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Synthesis and evaluation of near-infrared fluorescent sulfonamide derivatives for imaging of hypoxia-induced carbonic anhydrase IX expression in tumors

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ABSTRACT

A series of human carbonic anhydrase (hCA) IX inhibitors conjugated to various near-infrared fluorescent dyes was synthesized with the aim of imaging hypoxia-induced hCA IX expression in tumor cells in vitro, ex vivo and in vivo. The resulting compounds were profiled for inhibition of transmembrane hCA IX showing a range of potencies from 7.5 to 116 nM and up to 50-fold selectivity over the cytosolic form hCA II. Some of the compounds also showed inhibition selectivity for other transmembrane forms hCA XII and XIV as well. Compounds incubated in vitro with HeLa cells cultured under normoxic and hypoxic conditions detected upregulation of hCA IX under hypoxia by fluorescence microscopy. A pilot in vivo study in HT-29 tumor bearing mice showed significant accumulation of a fluorescent acetazolamide derivative in tumor tissue with little accumulation in other tissues. Approximately 10% of injected dose was non-invasively quantified in tumors by fluorescence molecular tomography (FMT), demonstrating the promise of these new compounds for quantitative imaging of hCA IX upregulation in live animals.

Human carbonic anhydrase (hCA) IX is a transmembrane cell surface enzyme which catalyzes the reversible interconversion of CO_2 to bicarbonate and a proton. hCA IX is overexpressed in response to tumor hypoxia in many common tumor types and plays a critical role in hypoxia associated tumor acidosis.[1–5] Tumor hypoxia and subsequent expression of hCA IX are also correlated to metastasis, poor prognosis and resistance to therapeutic intervention making hCA IX an important biomarker in the study of hypoxia and tumor cell proliferation and a valuable target for both therapeutics and novel diagnostics.[6–8] While hCA inhibitors have been in clinical use for many decades for conditions such as glaucoma, seizures, altitude sickness and as a diuretic, recently a number of inhibitors of hCA IX and the related transmembrane hCA XII have shown considerable antitumor and antimetastatic activity.[9–11] Furthermore, a CA IX specific antibody, girentuximab, is also being evaluated in Phase III clinical trials for the treatment of clear-cell renal cell cancer.[12] The ability to image this important enzyme with a targeted fluorescent agent would be of considerable value for further investigation of tumor hypoxia and hCA targeted therapeutics.

A veritable pharmacopeia of small molecule inhibitors for various hCAs has been described in the literature with sulfonamide based inhibitors standing out in particular as a robust class of hCA active molecules due to their high affinity, availability and ease of chemical manipulation.[11,13–19] As such, diverse arrays of sulfonamide inhibitors have been synthesized incorporating a variety of chemical functionalities for modifying properties such as selectivity among hCAs and cell permeability[15,20] and include several radiolabeled[21,22] and fluorescent derivatives.[9,16,23–27] Fluorescent sulfonamides have been reported to bind to hCAs on osteoclasts[23] as well as a number of tumor cell lines including CA IX transfected MDCK[28] HeLa, HT-29,[29] LS174T, SK-RC-52[9] and SiHa[26] cells. In vivo studies with fluorescent sulfonamides have also shown promise for detecting hCA expression in HT-29 and SK-RC-52 tumor xenografts.[9,30] However, to date, in vivo studies with fluorescent sulfonamides have been performed with fluorescein derivatives, whose green excitation and emission wavelengths are suitable for only ex vivo or superficial in vivo imaging applications and preclude accurate deep tissue quantification.

In contrast, optical imaging in the near-infrared (NIR) range allows efficient penetration of photons through living tissue and minimizes interference from tissue autofluorescence. Combined with quantitative fluorescence molecular tomography (FMT), NIR fluorescent agents have emerged as invaluable tools for

Abbreviations: hCA, human carbonic anhydrase; FMT, fluorescence molecular tomography; NIR, near-infrared.

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quantitative, deep tissue imaging of a complex array of biological processes across a range of important areas of disease research including oncology,[31,32] inflammation,[33–35] bone metabolism,[36,37] angiogenesis[38] and atherosclerosis.[39–41]

The aim of the present work was to prepare new NIR fluorescent hCA IX inhibitors for non-invasive in vivo detection and quantification of hCA IX expression. A series of NIR fluorescent sulfonamides was synthesized based on the well characterized inhibitors acetazolamide (**AAZ**) and benzenesulfonamide **1**. The compounds were screened for inhibition of hCA IX and isoforms II, XII and XIV. Lead compounds were then visualized on hypoxic cells in vitro by fluorescence microscopy and binding to hypoxic cells quantified by flow cytometry. Preliminary in vivo bio-distribution and tumor imaging was demonstrated in HT-29 tumor bearing mice.

The sulfonamide derivatives **2** and **3** were synthesized from acetazolamide by hydrolysis in refluxing ethanolic HCl followed by coupling to Boc-aminomethylbenzoic acid or FMOC-β-alanine using EDC and HOBT in DMF for 24 h as shown in Scheme 1. The protected intermediates were purified by HPLC[42] followed by deprotection with TFA/water or diethylamine, respectively. The resulting amines, as well as **1** and benzylamine **4** as a non-hCA binding control, were coupled to the succinimidyl esters of one or more of four commercially available hydrophilic indocyanine NIR fluorochromes (NIRF-OSu) to give the corresponding amide linked compounds **1a–d**, **2a–d**, **3c** and **4b**. The fluorochromes used in this study all have absorption maxima of about 665–675 nm and emission maxima of about 680–695 nm, ideal for in vivo imaging, and a significant net negative charge of −3 to −5 to reduce membrane permeability and nonspecific uptake in cells (Table 1). The compounds were purified by preparative HPLC and characterized by LCMS.[43]

The inhibition potency and selectivity of the new fluorescent inhibitors towards various hCAs was characterized by measuring inhibition of CO_2 hydration using the stopped flow method.[44] Inhibition data for four recombinant, purified hCA isozymes, hCA IX, the cytosolic form hCA II and two other transmembrane forms, hCA XII and XIV, along with that for the parent inhibitors, acetazolamide (**AAZ**) and 4-aminomethylbenzenesulfonamide **1** are shown in Table 2. Also shown are the selectivity ratios relative to hCA IX.

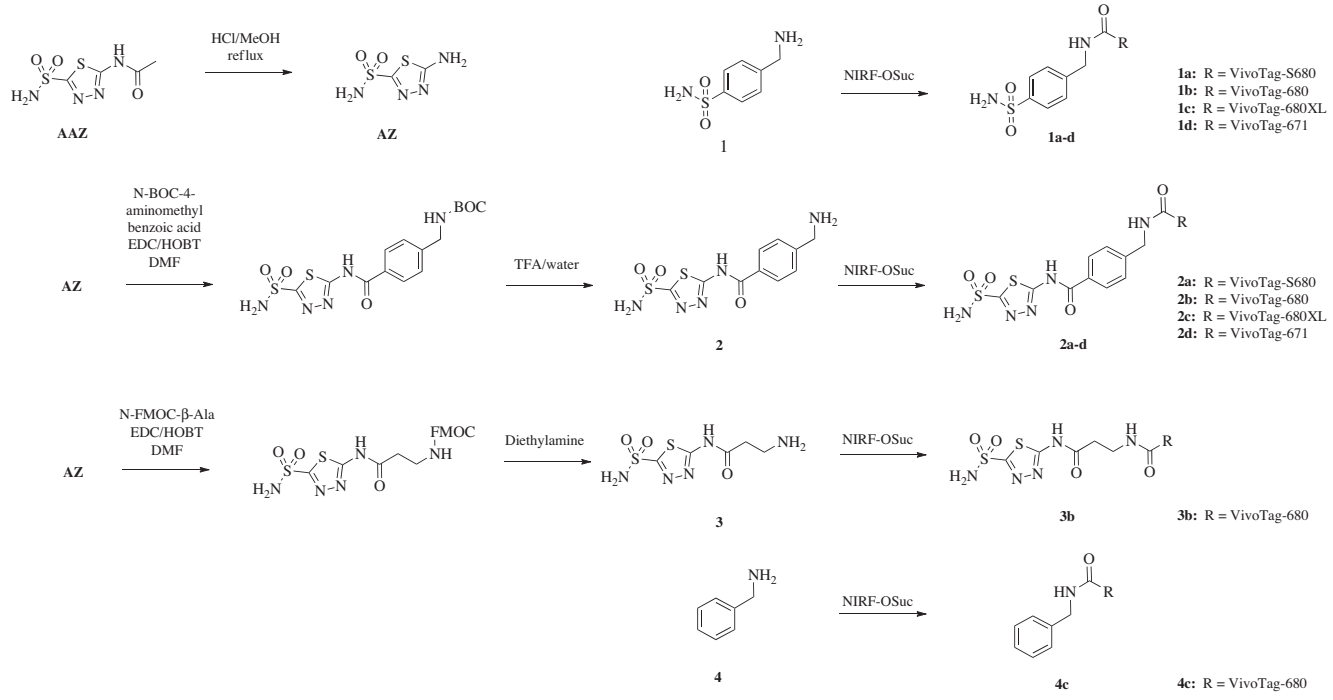
Table 1
Optical and charge properties of NIR fluorescent sulfonamides[a]

Compounds	**R** =	A_{max}/E_{max} (nm)	Net charge
1a, **2a**	VivoTag-S680	674/692	−3
1b, **2b**, **3b**	VivoTag-680	670/686	−5
1c, **2c**, **4c**	VivoTag-680XL	670/687	−5
1d, **2d**	VivoTag-671	667/683	−3

[a] All dyes were obtained from PerkinElmer as reactive succinimidyl esters. Absorbance and emission maxima were measured in 1XPBS.

Six of the new compounds tested showed less than 10 nM inhibition potencies toward hCA IX, including all of the acetazolamide derivatives and one of the benzenesulfonamides. Inhibition of hCA IX was generally slightly impaired for the fluorophore conjugated benzenesulfonamide derivatives **1a–d** relative to the parent inhibitor **1**, with the exception of **1d**, which had a K_i of less than 10 nM, three times lower than **1** and about 10 times lower than benzenesulfonamides **1a–c**. In contrast, hCA IX inhibition by the **AAZ** derivatives appeared insensitive to both the fluorophore structure and the linking moiety with all of the acetazolamide derivatives showing a K_i of 7-10 nM, an approximately threefold improvement over the parent **AAZ**.

Due to the cytosolic location of hCA II and the expected low membrane permeability of these highly charged fluorescent inhibitors, in vitro and in vivo selectivity is expected to be driven towards the membrane form hCA IX. However, hCA II is of considerable importance due to the ubiquitous expression of this enzyme in cells and the generally low selectivity of sulfonamide inhibitors and was thus also examined in this study. Four of the compounds tested, **1d**, **2a–b** and **3b** showed selectivity ratios for hCA IX over hCA II of greater than 30, a marked improvement over the parent inhibitors, especially **AAZ**, which actually shows a slight preference for hCA II. All of the benzenesulfonamides **1a–d** had



Scheme 1. Synthesis of NIR fluorescent sulfonamide derivatives.

Table 2
CA II, IX, XII and XIV inhibition data with sulfonamides **1a–d**, **2a–d**, **3b**, **4c** as well as parent inhibitors **AAZ** and **1** by stopped-flow CO_2 hydration method. K_i ratios relative to CA IX and K_d values to hypoxic HeLa cells for selected compounds are also shown

Compound	K_i[a] (nM)				K_i Ratios			K_d[b] (nM) HeLa
	hCA II	hCA IX	hCA XII	hCA XIV	hCA II/IX	hCA XII/IX	hCA XIV/IX	
AAZ[c]	12	25	5.7	41	0.5	0.2	1.6	–
1[d]	160	33	3.2	–	4.8	0.1	–	–
1a	336	88.1	297	407	3.8	3.4	4.6	–
1b	765	116	75	9.9	6.6	0.6	0.1	–
1c	564	103	8.1	60	5.5	0.1	0.6	–
1d	502	9.9	40	69	50.7	4.0	7.0	–
2a	288	8.4	57	77	34.3	6.8	9.2	–
2b	271	7.9	43	45	34.3	5.4	5.7	52
2c	9.8	7.6	36	57	1.3	4.7	7.5	49
2d	27.1	7.7	40	84	3.5	5.2	10.9	–
3b	248	7.5	35	66	33.1	4.7	8.8	8.3
4c	>100,000	>100,000	>100,000	>100,000	1.0	1.0	1.0	–

[a] Mean from three different determinations. Errors in the range of ±10% of the reported value.
[b] Dissociation constants for hypoxic HeLa cells minus normoxic background.
[c] From Ref. 6.
[d] From Ref. 20.

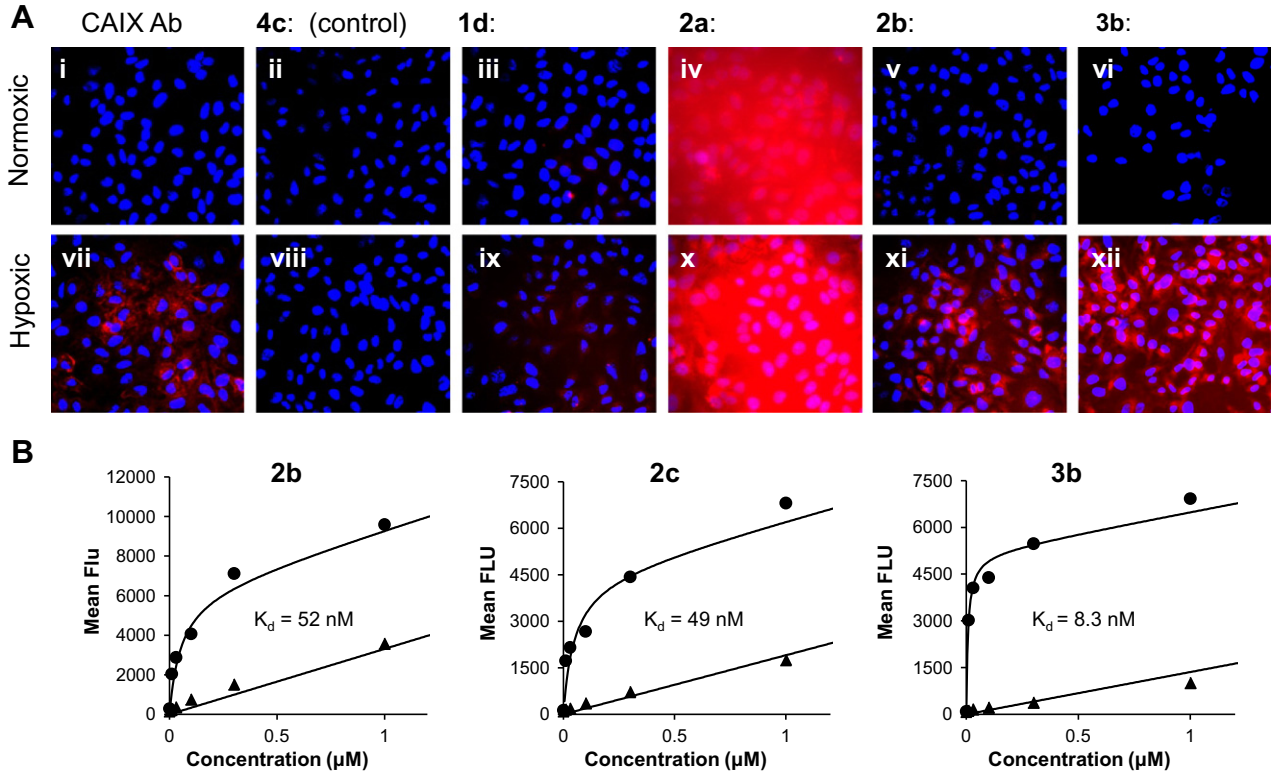


Figure 1. (A) Fluorescence microscopy of the uptake in normoxic (i–vi) or hypoxic (vii–xii) HeLa cells cultured with a CAIX antibody (i, vii), nonbinding control compound **4c** (ii, viii), **1d** (iii, ix), **2a** (iv, x), **2b** (v, xi), and **3b** (vi, xii). Magnification is 40× and exposure times are all 4000 ms except **F** and **L**, 6000 ms. Agent signal is shown in red, cell nuclear stain DAPI is shown in blue. (B) Fluorescence signal by flow cytometry for hypoxic (●) and normoxic (▲) HeLa cells for compounds **2b**, **2c** and **3b** over a range of concentrations from 10 nM to 1 μM along with curve fits for specific (hypoxic) and nonspecific (normoxic) binding.

comparatively low potencies toward hCA II in the range of 336–765 nM, 2–5 times less effective than **1**. Among the **AAZ** series **2a**–**b** and **3b**, the increase in selectivity for hCA IX is completely derived from a decrease in inhibition potency toward hCA II, possibly due to steric effects from the comparatively bulky (>1000 Da) fluorophores. However, acetazolamide derivatives **2c** and **2d**, had inhibition potencies not significantly different than the parent inhibitor **AAZ**.

hCA XII is another transmembrane CA isoform that is also associated with hypoxia and tumor growth that has been identified as a potential therapeutic target.[45] The parent inhibitors used both have single digit nM inhibition constants toward hCA XII, whereas the fluorescent derivatives synthesized in the current study were somewhat less potent. The **AAZ** series was 5–10 times less potent and the benzenesulfonamide series 10–100 times than the corresponding parent inhibitors. The exception was benzenesulfonamide **1c** which, with a K_i of 8.1 nM, had the highest potency toward hCA XII and also the highest selectivity for hCA XII among all of all the compounds tested.

For hCA XIV, the **AAZ** derivatives were all within a factor of two of the potency of the parent (45–84 nM), while the potencies for the benzenesulfonamide series varied more widely from 9.9 to 407 nM. The most selective hCA XIV inhibitor of the series was **1b**. As expected, the non-sulfonamide **4c**, which shares a

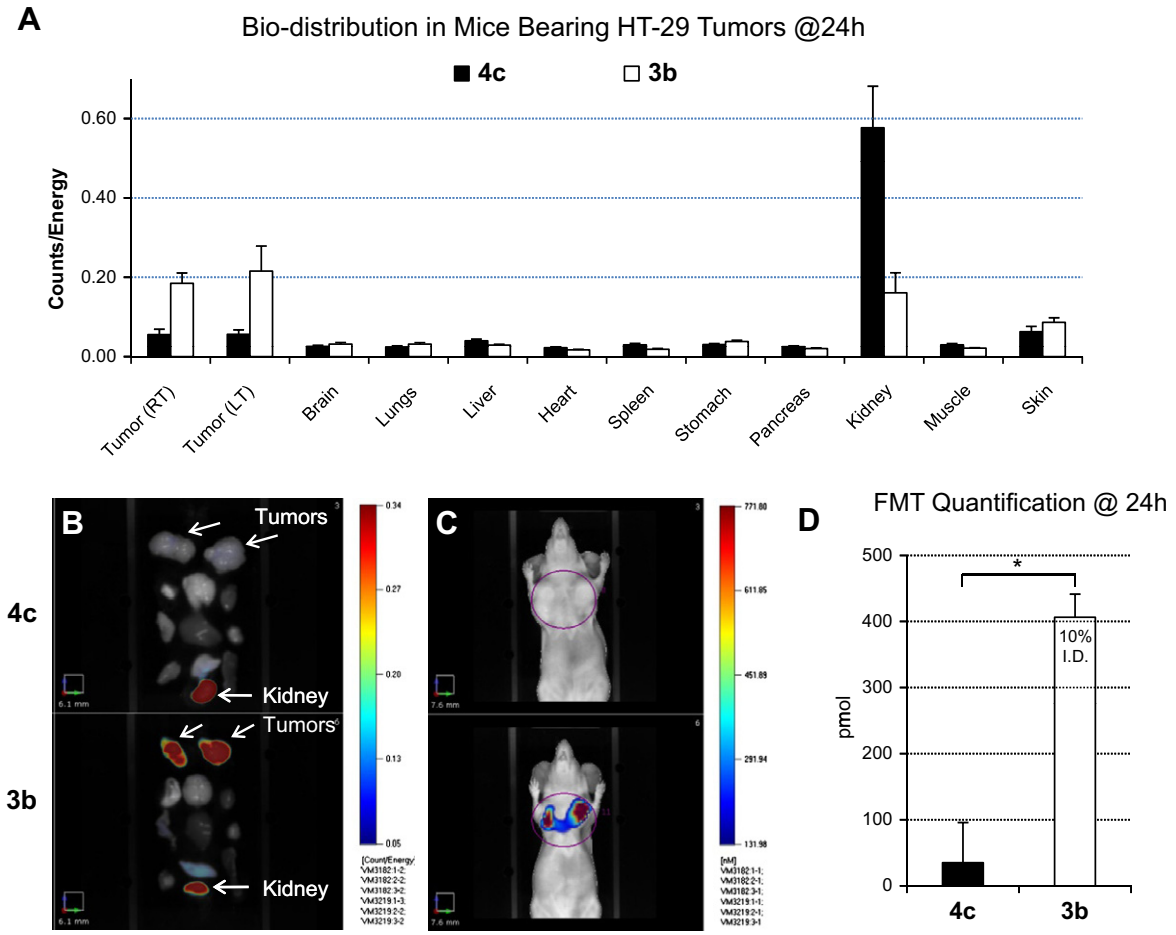


Figure 2. (A) Ex vivo bio-distribution of control compound **4c** and CA IX inhibitor **3b** in HT-29 tumor bearing mice 24 h post i.v. injection (4 nmol). (B) Ex vivo fluorescence reflectance images of HT-29 tumors and selected organs. (C) Noninvasive in vivo FMT tomographic images of **4c** and **3b** in HT-29 tumor bearing mice 24 h post injection and (D) FMT in vivo quantitation in pmol indicating 10% injected dose (400 pmol of 4 nmol injected) of **3b** accumulating in tumors relative <1% (35 pmol) for the control **4c**.

fluorochrome with **1c**, **2c**, and **3c**, had no inhibition potency toward any of the hCAs tested and thus provides an excellent control for background cell and tissue uptake properties of this class of fluorescent compounds.

Compounds **1d**, **2a**–**b**, **3b** and control compound **4c** were selected for in vitro uptake in hypoxic and normoxic HeLa cells. HeLa cells were chosen for in vitro screening of the NIR fluorescent inhibitors due to their very robust upregulation of hCA IX under hypoxic culture conditions and low background expression under normoxic conditions.[46]

HeLa cells were seeded in 8-well slide chambers and cultured under normoxia (air with 5% CO_2) for 24 h before hypoxic culture.[47] The five hCA IX targeted compounds **1d**, **2a**–**b** and **3b** and the control compound **4c** (1 μM final concentration in a well) were added to pre-designated well chambers during the last hour of culture. Anti-hCA IX antibody[48] was used as positive control for the assays. Slide chambers were then taken out from cultures and cell wells were rinsed with cold PBS after discarding the culture media. Well chambers were separated and the slides were dried at room temperature for 4–5 min. The cell nuclear staining marker (Dapi, Invitrogen) was dropped on the slides that were then covered with microscope slide cover glass. The slides were examined under fluorescence microscopy and images were acquired using an equal exposure time of 4000 ms for comparisons.[49] The fluorescence microscope images are shown in Figure 1A.

As shown in Figure 1A, fluorescence from the hCA IX antibody staining was significant for hypoxic cells, but low for normoxic cells (panels i and vii). The control compound **4c** (net charge −5) was nearly undetectable in both normoxic and hypoxic cells (panels ii and viii), reflecting the expected membrane impermeability of this highly charged molecule. In contrast, compound **2a** (net charge −3) showed a relatively high background signal in both hypoxic and normoxic cells, saturating the microscope under the exposure settings even in the normoxic cells (panels iv and x). Compounds **2b** (panels v and xi), **3b** (panels vi and xii), each with a net charge of −5, showed comparable or better fluorescence signal in hypoxic cells to that of the fluorescent CA IX antibody, with very low background in the normoxic cells similar to the control compound. **1d** (panels iii and ix), which bears a net charge of −3, also had low background and exhibited a modest preference for hypoxic cells, but the overall brightness was less than **2b** and **3b**.

The dissociation constants (K_d) of compounds **2b**, **2c**, and **3b** to live, hypoxic HeLa cells were also quantified by flow cytometry over a range of agent concentrations from 10 nM to 1 μM, using the normoxic background as a measure of nonspecific uptake.[50] The K_d values thus obtained[51] are shown in Figure 1B and Table 1. For compounds **2b** and **2c**, the K_d values of 52 nM and 49 nM, respectively, to live, hypoxic cells were about sixfold lower than the K_i values obtained for inhibition of the purified active site of the enzyme. The 8.3 nM K_d measured for compound **3b**, however, was essentially equal to the K_i of 7.5 nM toward purified hCA IX. Due to its strong binding to hypoxic HeLa cells, compound **3b** was selected for a pilot in vivo experiment.

To demonstrate the in vivo efficacy of the new fluorescent inhibitors, lead compound **3b** and control compound **4c** (4 nmol each) were injected i.v. into HT-29 tumor bearing mice followed

by FMT imaging 24 h later to allow sufficient time for unbound compound to clear from the animals. After imaging, the organs were excised to determine biodistribution by fluorescence reflectance. As shown in Figure 2A,B, ex vivo biodistribution revealed significant accumulations of **3b** in tumors, 10-fold over background signal in muscle tissue, with very little accumulation in other tissues except for the kidneys, the expected route of clearance. Animals injected with compound **4c** had fluorescence accumulation primarily in the kidneys, but not in the tumors with a mean reflectance signal fourfold less than **3b**. Tumor definition was very clear in the tomographic images, and maximum signal appeared to be coming from the center of the tumors (Fig. 2C). Quantification by FMT in the live animals revealed that approximately 400 pmol (10% of injected dose) was retained in the two implanted tumors after 24 h, approximately 10-fold more than **4c** (Fig. 2D).

In summary, we have synthesized a new series of NIR fluorescent, sulfonamide based hCA inhibitors some of which exhibit single digit nanomolar inhibition of hCA IX and significantly improved selectivity over hCA II relative to the parent inhibitors. The nature of the sulfonamide, the linker and the fluorochrome properties all influenced the inhibition and binding properties of these new agents. The lead compounds were found to selectively bind to hypoxic versus normoxic cells in vitro with comparable K_d values to K_i values toward purified enzyme while a non-binding control incorporating the same fluorophore showed little binding to either cells. A pilot in vivo experiment quantified significant accumulation (10% i.d.) in tumor tissues with little accumulation in other organs except the kidneys. These results illustrate the potential of NIR-fluorescent hCA IX inhibitors and FMT imaging to non-invasively quantify CAIX expression as an endogenous marker of tumor hypoxia, crucial to the study of the underlying biology of hypoxic tumors and the development and monitoring of novel anti-cancer therapies.

Disclosure statement: KG, BB, JZ, EH, PK, GC, WY, JDP, and MR are employed by PerkinElmer.

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43. *LCMS analyses determined on a Waters Micromass ZQ*: Compound **1a**: calcd 1206.2 (M+H+), m/z = 1206.3; compound **1b**: calcd 1308.1 (M+H+), m/z = 1308.2; compound **1c**: calcd 1421.2 (M+H+), m/z = 1421.1; compound **1d**: calcd 1120.2 (M+H+), m/z = 1120.2; compound **2a**: calcd 1333.2 (M+H+), m/z = 1333.1; compound **2b**: calcd 1435.1 (M+H+), m/z = 1435.1; compound **2c**: calcd 1548.2 (M+H+), m/z = 1548.3; compound **2d**: calcd 1247.2 (M+H+), m/z = 1247.3; compound **3b**: calcd 1373.1 (M+H+), m/z = 1373.2; compound **4c**: calcd 1342.2 (M+H+), m/z = 1342.1.
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46. As determined by flow cytometry using anti-CA IX antibody (rabbit polyclonal Anti-CA IX antibody, Santa Cruz Biotechnology) that was detected by Alexa Fluor 488 goat anti Rabbit IgG (Invitrogen).
47. To induce hypoxia, slide chambers were placed into a Modular Incubator Chamber (MIC-101, Billups-Rothenberg, Inc. CA) that was flushed with mixed-low oxygen gas (1.0% O_2, 5.0% CO_2, and 94% N_2). The modular chamber was then placed into an incubator and cultured for 24 h along with normoxic culture slide that was placed directly into incubator.
48. Phycoerythrin-conjugated Anti-Human Carbonic Anhydrase IX (hCA IX) monoclonal antibody (FAB2188P, R&D Systems.
49. Except compound **3b**, 6000 ms.
50. For flow cytometry, cells were harvested, centrifuged at 1000 rpm for 10 min, resuspended in 0.5 mL PBS and fluorescence quantified using a BD LSR2 flow cytometer (BD Biosciences, Rockville, MD) equipped with a solid-state 660 nm (60 mW) red laser and 712/21 nm bandpass filter.
51. K_d values of test agents were calculated using GraphPad Prism Curve Fitting Software; binding values to normoxic cells were considered as non-specific binding and binding values to hypoxic cells were used as total binding.