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Acetazolamide-based [^{18}F]-PET tracer: *In vivo* validation of carbonic anhydrase IX as a sole target for imaging of CA-IX expressing hypoxic solid tumors

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ABSTRACT

Carbonic anhydrase IX is overexpressed in many solid tumors including hypoxic tumors and is a potential target for cancer therapy and diagnosis. Reported imaging agents targeting CA-IX are successful mostly in clear cell renal carcinoma as SKRC-52 and no candidate was approved yet in clinical trials for imaging of CA-IX. To validate CA-IX as a valid target for imaging of hypoxic tumor, we designed and synthesized novel [^{18}F]-PET tracer (**1**) based on acetazolamide which is one of the well-known CA-IX inhibitors and performed imaging study in CA-IX expressing hypoxic tumor model as 4T1 and HT-29 *in vivo* models other than SKRC-52. [^{18}F]-acetazolamide (**1**) was found to be insufficient for the specific accumulation in CA-IX expressing tumor. This study might be useful to understand *in vivo* behavior of acetazolamide PET tracer and can contribute to the development of successful PET imaging agents targeting CA-IX in future. Additional study is needed to understand the mechanism of poor targeting of CA-IX, as if CA-IX is not reliable as a sole target for imaging of CA-IX expressing hypoxic solid tumors.

Tumor hypoxia is a salient feature of broad numbers of solid tumors including frequent observations in distant metastasis. Hypoxic tumors with low level of O_2 below 20 mmHg are more prone to aggressive nature with poor prognosis, resistance to cancer therapy and low survival rate of cancer patients.[1,2] Moreover, hypoxic cells are more resistant to radiation and chemotherapy than normoxic cells.[3] Thus, hypoxia becomes a sign of advanced stage tumor; detection of which might be helpful to locate the tumor site. Recent discovery suggested many biomarkers for detection of hypoxic tumors microenvironment such as HIF-1 for cellular processes, vascular endothelial growth factor (VEGF) for angiogenesis, carbonic anhydrase IX (CA-IX) for pH regulation, and glucose transporter 1 (GLUT-1) for metabolism.[4]

CA-IX is a highly active cell surface anchored enzyme involved in hypoxia-induced stress, and is regulated by transcription factor, hypoxia-inducible factor-1α (HIF-1α) activated in response to tumor hypoxia.[2,5] Hypoxia-induced stabilization of HIF-1α causes the expression of gene for CA-IX enzyme which alters cell homeostasis and consequently induces various oncogenic processes such as cell proliferation, angiogenesis, cell invasion, metastasis and metabolic changes in hypoxic tumors.[6,7] Among 15 human isozymes of carbonic anhydrase (CA), CA-IX is a most active CA for the CO_2 hydration reaction and prominently overexpressed in hypoxic tumors.[8] Overexpression of CA-IX plays a pivotal role in many cancers as non-small cell lung cancer (NSCLC),[9] breast,[10,11] renal cancer,[12] ovarian,[13] brain,[14] colon, and head and neck cancers.[13,15] Expression of CA-IX in different cancers with featured hypoxia and presence of extracellular active site make it a promising target for anticancer therapy as well as an attractive target for drug delivery purpose such as imaging and therapy.[16] The crystal structure of CA-IX enables to develop potent and selective small molecule inhibitors targeting CA-IX. Structurally, carbonic anhydrase is a metalloenzyme, and the active site is formed as distorted tetrahedral geometry with zinc ion as center in coordination with three imidazole groups of histidines and one hydroxide ion of substrate.[17] Recent paradigm in cancer therapeutics is the development of new CA-IX inhibitors for better efficacy in alone or in

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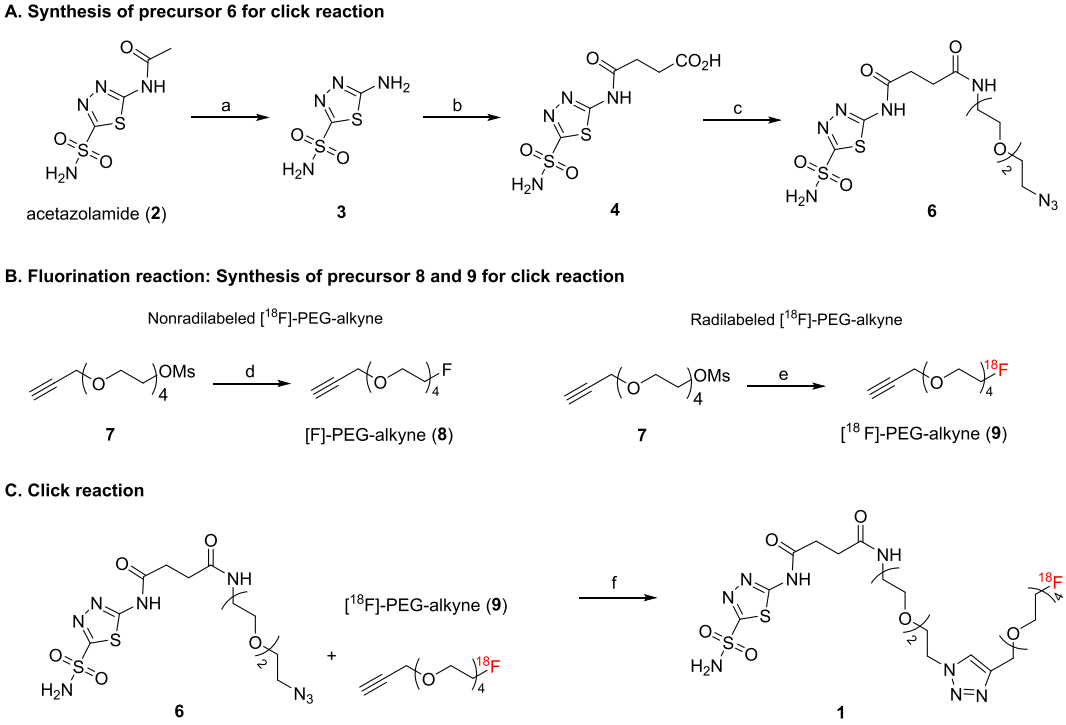
combination with chemotherapeutic agent or radiotherapy.[7,18,19] Small molecules inhibitors targeting CA-IX are either coumarin suicidal inhibitors or sulfonamides moiety.[13,20,21] Sulfonamide ligand with high affinity to central metal ion of an enzyme can be utilized for the targeted delivery of potent cytotoxic drugs into solid tumors, which is otherwise showed ineffective biodistribution to desired tumor site and accumulated in normal tissues.[19,22] Development of CA-IX inhibitors as an anticancer agent also enhanced the development of PET imaging ligand for diagnostics and small molecule-drug conjugates (SMDCs) for therapeutic purpose in both solid and metastatic tumors. Acetazolamide, a prototype[23] of sulfonamides, is a clinically approved *pan*-CA inhibitor with good profile,[21,24] and is also known to possess antitumor activity alone or in combination.[18,25] Suppression of tumor metastasis in lung carcinoma was also acquired by acetazolamide.[26] ^{99m}Tc-labeled acetazolamide was even used as a ligand for development of PET imaging radiotracer for imaging purpose[27] and SMDCs with acetazolamide ligand for pharmacodelivery of cytotoxic drug at tumor site.[28] However, most of the known radiotracers for imaging CA-IX as ^{99m}Tc-labeled acetazolamide ligand, ^{124}I-cG250 or ^{124}I- and ^{89}Zr-labeled antiCA-IX monoclonal antibodies were performed well predominantly for *in vivo* imaging of CA-IX in hypoxia independent renal cell carcinoma SKRC-52,[27,29] even though CA-IX is also expressed in various hypoxic tumor models.[30] Only few PET tracer as ^{68}Ga-NOTGA-(AEBSA)$_3$ and ^{18}F-AmBF3-(ABS)$_3$ with trimeric sulfonamide moieties achieved the proposed role. Surprisingly, the tracer with trimeric sulfonamide moieties showed higher uptake with selective targeting to CA-IX compared to monomeric and dimeric isoforms.[22] Reported studies raised a question that CA-IX is really a universal target for imaging of all solid tumors other than SKRC-52. One review was published performing validation of CA-IX target for hypoxic tumor imaging.[31] The review proposed that CA-IX is an unreliable target for hypoxic imaging due to the facts that CA-IX expression is cancer type-dependent, not all hypoxic tumors express CA-IX, expression of CA-IX is undetectable in certain cancer types, and re-oxygenation of previously hypoxic cancer cells induces expression of CA-IX. However, no *in vivo* experimental study was carried out to support this conclusion yet. Thus, further validation of CA-IX as a target for development of clinically feasible hypoxia imaging PET tracer is required to be performed. In the view of validation of CA-IX target for hypoxic tumor imaging, we designed and synthesized a new [^{18}F]-PET tracer (**1**) based on acetazolamide as shown in Scheme 1. *In vivo* PET imaging and biodistribution studies of [^{18}F]-PET tracer (**1**) were performed in CA-IX positive 4T1 and HT-29 skin xenograft Balb/c mice tumor models. 4T1 and HT-29 cancer cells are known to express CA-IX within hypoxic microenvironment. Imaging of PET tracer was also performed in CA-IX expressed lung metastatic tumors model formed by 4T1 breast cell lines.[11] The *in vivo* PET images and biodistribution profile provided insight about the behavior of our novel PET tracer [^{18}F]-acetazolamide (**1**) on tumor model.

The precursor **6** for [^{18}F]-labeling was synthesized to couple with [^{18}F]-PEG-alkyne (**9**) to form central triazole ring by copper (I)-catalyzed click chemistry as shown in Scheme 1. The precursor **6** was synthesized starting with hydrolysis of commercially available acetazolamide (**2**) giving compound **3**. Compound **3** was reacted with succinic anhydride in DMF with heating to obtain carboxylic acid (**4**). Compound **4** was subjected to amide coupling reaction with azide-PEG linker (2-(2-(2-azidoethoxy)ethoxy) ethan-1-amine) (**5**) using BOP which finally provided the precursor (**6**) for click reaction. This precursor was then used for synthesis of radiolabeled [^{18}F]-acetazolamide (**1**) through the click reaction with [^{18}F]-PEG-alkyne (**9**). Radiolabeling of the mesylated PEG-alkyne linker (**7**) successfully provided [^{18}F]-PEG-alkyne precursor (**9**) in 32.5% radiochemical yield with >99% of radiochemical purity. The click reaction of precursor (**6**) with [^{18}F]-PEG-alkyne (**9**) was performed to obtain final [^{18}F]-PET tracer (**1**) in radiochemical yield of 93.73% and radiochemical purity >99% after HPLC purification (see Fig. S1 and Table S1, ESI†).



Scheme 1. Reaction conditions and reagents: A. (a) conc. HCl, EtOH, reflux, quant. yield; (b) succinic anhydride, DMF, 100 °C, 96%; (c) 2-(2-(2-azidoethoxy)ethoxy)ethan-1-amine (5), BOP, *i*PrNEt$_2$, DMF, rt, 53%; B. (d) TBAF, THF, 80 °C, 33%; (e) ^{18}F/[2,2,2]-cryptand, CH$_3$CN, 100 or 80 °C, 33%; C. (f) CuI, *i*PrNEt$_2$, CH$_3$CN, rt, 94%.