ABSTRACT
Carbonic anhydrase IX (CA-IX) is a marker for tumor hypoxia, and its expression is negatively correlated with patient survival. CA-IX represents a potential target for eliminating hypoxic cancers. We synthesized fluorinated cationic sulfonamide inhibitors 1–3 designed to target CA-IX. The binding affinity for CA-IX ranged from 0.22 to 0.96 μM. We evaluated compound 2 as a diagnostic PET imaging agent. Compound 2 was radiolabeled with 18F in 10 ± 4% decay-corrected radiochemical yield with 85.1 ± 7.0 GBq/mmol specific activity and >98% radiochemical purity. 18F-labeled 2 was stable in mouse plasma at 37°C after 1 h incubation. PET/CT imaging was conducted at 1 h post-injection in a human colorectal cancer xenograft model. 18F-labeled 2 cleared through hepatobiliary and renal pathways. Tumor uptake was approximately 0.41 ± 0.06% ID/g, with a tumor-to-muscle ratio of 1.99 ± 0.25. Subsequently, tumor xenografts were visualized with moderate contrast. This study demonstrates the use of a cationic motif for conferring isomer selectivity for CA-IX imaging agents.

KEYWORDS
Carbonic anhydrase IX; fluorine-18; molecular imaging; positron emission tomography

Introduction
Tumor cells cycle through stages of being well-oxygenated or oxygen-deprived. Hypoxia occurs when oxygen level of the microenvironment is unable to sustain the metabolic demands of a growing tumor. Regardless of size, stage, grade, or histology, all solid tumors are susceptible to hypoxia. Although hypoxia may initially limit growth potential, it also promotes and regulates biological processes like angiogenesis, invasiveness, metastasis, resistance and insensitivity to conventional chemotherapy and/or components of the hypoxia-induced signaling pathway.

CA-IX is an attractive target because of its pathological expression in cancers, as well as the fact that it resides as a cell surface transmembrane protein. However, the highly conserved catalytic domain shared by CA isoforms poses as a challenge for synthesizing CA-IX selective imaging agents. Off-target binding to intracellular CAs, notably CA-I and CA-II expressed in erythrocytes, can reduce tumor targeting and contrast ratios. Therefore, strategies to confer CA-IX selectivity for small molecule inhibitors have focused on limiting transport across the plasma membrane.

Previously, our research group reported two approaches for sulfonamide-based CA-IX imaging agents that successfully targeted human colorectal cancer xenografts. In one approach, we leveraged a multimeric design to synthesize 18F-labeled trivalent tracers (Figure 1(A)) that were of sufficient bulk (MW > 1 kDa) to be cell-impermeable. In the other approach, we conjugated pharmacophores to different polyaminocarboxylate chelators for 68Ga-radiolabeling (Figure 1(B)). The hydrophilicity of the metal/chelator complex facilitated selective targeting of CA-IX in vivo.

In addition to factors like size and hydrophilicity, the overall charge of a molecule can modulate the property to permeate through the plasma membrane. Previous work published by Casey et al. demonstrated that cationic sulfonamide derivatives showed favorable CA-IX inhibition profiles (Figure 1(C)). As charged molecules, these inhibitors have limited transcellular uptake. In this paper, we investigated if fluorinated sulfonamides...
derivatives engrafted with a cationic motif can be used as potential PET tracers for imaging CA-IX expression in tumors (Figure 2).

Materials and methods

Chemicals and instrumentation

All chemicals and solvents were obtained from commercial sources, and used without further purification. Triethylammonium phosphate buffer (TEA-PB, pH 7.29) was prepared by titrating a solution of triethylamine (8 mL) in deionized water (1 L) with o-phosphoric acid monitored using a Denver Instrument (Bohemia, NY) UltraBasic Benchtop pH meter. Proton NMR spectra were obtained using a Bruker (Billerica, MA) Avance 400inv Spectrometer, and are reported in parts per million. Mass analyses were performed using an AB SCIEX (Framingham, MA) 4000 QTRAP mass spectrometer system with an ESI ion source. Melting points were taken on a Fisher–Johns melting point apparatus (Fisher Scientific, Pittsburgh, PA) and were uncorrected. The quaternary methyl ammonium (QMA) anion exchange and C18 light Sep-Pak cartridges (1 cm³, 50 mg) were obtained from Waters (Milford, MA). Purification and quality control of 18F-labeled 2 were performed on an Agilent (Santa Clara, CA) HPLC system equipped with a model 1200 quaternary pump, a model 1200 UV absorbance detector, and a Bioscan (Washington, DC) NaI scintillation detector. The radio-detector was connected to a Bioscan B-FC-1000 Flow-count system, and the output from the Bioscan Flow-count system was fed into an Agilent 35900E Interface, which converted the analog signal to digital signal. The operation of the Agilent HPLC system was controlled using the Agilent ChemStation software. The HPLC columns used were a semi-preparative column (Phenomenex C18, 5 μm, 250 × 10 mm) and an analytical column (Phenomenex C18, 5 μm, 250 × 4.6 mm). 18F-Fluoride was produced by the 18O(p, n)18F reaction using an Advanced Cyclotron Systems Inc. (Richmond, BC, Canada) TR19 cyclotron. Radioactivity of 18F-labeled 2 was measured using a Capintec (Ramsey, NJ) CRC-25 R/W dose calibrator, and the radioactivity of mouse tissues collected from biodistribution studies were counted using using a Perkin Elmer (Waltham, MA) Wizard2 2480 automatic gamma counter. PET imaging experiments were conducted using a Siemens (Knoxville, TN) Inveon microPET/CT scanner.

Syntheses of precursors and standards

2,3,5,6-Tetrafluorophenyl 4-([dimethylamino) methyl] benzoate (4)

A mixture of 4-([dimethylamino) methyl] benzoic acid hydrochloride (2.37 g, 11 mmol), 2,3,5,6-tetrafluorophenol (1.99 g, 12 mmol), and N,N-diisopropylethylamine (1.42 g, 11 mmol) in dichloromethane (80 mL) was added N,N-dicyclohexylcarbodiimide (2.06 g, 10 mmol). After stirring at room temperature for 27 h, the reaction mixture was filtered, and the filtrate was extracted with 2 M NaOH aqueous solution (100 mL). The organic phase was dried with anhydrous magnesium sulfate, and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (CH2Cl2:MeOH = 9:1) to afford 4 (2.14 g, 76%) as a white solid.
N,N-dimethyl-N-[(4-fluorobenzyl)-4-[(2,3,5,6-tetrafluorophenoxy) carbonyl] benzylationmonium bromide (5)

4-Fluorobenzyl bromide (236 µL, 359 mg, 1.9 mmol) was added to a solution of 4 in acetonitrile (6 mL). The reaction mixture was stirred at room temperature for 3 days. The precipitated product was filtered, and washed with diethyl ether (2 mL x 3) to yield 5 as white powder (852 mg, 87%). 1H NMR (300 MHz, DMSO) δ 7.67 (dd, J = 8.6, 5.5 Hz, 2H), 7.58–7.51 (m, 2H), 7.44–7.29 (m, 4H), 4.65 (s, 2H), 4.64 (s, 2H), 4.57 (d, J = 5.8 Hz, 2H), 2.89 (s, 6H). MS (ESI) calculated for C18H13F2NO24 374.2, found (M+H)+ 374.2. Melting point: 198 °C.

N,N-dimethyl-N-((2-aminosulfonyl) benzyl) amino) carbonyl] benzylationmonium trifluoromethanesulfonate (7)

A solution of silver trifluoromethanesulfonate (232 mg, 0.9 mmol) in acetonitrile (3 mL) was added dropwise to a solution of 6 (562 mg, 0.9 mmol) in a mixture of acetonitrile (6 mL) and methanol (6 mL). The resulting solution was stirred for 4 h. The formed silver bromide precipitate was filtered through celite, and washed with methanol (5 mL x 2). The filtrate was concentrated under reduced pressure to yield 7 as light yellow solid (625 mg, 100%). 1H NMR (300 MHz, DMSO) δ 8.23 (d, J = 8.2 Hz, 2H), 7.87 (d, J = 7.9 Hz, 2H), 7.79 (d, J = 8.3 Hz, 2H), 7.50 (d, J = 7.9 Hz, 2H), 7.16–6.94 (m, 1H), 4.95 (s, 2H), 4.75 (s, 2H), 2.99 (s, 6H), 1.34 (s, 12H). MS (ESI) calculated for C29H27BrF4NO4+ 544.2, found (M+H)+ 544.3. Melting point: 149.0 °C.
assay buffer. The inhibition constant ($K_i$) was obtained by considering the classical Michaelis–Menten equation, which has been fitted by non-linear least squares by using PRISM 3. All CA isozymes used in the experiments were purified human recombinant proteins obtained as reported earlier by our group.

Radiosynthesis of [18F]2

The $^{18}$F-fluoride in $\text{H}_2^{18}$O was passed through the QMA cartridge, and $^{18}$F-fluoride was trapped and then eluted out with 0.3 mL aqueous solution of 5 mg tetrabutylammonium triflate into a 4-mL V-shaped reaction vial. Acetonitrile (1 mL) was added and the reaction vial was placed in a heating block and heated at 110 °C under vacuum for 6 min and subsequently under N$_2$ flow for another 6 min. A mixture of Cu(OTf)$_2$ (100 $\mu$L, 0.2 M), pyridine (500 $\mu$L, 1 M), and precursor (100 $\mu$L, 40 mM) solutions in N,N-dimethylformamide was added to the reaction vial. The reaction mixture was incubated at 110 °C for 20 min. The reaction was quenched with water (1 mL) and the resulted mixture was purified by HPLC using the semi-preparative column eluted with 24% CH$_3$CN and 76% TEA-PB at a flow rate of 4.5 mL/min. The retention time of $^{18}$F-labeled 2 was 17.6 min. The collected $^{18}$F-labeled tracer was diluted with ammonium formate (50 mM, 50 mL), and trapped on a C$_{18}$ light Sep-Pak cartridge. The final product was eluted out with ethanol (0.4 mL), and formulated with saline (4 mL) for plasma stability, biodistribution, and PET/CT imaging studies. Quality control was performed by HPLC on the analytical column eluted with 25% CH$_3$CN and 75% PBS at a flow rate of 2.0 mL/min. The retention time of $^{18}$F-labeled 2 was 6.9 min. The specific activity of $^{18}$F-labeled 2 was measured using the analytical HPLC system. It was calculated via dividing the injected radioactivity of $^{18}$F-labeled tracer solution by the amount of the tracer in the injected solution. The amount of the tracer was calculated from the UV absorbance standard curve of non-radioactive 2.

LogD$_{7,4}$ measurement

The LogD$_{7,4}$ value of $^{18}$F-labeled 2 was measured using the shake flask method as previously reported. Briefly, an aliquot (2 $\mu$L) of $^{18}$F-labeled 2 was added to a vial containing 3 mL of n-octanol and 3 mL of phosphate buffer (0.1 M, pH 7.4). The mixture was vortexed for 1 min and then centrifuged at 3000g for 10 min. Samples of the n-octanol (1 mL) and buffer (1 mL) layers were taken and counted in a well counter. LogD$_{7,4}$ was calculated using the following equation: LogD$_{7,4} = \log_{10}$(counts in n-octanol phase)/ (counts in buffer phase).

Stability in mouse plasma

Stability in plasma was performed following published procedures. Aliquots (100 $\mu$L) of $^{18}$F-labeled 2 were incubated with 400 $\mu$L of balb/c mouse plasma (Innovative Research, Novi, MI) for upwards of 60 min at 37 °C. At the end of each incubation period, samples were quenched by addition of acetonitrile (0.5 mL), centrifuged to remove proteins, and finally passed through a 0.2 micron filter. The filtered samples were loaded onto the analytical radio-HPLC to check for metabolite formation, and analyses were conducted using Agilent ChemStation software.

In vivo experiments

In vivo experiments were conducted in accordance with the guidelines established by the Canadian Council on Animal Care and approved by the Animal Ethics Committee of the University of British Columbia. Male immunodeficient NOD.Cg-Prkdcs(Scid)Il2rgtm1Wjl/SzJ (NSG) mice were obtained from a breeding colony at the Animal Resource Centre of the BC Cancer Research Centre.

Tumor implantation

Animal model was established following previously published procedures. Under anesthesia with 2.5% isoflurane in 2.0 L/min of oxygen, mice were subcutaneously inoculated with $5 \times 10^6$ HT-29 cells (in 100 $\mu$L PBS and Matrigel at 1:1 ratio) under the right dorsal flank. Biodistribution studies and PET/CT imaging were performed when tumors reached 7–9 mm in diameter.

PET imaging and biodistribution studies

For dynamic imaging study, tumor-bearing mice were sedated with 2% isoflurane inhalation and positioned prone onto the scanner bed. A 10 min baseline CT scan was obtained for localization and attenuation correction before radiotracer injection, using 60 kV X-rays at 500 mA, three sequential bed position with 33% overlap, and 220 degree continuous rotation. PET data were acquired in list mode acquisition, reconstructed using the 3d-OSEM-MAP algorithm with CT-based attenuation correction. The dynamic acquisition of 60 min was started at the time of intravenous injection with 6–8 MBq of the radiotracer. The list mode data were rebinned into time intervals ($12 \times 10$, $6 \times 30$, $5 \times 60$, $6 \times 300$, and $2 \times 600$ s) to obtain tissue time-activity curves. The mice were kept warm by a heating pad during acquisition. For static imaging study, the mice were briefly sedated for intravenous injection of the radiotracer (6–8 MBq), and allowed to recover and roam freely in their cages for 45 min. At that point, the mice were sedated with 2% isoflurane inhalation, placed on the scanner, and an attenuation correction CT scan was obtained as described above. A single static emission scan was subsequently acquired for 10 min.

Biodistribution studies were performed to confirm the quantitative ROI uptake values observed from PET scans. At 1 h p.i., mice were euthanized. Blood was promptly withdrawn, and the organs/tissues of interest were harvested, rinsed with normal saline, blotted dry, and weighed. The radioactivity of the collected mouse tissues was counted and expressed as the percentage of the injected dose per gram of tissue (%ID/g).

Results and discussion

As shown in Figure 2, the three cationic sulfonamides 1–3 were designed using 4-dimethylaminobenzoic acid as the linker to connect a 4-fluorobenzyl group and a benzenesulfonamide motif for CA-IX targeting. This design also generated the needed cationic quaternary ammonium group that would prevent free diffusion of 1–3 into cells and binding to intracellular CA isoforms including CA-I and CA-II. In addition, $^{18}$F-labeled 1–3 could be prepared via copper-mediated aromatic radiofluorination reaction using an arylboronic pinacol ester precursor as recently demonstrated by us for the preparation of $^{18}$F-fluorobenzyltriphenylphosphonium ($^{18}$F-FbNTP), a myocardial perfusion PET tracer. The preparation of compounds 1–3 followed the procedures depicted in Scheme 1. The activated ester 4 was obtained in 99% yield by reacting 4-[(dimethylamino)methyl] benzoic acid with 2,3,5,6-tetrafluorophenol using N,N-dicyclohexylcarbodiimide (DCC) as the coupling reagent. Reacting 4 with 4-fluorobenzyl bromide in acetonitrile afforded the quaternary ammonium bromide salt 5 as a precipitate which was isolated in 87% yield by
in vivo evaluations. Renal cell carcinomas commonly overexpress CA-IX, typically with $K_i$ values in the low 0.52–0.07 $\mu$M range of 0.52–0.07 $\mu$M for CA-I, -II, -IV, and -IX, respectively. Relative to acetazolamide ($K_i$(CA-IX) = 0.03 $\mu$M), a pan CA inhibitor, the binding affinities of compounds 1–3 for CA-IX were substantially lower. With the best affinity towards CA-IX, compound 2 was selected for radiolabeling and in vivo experiments.

The preparation of the $^{18}$F-fluorination precursor 8 is shown in Scheme 2(A). Reacting the activated ester 4 with 4-bromomethylphenylboronic acid pinacol ester afforded the quaternary ammonium bromide salt 6 in 14% yield. The ammonium bromide salt 6 was treated with silver trflate and converted quantitatively to the ammonium triflate salt 7. The desired precursor 8 was obtained in 64% by coupling 7 with 4-(2-aminoethyl)benzenesulfonamide.

Synthesis of $^{18}$F-labeled 2 was performed by nucleophilic substitution of the precursor 8 with $^{18}$F-fluoride via copper-mediated aromatic fluorination reaction (Scheme 2B)46–51. Purification and quality control of $^{18}$F-labeled 2 was performed by HPLC and $^{18}$F-labeled 2 was isolated in 10 ± 4% (n = 3) decay-corrected radiochemical yield with >98% radiochemical purity and 85.1 ± 70.3 GBq/umol specific activity. An in vitro stability study was conducted by incubating $^{18}$F-labeled 2 at 37 °C in mouse plasma, and monitored by HPLC. As shown in Figure 3, no noticeable degradation of $^{18}$F-labeled 2 was observed after 60 min incubation, suggesting high stability of $^{18}$F-labeled 2 in mouse plasma.

Lipophilicity of $^{18}$F-labeled 2 was measured using traditional shake flask method46. The obtained LogD$_{7.4}$ ($D_{7.4}$: distribution coefficient between n-octanol and pH 7.4 phosphate buffer) value was $-0.79 ± 0.02$, indicating that the tracer was hydrophilic.

In vivo imaging experiments were conducted in immunodeficient NSG mice bearing HT-29 human colorectal cancer xenografts. Biodistribution data and representative PET/CT images acquired at 1 h post-injection are shown in Figures 4 and 5, respectively. Tracer uptake was predominantly observed in the excretory organs, liver (10.7 ± 0.96%ID/g) and kidneys (13.7 ± 3.96%ID/g). Moderate uptake was observed in HT-29 tumor xenografts (0.41 ± 0.06%ID/g), which corresponded to tumor-to-muscle ratio of 1.99 ± 0.25. The lowest uptake was observed for the brain (0.02 ± 0.00%ID/g), indicating that the tracer was unable to penetrate the blood–brain barrier. The tracer was stable against in vivo defluorination as uptake in bone was observed in negligible amount at 0.13 ± 0.02%ID/g. PET images are consistent with biodistribution data, as the gastrointestinal tract and kidneys showed the highest accumulation of activity. HT-29 xenografts were visualized in PET images with moderate tumor-to-background contrast. Analyzing the time activity curve for $^{18}$F-labeled 2 (Figure 6), tracer was rapidly cleared through the kidneys and hepatobiliary tract. Despite moderate uptake, the uptake in tumor xenograft was higher compared to non-target tissues like bone, brain, and muscle, enabling its visualization in PET images.

The development of CA-IX targeting agents, particularly those derived from small molecule inhibitors has seen marked improvement in terms of tumor targeting and visualization. Initial efforts were hampered by the lack of target specificity, poor pharmacokinetics, and/or tracer instability24,25. Subsequently, our group and others have leveraged a radiometal-based approach to develop cell-impermeable tracers targeting CA-IX in vivo. Most recently, Pomper’s group radiolabeled a dual motif CA-IX inhibitor consisting of a 4,4-bis(4-hydroxyphenyl)valeric acid and a succinyl acetylazolamide group with $^{111}$In (via DOTA chelator) and $^{64}$Cu (via NOTA chelator) and achieved tumor uptakes values of 14.5–26.0%ID/g at 1 h p.i.29,30. Krall et al31 reported the synthesis and evaluation of a $^{99m}$Tc-labeled acetylazolamide derivative with excellent tumor targeting (22%ID/g at 3 h p.i.) and image contrast. Notably, both research groups used the human renal-cell carcinoma SK-RK-52 model for in vivo evaluations. Renal cell carcinomas commonly overexpress CA-IX due to perturbations of the von Hippel-Lindau (VHL) gene, which in turn regulates HIF-1α52–54. The expression of CA-IX in this model is not necessarily driven by hypoxia.

Beyond the use of radiometals, another major commonality shared by these successful tracers is the high affinity that they exhibit for CA-IX, typically with $K_i$ values in the low

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**Table 1.** Inhibition constants ($K_i$) of cationic inhibitors 1–3 to carbonic anhydrases I, II, IV, and IX as determined by a stopped-flow CO$_2$ hydration assay. Errors in the range of 5–10% of the reported value from three different assays.

| Compound    | CA-I | CA-II | CA-IV | CA-IX |
|-------------|------|-------|-------|-------|
| 1           | 0.89 | 0.72  | >50.0 | 0.44  |
| 2           | 0.52 | 0.07  | 9.54  | 0.22  |
| 3           | 0.78 | 0.48  | 3.63  | 0.96  |
| Acetazolamide| 0.25 | 0.01  | 0.08  | 0.03  |

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filtration. The desired compounds 1–3 were obtained by coupling 5 in methanol with excess 3-(aminomethyl)benzenesulfonamide, 4-(2-aminoethyl)benzenesulfonamide, and 4-(aminomethyl)benzenesulfonamide, respectively. After evaporating methanol, the salt was triturated with tetrahydrofuran to obtain compounds 1–3 in 93–99% yields.

After synthesis, the binding affinity of compounds 1–3 were evaluated for four physiologically relevant CAs: CA-I, -II, -IV, and -IX (Table 1). CA-I and CA-II are cytosolic isozymes that are found primarily in the eyes, lungs, and kidneys6. Compounds 1–3 exhibited different inhibitory profiles. The derivatives have inhibition constants ($K_i$) in the ranges of 0.52–0.89, 0.07–0.72, 3.63–>50, and 0.22–0.96 $\mu$M for CA-I, -II, -IV, and -IX, respectively. Relative to acetazolamide ($K_i$(CA-IX) = 0.03 $\mu$M), a pan CA inhibitor, the binding affinities of compounds 1–3 for CA-IX were substantially lower. With the best affinity towards CA-IX, compound 2 was selected for radiolabeling and in vivo experiments.
Scheme 2. (A) Synthesis of the radiolabeling precursor 8. (B) Synthesis of $^{18}$F-labeled 2.

Figure 3. HPLC chromatograms of $^{18}$F-labeled 2 from (A) QC sample, or plasma sample after being incubated at 37°C for (B) 5 min, (C) 15 min, or (D) 60 min.

Figure 4. Biodistribution of $^{18}$F-labeled 2 at 1 h post-injection in HT-29 tumor-bearing mice. Values (%ID/g) are presented as mean ± standard deviation ($n = 5$).

Figure 5. Representative PET and CT images acquired at 1 h post-injection with $^{18}$F-labeled 2 in HT-29 colorectal cancer xenograft-bearing mice. Arrow indicates location of tumor.
nanomolar range. On the contrary, compound \( \text{2} \) selected for radio-labeling and evaluated in this study had only moderate binding affinity to CA-IX \( (K_i = 0.22 \, \mu \text{M}) \). A high binding affinity to the target of interest is one of many factors (stability, selectivity, target density, target accessibility, etc.) that determine efficient tumor targeting and accumulation\(^{55}\). Future studies leveraging the use of cationic sulfonamides to synthesize diagnostic agents targeting CA-IX require better understanding of the structure activity relationship to improve tracer affinity. The ability to visualize tumor notwithstanding the moderate uptake value suggests that cationic sulfonamides can potentially be used as pharmacophores for CA-IX imaging agents.

**Conclusion**

We designed three cationic sulfonamide inhibitors \( \text{1–3} \) to potentially target CA-IX for PET applications. Imaging and biodistribution data for \( \text{18F} \)-labeled \( \text{2} \) showed clear visualization of tumor xenografts despite moderate uptake and tumor-to-background contrast. This is encouraging considering the relatively modest binding affinity of \( \text{2} \) to CA-IX. Therefore, our data demonstrate the use of cationic motif may be useful for designing future CA-IX tracers assuming high affinity binders can be obtained.

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**Disclosure statement**

The authors report no conflicts of interest.

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