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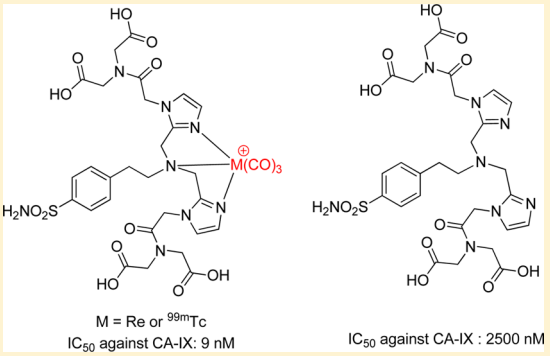
Synthesis and SAR of Novel Re/^{99m}Tc-Labeled Benzenesulfonamide Carbonic Anhydrase IX Inhibitors for Molecular Imaging of Tumor Hypoxia

Genliang Lu, Shawn M. Hillier, Kevin P. Maresca, Craig N. Zimmerman, William C. Eckelman, John L. Joyal, and John W. Babich*

Molecular Insight Pharmaceuticals, Inc., 160 Second Street, Cambridge, Massachusetts 02142, United States

ABSTRACT: Carbonic anhydrase IX (CA-IX) is upregulated in cancer in response to the hypoxic tumor microenvironment, making it an attractive molecular target for the detection of hypoxic solid tumors. A series of small molecule benzenesulfonamide based CA-IX inhibitors containing novel tridentate chelates complexed with the $M(CO)_3$ core (M = Re or ^{99m}Tc) were designed and synthesized. The in vitro binding affinity of the benzenesulfonamide rhenium complexes yielded IC_{50} values ranging from 3 to 116 nM in hypoxic CA-IX expressing HeLa cells. One of the most potent compounds, **3d** (IC_{50} = 9 nM), was radiolabeled with technetium tricarbonyl ($\{^{99m}Tc(CO)_3\}^+$) to afford the $\{^{99m}Tc(CO)_3\}^+$ complex in excellent yield and high purity. ^{99m}Tc-$(CO)_3$–**3d** bound specifically to CA-IX expressing hypoxic HeLa cells. This effort led to the identification of a diverse series of promising high affinity $\{^{99m}Tc(CO)_3\}^+$ radiolabeled CA-IX inhibitors with the potential to significantly impact diagnosis, staging, and treatment selection of hypoxic solid tumors.



INTRODUCTION

Carbonic anhydrases (CAs) are a family of enzymes comprised of 16 isoenzymes that catalyze the hydration of carbon dioxide to a proton and water represented by the chemical reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ and therefore play an important role in the regulation of intracellular and extracellular pH. Specific isozymes are either cytosolic (CA-I, CA-II, CA-III, CA-VII, and CA-XIII), anchored to the membrane (CA-IV, CA-IX, CA-XII, and CA-XIV), found within the mitochondria (CA-VA and CA-VB), or secreted from the cell (CA-VI).[1] Inhibitors of CAs, including acetazolamide, methazolamide, and ethoxazolamide, have been utilized for the treatment of a range of human diseases.[2−4] CA-IX is a transmembrane isoform with an extracellular catalytic domain. It has a limited tissue distribution and is found at low levels primarily in the gastrointestinal tract.[1] Importantly, the expression of CA-IX is under the control of hypoxia inducible factor-1α and is highly expressed in tumor cells exposed to hypoxia both in vitro and in vivo.[5] Increased CA-IX expression (greater than 20-fold) has been detected in selected tumor cell lines in vitro[6] and by immunohistochemistry in carcinomas of the cervix, ovaries, kidneys, esophagus, lung, breast, and brain.[1] Additionally, CA-IX is constitutively expressed in the majority of clear cell renal carcinomas as a result of a mutation in the Von Hippel−Lindau tumor suppressor.[4,5]

CA-IX has been shown by immunohistochemistry and by a variety of molecular techniques to be correlated with tumor progression and poor survival and has been proposed as a clinical diagnostic and prognostic marker for breast, renal, and non-small cell lung cancers.[7−9] An ^{125}I-labeled anti-CA-IX antibody, M75, has been used to monitor tumor hypoxia in both HeLa[10] and HT-29[6] xenografts in mice. Moreover, a chimeric ^{124}I-labeled anti-CA-IX antibody, G250, is currently undergoing clinical trials for the detection of clear cell renal carcinoma, validating CA-IX as a promising cancer target.[11]

While intact antibodies such as G250 offer the potential for tumor targeting with a radioisotope, the long circulating half-life and poor tissue penetrability limit the effectiveness of antibodies as radiodiagnostic and radiotherapeutic agents for solid tumors.[12] Radiolabeled small molecule radiotracers that bind CA-IX offer the preferred approach over antibodies.[13,14] While many series of CA-IX inhibitors have been reported,[15−22] there are only a few examples of small molecule CA-IX inhibitors with the potential to be used as cancer diagnostic agents.[23−26] For example, a fluorescent compound, 4-(sulfamoylphenylethylthioureido)fluorescein, has been shown to bind hypoxic tumor cells containing CA-IX.[23−25] In addition, the synthesis of membrane-impermeable CA-IX inhibitors that utilize click chemistry as an efficient method for incorporation of the radioisotope, fluorine-18 (^{18}F) have been described.[26] These analogues are being investigated as positron emission tomography (PET) imaging agents for characterizing various cancers.

^{99m}Tc has become the mainstay of diagnostic nuclear medicine employing SPECT and in various chemical forms is

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used in more than 85% of the diagnostic scans performed each year in hospitals.[27−29] The preferential use of ^{99m}Tc radiopharmaceuticals reflects the ideal nuclear properties of the isotope ($t_{1/2}$ = 6 h, E = 140 keV) as well as its convenient availability from commercial generator columns.[30,31] The development of ^{99m}Tc complexes as radiopharmaceuticals is facilitated by the use of rhenium, the group VIIB congener of technetium. Rhenium generally produces complexes with similar physical properties to those of technetium and is often used as a nonradioactive alternative to technetium for structure−activity relationship (SAR) analysis and structural characterization.

We have recently described a series of tridentate pyridine and carboxyl functionalized imidazole chelators for the $M(CO)_3$ core (M = Re or ^{99m}Tc) which can alter the binding properties and pharmacokinetics of small molecules and peptides to which they are attached.[32−34] Herein we describe the design, synthesis, and in vitro evaluation of several novel benzenesulfonamide rhenium tricarbonyl complexes ($Re(CO)_3$) incorporating these chelators derived from 4-(2-**Y**-alkyl)-benzenesulfonamide where **Y** = bis(pyridin-2-ylmethyl)amino (DPA), (pyridin-2-ylmethyl)(carboxymethyl)amino (PAMA), bis((1-methyl-1*H*-imidazol-2-yl)methyl)amino (NMI), bis((1-(carboxymethyl)-1*H*-imidazol-2-yl)methyl)amino (CIM), ((1-(carboxymethyl)-1*H*-imidazol-2-yl)methyl)((1-(2-(bis-(carboxymethyl)amino)-2-oxoethyl)-1*H*-imidazol-2-yl)-methyl)amino (CIM/TIM), bis((1-(2-(bis(carboxymethyl)-amino)-2-oxoethyl)-1*H*-imidazol-2-yl)methyl)amino (TIM), ((1-(2-(bis(carboxymethyl)amino)-2-oxoethyl)-1*H*-imidazol-2-yl)methyl)((1-(2-((1,5-dicarboxy-3-(2-carboxyethyl)pentan-3-yl)amino)-2-oxoethyl)-1*H*-imidazol-2-yl)methyl)amino (TIM/HIM), and bis((1-(2-((1,5-dicarboxy-3-(2-carboxyethyl)-pentan-3-yl)amino)-2-oxoethyl)-1*H*-imidazol-2-yl)methyl)-amino (HIM) (Figure 1). One of the most active molecules, 4-(2-TIM-ethyl)benzenesulfonamide rhenium complex **3d**, was successfully radiolabeled with ^{99m}Tc in high yield and specific activity and evaluated for binding to CA-IX expressing cells.

RESULTS AND DISCUSSION

Synthesis of Benzenesulfonamide Rhenium Complexes with Homogeneous Ligands. The preparation of homogeneous 4-aminoalkylbenzenesulfonamide rhenium tricarbonyl complexes **3a−j** commenced with double reductive amination of benzenesulfonamide **1a−d** with two molecules of the appropriate picolinaldehyde or *N*-substituted imidazole-2-carbaldehyde to afford the desired 4-aminoalkylbenzenesulfonamide ligand **2a−j** (Scheme 1).[34,35] 4-(2-DPA-ethyl) and 4-(2-NMI-ethyl)benzenesulfonamide rhenium complexes **3a** and **3b** were prepared by heating a solution of **2a** and **2b** in methanol to 90 °C in a pressure tube for 4 h in the presence of the rhenium tricarbonyl precursor $[NEt_4]_2[ReBr_3(CO)_3]$ and purified by flash chromatography over silica gel with yields ranging from 96 to 98%. Synthesis of the *tert*-butyl protected benzenesulfonamide $Re(CO)_3$ analogues was accomplished by heating a solution of **2c−j** in methanol or acetonitrile to 90 °C in a pressure tube for 4 h in the presence of the rhenium tricarbonyl precursor $[NEt_4]_2[ReBr_3(CO)_3]$. The crude *tert*-butyl protected benzenesulfonamide rhenium complexes were not characterized but directly deprotected with TFA/DCM at room temperature. Upon completion of the deprotection the reaction was concentrated under a stream of nitrogen to afford the crude residue, which was purified by HPLC using a C18 column to afford the desired complexes **3c−j**.

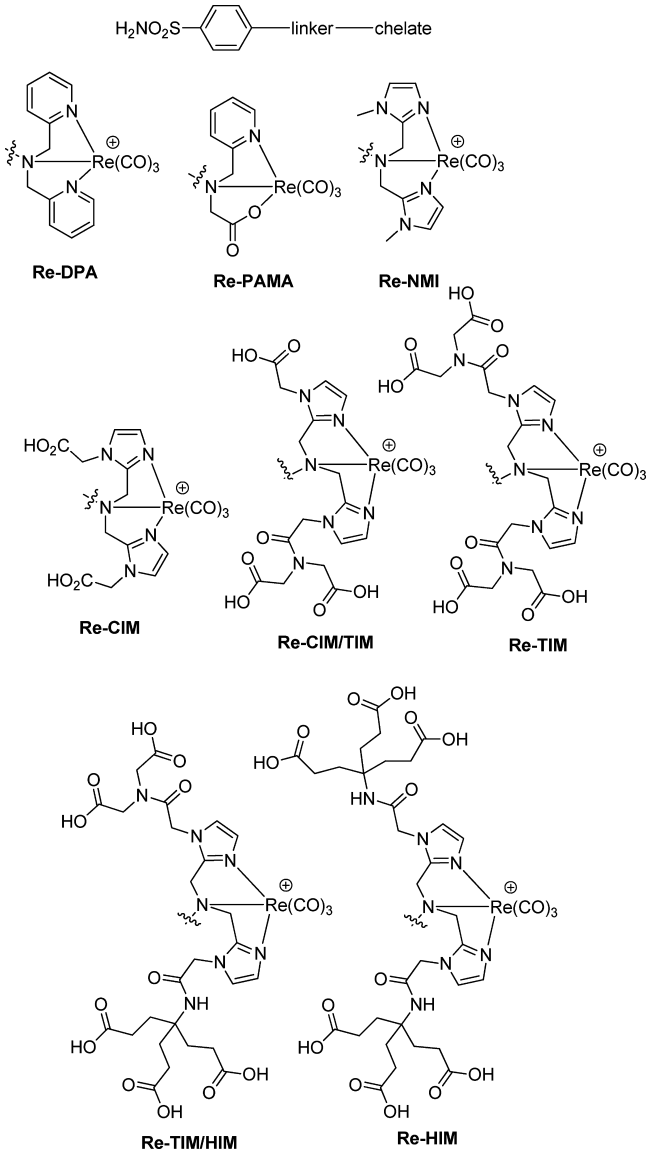

Figure 1. General structure of benzenesulfonamide rhenium tricarbonyl complexes.

Synthesis of Benzenesulfonamide Rhenium Complexes with Heterogeneous Ligands. The heterogeneous ligands **4a−c** were prepared using sequential double reductive alkylation on 4-aminoethylbenzenesulfonamide **1a** (Scheme 2).[36,37] Addition of 1 equiv of the first aldehyde (picolinaldehyde or *N*-substituted imidazole-2-carbaldehyde) followed by sodium borohydride afforded the intermediate. Subsequent treatment of intermediate with *t*-butylglyoxalate or different *N*-substituted imidazole-2-carbaldehyde afforded benzenesulfonamide **4a−c**. 4-(2-PAMA-ethyl)benzenesulfonamide rhenium complexes **5a** was readily prepared by deprotection of *tert*-butyl group with TFA, followed by heating a solution of **4a** in methanol to 100 °C in a pressure tube for 5 h in the presence of the rhenium tricarbonyl precursor $[NEt_4]_2[ReBr_3(CO)_3]$. 4-(2-CIM/TIM-ethyl) or 4-(2-TIM/HIM-ethyl)-benzenesulfonamide rhenium complexes **5b** or **5c** was readily prepared by heating a solution of **4b** or **4c** in acetonitrile to 90 °C in a pressure tube for 4 h in the presence of the rhenium tricarbonyl precursor $[NEt_4]_2[ReBr_3(CO)_3]$ followed by deprotection of *tert*-butyl group with TFA.

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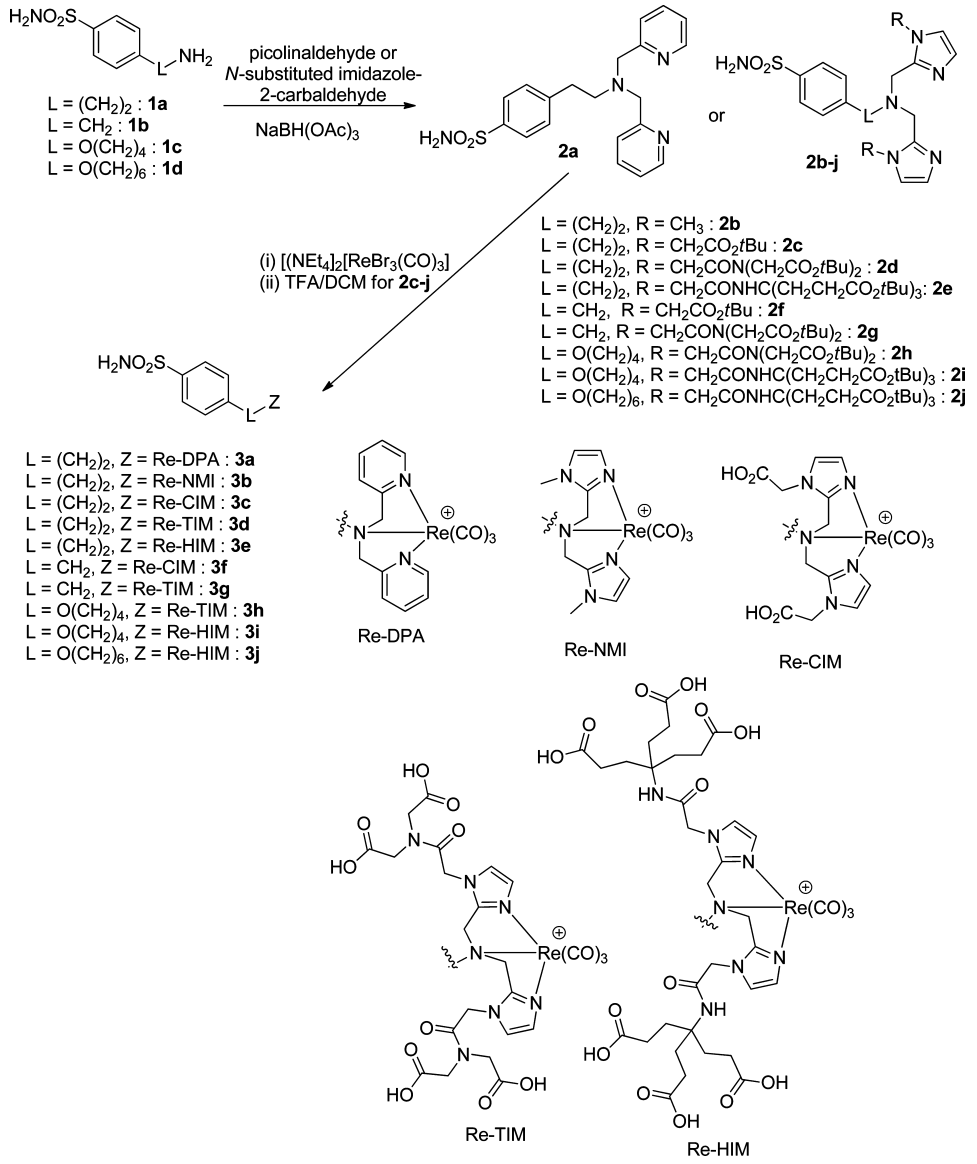
Scheme 1. Synthesis of Benzenesulfonamide Rhenium Complexes 3a–j with Homogeneous Ligands



To compare free ligands with benzenesulfonamide rhenium complexes for the binding affinity against CA-IX, the nonmetalated free ligands **6**, **7**, **8**, and **9** (Figure 2) were conveniently prepared via removal of *tert*-butyl group of **2d**, **2e**, **4c**, and **2i** by TFA/DCM solution.

Competitive Binding to Hypoxic CA-IX Expressing HeLa Cell. All compounds were evaluated in a competitive binding assay using the 4-(2-TIM-ethyl)benzenesulfonamide $^{99m}Tc(CO)_3$ complex (^{99m}Tc–**3d**) as the radioligand for binding to hypoxic CA-IX expressing HeLa cells. Acetazolamide (AZO) was included in all assays as the positive control. As illustrated in Table 1, all rhenium analogues demonstrated moderate to high affinity for CA-IX (IC_{50} = 3–116 nM).

The SAR of the rhenium complexes displayed several interesting trends. The evaluation of several different chelates was first investigated within the 4-(2-Y-ethyl)-benzenesulfonamide rhenium complexes **3a–e** and **5a–c**. The very hydrophobic dipyridylmethylamine complex, Re-DPA **3a** (IC_{50} = 116 nM), displayed only modest affinity for CA-IX. For the less hydrophobic pyridylamine monoacetic acid complex (Re-PAMA) **5a** (IC_{50} = 28 nM), a 4-fold increase in affinity for CA-IX was observed. The *N*-methylimidazole complex (Re-NMI) **3b** with hydrophobicity that was intermediate between Re-DPA **3a** and Re-PAMA **5a** displayed affinity (IC_{50} = 53 nM) for CA-IX that was 2-fold better than the more hydrophobic **3a** but 2-fold poorer affinity than the less hydrophobic **5a**. These results established a working hypothesis that the hydrophobicity as measured by HPLC retention times and experimental partition coefficients (clogP) of similarly related complexes[34] influenced the binding affinity.

Therefore, benzenesulfonamides incorporating polar imidazole ligands CIM, CIM/TIM, and TIM were designed and synthesized. The rhenium complexes demonstrated between a 3- and 29-fold improvement in affinity over the hydrophobic complexes **3a**, **3b**, and **5a**. Complexes with the highest affinity for CA-IX in the carboxylic acid substituted imidazole series, Re-CIM **3c** (IC_{50} = 4 nM), Re-CIM/TIM **5b** (IC_{50} = 3 nM), and Re-TIM **3d** (IC_{50} = 9 nM) tended to be molecules that were significantly less hindered than the complexes Re-TIM/HIM **5c** (IC_{50} = 44 nM) and Re-HIM **3e** (IC_{50} = 109 nM).

An effect of the linker length and composition of the linker between benzenesulfonamide and chelate portion of the

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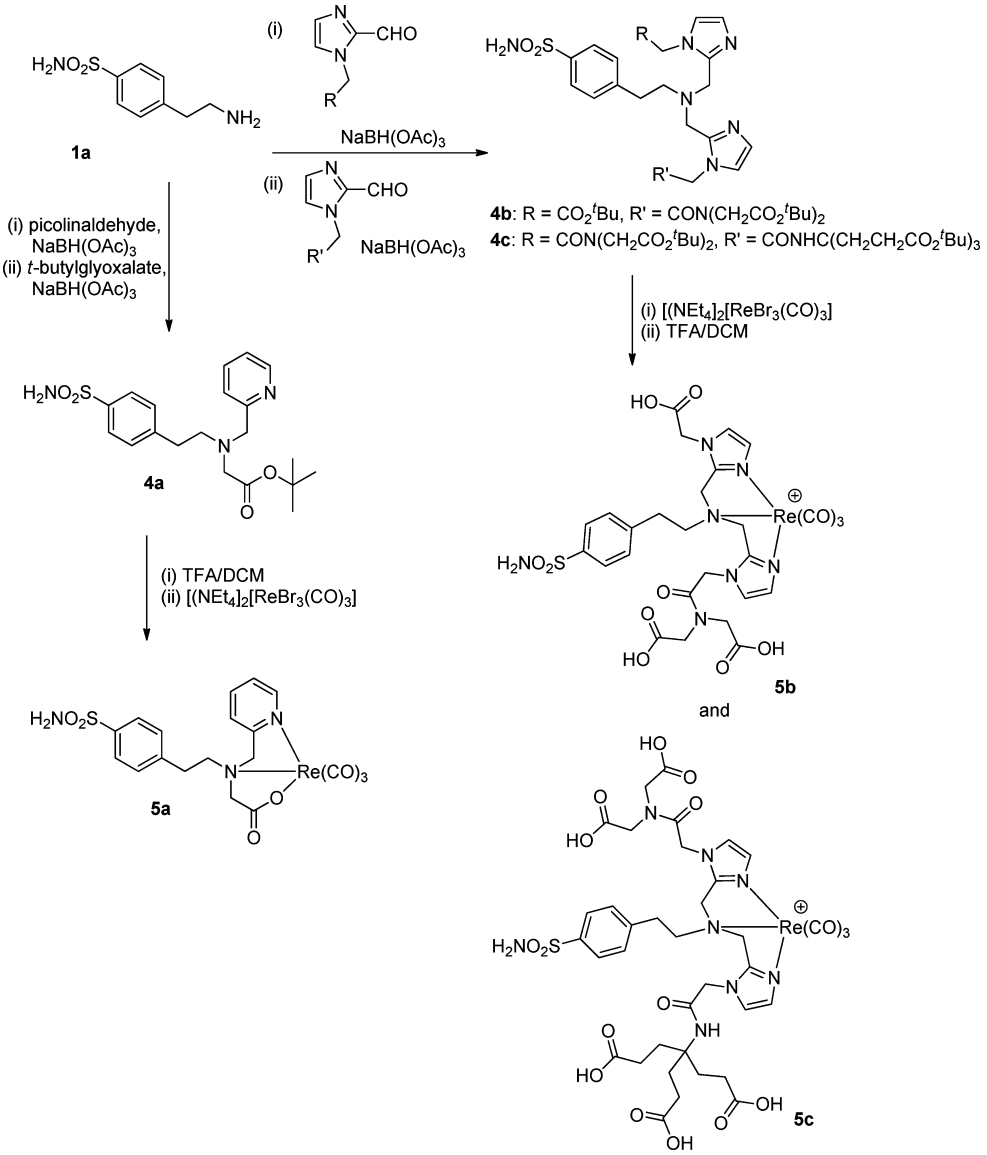
Scheme 2. Synthesis of Benzenesulfonamide Rhenium Complexes with Heterogeneous Ligands 5a−c



molecules was also observed. For the polar analogues Re-CIM **3f** (IC_{50} = 51 nM) and Re-TIM **3g** (IC_{50} = 116 nM), a methylene ($-CH_2-$) linker displayed 13-fold lower affinity than the corresponding homologues **3c** and **3d** which contained the ethylene ($-CH_2CH_2-$) linker. Additionally complex **3h** (IC_{50} = 43 nM) with the longer oxygen containing ($-O(CH_2)_4-$) linker displayed lower affinity than **3d** (IC_{50} = 9 nM) which contained the ethylene ($-CH_2CH_2-$) linker. However, a different trend was observed with the polar chelate that contained additional carboxylic acids, HIM, where the longer length linkers ($-O(CH_2)_4-$) **3i** (IC_{50} = 35 nM) and ($-O(CH_2)_6-$) **3j** (IC_{50} = 33 nM) provided complexes with better affinity for CA-IX than complexes like 4-(2-HIM-ethyl) benzenesulfonamide rhenium complex **3e** (IC_{50} = 109 nM) with the shorter linker ($-CH_2CH_2-$).

All of the nonmetalated free ligands analyzed displayed poor affinity for CA-IX as demonstrated by **6** (IC_{50} = 2500 nM), **7** (IC_{50} = 1600 nM), **8** (IC_{50} = 2500 nM), and **9** (IC_{50} = 782 nM). This suggested that the metal complex is required for high affinity binding to CA-IX. This may be attributed to the positive charge of the metal complexes or a more constrained structure of the metal complexes compared to the corresponding free ligands.

Radiochemistry. The ability of these novel benzenesulfonamide CA-IX inhibitors to be converted to the radioactive ^{99m}Tc complexes was demonstrated by employing one of the highest affinity compounds, **3d** (IC_{50} = 9 nM). The radiolabeling was accomplished using the fully protected *tert*-butyl ester derivative **2d**. The radiolabeling of *tert*-butyl ester derivative **2d** with $\{^{99m}Tc(CO)_3\}^+$ to form the desired metal complex was achieved in two steps utilizing standard methodology.[36] IsoLink kits (Covidien) were used to generate the desired intermediate $[^{99m}Tc(CO)_3(H_2O)_3]^+$, which following neutralization with hydrochloric acid, was reacted with **2d** in a sealed vial at 75 °C for 60 min with a ligand concentration of 10^{-4} M in an equal volume mixture of acetonitrile and water. After cooling and evaporation of solvent, the *tert*-butyl ester protecting groups were removed by treatment with 50% TFA in DCM at room temperature. Upon completion of the deprotection, the reaction was analyzed for purity via HPLC and subsequently purified by HPLC to afford the desired conjugate ^{99m}Tc−**3d** in 90% RCY and >95% RCP (Figure 3).

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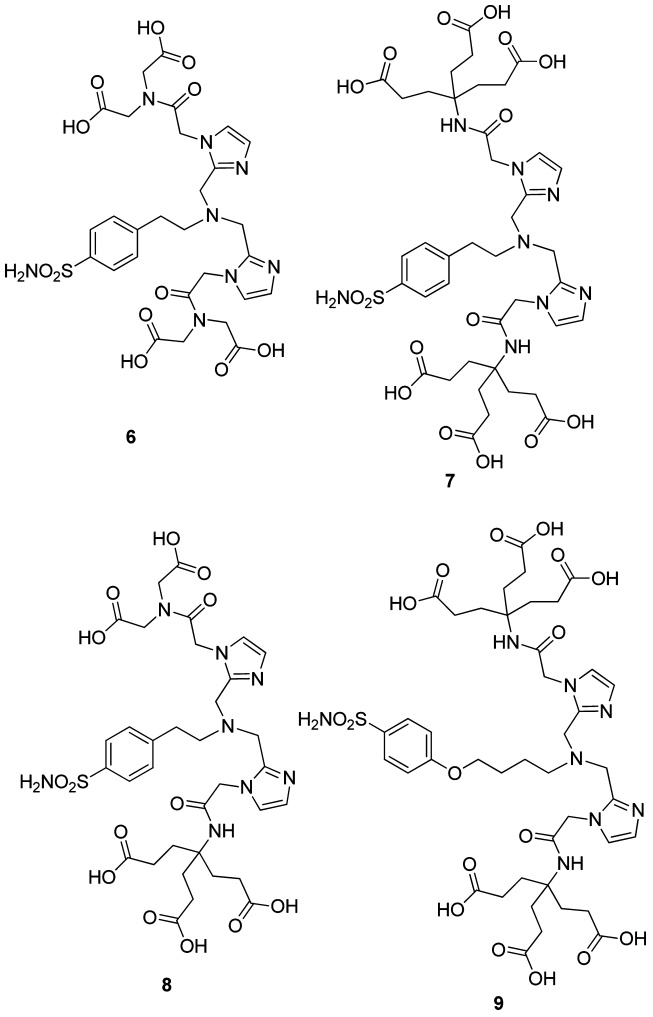
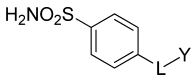


Figure 2. Structure of nonmetal free ligands **6**, **7**, **8**, and **9**.

Table 1. Competitive Binding of Benzenesulfonamide Analogues



compd	L	Y	IC_{50} (nM)
AZO			7
3a	$(CH_2)_2$	Re-DPA	116
5a	$(CH_2)_2$	Re-PAMA	28
3b	$(CH_2)_2$	Re-NMI	53
3c	$(CH_2)_2$	Re-CIM	4
5b	$(CH_2)_2$	Re-CIM/TIM	3
3d	$(CH_2)_2$	Re-TIM	9
5c	$(CH_2)_2$	Re-TIM/HIM	44
3e	$(CH_2)_2$	Re-HIM	109
3f	CH_2	Re-CIM	51
3g	CH_2	Re-TIM	116
3h	$O(CH_2)_4$	Re-TIM	43
3i	$O(CH_2)_4$	Re-HIM	35
3j	$O(CH_2)_6$	Re-HIM	33
6	$(CH_2)_2$	TIM	2500
7	$(CH_2)_2$	HIM	1600
8	$(CH_2)_2$	TIM/HIM	2500
9	$O(CH_2)_4$	HIM	782

This procedure utilized HPLC to remove excess free ligand. Because the free ligand has poor affinity for CA-IX compared to the radiolabeled final product, current research is directed at production of a "kit" formulation where the product can be utilized directly without purification from the excess free ligand.

Binding to CA-IX. To ensure that the binding of ^{99m}Tc−**3d** to CA-IX on HeLa cells was specific and saturable, cells were incubated with 3 nM ^{99m}Tc−**3d** alone or in the presence of 10 μM acetazolamide, a structurally unrelated carbonic anhydrase inhibitor, after exposure of the cells to hypoxic or normoxic conditions for 24 h. As illustrated in Figure 4A, ^{99m}Tc-3d bound only to hypoxic HeLa cells and not to HeLa cells that remained under normoxic conditions. Further, the binding of ^{99m}Tc−**3d** to the hypoxic HeLa cells was abrogated by coincubation with 10 μM acetazolamide. A dose curve of 1−10000 nM acetazolamide or nonradiolabeled **3d** demonstrated IC_{50} values of 7 and 9 nM, respectively (Figure 4B).

CONCLUSIONS

In summary, a series of benzenesulfonamide CA-IX inhibitors incorporating tridentate chelates DPA, NMI, CIM, CIM/TIM, TIM, TIM/HIM, and HIM with the $M(CO)_3$ core (M = Re or ^{99m}Tc) were designed, prepared, and evaluated for binding to hypoxic CA-IX expressing HeLa cells to generate a SAR. All rhenium analogues demonstrated moderate to high affinity for CA-IX (IC_{50} = 3−116 nM), with compounds containing the less hindered polar imidazole derivatized chelates, CIM, CIM/TIM, and TIM, and ethylene linker exhibiting the highest affinity. This effort has led to the identification of a diverse series of promising high affinity $\{^{99m}Tc(CO)_3\}^+$ radiolabeled CA-IX inhibitors which, because of the variation in chemical composition, will likely exhibit divergence in tissue distribution and pharmacokinetic properties. These compounds will be further evaluated in animal models of tumor hypoxia to select a lead compound with adequate tumor uptake and acceptable tumor-to-background for advancement to clinical trials.

EXPERIMENTAL SECTION

General Procedures. All reactions were carried out in dry glassware under an atmosphere of argon unless otherwise noted. Reactions were purified by flash chromatography under medium pressure using a Biotage SP4 or by preparative high pressure liquid chromatography using a Varian HPLC Prostar 210 Instrument. ^{1}H NMR was recorded on a Bruker 400 MHz instrument. Spectra are reported as ppm and are referenced to the solvent resonances in $CDCl_3$, DMSO-d_6, or methanol-d_4. Low resolution ESI mass spectra were obtained on Agilent G1956B LC/MS with an Agilent 1100 HPLC system. Higher resolution ESI mass spectra were obtained on Agilent G6510A Q-TQF LC/MS. All compounds evaluated for binding affinity toward CA-IX (**3c−j**, **5a−c**, **6**, **7**, **8**, and **9**) were purified on a Varian Prostar 210 preparative HPLC system using a Vydac C18 reverse-phase column (250 mm × 10 mm × 5 μm) connected to a Varian Prostar model 340 UV−vis detector monitoring at 220 nm or Biotage SP4 system over C18 catridge. Final product purification by HPLC was achieved using a binary solvent gradient of 5−85% mobile phase B where mobile phase A is water containing 0.1% TFA and mobile phase B is acetonitrile containing 0.1% TFA. The analytical purity of test compounds was obtained on an Agilent 1100 HPLC system with ZORBAX SB-Aq column (4.6 × 50 mm × 5 μm) using a binary solvent gradient of 15−100% mobile phase B where mobile phase A is water containing 0.1% formic acid and mobile phase B is acetonitrile containing 0.1% formic acid. The purity of all compounds evaluated in the biological assay was >95% purity as judged by HPLC. Analytical HPLC of ^{99m}Tc−**3d** was performed using

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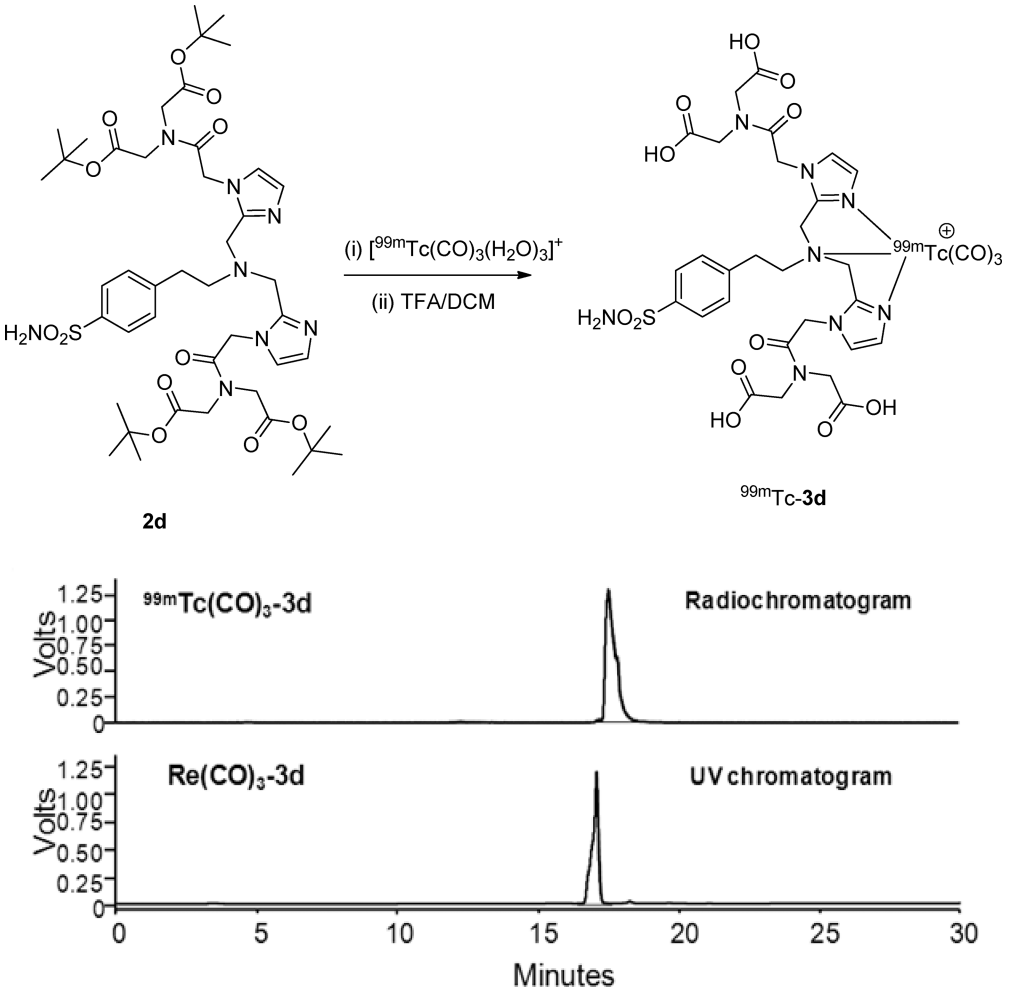

Figure 3. Radiochromatogram of $^{99m}Tc(CO)_3$–**3d** (50 μCi injection) demonstrating 98% RCP, 90% RCY (top), and the corresponding UV–visible chromatogram of the rhenium complex **3d** used for confirmation of identity (bottom).

the same method described above with an analytical Vydac C18 reverse-phase column (250 mm × 4.6 mm × 5 μm).

General Synthesis of Benzenesulfonamide Analogues 2a–j. A suspension of benzenesulfonamide **1a–d** (1 equiv), appropriate pyridine-2-carboxaldehyde or *N*-substituted imidazole-2-carbaldehyde (2 equiv), and AcOH in DCE was stirred at 75 °C for 30 min under nitrogen. The reaction mixture was cooled to 0 °C and treated with $NaBH(OAc)_3$ (3 equiv). The reaction mixture was stirred at room temperature overnight and quenched by methanol. Solvents were concentrated under reduced pressure to give a residue, which was purified by flash chromatography over silica gel with a gradient of 0–50% methanol in DCM to afford desired product **2a–j**.

*4-(2-(Bis(pyridin-2-ylmethyl)amino)ethyl)benzenesulfonamide (**2a**).* Treatment of 4-(2-aminoethyl)benzenesulfonamide **1a** (200 mg, 1.0 mmol), pyridine-2-carboxaldehyde (214 mg, 2.0 mmol), and AcOH (0.05 mL, 0.87 mmol) in DCE (20 mL) according to the general procedure described above afforded **2a** (370 mg, 97%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) 8.46 (d, J = 4.0 Hz, 2 H), 7.69–7.65 (m, 4 H), 7.30–7.20 (m, 6 H), 3.79 (s, 4 H), 2.87 (t, J = 7.0 Hz, 2 H), 2.69 (t, J = 7.0 Hz, 2 H). MS (ESI), 383.1 $(M + H)^+$.

*4-(2-(Bis((1-methyl-1H-imidazol-2-yl)methyl)amino)ethyl)-benzenesulfonamide (**2b**).* Treatment of 4-(2-aminoethyl)-benzenesulfonamide **1a** (200 mg, 1.0 mmol), 1-methyl-1*H*-imidazole-2-carbaldehyde (220 mg, 2.0 mmol), and AcOH (0.05 mL, 0.87 mmol) in DCE (20 mL) according to the general procedure described above afforded **2b** (389 mg, 100%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) 7.62 (d, J = 8.0 Hz, 2 H), 7.25 (s, 2 H), 7.04 (s, 2 H), 7.02 (d, J = 8.0 Hz, 2 H), 6.79 (d, J = 0.8 Hz, 2 H), 3.64 (s, 4 H), 3.26 (s, 6 H), 2.72 (t, J = 6.8 Hz, 2 H), 2.66 (t, J = 6.8 Hz, 2 H). MS (ESI), 389.1 $(M + H)^+$.

*tert-Butyl 2,2′-(2,2′-(4-Sulfamoylphenethylazanediyl)bis-(methylene)bis(1H-imidazole-2,1-diyl))diacetate (**2c**).* Treatment of 4-(2-aminoethyl)benzenesulfonamide **1a** (110 mg, 0.55 mmol), *tert*-butyl 2-(2-formyl-1*H*-imidazol-1-yl)acetate (250 mg, 1.19 mmol), and AcOH (0.10 mL, 1.75 mmol) in DCE (20 mL) according to the general procedure described above afforded **2c** (132 mg, 41%). ^{1}H NMR (400 MHz, CD_3OD) 7.75 (d, J = 8.4 Hz, 2 H), 7.18 (d, J = 8.4 Hz, 2 H), 7.07 (s, 2 H), 6.93 (s, 2 H), 4.58 (s, 4 H), 3.68 (s, 4 H), 2.84–2.74 (m, 4 H), 1.44 (s, 18 H). MS (ESI), 589.4 $(M + H)^+$.

*tert-Butyl 2,2′-(2,2′-(4-Sulfamoylphenethylazanediyl)bis-(methylene)bis(1H-imidazole-2,1-diyl-acetylazanediyl))diacetate (**2d**).* Treatment of 4-(2-aminoethyl)benzenesulfonamide **1a** (100 mg, 0.50 mmol), *tert*-butyl 2,2′-(2-(2-formyl-1*H*-imidazol-1-yl)-acetylazanediyl)diacetate (457 mg, 1.2 mmol), and AcOH (0.10 mL, 1.75 mmol) in DCE (30 mL) according to the general procedure described above afforded **2d** (465 mg, 100%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.63 (d, J = 8.0 Hz, 2 H), 7.23–7.21 (m, 4 H), 6.96 (s, 2 H), 6.79 (s, 2 H), 5.00 (s, 4 H), 4.30 (s, 4 H), 3.95 (s, 4 H), 3.59 (s, 4 H), 2.70–2.66 (m, 2H), 2.59–2.55 (m, 2 H), 1.42 (s, 18 H), 1.33 (s, 18 H). MS (ESI), 466.4 $(M/2 + H)^+$.

*Tetra-tert-butyl 4,4′-((2,2′-(2,2′-(((4-Sulfamoylphenethyl)-azanediyl)bis(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis-(azanediyl))bis(4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate) (**2e**).* Treatment of 4-(2-aminoethyl)benzenesulfonamide **1a** (80 mg, 0.40 mmol), di-*tert*-butyl 4-(2-bromoacetamido)-4-(3-(*tert*-butoxy)-3-oxopropyl)heptanedioate (447 mg, 0.81 mmol), and AcOH (0.05 mL, 0.87 mmol) in DCE (20 mL) according to the general procedure

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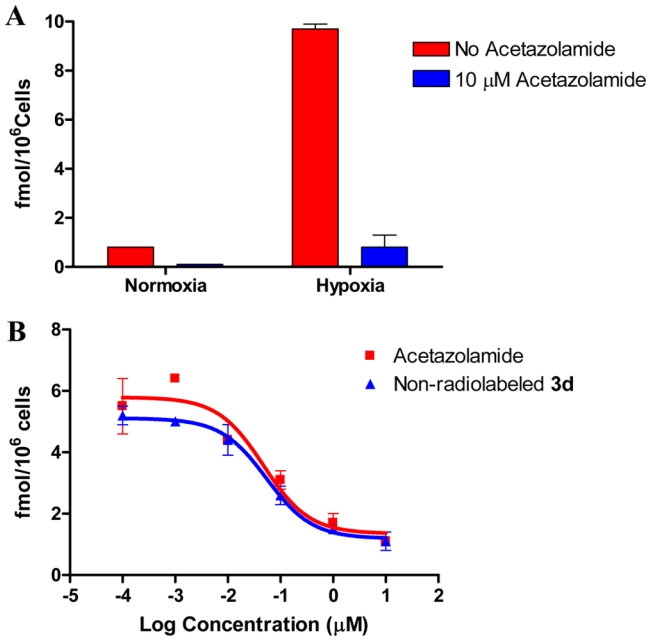

Figure 4. Binding of ^{99m}Tc−**3d** to HeLa cells. (A) ^{99m}Tc−**3d** (3 nM) was incubated with hypoxic or normoxic HeLa cells in the presence or absence of 10 μM acetazolamide. (B) ^{99m}Tc−**3d** (3 nM) was incubated with hypoxic HeLa cells in the presence of 1−10000 nM acetazolamide or nonradiolabeled **3d**.

described above afforded **2e** (322 mg, 63%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.77 (s, 2 H), 7.64 (d, J = 8.0 Hz, 2 H), 7.23 (s, 2 H), 7.21 (d, J = 8.4 Hz, 2 H), 7.01 (s, 2 H), 6.80 (s, 2 H), 4.57 (s, 4 H), 3.61 (s, 4 H), 2.79−2.62 (m, 4 H), 2.09 (t, J = 8.0 Hz, 12 H), 1.76 (t, J = 8.0 Hz, 12 H), 1.32 (s, 54 H). MS (ESI), 636.5 (M/2 + H)$^+$.

*tert-Butyl 2,2′-(2,2′-(4-Sulfamoylbenzylazanediyl)bis(methylene)-bis(1H-imidazole-2,1-diyl))diacetate (**2f**).* Treatment of 4-(2-aminomethyl)benzenesulfonamide hydrochloride (**1b**) (223 mg, 1.0 mmol) and *tert*-butyl 2-(2-formyl-1*H*-imidazol-1-yl)acetate (441 mg, 2.1 mmol) in DCE (20 mL) according to the general procedure described above afforded **2f** (569 mg, 99%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) 7.83 (d, J = 8.4 Hz, 2 H), 7.41 (d, J = 8.4 Hz, 2 H), 6.97 (s, 2 H), 6.82 (s, 2 H), 4.66 (s, 2 H), 4.43 (s, 2 H), 3.83 (s, 1 H), 3.73 (s, 1 H), 3.61 (s, 2 H), 3.48 (s, 4 H), 1.39 (s, 18 H). MS (ESI), 575.3 (M + H)$^+$.

*tert-Butyl 2,2′-(2,2′-(4-Sulfamoylbenzylazanediyl)bis(methylene)-bis(1H-imidazole-2,1-diyl-acetylazanediyl))diacetate (**2g**).* Treatment of 4-(2-aminomethyl)benzenesulfonamide hydrochloride (**1b**) (111 mg, 0.50 mmol) and *tert*-butyl 2,2′-(2-(2-formyl-1*H*-imidazol-1-yl)acetylazanediyl)diacetate (419 mg, 1.1 mmol) in DCE (40 mL) according to the general procedure described above afforded **2g** (322 mg, 70%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.71 (d, J = 8.4 Hz, 2 H), 7.46 (d, J = 8.4 Hz, 2 H), 7.24 (s, 2 H), 6.94 (s, 2 H), 6.80 (s, 2 H), 5.04 (s, 4 H), 4.30 (s, 4 H), 3.92 (s, 4 H), 3.64 (d, J = 16.8 Hz, 1 H), 3.60 (d, J = 16.8 Hz, 1 H), 3.42 (s, 4 H), 1.42 (s, 18 H), 1.34 (s, 18 H). MS (ESI), 459.3 (M/2 + H)$^+$.

*Tetra-tert-butyl 2,2′,2″,2‴-((2,2′-(2,2′-(((4-(4-Sulfamoylphenoxy)-butyl)azanediyl)bis(methylene))bis(1H-imidazole-2,1-diyl))bis-(acetyl))bis(azanetriyl))tetraacetate (**2h**).* Treatment of 4-(4-aminobutoxy)benzenesulfonamide TFA salt (**1c**) (437 mg, 1.0 mmol), *tert*-butyl 2,2′-(2-(2-formyl-1*H*-imidazol-1-yl)acetylazanediyl)-diacetate (838 mg, 2.2 mmol), and AcOH (0.10 mL, 1.75 mmol) in DCE (40 mL) according to the general procedure described above afforded **2h** (560 mg, 57%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.70 (d, J = 8.8 Hz, 2 H), 7.17 (s, 2 H), 6.99−6.97 (m, 4 H), 6.82 (s, 2 H), 5.06 (s, 4 H), 4.31 (s, 4 H), 3.96 (s, 4 H), 3.87 (t, J = 6.2 Hz, 2 H), 3.38 (s, 4 H), 2.42 (t, J = 6.6 Hz, 2 H), 1.56−1.47 (m, 4 H), 1.44 (s, 18 H), 1.36 (s, 18 H). MS (ESI), 975.3 (M + H)$^+$.

*Tetra-tert-butyl 4,4′-((2,2′-(2,2′-(((4-(4-Sulfamoylphenoxy)butyl)-azanediyl)bis(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis-(azanediyl))bis(4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate (**2i**).* Treatment of 4-(4-aminobutoxy)benzenesulfonamide TFA salt (**1c**) (216 mg, 0.59 mmol) and di-*tert*-butyl 4-(3-(*tert*-butoxy)-3-oxopropyl)-4-(2-(2-formyl-1*H*-imidazol-1-yl)acetamido)heptanedioate (570 mg, 1.033 mmol) in DCE (20 mL) according to the general procedure described above afforded **2i** (186 mg, 28%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.73−7.70 (m, 4 H), 7.17 (s, 2 H), 7.00−6.98 (m, 4 H), 6.78 (s, 2 H), 4.61 (s, 4 H), 3.87 (t, J = 7.2 Hz, 2 H), 3.51 (s, 4 H), 2.50−2.48 (m, 2 H), 2.11 (t, J = 8.0 Hz, 12 H), 1.78 (t, J = 8.0 Hz, 12 H), 1.60−1.35 (m, 58 H). MS (ESI), 658.4 (M/2 + H)$^+$.

*Tetra-tert-butyl 4,4′-((2,2′-(2,2′-(((6-(4-Sulfamoylphenoxy)hexyl)-azanediyl)bis(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis-(azanediyl))bis(4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate) (**2j**).* Treatment of 4-((6-aminohexyl)oxy)benzenesulfonamide containing TFA **1d** (346 mg, 0.659 mmol), di-*tert*-butyl 4-(3-(*tert*-butoxy)-3-oxopropyl)-4-(2-(2-formyl-1*H*-imidazol-1-yl)acetamido)heptanedioate (727 mg, 1.32 mmol), and AcOH (0.05 mL, 0.87 mmol) in DCE (50 mL) according to the general procedure described above afforded **2j** (153 mg, 17%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.76 (s, 2 H), 7.71 (d, J = 8.8 Hz, 2 H), 7.17 (s, 2 H), 7.04 (s, 2 H), 7.02 (d, J = 8.8 Hz, 2 H), 6.82 (s, 2 H), 4.62 (s, 4 H), 3.97 (t, J = 6.6 Hz, 2 H), 3.53 (s, 4 H), 2.44−2.40 (m, 2 H), 2.11 (t, J = 7.8 Hz, 12 H), 1.78 (t, J = 7.8 Hz, 12 H), 1.67−1.19 (m, 62 H). MS (ESI), 672.4 (M/2 + H)$^+$.

*tert-Butyl 2-((Pyridin-2-ylmethyl)(4-sulfamoylphenethyl)amino)-acetate (**4a**).* A solution of 4-(2-aminoethyl)benzenesulfonamide (**1a**) (1.60 g, 8.0 mmol), AcOH (0.30 mL, 5.24 mmol) and 2-pyridinecarboxaldehyde (0.76 mL, 8.0 mmol) in DCE (50 mL) was heated at 75 °C for 30 min under nitrogen. The reaction mixture was cooled to 0 °C and treated sequentially with $NaBH(OAc)_3$ (6.36 g, 30 mmol) and crude *tert*-butyl glyoxalate[37] (2.08 g). The reaction mixture was stirred at room temperature overnight and quenched by water. The reaction mixture was extracted with DCM. The organic layer was dried and concentrated under reduced pressure to give a residue, which was purified by flash chromatography over silica gel with a gradient of 0−10% methanol in DCM afforded **4a** (1.04 g, 32%). ^{1}H NMR (400 MHz, DMSO-d_6) 8.45−8.43 (m, 1 H), 7.70−7.63 (m, 3 H), 7.33 (d, J = 8.4 Hz, 2 H), 7.27 (s, 2 H), 7.24−7.20 (m, 2 H), 3.86 (s, 2 H), 3.31 (s, 2 H), 2.85−2.77 (m, 4 H), 1.40 (s, 9 H). MS (ESI), 406 (M + H)$^+$.

*tert-Butyl 2,2′-(2-(2-((((1-(2-tert-Butoxy-2-oxoethyl)-1H-imidazol-2-yl)methyl)(4-sulfamoylphenethyl)amino)methyl)-1H-imidazol-1-yl)acetylazanediyl)diacetate (**4b**).* A solution of 4-(2-aminoethyl)-benzenesulfonamide (**1a**) (600 mg, 3.0 mmol), AcOH (0.10 mL, 1.75 mmol), and *tert*-butyl 2,2′-(2-(2-formyl-1*H*-imidazol-1-yl)-acetylazanediyl)diacetate (381 mg, 1.0 mmol) in DCE (25 mL) was stirred at 75 °C for 30 min under nitrogen. The reaction mixture was cooled to 0 °C and treated with $NaBH(OAc)_3$ (0.422 g, 2.0 mmol). The reaction mixture was stirred at room temperature overnight and quenched with water. Solvent were concentrated under reduced pressure to give a residue, which was purified by flash chromatography over silica gel eluting with 1% MeOH in DCM to 20% MeOH in DCM to afford *tert*-butyl 2,2′-(2-(2-((4-sulfamoylphenethylamino)-methyl)-1*H*-imidazol-1-yl)acetylazanediyl)diacetate (191 mg). To a solution of the above product, *tert*-butyl 2,2′-(2-(2-((4-sulfamoylphenethylamino)methyl)-1*H*-imidazol-1-yl)acetylazanediyl)-diacetate (191 mg, 0.33 mmol), AcOH (0.10 mL, 1.75 mmol), and *tert*-butyl 2-(2-formyl-1*H*-imidazol-1-yl)acetate (105 mg, 0.50 mmol) in DCE (10 mL) at 0 °C was added $NaBH(OAc)_3$ (0.212 g, 1.0 mmol). The reaction mixture was stirred at room temperatureover-night and quenched by water. Solvent was concentrated under reduced pressure to give a residue, which was purified by flash chromatography over silica gel eluting with DCM to 10% MeOH in DCM afforded **4b** (143 mg, 19% over 2 steps). ^{1}H NMR (400 MHz, DMSO-d_6) 7.63 (d, J = 8.4 Hz, 2 H), 7.23 (s, 2 H), 7.21 (d, J = 8.0 Hz, 2 H), 7.03 (s, 1 H), 6.97 (s, 1 H), 6.78 (s, 2 H), 4.93 (s, 2 H), 4.72 (s, 2 H), 4.26 (s, 2 H), 3.94 (s, 2 H), 3.62 (s, 2 H), 3.57 (s, 2 H), 2.71−2.56 (m, 4 H), 1.41 (s, 18 H), 1.34 (s, 9 H). MS (ESI), 760.3 (M + H)$^+$.

Di-tert-butyl 4-(2-(2-((((1-(2-(Bis(2-(tert-butoxy)-2-oxoethyl)-amino)-2-oxoethyl)-1H-imidazol-2-yl)methyl)(4-

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*sulfamoylphenethyl)amino)methyl)-1H-imidazol-1-yl)acetamido)-4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate (**4c**).* A solution of 4-(4-aminobutoxy)benzenesulfonamide (2.40, 12.0 mmol), AcOH (0.40 mL, 7.0 mmol), and *tert*-butyl 2,2′-(2-(2-formyl-1*H*-imidazol-1-yl)-acetylazanediyl)diacetate (1.524 g, 4.0 mmol) in DCE (100 mL) was stirred at 75 °C for 30 min under nitrogen. The reaction mixture was cooled to 0 °C, treated with $NaBH(OAc)_3$ (1.64 g, 8.0 mmol), and stirred at room temperature overnight and quenched by water. Solvent was concentrated under reduced pressure to give a residue, which was purified by flash chromatography over silica gel with a gradient of 0−20% methanol in DCM afforded di-*tert*-butyl 2,2′-((2-(2-(((4-sulfamoylphenethyl)amino)methyl)-1*H*-imidazol-1-yl)acetyl)-azanediyl)diacetate as a white solid (547 mg, 24%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.68 (d, J = 8.0 Hz, 2 H), 7.32 (d, J = 8.0 Hz, 2 H), 7.24 (s, 2 H), 6.94 (s, 1 H), 6.72 (s, 1 H), 4.95 (s, 2 H), 4.25 (s, 2 H), 3.95 (s, 2 H), 3.61 (s, 2 H), 2.70−2.67 (m, 4 H), 1.43 (s, 9 H), 1.35 (s, 9 H). MS (ESI), 566.2 $(M + H)^+$. To a solution of above product, di-*tert*-butyl 2,2′-((2-(2-(((4-sulfamoylphenethyl)amino)methyl)-1*H*-imidazol-1-yl)acetyl)azanediyl)diacetate (200 mg, 0.353 mmol), di-*tert*-butyl 4-(3-(*tert*-butoxy)-3-oxopropyl)-4-(2-(2-formyl-1*H*-imidazol-1-yl)acetamido)heptanedioate (195 mg, 0.353 mmol), and AcOH (0.10 mL, 1.75 mmol) in DCE (10 mL) at 0 °C was treated with $NaBH(OAc)_3$ (148 mg, 0.70 mmol). The reaction mixture was stirred at 0 °C for 30 min and at room temperature overnight and quenched by water. The reaction mixture was concentrated under reduced pressure to give a residue, which was purified by flash chromatography over silica gel with a gradient of 0−10% methanol in DCM afforded **4c** (237 mg, 61%) as yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) 7.76 (s, 1 H), 7.66 (d, J = 8.4 Hz, 2 H), 7.23 (d, J = 8.4 Hz, 2 H), 7.22 (s, 2 H), 7.01 (d, J = 1.2 Hz, 1 H), 6.99 (d, J = 1.6 Hz, 1 H), 6.83 (d, J = 0.8 Hz, 1 H), 6.80 (d, J = 0.8 Hz, 1 H), 5.02 (s, 2 H), 4.55 (s, 2 H), 4.31 (s, 2 H), 3.97 (s, 2 H), 3.63 (s, 2 H), 3.60 (s, 2 H), 2.77−2.73 (m, 2 H), 2.66−2.60 (m, 2 H), 2.10 (t, J = 8.2 Hz, 6 H), 1.78 (t, J = 8.2 Hz, 6 H), 1.36 (s, 45 H). MS (ESI), 551.4 $(M/2 + H)^+$.

General Procedure for Synthesis of Rhenium Tricarbonyl Complexes 3c−j, 5b, and 5c. A solution of **2c−i**, **4b**, or **4c** and $[NEt_4]_2[ReBr_3(CO)_3]$ in methanol or acetonitrile in sealed pressure tube was stirred at 90 °C for 4 h. The solvent was evaporated to give a residue, which was dissolved in DCM (1.0−3.0 mL) and TFA (1.0−3.0 mL) and the reaction mixture was stirred at room temperature overnight. The solvent was evaporated and afforded the crude product, which was purified by HPLC using a binary solvent gradient of 0−50% mobile phase B where mobile phase A is water containing 0.1% TFA and mobile phase B is acetonitrile containing 0.1% TFA and lyophilized to afford the desired product **3c−j**, **5b**, or **5c**.

*4-(2-(Bis(pyridin-2-ylmethyl)amino)ethyl)benzenesulfonamide Rhenium Tricarbonyl Complex (**3a**).* A solution of **2a** (107 mg, 0.279 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (216 mg, 0.279 mmol) in methanol (5.0 mL) in sealed pressure tube was stirred at 90 °C for 4 h. The solvent was evaporated to give a residue, which was purified by flash chromatography over silica gel with a gradient of 0−10% methanol in DCM afforded **3a** (174 mg, 96%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) 8.82 (d, J = 5.2 Hz, 2H), 8.02 (td, J = 7.8, 1.2 Hz, 2 H), 7.83 (d, J = 8.0 Hz, 2 H), 7.63 (d, J = 8.4 Hz, 2 H), 7.60 (d, J = 8.4 Hz, 2 H), 7.41 (t, J = 6.6 Hz, 2 H), 7.32 (s, 2 H), 5.18 (d, J = 16.8 Hz, 2 H), 5.04 (d, J = 16.4 Hz, 2 H), 3.97−3.92 (m, 2 H), 3.29−2.20 (m, 2 H). HRMS (ESI) calcd for $C_{23}H_{22}N_4O_5ReS$ $(M)^+$, 653.0868; found, 653.0866.

*4-(2-(Bis((1-methyl-1H-imidazol-2-yl)methyl)amino)ethyl)-benzenesulfonamiderhenium Tricarbonyl Complex (**3b**).* A solution of **2b** (79.7 mg, 0.205 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (158 mg, 0.205 mmol) in methanol (5.0 mL) in a sealed pressure tube was stirred at 90 °C for 4 h. The solvent was evaporated to give a residue, which was purified by flash chromatography over silica gel eluting with 0−10% methanol in DCM afforded **3b** (118 mg, 98%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) 7.82 (d, J = 8.4 Hz, 2H), 7.62 (d, J = 8.0 Hz, 2 H), 7.31 (s, 2 H), 7.29 (d, J = 1.6 Hz, 2 H), 7.07 (d, J = 1.6 Hz, 2 H), 5.05 (d, J = 16.4 Hz, 2 H), 4.83 (d, J = 16.4 Hz, 2 H), 3.70 (s, 6 H), 2.50−2.48 (m, 4 H). HRMS (ESI) calcd for $C_{21}H_{24}N_6O_5ReS$ $(M)^+$, 659.1086; found, 659.1084.

*2,2′-(2,2′-(4-Sulfamoylphenethylazanediyl)bis(methylene)bis(1H-imidazole-2,1-diyl))diacetic Acid Rhenium Tricarbonyl Complex (**3c**).* Treatment of **2c** (65 mg, 0.11 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (92.4 mg, 0.12 mmol) in methanol (3.0 mL) according to the general procedure described above afforded **3c** (60 mg, 53%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 7.81 (d, J = 8.0 Hz, 2 H), 7.60 (d, J = 8.4 Hz, 2 H), 7.30 (s, 2 H), 7.23 (d, J = 1.2 Hz, 2 H), 7.08 (d, J = 1.2 Hz, 2 H), 4.91 (s, 4 H), 4.72 (s, 4 H), 3.89−3.85 (m, 2 H), 3.18−3.14 (m, 2 H). HRMS (ESI) calcd for $C_{23}H_{24}N_6O_9ReS$ $(M)^+$, 747.0883; found, 747.0873.

*2,2′-(2,2′-(4-Sulfamoylphenethylazanediyl)bis(methylene)bis(1H-imidazole-2,1-diyl-acetylazanediyl))diacetic Acid Rhenium Tricarbonyl Complex (**3d**).* Treatment of **2d** (32 mg, 0.034 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (30 mg, 0.039 mmol) in methanol (3.0 mL) according to the general procedure described above afforded **3d** as a white solid (33 mg, 27%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.79 (d, J = 8.4 Hz, 2 H), 7.58 (d, J = 8.0 Hz, 2 H), 7.29 (s, 2 H), 7.13 (s, 2 H), 7.06 (s, 2 H), 5.06 (s, 4 H), 4.65 (d, J = 16.4 Hz, 2 H), 4.40 (d, J = 16.4 Hz, 2 H), 4.29 (s, 4 H), 4.08 (d, J = 17.6 Hz, 2 H), 4.02 (d, J = 17.6 Hz, 2 H), 3.87−3.83 (m, 2 H), 3.11−3.08 (m, 2 H). HRMS (ESI) calcd for $C_{31}H_{34}N_8O_{15}ReS$ $(M)^+$, 977.1416; found, 977.1411.

*4,4′-((2,2′-(2,2′-(((4-Sulfamoylphenethyl)azanediyl)bis-(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis(azanediyl))-bis(4-(2-carboxyethyl)heptanedioic Acid Rhenium Tricarbonyl Complex (**3e**).* Treatment of **2e** (167 mg, 0.131 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (116 mg, 0.15 mmol) in methanol (3.0 mL) according to the general procedure described above afforded **3e** (75 mg, 48%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 12.10 (brs, 6 H), 7.78 (d, J = 8.4 Hz, 2 H), 7.71 (s, 2 H), 7.55 (d, J = 8.0 Hz, 2 H), 7.27 (s, 2 H), 7.17 (s, 2 H), 7.03 (s, 2 H), 4.72 (s, 4 H), 4.64 (d, J = 16.4 Hz, 2 H), 4.52 (d, J = 16.4 Hz, 2 H), 3.86−3.82 (m, 2 H), 3.13−3.09 (m, 2 H), 2.14 (t, J = 8.0 Hz, 12 H), 1.85 (t, J = 8.0 Hz, 12 H). HRMS (ESI) calcd for $C_{43}H_{54}N_8O_{19}ReS$ $(M)^+$, 1205.2778; found, 1205.2776.

*2,2′-(2,2′-(4-Sulfamoylbenzylazanediyl)bis(methylene)bis(1H-imidazole-2,1-diyl))diacetic Acid Rhenium Tricarbonyl Complex (**3f**).* Treatment of **2f** (40 mg, 0.070 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (60 mg, 0.077 mmol) in methanol (3.0 mL) according to the general procedure described above afforded **3f** (23 mg, 45%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 7.93 (d, J = 8.0 Hz, 2 H), 7.86 (d, J = 8.4 Hz, 2 H), 7.47 (s, 2 H), 7.14 (d, J = 1.2 Hz, 2 H), 7.06 (d, J = 1.2 Hz, 2 H), 4.92 (s, 2 H), 4.79 (d, J = 16.0 Hz, 2 H), 4.76 (s, 4 H), 4.20 (d, J = 16.0 Hz, 2 H). HRMS (ESI) calcd for $C_{22}H_{22}N_6O_9ReS$ $(M)^+$, 733.0726; found, 733.0715.

*2,2′,2″,2‴-((2,2′-(2,2′-(((4-Sulfamoylbenzyl)azanediyl)bis-(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis(azanetriyl))-tetraacetic Acid Rhenium Tricarbonyl Complex (**3g**).* Treatment of **2g** (75 mg, 0.082 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (75 mg, 0.097 mmol) in methanol (4.0 mL) according to the general procedure described above afforded **3g** (56 mg, 70%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 7.91 (d, J = 8.4 Hz, 2 H), 7.87 (d, J = 8.4 Hz, 2 H), 7.43 (s, 2 H), 7.09 (d, J = 1.6 Hz, 2 H), 7.06 (d, J = 1.6 Hz, 2 H), 5.03−4.92 (m, 6 H), 4.75 (d, J = 15.6 Hz, 2 H), 4.21 (s, 4 H), 4.08−3.92 (m, 6 H). HRMS (ESI) calcd for $C_{30}H_{32}N_8O_{15}ReS$ $(M)^+$, 963.1260; found, 963.1255.

*2,2′,2″,2‴-((2,2′-(2,2′-(((4-(4-Sulfamoylphenoxy)butyl)azanediyl)-bis(methylene))-bis(1H-imidazole-2,1-diyl))bis(acetyl))bis-(azanetriyl))tetraacetic Acid Rhenium Tricarbonyl Complex (**3h**).* Treatment of **2h** (97.4 mg, 0.10 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (80 mg, 0.10 mmol) in acetonitrile (5.0 mL) according to the general procedure described above afforded **3h** as a white solid (70 mg, 69%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.73 (d, J = 9.2 Hz, 2 H), 7.18 (s, 2 H), 7.11 (d, J = 2.4 Hz, 2 H), 7.10 (d, J = 9.2 Hz, 2 H), 7.03 (d, J = 1.6 Hz, 2 H), 5.00 (s, 4 H), 4.39 (d, J = 16.4 Hz, 2 H), 4.29 (d, J = 16.4 Hz, 2 H), 4.23 (s, 4 H), 4.12 (t, J = 6.0 Hz, 2 H), 4.02 (s, 4 H), 3.79−3.75 (m, 2 H), 1.92−1.80 (m, 4 H). HRMS (ESI) calcd for $C_{33}H_{38}N_8O_{16}ReS$ $(M)^+$, 1021.1684; found, 1021.1676.

*4,4′-((2,2′-(2,2′-(((4-(4-Sulfamoylphenoxy)butyl)azanediyl)bis-(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis(azanediyl))-bis(4-(2-carboxyethyl)heptanedioic acid) Rhenium Tricarbonyl Complex (**3i**).* Treatment of **2i** (114 mg, 0.0867 mmol) and

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$[NEt_4]_2[ReBr_3(CO)_3]$ (66.8 mg, 0.0867 mmol) in acetonitrile (5.0 mL) according to the general procedure described above afforded **3i** as a white solid (57.7 mg, 53%). ^{1}H NMR (400 MHz, DMSO-d_6) 12.11 (brs, 6 H), 7.73 (d, J = 8.8 Hz, 2 H), 7.71 (s, 2 H), 7.18 (s, 2 H), 7.16 (d, J = 1.6 Hz, 2 H), 7.06 (d, J = 9.2 Hz, 2 H), 7.03 (d, J = 1.6 Hz, 2 H), 4.72 (d, J = 17.6 Hz, 2 H), 4.67 (d, J = 16.8 Hz, 2 H), 4.45 (d, J = 16.4 Hz, 2 H), 4.36 (d, J = 16.4 Hz, 2 H), 4.09 (t, J = 6.0 Hz, 2 H), 3.78−3.70 (m, 2 H), 2.13 (t, J = 7.8 Hz, 12 H), 1.90−1.70 (m, 16 H). HRMS (ESI) calcd for $C_{45}H_{58}N_8O_{20}ReS$ $(M)^+$, 1249.3046; found, 1249.3031.

*4,4′-((2,2′-(2,2′-(((6-(4-Sulfamoylphenoxy)hexyl)azanediyl)bis(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis(azanediyl))bis(4-(2-carboxyethyl)heptanedioic acid) Rhenium Tricarbonyl Complex (**3j**).* Treatment of **2j** (81 mg, 0.060 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (47 mg, 0.060 mmol) in acetonitrile (5.0 mL) according to the general procedure described above afforded **3j** as a white solid (12.2 mg, 16%). ^{1}H NMR (400 MHz, DMSO-d_6) 12.11 (brs, 6 H), 7.72 (d, J = 8.8 Hz, 2 H), 7.71 (s, 2 H), 7.18 (s, 2 H), 7.16 (d, J = 1.6 Hz, 2 H), 7.05 (d, J = 8.8 Hz, 2 H), 7.03 (d, J = 1.6 Hz, 2 H), 4.73 (d, J = 16.8, 2 H), 4.67 (d, J = 16.8, 2 H), 4.42 (d, J = 16.8 Hz, 2 H), 4.34 (d, J = 16.4 Hz, 2 H), 4.04 (t, J = 6.6 Hz, 2 H), 3.64−3.60 (m, 2 H), 2.13 (t, J = 7.8 Hz, 12 H), 1.84 (t, J = 7.8 Hz, 12 H), 1.76−1.35 (m, 8 H). HRMS (ESI) calcd for $C_{47}H_{62}N_8O_{20}ReS$ $(M)^+$, 1277.3359; found, 1277.3351.

*2-((Pyridin-2-ylmethyl)(4-sulfamoylphenethyl)amino)acetic Acid Rhenium Tricarbonyl Complex (**5a**).* To a solution of **4a** (150 mg, 0.37 mmol) in DCM (3.0 mL) and TFA (3.0 mL) was stirred at room temperature overnight. Solvent was removed under reduced pressure to give a residue. To a solution of the above residue in MeOH (6.0 mL) was added $[NEt_4]_2[ReBr_3(CO)_3]$ (384 mg, 0.50 mmol) and K_2CO_3 (60 mg). The reaction mixture was stirred at 100 °C for 5 h at a pressure tube. Solvent was evaporated under reduced pressure to a residue, which was purified by HPLC afforded **5a** (38 mg, 17%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 8.77 (d, J = 5.6 Hz, 1 H), 8.17 (t, J = 7.8 Hz, 1 H), 7.79 (d, J = 8.0 Hz, 2 H), 7.74 (d, J = 7.6 Hz, 1 H), 7.59−7.56 (m, 3 H), 7.29 (s, 2 H), 4.92 (d, J = 16.0 Hz, 1 H), 4.77 (d, J = 16.0 Hz, 1 H), 4.10 (d, J = 16.4 Hz, 1 H), 3.74−3.68 (m, 1 H), 3.64−3.58 (m, 1 H), 3.53 (d, J = 16.8 Hz, 1 H), 3.14−3.08 (m, 2 H). HRMS (ESI) calcd for $C_{19}H_{19}N_3O_7ReS$ $(M + H)^+$, 620.0501; found, 620.0496.

*2,2′-(2-(2-((((1-(Carboxymethyl)-1H-imidazol-2-yl)methyl)(4-sulfamoylphenethyl)amino)methyl)-1H-imidazol-1-yl)-acetylazanediyl)diacetic Acid Rhenium Tricarbonyl Complex (**5b**).* Treatment of **4b** (91 mg, 0.12 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (93 mg, 0.12 mmol) in methanol (5.0 mL) according to the general procedure described above afforded **5b** as a white solid (72 mg, 70%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.80 (d, J = 8.4 Hz, 2 H), 7.58 (d, J = 8.0 Hz, 2 H), 7.29 (s, 2 H), 7.22 (d, J = 1.2 Hz, 1 H), 7.15 (d, J = 1.2 Hz, 1 H), 7.07 (d, J = 1.2 Hz, 1 H), 7.04 (d, J = 1.2 Hz, 1 H), 5.09 (s, 2 H), 4.86 (s, 2 H), 4.74 (d, J = 16.4 Hz, 1 H), 4.60 (dd, J = 16.0, 6.4 Hz, 2 H), 4.42 (d, J = 16.0 Hz, 1 H), 4.29 (s, 2 H), 4.10 (d, J = 17.6 Hz, 1 H), 4.02 (d, J = 17.2 Hz, 1 H), 3.85−3.80 (m, 2 H), 3.15−3.07 (m, 2 H). HRMS (ESI) calcd for $C_{27}H_{29}N_7O_{12}ReS$ $(M)^+$, 862.1147; found, 862.1111.

*4-(2-(2-((((1-(2-(Bis(carboxymethyl)amino)-2-oxoethyl)-1H-imidazol-2-yl)methyl)(4-sulfamoylphenethyl)amino)methyl)-1H-imidazol-1-yl)acetamido)-4-(2-carboxyethyl)heptanedioic Acid Rhenium Tricarbonyl Complex (**5c**).* Treatment of **4c** (80 mg, 0.0726 mmol) and $[NEt_4]_2[ReBr_3(CO)_3]$ (58 mg, 0.075 mmol) in acetonitrile (5.0 mL) according to the general procedure described above afforded **5c** as a white solid (62.8 mg, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) 7.80 (d, J = 8.4 Hz, 1 H), 7.71 (s, 1 H), 7.58 (d, J = 8.4 Hz, 2 H), 7.29 (s, 2 H), 7.20 (d, J = 1.6 Hz, 1 H), 7.14 (d, J = 1.2 Hz, 2 H), 7.14−7.05 (m, 2 H), 5.09 (s, 2 H), 4.71 (s, 2 H), 4.67−3.86 (m, 10 H), 3.13−3.11 (m, 2 H), 2.19 (t, J = 8.2 Hz, 6 H), 1.86 (t, J = 8.2 Hz, 6 H). HRMS (ESI) calcd for $C_{37}H_{44}N_8O_{17}ReS$ $(M)^+$, 1091.2103; found, 1091.2090.

General Procedure for Synthesis of Compounds 6, 7, 8, and 9. A solution of **2d** (**2e**, **4c**, or **2i**) in TFA and DCM was stirred at room temperature overnight. Solvent was removed under a stream of nitrogen to give a residue, which was purified by medium pressure column chromatography utilizing Biotage SP4 over C18 cartridge using a binary solvent gradient of 0−50% mobile phase B where mobile phase A is water containing 0.1% TFA and mobile phase B is 50% acetonitrile and 50% water containing 0.1% TFA and lyophilized to give the desired product **6** (**7**, **8** or **9**).

*2,2′,2″,2‴-((2,2′-(2,2′-(((4-Sulfamoylphenethyl)azanediyl)bis(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis(azanetriyl))-tetraacetic Acid (**6**).* Treatment of **2d** (753 mg, 0.809 mmol) in TFA (5.0 mL) and DCM (5.0 mL) according to the general procedure described above afforded **6** (584 mg, 99%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 7.68 (d, J = 7.6 Hz, 2 H), 7.61 (s, 2 H), 7.60 (s, 2 H), 7.32 (d, J = 8.0 Hz, 2 H), 7.26 (s, 2 H), 5.34 (s, 4 H), 4.35 (s, 4 H), 4.07 (s, 8 H), 2.71−2.68 (m, 4H). HRMS (ESI) calcd for $C_{28}H_{35}N_8O_{12}S$ $(M + H)^+$, 707.2095; found, 707.2093.

*4,4′-((2,2′-(2,2′-(((4-Sulfamoylphenethyl)azanediyl)bis(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis(azanediyl))-bis(4-(2-carboxyethyl)heptanedioic Acid) (**7**).* Treatment of **2e** (400 mg, 0.314 mmol) in TFA (4.0 mL) and DCM (4.0 mL) according to the general procedure described above afforded **7** (180 mg, 61%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 7.70 (s, 2 H), 7.66 (d, J = 8.4 Hz, 2 H), 7.23 (d, J = 8.8 Hz, 2 H), 7.22 (s, 2 H), 7.02 (s, 2 H), 6.82 (s, 2 H), 4.58 (s, 4 H), 3.64 (s, 4 H), 2.74−2.64 (m, 4 H), 2.13 (t, J = 8.0 Hz, 12 H), 1.83 (t, J = 7.8 Hz, 12 H). HRMS (ESI) calcd for $C_{40}H_{55}N_8O_{16}S$ $(M + H)^+$, 935.3457; found, 935.3452.

*4-(2-(2-((((1-(2-(Bis(carboxymethyl)amino)-2-oxoethyl)-1H-imidazol-2-yl)methyl)(4-sulfamoylphenethyl)amino)methyl)-1H-imidazol-1-yl)acetamido)-4-(2-carboxyethyl)heptanedioic Acid (**8**).* Treatment of **4c** (65.5 mg, 0.0595 mmol) in TFA (1.0 mL) and DCM (1.0 mL) according to the general procedure described above afforded **8** (38.1 mg, 78%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 12.43 (brs, 5 H), 7.89 (s, 1 H), 7.68 (d, J = 8.0 Hz, 2 H), 7.55 (s, 1 H), 7.49 (s, 2 H), 7.33 (s, 1 H), 7.32 (d, J = 8.4 Hz, 2 H), 7.26 (s, 2 H), 5.29 (s, 2 H), 4.90 (s, 2 H), 4.34 (s, 2 H), 4.09 (s, 2 H), 4.07 (s, 2 H), 4.02 (s, 2 H), 2.74 (s, 4 H), 2.17 (t, J = 8.2 Hz, 6 H), 1.85 (t, J = 8.0 Hz, 6 H). HRMS (ESI) calcd for $C_{34}H_{45}N_8O_{14}S$ $(M + H)^+$, 821.2776; found, 821.2768.

*4,4′-((2,2′-(2,2′-(((4-(4-Sulfamoylphenoxy)butyl)azanediyl)bis(methylene))bis(1H-imidazole-2,1-diyl))bis(acetyl))bis(azanediyl))-bis(4-(2-carboxyethyl)heptanedioic Acid) (**9**).* Treatment of **2i** (23 mg, 0.0175 mmol) in TFA (1.0 mL) and DCM (1.0 mL) according to the general procedure described above afforded **9** (17.6 mg, 100%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 12.12 (brs, 6 H), 7.87 (s, 2 H), 7.71 (d, J = 8.8 Hz, 2 H), 7.52 (brs, 4 H), 7.17 (s, 2 H), 7.00 (d, J = 8.8 Hz, 2 H), 4.88 (s, 4 H), 3.96 (s, 4 H), 3.94 (t, J = 6.4 Hz, 2 H), 2.59−2.56 (m, 2 H), 2.16 (t, J = 8.0 Hz, 12 H), 1.84 (t, J = 7.6 Hz, 12 H), 1.65−1.49 (m, 4 H). HRMS (ESI) calcd for $C_{42}H_{59}N_8O_{17}S$ $(M + H)^+$, 979.3719; found, 979.3716.

Preparation of ^{99m}Tc−3d. The $^{99m}Tc(I)(CO)_3^+$ radiolabeling of **2d** was accomplished in two steps using commercially available IsoLink kits (Covidien) and $[Na][^{99m}TcO_4]$ in 1 mL of 0.9% sodium chloride solution (50 mCi/mL) to form the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ intermediate, which was neutralized and reacted with **2d** at a concentration of 10^{-4} M in an equal volume mixture of acetonitrile and water in a sealed vial. The sealed vial was heated in an oil bath at 75 °C for 60 min. After cooling and evaporation of solvent by a stream of nitrogen, the *tert*-butyl ester protecting groups were removed by treatment with 50% TFA in DCM (4 mL) at room temperature for 30 min. After subsequent evaporation and formulation in 0.9% sodium chloride solution, the reaction was analyzed for purity via reverse-phase HPLC. Purification by HPLC resulted in the desired conjugate ^{99m}Tc−**3d** in 90% RCY and >95% RCP as a "carrier free" product. The final formulation was composed of 10% ethanol in 0.9% sodium chloride solution.

Cell Culture. The human cervical cancer cell line, HeLa, was obtained from the American Type Culture Collection. HeLa cells were maintained in Minimal Essential Medium supplemented with 10% fetal bovine serum (Hyclone), 4 mM glutamine, 1 mM sodium pyruvate, and 50 μg/mL gentamicin. Media components were from Invitrogen unless otherwise noted. Cells were grown in a humidified incubator at 37 °C/5% CO_2 and removed from flasks for passage or for transfer to 12-well assay plates by incubating them with 0.25% trypsin/EDTA (Invitrogen).

Binding to CA-IX. The ability of the CA-IX inhibitors to compete with ^{99m}Tc−**3d** for binding to hypoxic HeLa cells was examined. HeLa cells were plated in 12-well plates at approximately 2.5 × 10^5 cells/well and allowed to adhere to the plate for 24 h. Cells were then incubated under hypoxic conditions (0.1% O_2/5% CO_2 at 37 °C) for an additional 24 h. The cells were then removed from hypoxia and incubated for 1 h in Hank's Balanced Salt Solution (HBSS) with 0.5% BSA and 3nM ^{99m}Tc−**3d** in the presence of 1−10000 nM test CA-IX inhibitor, or acetazolamide. The assay media was then removed and the cells were washed 2× with cold HBSS/0.5% BSA, collected by adding 0.25 mL of 1% SDS, and transferred to a 1.5 mL tube for counting the amount of radioactivity bound in a Wallac 1282 automated gamma counter. IC_{50} values were determined by nonlinear regression using GraphPad Prism software.

AUTHOR INFORMATION

Corresponding Author

*Phone: (617) 492-5554. Fax: (617) 492-5664. E-mail: jbabich@molecularinsight.com.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

RCY, radiochemical yield; RCP, radiochemical purity; SPECT, single-photon emission computed tomography

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