Between the A-form and B-form of all [^{99m}Tc]Ham complexes, cellular uptakes were not markedly different. The uptake of each [^{99m}Tc]Ham complex with a bivalent CA-IX ligand ([^{99m}Tc]SAB2A, [^{99m}Tc]SAB2B, [^{99m}Tc]URB2A, and [^{99m}Tc]URB2B) into HT-29 cells was significantly greater than that into MDA-MB-231 cells. Moreover, the uptake into RCC4-VA cells was also significantly greater than that into RCC4-VHL cells, indicating marked selectivity for the CA-IX high-expressing cells (Figure 2). However, [^{99m}Tc]BHamA and [^{99m}Tc]-

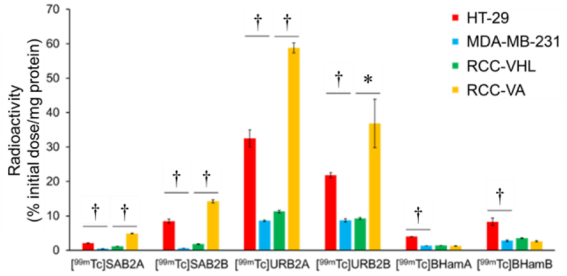


Figure 2. In vitro uptake of [^{99m}Tc]Ham complexes into cells. Values are expressed as the mean ± standard error of six independent experiments. *$P < 0.05$, and $^{\dagger}P < 0.005$ (Student's *t*-test).

BHamB, [^{99m}Tc]Ham complexes with no CA-IX ligand, also bound to HT-29 cells. In contrast, both complexes showed almost no binding to RCC4-VA cells, suggesting that uptakes of [^{99m}Tc]BHamA and [^{99m}Tc]BHamB into HT-29 cells would not be due to CA-IX. The uptakes of [^{99m}Tc]URB2A and [^{99m}Tc]URB2B were greater than those of [^{99m}Tc]SAB2A and [^{99m}Tc]SAB2B. These results corresponded with a report that UR showed a much greater affinity for CA isozymes as compared with nonsubstituted SA.[33]

After the most effective compound ([^{99m}Tc]URB2) was identified, we evaluated the CA-IX-specific binding affinity of [^{99m}Tc]MURB2 and [^{99m}Tc]MBHam (a negative control of [^{99m}Tc]MURB2) (Figure 3). The uptake of [^{99m}Tc]MURB2 into HT-29 cells was significantly reduced by the addition of acetazolamide, indicating that [^{99m}Tc]MURB2 CA-specifically bound to HT-29 cells. On the other hand, [^{99m}Tc]MBHam also bound to HT-29 cells; however, this uptake was not reduced by the addition of acetazolamide, suggesting that the uptake of [^{99m}Tc]MBHam into HT-29 cells is not CA-IX-specific, as with [^{99m}Tc]BHamA and [^{99m}Tc]BHamB. The uptakes of [^{99m}Tc]URB2A and [^{99m}Tc]URB2B were also reduced by the addition of acetazolamide, supporting the CA-IX-specific binding.

In the acetazolamide inhibition assay, IC_{50} values of acetazolamide in the presence of [^{99m}Tc]MURB2, [^{99m}Tc]-URB2A, and [^{99m}Tc]URB2B were not significantly different (22.7, 38.2, and 33.5 nM, respectively), suggesting that *N*-methylation of Ham would not affect the CA-IX binding affinity (Table 1 and Figure 4).

For application to further studies, we selected two effective compounds, [^{99m}Tc]URB2A and [^{99m}Tc]MURB2, which

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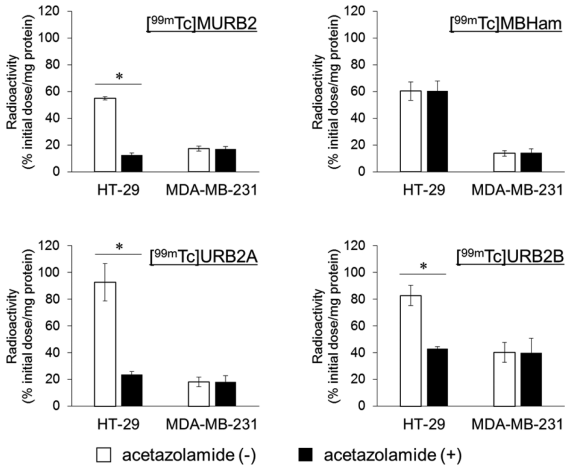

Figure 3. In vitro uptake of [99mTc]MURB2, [99mTc]MBHam, [99mTc]URB2A, and [99mTc]URB2B into cells. Values are expressed as the mean ± standard error of six independent experiments. *P < 0.005 (Student's t-test).

Table 1. Half-Maximal Inhibitory Concentration (IC_{50}, nM) for the Binding of Acetazolamide to CA-IX Determined Using [99mTc]Ham and [99mTc]MHam Complexes as Ligands

compound	IC_{50} of acetazolamide (nM)[a]
[99mTc]MURB2	22.7 ± 7.5
[99mTc]URB2A	38.2 ± 10.5
[99mTc]URB2B	33.5 ± 6.8

[a]Values are expressed as the mean ± standard error of six independent experiments.

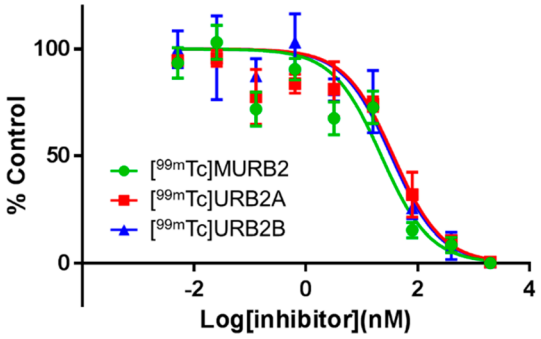

Figure 4. Inhibition curves of [99mTc]MURB2, [99mTc]URB2A, and [99mTc]URB2B from the inhibition assay for binding acetazolamide to CA-IX. Values are expressed as the mean ± standard error of six independent experiments.

showed higher CA-IX-specificity, determined by the ratio of uptake without acetazolamide to that with 2.0 μM acetazolamide into HT-29 cells (4.4, 3.9, and 1.9 for [99mTc]MURB2, [99mTc]URB2A, and [99mTc]URB2B, respectively).

Stability in Murine Plasma Assay. The stability of [99mTc]URB2 and [99mTc]MURB2 in murine plasma was examined after incubation at 37 °C for 1, 4, and 7 h. The recovery rates of radioactivity in the supernatant after centrifugation were 86.9 and 83.7% for [99mTc]URB2 and [99mTc]MURB2, respectively (n = 3). In other words, the protein-bound rates of [99mTc]URB2 and [99mTc]MURB2 were 13.1 and 16.3%, respectively (n = 3). Both compounds showed over 80% radiochemical purity until 7 h (Table 2).

Table 2. In Vitro Stability of [99mTc]MURB2 and [99mTc]URB2 in Murine Plasma[a]

	radiochemical purity (%) at given incubation times		
compound	1 h	4 h	7 h
[99mTc]MURB2	93.9 ± 1.4	90.2 ± 2.3	86.9 ± 4.1
[99mTc]URB2	90.7 ± 2.3	83.5 ± 2.3	80.0 ± 3.1

[a]Values are expressed as the mean ± standard error of six independent experiments.

Biodistribution. We previously confirmed that high and low levels of CA-IX expression were present in HT-29 and MDA-MB-231 tumor lysate from model mice, respectively. Thus, HT-29 and MDA-MB-231 tumors were used as CA-IX high-expressing and low-expressing tumors in vivo, respectively.[26] We examined the stability of [99mTc]URB2A and [99mTc]MURB2 in saline supplemented with 0.1% Tween 80. The HPLC analysis after incubation for 1 h at room temperature showed no marked decomposition of [99mTc]-URB2A or [99mTc]MURB2, indicating that they were stable in the injectate. The uptake of [99mTc]URB2A and [99mTc]-MURB2 in each organ was expressed as % ID/g (Figure 5, and

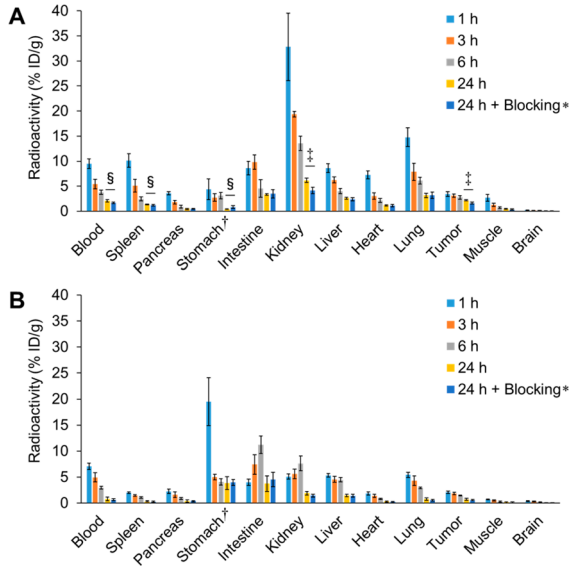

Figure 5. Radioactivity of extracted organs after intravenous injection of [99mTc]URB2A (A) and [99mTc]MURB2 (B) in the HT-29 tumor-bearing mice. Values are expressed as the mean ± standard deviation of five mice. *Coinjection of acetazolamide (10 mg/kg mouse). ‡Values are expressed as % injected dose. §P < 0.05, and ‡P < 0.001 (Student's t-test).

Tables S2 and S3). Tumor uptake of [99mTc]URB2A was 3.44, 3.13, 2.75, and 2.18% ID/g at 1, 3, 6, and 24 h p.i., respectively. This uptake was markedly greater than that previously reported using 99mTc-labeled CA-IX probes in HT-29 tumor-bearing mice.[36,37] The in vivo blocking studies were performed at the latest time to minimize the possibility of nontargeted accumulation of the 99mTc-labeled compound. Coinjection with acetazolamide (10 mg/kg mouse) significantly reduced the accumulation of [99mTc]URB2A in the HT-29 tumor at 24 h p.i. (1.62% ID/g), indicating CA-specificity of [99mTc]-

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URB2A. Moreover, the accumulation of [^{99m}Tc]URB2A in the HT-29 tumor (2.18% ID/g at 24 h p.i.) was significantly greater than that in the MDA-MB-231 tumor (0.77% ID/g at that time), suggesting marked in vivo selectivity for the CA-IX high-expressing tumor (Figure 6). On the other hand, high

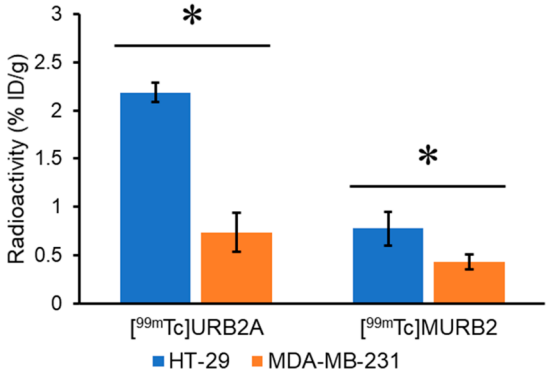

Figure 6. Radioactivity of extracted tumors at 24 h p.i. of [^{99m}Tc]URB2A or [^{99m}Tc]MURB2 in the HT-29 or MDA-MB-231 tumor-bearing mice. Values are expressed as the mean ± standard deviation of five mice. *P < 0.01 (Student's t-test).

blood retention was observed at all times, and the tumor/blood ratio was 0.4, 0.6, 0.7, and 1.0 at 1, 3, 6, and 24 h p.i., respectively. The low ratio should be improved because it causes a high background signal on in vivo imaging. One strategy to solve this problem is connecting two Ham by an ethylene or propylene linker, which showed shorter blood retention in a previous report.[38] Meanwhile, [^{99m}Tc]URB2A showed high kidney uptake, which was significantly blocked by acetazolamide coinjection. It was reported, that other transmembrane CA isozymes (CA-IV and CA-XIV) are expressed in the kidney;[39] therefore, the kidney uptake of [^{99m}Tc]URB2A might be caused by binding to these CA isozymes. A recent report suggested that expression levels of CA-IV and CA-XIV might be lower than that of CA-IX, and that blocking of binding sites in the kidney and other normal organs with nonradioactive CA ligands at suitable concentrations led to a higher tumor/normal organ ratio.[40] Moreover, the decrease in kidney uptake of [^{99m}Tc]URB2A might be influenced by the diuretic effect of acetazolamide.

Tumor uptake of [^{99m}Tc]MURB2 was 2.10, 1.87, 1.45, and 0.73% ID/g at 1, 3, 6, and 24 h p.i., respectively. In contrast to [^{99m}Tc]URB2A, coinjection of acetazolamide did not significantly reduce the accumulation of [^{99m}Tc]MURB2 in the HT-29 tumor at 24 h p.i. (0.57% ID/g). Because the in vitro CA-IX-selective binding affinity of [^{99m}Tc]MURB2 was confirmed by a cell binding assay, this nonsignificance is caused by a change in pharmacokinetics through the N-methyl-substitution of Ham. The accumulation of radioactivity was decreased in not only the tumor but also the normal organs, suggesting that radioactivity circulation was insufficient to specifically accumulate in the tumor at 24 h p.i. On the other hand, the in vivo selectivity of [^{99m}Tc]MURB2 for the HT-29 tumor as compared with the MDA-MB-231 tumor was demonstrated at 24 h p.i. (0.73 and 0.43% ID/g for HT-29 and MDA-MB-231 tumors, respectively) (Figure 6). Interestingly, the slight structural difference of Ham markedly reduced the kidney uptake at 1 and 3 h p.i., and alternatively increased the stomach uptake at 1 h p.i. Although it is well-known that $^{99m}TcO_4^-$ predominantly accumulates in the stomach, the high radioactivity in the stomach would not be caused by the accumulation of $^{99m}TcO_4^-$ following collapse of the ^{99m}Tc complex, because high in vitro stability in murine plasma was confirmed. In addition, no marked accumulation of [^{99m}Tc]-URB2A or [^{99m}Tc]MURB2 in the thyroid at 24 h p.i. was observed (<0.1% injected dose for all evaluated mice), suggesting their limited decomposition to pertechnetate. Moreover, in our previous study, a [^{99m}Tc]MHam complex showed marked accumulation in the stomach as compared with the corresponding [^{99m}Tc]Ham complex (unpublished data), suggesting that [^{99m}Tc]MHam complexes may generally accumulate in the stomach by an active system, although the details are unclear. On the other hand, radioactivity retention in the blood was not significantly changed by N-methyl-substitution of Ham, and the tumor/blood ratio was 0.3, 0.4, 0.5, and 1.0 at 1, 3, 6, and 24 h p.i., respectively. These results suggest that [^{99m}Tc]URB2A is more effective for in vivo CA-IX imaging than [^{99m}Tc]MURB2.

CONCLUSION

We designed and synthesized [^{99m}Tc]Ham and [^{99m}Tc]MHam complexes with bivalent SA and UR and evaluated their binding to CA-IX-expressing cells. An in vitro cellular binding assay showed that bivalent [^{99m}Tc]Ham and [^{99m}Tc]MHam complexes exhibited specific binding affinity for CA-IX. After that, we evaluated the biodistribution of [^{99m}Tc]URB2A, which showed the highest cellular binding affinity, and moderate tumor uptake of [^{99m}Tc]URB2A was observed and reduced by coinjection with the CA inhibitor, acetazolamide. These studies provided key information for the design of novel Ham-based imaging probes targeting CA-IX.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.8b01120.

Tables of radiochemical yield and radiochemical purity of [^{99m}Tc]Ham and [^{99m}Tc]MHam complexes, and data on the biodistribution of [^{99m}Tc]URB2A and [^{99m}Tc]-MURB2 in HT-29 tumor-bearing mice (PDF)

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Notes

The authors declare no competing financial interest.

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