Carbonic Anhydrase Inhibitors Developed Through 'click tailing'

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Running Title: Carbonic Anhydrase Inhibitors Synthesised using Click Chemistry

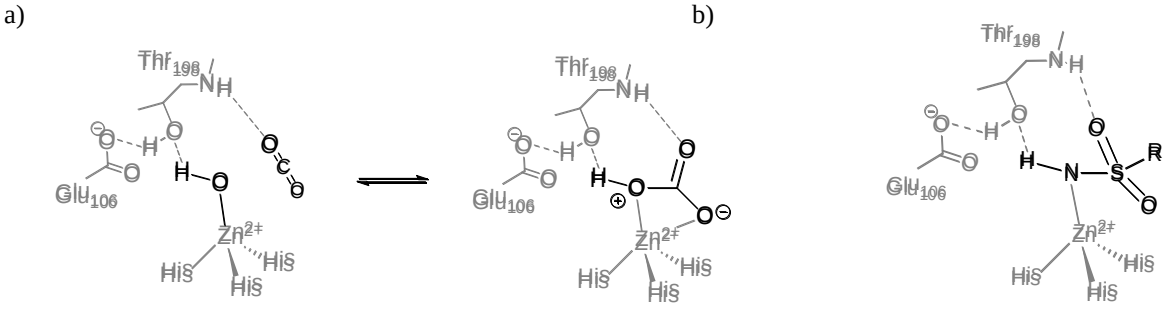
Keywords. Carbonic anhydrase, click chemistry, hypoxia, CA IX, glycoconjugates, sulfonamide, medicinal chemistry, metallocene

List of Abbreviations. DCR, dipolar cycloaddition reaction; CA, carbonic anhydrase; PET, positron emission topography; ZBG, zinc binding group; PDB, Protein Data Bank; K_i, inhibition constant; SPR, structure-property relationships; SAR, structure-activity relationships; CuAAC, copper-catalyzed azide-alkyne cycloaddition; pH_e, extracellular pH; pH_i, intracellular pH; HIF, hypoxia-inducible factor; shRNA, short hairpin RNA; cLog P, calculated Log P; P_e, effective permeability; PAMPA, parallel artificial membrane permeability assay; ZNS, zonisamide; TPM, topiramate; K_d, dissociation constant.

Abstract. In recent years there has been renewed activity in the literature concerning the 1,3-dipolar cycloaddition reaction (1,3-DCR) of organic azides (R-N_3) with alkynes (R′-C≡CH) to form 1,2,3-triazoles, *i.e.* the Huisgen synthesis. The use of catalytic Cu(I) leads to a dramatic rate enhancement (up to 10^7-fold) and exclusive synthesis of the 1,4-disubstituted 1,2,3-triazole product. The reaction, now referred to as the copper-catalyzed azide-alkyne cycloaddition (CuAAC), meets the stringent criteria of a click-reaction in that it is modular, wide in scope, high yielding, has no byproducts, operates in water at ambient temperature, product purification is simple and the starting materials are readily available. The 1,3-DCR reaction has rapidly become the premier click chemistry reaction with applications spanning modern chemistry disciplines, including medicinal chemistry. Recently the 'tail' approach initiative for the development of carbonic anhydrase inhibitors (CAIs) has been combined with the synthetic versatility of click chemistry. This has proven a powerful combination leading to the synthesis of CAIs with useful biopharmaceutical properties and activities. This review will discuss complementary and contrasting applications that have utilized 'click tailing' for the development of CAIs. Applications encompass i) medicinal chemistry and drug discovery; ii) radiopharmaceutical development of positron emission topography (PET) chemical probes; and iii) *in situ* click chemistry.

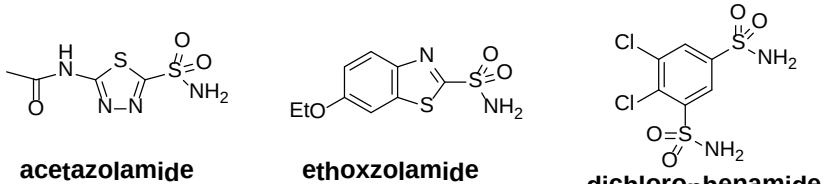
Introduction. Carbonic anhydrase (CA, EC 4.2.1.1) enzymes catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton: $CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$ [1]. Recent advances in our understanding of the biology of α-CAs and the impact of this simple, yet critical, equilibrium on human health augments a need for small molecule inhibitors that can effectively interrogate the biological hypotheses associated with this enzyme class. Catalytically active α-CAs comprise a tetrahedral Zn^{2+} cation coordinated to the imidazole sidechains of three histidine residues located at the base of a funnel shaped active site cavity. In addition to this structural role, the Zn^{2+} cation plays a catalytic role, binding to the substrate H_2O molecule and lowering its pK_a to form zinc bound hydroxide, hydroxide is the nucleophile that reacts with CO_2, Figure 1a [2]. The Zn^{2+} cation is the implied target for drug design efforts and almost all reported small molecule CAIs comprise a zinc binding group (ZBG) [1]. The primary sulfonamide moiety ($-SO_2NH_2$) is recognized as the foremost example of a ZBG for CAIs. The sulfonamide anion ($-SO_2NH^-$) coordinates to the active site Zn^{2+} similarly to the transition state of the endogenous reaction, Figure 1b [2]. At the time of writing the Protein Data Bank (PDB) contained X-ray structures of ~100 unique sulfonamide ligands (R- SO_2NH_2) in complex with CAs, mostly with hCA II (h = human). Isosteres of sulfonamides, such as the sulfamates (R-O-SO_2NH_2) and sulfamides (R-NH-SO_2NH_2), are also well known CAIs – these contribute an additional ~20 X-ray structures of ligands in complex with CA that have been deposited in the PDB. The binding mode of the sulfonamide anion to the zinc ion is invariant and the canonical sulfonamide:CA II interactions are as shown in Figure 1b.

Figure 1. Schematic of the CA active site of catalytically active hCA isozymes: a) showing CO_2 hydration to HCO_3^- and b) with zinc bound sulfonamide anion as a transition state isostere.



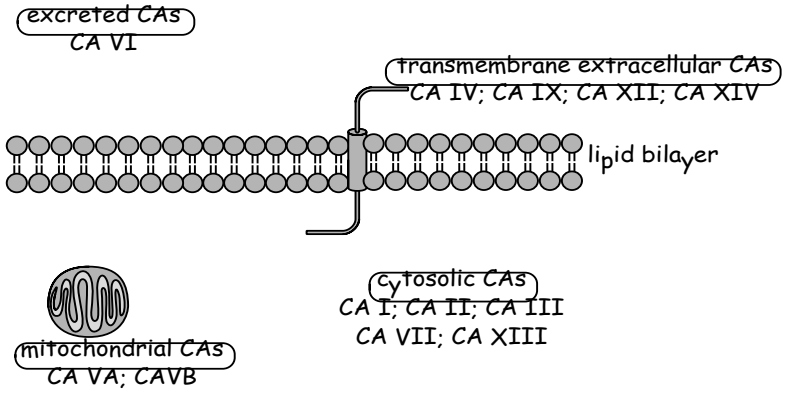
It is relatively straightforward to develop CAIs of the type R-SO_2NH_2 that have low nanomolar inhibition constants (K_is). The conservation of active site architecture amongst human CAs has however meant that the development of isozyme selective inhibitors presents a more difficult challenge for medicinal chemists. The CAIs acetazolamide, ethoxzolamide and dichlorophenamide, entered clinical use many years before the characterization of most CA isozymes, Figure 2. It is not surprising that the systemic administration of these drugs have side effects that now are attributed to the inhibition of off-target CA isozymes [1].

Figure 2. Examples of classical clinically used CA inhibitors.



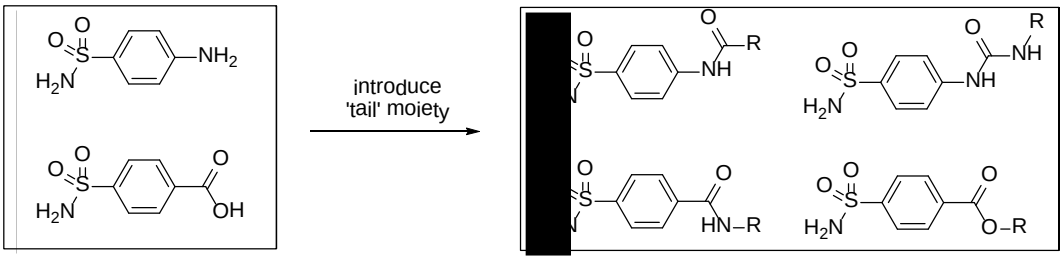
Our growing knowledge of CA biology changes the contemporary medicinal chemistry landscape surrounding CA inhibition. It has generally proven difficult to target subtle isozyme differences by rational drug design despite the available PDB structures of CA in complex with ligands. We now know that different hCA isozymes exhibit variable tissue distribution, subcellular locations and expression profiles in healthy versus diseased cells. For example, five isozymes are cytosolic, two are found in mitochondria, one is secreted and four are transmembrane with an extracellular active site, Figure 3 [1]. We also know that CA IX and XII are highly overexpressed in hypoxic tumors (see later) [3, 4]. These differences underpin clear opportunities for medicinal chemistry programs to inhibit specific CA isozymes by manipulation and finetuning of the physicochemical properties of the small molecule inhibitor to target a tissue/cellular location selectively. The role of medicinal chemistry in the CA field is the development of small molecule inhibitors with both activities and properties that are useful *in vivo* and that have potential for downstream development as first-in-class CA-based therapies.

Figure 3. The subcellular localization of catalytically active human CA isozymes.



The concept of the 'tail approach' for developing CAIs was first described by Supuran and co-workers [5]. Here 'tail' moieties were tethered to a known R-SO_2NH_2 (R = aromatic/heteroaromatic) CA pharmacophore to give inhibitors with a balanced physicochemical property profile of aqueous solubility and lipophilicity to allow topical administration as eye drops. Since this initial report it has become increasingly evident that structure-property relationships (SPR) of CAIs are on an equal footing with structure-activity relationships (SAR) in the pursuit of compounds with appropriate biopharmaceutical properties for eventual *in vivo* use. The focus on SPR necessarily takes into account the biological environment for both normal and diseased cells/tissues of the CA isozyme of interest. Appending tail groups to the Ar-SO_2NH_2 CA 'anchor' to give the extended pharmacophore [tail]-[aromatic]-[ZBG] has proven effective in enhancing the physicochemical properties such as lipophilicity, pK_a, solubility, permeability, stability and cytotoxicity of a large number of CAIs [6, 7]. The attachment of the tail moiety to the Ar-SO_2NH_2 CA anchor has typically been achieved using straightforward chemistry to form covalent links such as esters, amides, imines, ureas and thioureas, Figure 4 [6].

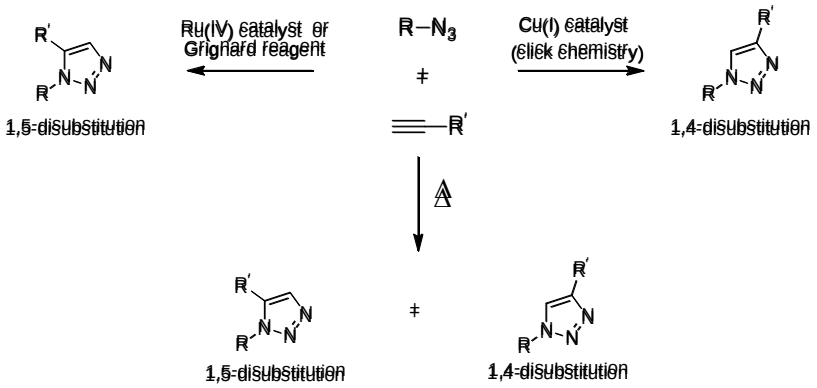
Figure 4. Examples of the tail approach for development of CAIs. Tail groups (R) are linked to the Ar-SO_2NH_2 scaffold to give the CA pharmacophore [tail]-[aromatic]-[ZBG].



Click chemistry.

In recent years there has been renewed activity in the literature concerning the 1,3-dipolar cycloaddition reaction (1,3-DCR) of organic azides (R-N_3) with alkynes (R′-C≡CH) to form 1,2,3-triazoles, *i.e.* the Huisgen reaction [8]. Typically equimolar amounts of 1,4- and 1,5- disubstituted triazoles are obtained by heating neat mixtures of the parent azide and alkyne at elevated temperature, while there is virtually no conversion to triazoles under ambient conditions, Scheme 1 [8]. Both Meldal [9] and Sharpless [10] discovered that catalytic Cu(I) leads to a dramatic rate enhancement (up to 10^7-fold) and exclusive synthesis of the 1,4-disubstituted 1,2,3-triazole product. The reaction, now referred to as the copper-catalyzed azide-alkyne cycloaddition (CuAAC), meets the stringent criteria of a click-reaction [11] - it is modular, wide in scope, high yielding, has no byproducts, operates in water at ambient temperature, product purification is simple and the starting materials are readily available. The CuAAC has rapidly become the premier click chemistry reaction with applications spanning modern chemistry disciplines, including medicinal chemistry [12, 13]. The synthesis of the other possible product of this cycloaddition, the 1,5-disubstituted-1,2,3-triazole, has been achieved regioselectively more recently by catalysis with ruthenium complexes such as [Cp*RuCl(PPh_3)$_2$] [14, 15]. In comparison with the CuAAC there are few examples of these variants and the scope much less investigated.

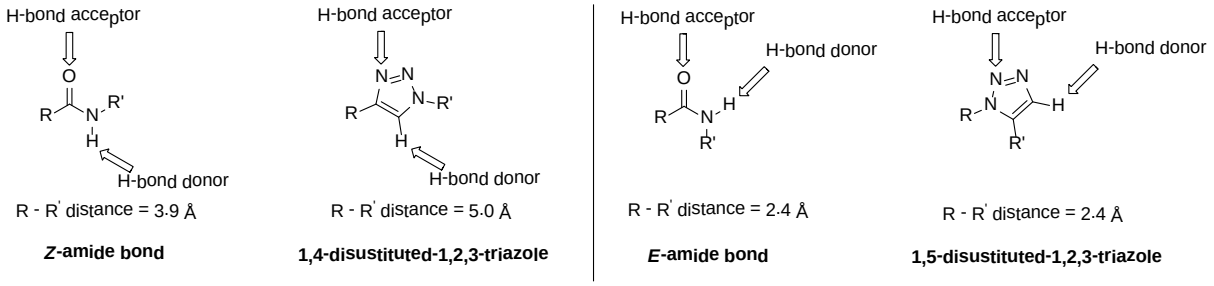
Scheme 1. Synthetic options towards 1,2,3-triazoles using the 1,3-DCR of organic azides (R-N_3) with terminal alkynes (R′-C≡CH).



In drug development some functional groups, while synthetically attractive, can be a source of liability to the biopharmaceutical performance of a compound *in vivo*. Bioisosteres, either classical or non-classical, are often interchanged with such groups. The rationale is to address the liability over the parent molecule whilst retaining the biological activity [16]. The potential of the 1,4- and 1,5- disubstituted-1,2,3-triazole as a nonclassical

bioisostere of a *Z*- and *E*- amide bond, respectively, has been described, Figure 5 [17]. The 1,2,3-triazole has a large dipole and the lone pair electrons of N-2 and N-3 are able to act as hydrogen bond acceptors [18, 19]. The stability attributes of the triazole are also favorable, with stability to chemical challenges encountered by small molecules when in the biological milieus such as acidic, basic, reductive and oxidative conditions as well as enzymatic degradation [17]. The 1,2,3-triazole mimicry of a peptide bond and its chemical robustness have no doubt aided the enthusiastic adoption of this moiety into medicinal chemistry research programs. The triazole moiety may either play a passive or active role when incorporated into a pharmacophore. When operating passively the triazole provides a non-labile covalent spacer between discrete N-1 and C-4 or C-5 substituents. Alternatively, when operating in an active capacity the triazole participates through direct interactions with the biological target and contributes to the overall pharmacophore, Figure 5 [17].

Figure 5. The 1,4 and 1,5 disubstituted-1,2,3 triazoles as a bioisosteres of a *Z*- and *E*- amide bond, respectively.



Carbonic anhydrase inhibitors developed through "click tailing".

This review will discuss complementary and contrasting applications where the tail approach has been combined with the versatility of click chemistry for the development of CAIs. Applications encompass i) medicinal chemistry and drug discovery; ii) radiopharmaceutical development of PET chemical probes and iii) *in situ* click chemistry.

i) Medicinal chemistry and drug discovery.

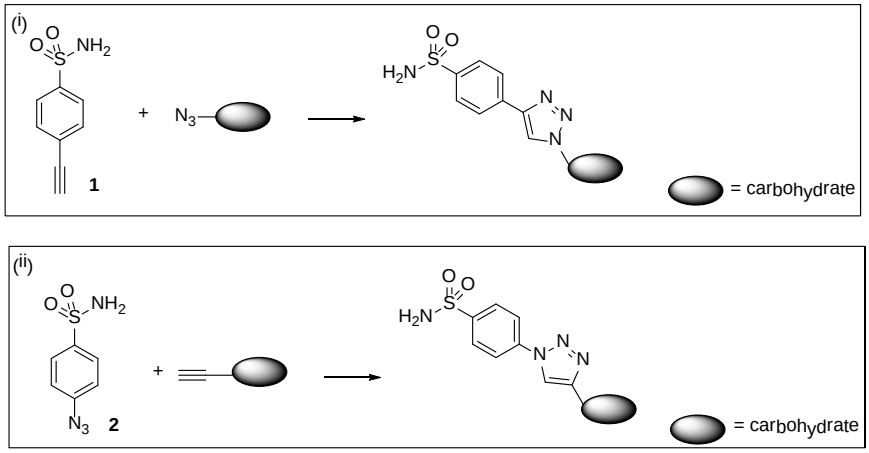
At a molecular level solid tumors are hypoxic (i.e. low $O_2 \equiv 0.1–5\%\ O_2$) owing to inadequate vasculature around the solid tumor. The increased metabolism of cells in solid tumors leads to an elevated level of secreted lactic acid and CO_2, which when combined with poor clearance, causes increased extracellular acidity (lower pH_e) [20, 21]. Lowered pH_e promotes tumor invasiveness [22], however a variation to intracellular pH (pH_i) represents a severe threat to cancer cell survival through disruption of critical biological functions [21]. Hypoxia induces the activation of hypoxia-inducible factor or HIF, HIF regulates a signaling cascade involving ~hundred genes that initiate adaptive cellular functions allowing solid tumor cells to not only survive hypoxia, but to proliferate and metastasize [23]. CA IX and XII are amongst the most highly induced HIF responsive genes and are overexpressed and sustained in a number of solid tumors including breast, brain (glioblastoma), clear cell renal, colorectal, head and neck, bladder and non-small cell lung carcinomas [3, 4, 24]. Using shRNA (short hairpin RNA) manipulation the roles of CA IX and XII have recently been shown to regulate and maintain normal pH_i under hypoxic conditions – this action promotes tumor cell survival during hypoxia [20]. Furthermore, *in vivo* studies in transfected LS174T colorectal cancer xenograft models wherein *ca9* and *ca12* gene expression were

silenced delivered an impressive 85% reduction in tumor volume [20]. These studies have validated the targeting of CA IX and XII as a potential anticancer therapy, particularly for the benefit of patients with hypoxic tumors.

Click tailing to generate glycoconjugates targeting tumor-associated CAs. CA IX and XII possess two discriminating attributes that provide opportunities for selective targeting with small molecule inhibitors. First, their expression in hypoxic tumors occurs within tissues that normally lack these isozymes [25] and second, unlike the physiologically dominant cytosolic CAs, CA IX and XII are transmembrane proteins with an *extracellular* enzyme active site [1], Figure 3. In order to selectively target CA IX and XII isozymes for *in vivo* applications our group has focused on optimizing both SAR and SPR of CAIs to provide chemical probes and/or drug leads [26-33]. The physical barrier of the cell membrane is central to the behaviour of compounds *in vivo*. In general, passive diffusion across a biological membrane correlates directly with the lipophilicity of the small molecule, with membrane permeability generally decreasing with increasing small molecule polarity or with increasing capacity for hydrogen bonding [34]. We first articulated the term "click tailing" in 2006 for the development of cell membrane impermeable CAIs [26]. Our rationale was to use click chemistry to append carbohydrate tails onto the $ArSO_2NH_2$ pharmacophore to give the motif [sugar]-[aromatic]-[ZBG]. The polar glycoconjugate triazole CAIs have a lowered cLog P, enhanced aqueous solubility and limited passive membrane permeability. These properties enable them to specifically target the extracellular CAs. It had been demonstrated already that CA IX and XII display high affinity for primary aryl and heteroaryl sulfonamides, as expected [35, 36].

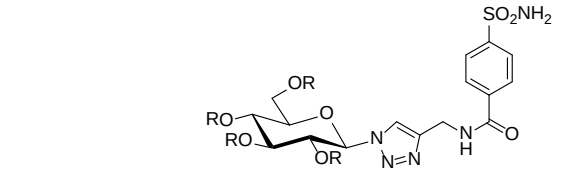
From a synthetic viewpoint the reactions used to generate molecular diversity in carbohydrate-based libraries need to be facile, mild, high yielding and ideally give a predictable stereochemical outcome. We have shown that click chemistry using CuAAC fulfils these demands in carbohydrate chemistry [37]. For synthesis of glycosyltriazole CAIs, the CA anchor $ArSO_2NH_2$ pharmacophore can incorporate either a terminal alkyne or azide functionality as the reaction partner for click chemistry. The library formats trialled were a combination of either: (i) an acetylenic Ar-SO_2NH_2 fragment (eg. **1**) with a panel of azido sugars, or ii) an azido Ar-SO_2NH_2 fragment (eg. **2**) with a panel of sugar acetylenes, Scheme 2.

Scheme 2. Click-tailing strategy to generate glycosyltriazole CAIs. Two library formats were utilized: (i) an acetylene containing benzenesulfonamide fragment (eg. **1**) with a panel of azido sugars, or (ii) an azide containing benzenesulfonamide fragment (eg. **2**) with a panel of sugar acetylenes.

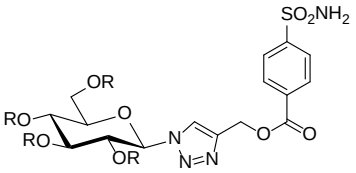


The first series of CAIs were prepared using format (i) and encompassed benzenesulfonamides comprising anomeric triazole tails in combination with either an ester or amide functionality [26]. A second series using format (ii) was undertaken in combination with an *O*-glycoside linkage [27]. Subsequent libraries have followed to give a non-native covalent linkage to the carbohydrate to promote *in vivo* enzymatic stability. For example a library of *S*-linked glycosyltriazoles in three oxidation states (-*S*-, -*SO*- and –SO_2-) were synthesized [32]. The *in vitro* inhibition of hCA I, II, IX, and in some cases CA XII, for triazole glycoconjugates using the CO_2 hydration assay have been determined. Table 1 presents data for selected glucose-based inhibitors from these studies, wherein the glucosyl moiety is either a peracetylated sugar (**3a-9a**) or a free sugar (**3b-9b**). The inhibitors encompass 1,2,3-triazoles with amide (**3a**, **3b**), ester (**4a**, **4b**) *O*-glucoside (**5a**, **5b**) and *S*-glucoside (**7-9a**, **7-9b**) covalent linkages as well as anomeric triazoles where the triazole and sugar are directly linked (**6a**, **6b**), Figure 6. The inhibition results for all compounds have provided a comparative surveillance of the active sites of these clinically relevant CA isozymes, and have proven immensely informative in advancing towards CA-based therapeutics and/or chemical probes displaying improved potency and selectivity. The impressive potency and selectivity of several glycoconjugate triazoles has confirmed the effectiveness of the carbohydrate tail at differentiating amongst isozymes.

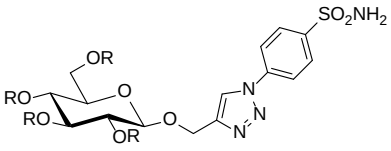
Figure 6. Structures of selected glucoconjugate CAIs prepared by click tailing using CuCAAC: **3a-9a** (R = Ac) and **3b-9b** (R = H).



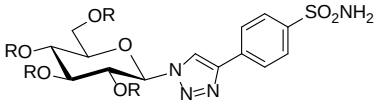
3a, 3b



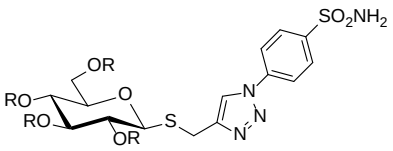
4a, 4b



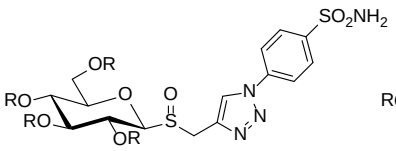
5a, 5b



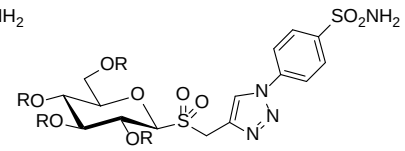
6a, 6b



7a, 7b



8a, 8b



9a, 9b

Table 1. Enzyme inhibition and cLog P data for hCA isozymes I, II, IX and XII with selected glucoconjugate triazole CAIs **3a-9a**, **3b-9b**.

Compound	K_i (nM)[a]				
	cLog P[b]	hCA I[c]	hCA IIc[b]	hCA IX[d]	hCA XII[d]
3a	-0.462	5 600	384	430	4.3
3b	-2.606	2 000	8.2	442	11.4
4a	+0.563	2 300	119	1238	7.7
4b	-1.581	4 400	7.0	183	7.1
5a	+0.499	1 500	46	107	n.d.
5b	-1.642	7.0	8.7	101	n.d.
6a	+0.539	4 400	9.1	120	n.d.

6b	-1.604	9.4	380	89	n.d.
7a	+1.098	91	5.3	8.6	9.5
7b	-1.042	114	5.6	257	9.5
8a	-0.277	103	5.4	9.9	8.4
8b	-2.417	102	7.8	9.8	10.2
9a	-0.343	97	6.9	9.3	9.1
9b	-2.483	101	7.6	9.5	10.3

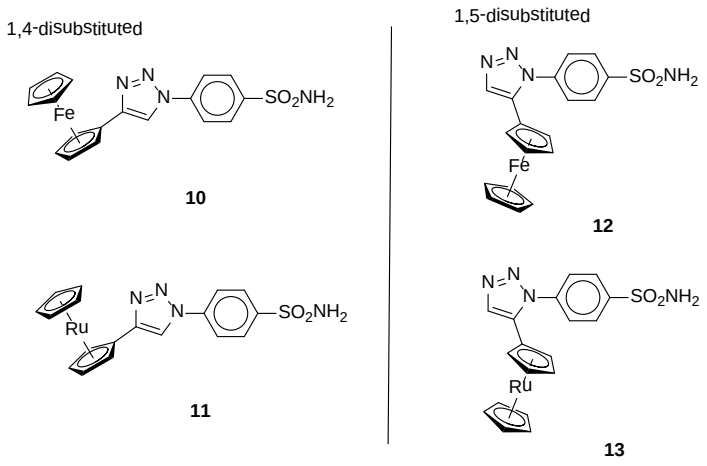
[a]Errors in the range of ± 5-10 % of the reported value, from three determinations. [b]cLog P data calculated using ChemBioDraw Ultra 11.0. [c]Human (cloned) isozymes. [d]Catalytic domain of human (cloned) isozymes. n.d. not determined.

Calculated Log P (cLog P) values are a very useful guide in the prediction of passive membrane permeability, with Log P prediction software one of the most reliable in silico tools for property profiling [38]. Table 1 also includes cLog P values for the glucoconjugates. As expected the cLog P trend is acetylated sulfonamides > free sugar sulfonamides, consistent with the four incorporated acetate groups increasing the lipophilicity of the compound. All free sugar sulfonamides have cLog P values that fall within the range indicative of molecules with poor membrane permeability, i.e. cLog P < 0. Permeability measurements through artificial lipid membrane biolayers have been extensively used to classify small molecules for their passive membrane permeability characteristics [34, 39]. The PAMPA (parallel artificial membrane permeability assay) technique provides a measurement for apparent *in vitro* effective permeability (P_e), high permeability compounds typically have P_e values in excess of 3×10^{-6} cm s^{-1} while low permeability compounds typically have P_e values less than 3×10^{-6} cm s^{-1} [40, 41]. The PAMPA results for glycosyltriazole sulfonamides again confirm that this class of compound would be expected to have poor passive membrane permeability [33]. This characteristic of the molecules is expected to lead to preferential inhibition of the transmembrane CAs IX and XII over cytosolic CAs, however further *in vivo* studies are required to confirm this hypothesis. In summary, this combined SAR and SPR approach has provided valuable chemical tools and potential drug leads for the study of CAs. The interested reader is directed to the original research papers for more detail [26-33].

Click tailing to generate metallocenes targeting tumor-associated CAs. The versatility of the 'click-tailing' strategy with carbohydrates may be extended to other tail groups. Metal-based sulfonamide complexes have been reported previously, wherein various metal ions are coordinated to the sulfur or nitrogen of the heterocyclic sulfonamide scaffolds such as acetazolamide [42, 43]. Inhibitor complexes of this nature were observed to be 10-

to 100-fold more potent than the parent sulfonamide, a remarkable potency enhancement that makes this class of CA inhibitor among the most potent known to date. A modern strategy that has been adopted to improve the therapeutic properties of organic drugs is to covalently attach an air and aqueous stable organometallic fragment to the drug molecule [44, 45]. Examples include compounds such as the antimalarial agent ferroquine - generated from chloroquine and the ferrocene fragment [46]; and the anti-breast cancer therapy ferrocifen - generated from tamoxifen and the ferrocene fragment [47]. Our group has adopted this strategy and combined it with the 1,3-DCR to append metallocene tails onto the CA $ArSO_2NH_2$ scaffold fragment to generate metallocene-based CAIs [48]. This has provided a new class of organometallic-based CAIs that are chemically stable in acqueous solutions, unlike early inorganic-based CA inhibitor complexes. The regioselective synthesis of both 1,4- and 1,5-disubstituted-1,2,3-triazole benzenesulfonamides were achieved by reaction of the azide containing $ArSO_2NH_2$ scaffold (**2**) with either ethnylferrocene or ethynylruthenocene, Figure 7. Specifically, the 1,4-disubstituted 1,2,3-triazole regioisomers **10** and **11** were prepared by the CuAAC while the 1,5-disubstituted 1,2,3-triazole regioisomers **12** and **13** were prepared by the ruthenium-catalyzed 1,3-DCR using $[Cp^*RuCl(PPh_3)_2]$ as catalyst.

Figure 7. Structures of metallocene-based CAIs prepared by click tailing, using either CuCAAC (**10**, **11**) or $[Cp^*RuCl(PPh_3)_2]$ as catalyst (**12**, **13**).



The *in vitro* inhibition of hCA I, II and IX using the CO_2 hydration assay was carried out and the data are shown in Table 2. These results demonstrate that both the metal (Ru or Fe) and the triazole substitution pattern (1,4- or 1,5-) influence the CA inhibition profiles. The high potency of these inhibitors has ensured that work on this organometallic class of inhibitors is of ongoing interest with efforts now taking place to improve selectivity for CA IX over CA II.

Table 2. Enzyme Inhibition Data for hCA Isozymes I, II and IX with Metallocene-based CAIs (**10**-**13**).

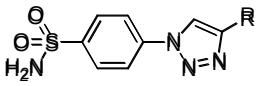
Compound	K_i **(nM)**		
	hCA I	hCA II	hCA IX

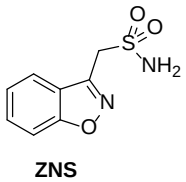
10	3900	80	85
11	44	9.7	10.3
12	160	36	65
13	9	12.3	64

[a]Errors in the range of ± 5-10 % of the reported value, from three determinations. [b]Human (cloned) isozymes. [c]Catalytic domain of human (cloned) isozymes.

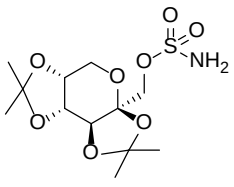
Click tailing to target mitochondrial CAs. The mitochondrial membrane is impermeable to HCO_3^-. Mitochondrial CAs (isozymes VA and VB) provide HCO_3^- as substrate for the mitochondrial enzyme pyruvate carboxylase. Eventually this biosynthetic pathway leads to the formation of citrate from pyruvate. Citrate is translocated from mitochondria to the cytoplasm, and through another biosynthetic pathway leads to *de novo* lipogenesis [1]. Mitochondrial CAs are thus critical to fatty-acid biosynthesis and have been flagged as potential targets for anti-obesity therapies that act through a novel mechanism of action. A library of 10 novel benzenesulfonamides containing triazole-tethered phenyl 'tail' moieties was synthesized by CuAAC reaction between 4-azido benzenesulfonamide **2** and variously substituted phenyl acetylenes. The inhibition data of **2** and triazole sulfonamides **14–23** against the human cytosolic isozymes CA I and II, and the mitochondrial isozymes CA VA and VB, are presented in Table 3. Data for the clinically used sulfonamide and sulfamate therapeutics, zonisamide (ZNS) and topiramate (TPM), for which reduced lipogenesis and weight loss is an observed side effect, are also included. The triazole compounds were low- to mid- nanomolar inhibitors of CA II, VA and VB, and weaker micromolar inhibitors of CA I. These compounds represent some of the first potent mitochondrial CAIs reported and their inhibition profiles should prove valuable lead work in the discovery of isozyme selective CAIs targeting the mitochondrial CA isozymes with potential application as anti-obesity agents.

Table 3. Inhibition data for 4-azido benzenesulfonamide (**2**), benzenesulfonamides containing a triazole-tethered phenyl 'tail' (**14–23**) and standard CAIs **ZNS** and **TPM** against human isozymes CA I, II, VA and VB.



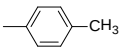
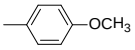
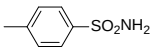
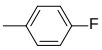
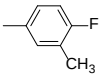
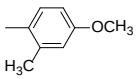


ZNS



TPM

Compounds	**R**	***K*i (nM)**[a]			
		hCA I[b]	hCA II[b]	hCA VA[c]	hCA VB[c]
ZNS	-	56	35	20	6 033

TPM	-	250	10	63	30
1	-	3900	47.0	56.0	55.8
14		5100	7.9	17.8	10.6
15		2100	18.6	12.8	10.6
16		4000	33.8	19.6	54.2
17		3000	7.7	9.3	11.4
18		2200	8.4	10.6	51.8
19		100	8.1	15.1	11.9
20		4600	40.3	14.2	11.2
21		6500	10.7	19.1	52.3
22		5800	11.7	16.9	10.5
23		5500	8.3	17.1	12.9

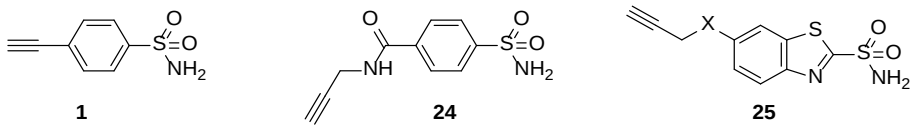
[a]Errors in the range of ± 5-10 % of the reported value, from three determinations. [b]Human (cloned) isozymes. [c]Catalytic domain of human (cloned) isozymes.

ii) Click tailing to generate radiopharmaceuticals targeting CA IX.

Siemens Medical Solutions USA, Inc. has described the synthesis of CAIs using click chemistry for radiopharmaceutical applications [49]. Their intention was to target CA IX expression *in vivo* as a diagnostic tool for hypoxic tumor imaging using PET. Similarly to the click-tailing described above, scaffolds comprising a primary sulfonamide (needed for CA inhibition) and an alkyne (needed to participate in click chemistry) were utilized, Figure 8. The three scaffolds **1**, **24** and **25**, were reacted in parallel using CuAAC with a large panel of azides (R-N_3) to generate three combinatorial libraries of CAIs. Following the 'click tailing' the tail moieties were further elaborated synthetically. The 'cold' [^{19}F] containing 1,2,3-triazole CAIs were evaluated for CA II and CA IX binding at a test compound concentration of 1 μM using fluorescence based assay with the napthalene sulfonamide dansylamide as a competitive CA binder. Selected compounds were followed up to determine dissociation constants (K_ds). A number of compounds with K_d values in the low nM range at both CA IX and II were presented and these were typically 10-fold more potent binders at CA II than at CA IX. Further to

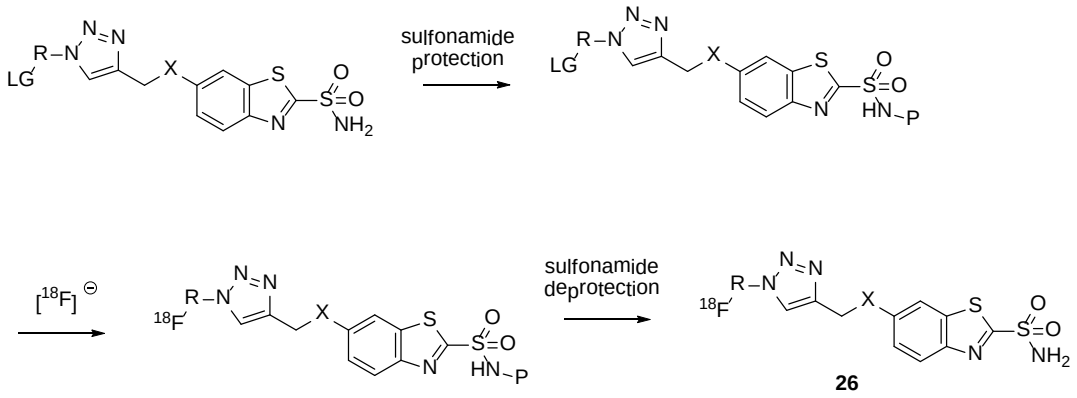
CA binding, a red blood cell membrane permeability assay was conducted for selected inhibitors. The significance of this assay is that CAIs that lack red blood cell permeability are less likely to have off-target CA II inhibition side effects (red blood cells contain high levels of CA II). The majority of compounds presented were cell permeable to an extent similar to the standard CA inhibitor ethoxzolamide, used as a control. There were however several compounds with limited permeability, the structures of these membrane impermeant compounds were not revealed in this patent application. It has been shown that due to the electrophilic character of the benzothizole ring of scaffold **25**, the SO_2NH_2 moiety can be displaced by nucleophiles encountered *in vivo* such as the thiol group of cysteine or glutathione [50]. This poor *in vivo* stability renders the utility of compounds derived from scaffold **25** as questionable.

Figure 8. CA inhibitor scaffolds **1**, **24** and **25** with alkyne functional groups for click chemistry.



The typical synthesis of a radiolabelled analogue (for example **26**) for PET applications is shown in Scheme 3. The primary sulfonamide group was first protected, followed by the nucleophilic displacement of a leaving group (eg. tosylate) elsewhere in the CA inhibitor molecule with a radionuclide such as [^{18}F] fluoride anion. The final step was the deprotection of the sulfonamide moiety to give the target PET ligand.

Scheme 3. Example synthesis of a triazole CA inhibitor **26** as a PET ligand for imaging CA IX expressing tumors. P = protecting group, LG = leaving group.

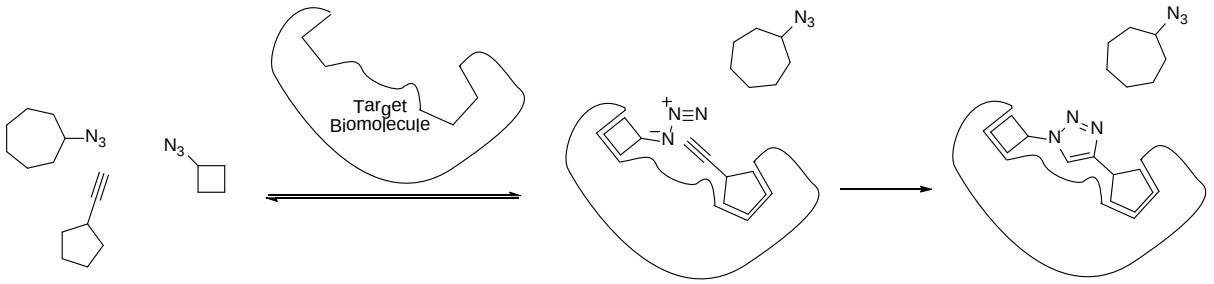


iii) *In situ* click chemistry synthesis of CA inhibitors.

In situ medicinal chemistry is a novel means to combine the synthesis and screening of small molecule ligands for medically relevant biomolecules into a single process. The approach is readily conceptualized by the familiar 'lock and key' icon wherein the biomolecular target acts as the 'lock' and templates the correct assembly of fragments leading to synthesis of a complementary small molecule 'key'. The formation of covalent bonds between fragments occurs within the targets binding site and in principle *in situ* medicinal chemistry represents

an extension of fragment-based drug discovery towards target-guided fragment optimization [51]. The pioneering application of *in situ* click chemistry was reported by Sharpless, Finn and co-workers in 2002 [52]. Azides and acetylenes are inert to the highly functionalized biological milieus (i.e. they are bioorthogonal functional groups) and when combined in the presence of a target biomolecule under native state conditions the templated synthesis of high affinity compounds comprising a 1,2,3-triazole moiety is possible. Of significance with this methodology is that the target active site templates the synthesis of high affinity triazole products without Cu(I) (or thermal) catalysis under native state conditions, Figure 9.

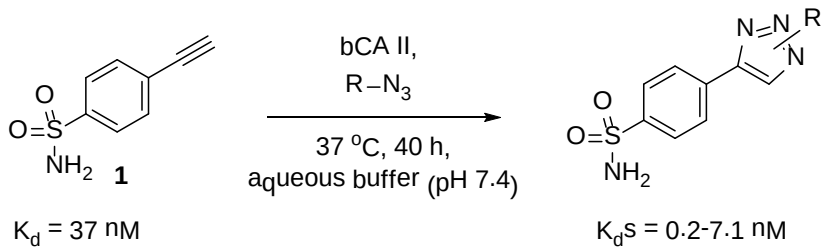
Figure 9. Schematic representation of target-templated *in situ* click chemistry.



When targeting CA, the alkyne fragment 4-ethynyl benzenesulfonamide (**1**) was combined with an azide 'tail' fragment (24 examples in parallel) and incubated with bCA II (b = bovine) at 37 ºC for 40 h prior to analysis by HPLC-SIM-MS using electrospray ionization [53]. *In situ* formed triazoles were detected for 12 of the 24 combinations and each triazole inhibitor was shown to have a higher binding affinity for bCA II (K_ds = 0.2-7.1 nM) than the parent **1** (K_d = 37 nM). Ample evidence was included in the study to confirm that the observed triazole formation was indeed induced by the presence of the bCA II template, and that the click reaction occurs exclusively within the bCA II active site. The *in situ* synthesis of a triazole product virtually guarantees that the compound will be a potent lead for drug discovery [54]. The experiment reports that the level of *in situ* triazole formation is very small, certainly << 2%, likely << 1%, but necessarily greater than any background reaction. The ability to detect the small quantities of formed triazole inhibitors is limiting and false negatives are a potential problem, with several traizoles shown also to have low nM K_d values yet not detected *in situ*.

More recently the *in situ* click chemistry targeting bCA II was miniaturized by use of a microfluidic chemical reaction circuit in which the consumption of enzyme, azide and alkyne fragments alike was significantly reduced [55]. This approach is automated and significant for drug discovery applications is that the 32 *in situ* reactions were prepared in ~30 minutes (57 s/reaction cycle), this is a remarkably short operation time. Substantially less protein (3.8 μL of 5 mg/mL protein compared to 94 μL of 1 mg/mL protein for microtitre plate used in earlier work) is used. The speed and smaller sample requirements have improved the compatibility of this approach with drug discovery, the interested reader is directed to the original research papers for specific protocol details [55].

Scheme 4. *In situ* click chemistry for the development of CA inhibitors.



R-N_3: 24 examples ⟶ 12 *in situ* hits with K_ds < **1**

Conclusion

Click tailing combines the 'tail' approach for CA inhibitor development with click chemistry for the straightforward covalent linking of selected fragments onto a known CA pharmacophore scaffold. The click tailing examples presented have demonstrated both the power and versatility of this approach for generating CAIs with useful biopharmaceutical properties and CA enzyme inhibition activity.

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References

[1] Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov. 2008;7(2):168-81.

[2] Krishnamurthy VM, Kaufman GK, Urbach AR, Gitlin I, Gudiksen KL, Weibel DB, et al. Carbonic Anhydrase as a Model for Biophysical and Physical-Organic Studies of Proteins and Protein-Ligand Binding. Chem Rev. 2008;108(3):946-1051.

[3] Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, et al. Hypoxia-inducible Expression of Tumor-associated Carbonic Anhydrases. Cancer Res. 2000;60(24):7075-83.

[4] Kaluz S, Kaluzová M, Liao S-Y, Lerman M, Stanbridge EJ. Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: A one transcription factor (HIF-1) show? Biochimica et Biophysica Acta - Reviews on Cancer. 2009;1795(2):162-72.

[5] Scozzafava A, Menabuoni L, Mincione F, Briganti F, Mincione G, Supuran CT. Carbonic Anhydrase Inhibitors. Synthesis of Water-Soluble, Topically Effective, Intraocular Pressure-Lowering Aromatic/Heterocyclic Sulfonamides Containing Cationic or Anionic Moieties: Is the Tail More Important than the Ring? J Med Chem. 1999;42(14):2641-50.

[6] Supuran C, Casini A, Scozzafava A. Development of sulfonamide carbonic anhydrase inhibitors. In: Supuran CT, Scozzafava A, Conway J, eds. *Carbonic anhydrase: Its inhibitors and activators*. Florida: CRC Press LLC 2004.

[7] Winum J-Y, Poulsen S-A, Supuran C. Therapeutic applications of glycosidic carbonic anhydrase inhibitors. Med Res Rev. 2009;29:419-35.

[8] Huisgen R, Szeimies G, Moebius L. 1,3-Dipolar cycloadditions XXXII. Kinetics of the addition of organic azides to carbon-carbon multiple bonds. Chem Ber. 1967 1967;100:2494-507.

[9] Tornoe CW, Christensen C, Meldal M. Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. J Org Chem. 2002 04/02/;67(9):3057-64.

[10] Rostovtsev V, Green LG, Fokin V, Sharpless K. A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective “ligation” of azides and terminal alkynes. Angew Chem Int Ed. 2002;41:2596-9.

[11] Kolb HC, Finn M, Sharpless KB. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. Angew Chem Int Ed. 2001;40(11):2004-21.

[12] Moses JE, Moorhouse AD. The growing applications of click chemistry. Chem Soc Rev. 2007;36(8):1249-62.

[13] Tron GC, Pirali T, Billington RA, Canonico PL, Sorba G, Genazzani AA. Click Chemistry Reactions in Medicinal Chemistry: Applications of the 1,3-dipolar Cycloaddition Between Azides and Alkynes. Med Res Rev. 2008;28(2):278-308.

[14] Zhang L, Chen X, Xue P, Sun HH, Williams ID, Sharpless KB, et al. Ruthenium-Catalyzed Cycloaddition of Alkynes and Organic Azides. J Am Chem Soc. 2005 10/28/;127(46):15998-9.

[15] Majireck MM, Weinreb SM. A Study of the Scope and Regioselectivity of the Ruthenium-Catalyzed [3 + 2]-Cycloaddition of Azides with Internal Alkynes. J Org Chem. 2006;71(22):8680-3.

[16] Patani GA, LaVoie EJ. Bioisosterism: A Rational Approach in Drug Design. Chem Rev. 1996;96(8):3147-76.

[17] Tron G, Pirali T, Billington R, Canonico P, Sorba G, Genazzani A. Click chemistry reactions in medicinal chemistry: Applications of the 1,3-dipolar cycloaddition between azides and alkynes. Med Res Rev. 2008;28(2):278-308.

[18] Kolb HC, Sharpless KB. The growing impact of click chemistry on drug discovery. Drug Disc Today. 2003;8(24):1128-37.

[19] Bourne Y, Kolb H, Radic Z, Sharpless K, Taylor P, Marchot P. Freeze-frame inhibitor captures acetylcholinesterase in a unique conformation. PNAS. 2004;101:1449-54.

[20] Chiche J, Ilc K, Laferriere J, Trottier E, Dayan F, Mazure NM, et al. Hypoxia-Inducible Carbonic Anhydrase IX and XII Promote Tumor Cell Growth by Counteracting Acidosis through the Regulation of the Intracellular pH. Cancer Res. 2009;69(1):358-68.

[21] Swietach P, Vaughan-Jones R, Harris A. Regulation of tumor pH and the role of carbonic anhydrase 9. Cancer Metastasis Rev. 2007;26(2):299-310.

[22] Martinez-Zaguilan R, Seftor E, Seftor R, Chu Y, Gillies R, Hendrix M. Acidic pH enhances the invasive behavior of human melanoma cells. Clin Exp Metastasis. 1996;14:176–86.

[23] Brahimi-Horn MC, Pouyssegur J. HIF at a glance. J Cell Sci. 2009;122(8):1055-7.

[24] Potter CPS, Harris AL. Diagnostic, prognostic and therapeutic implications of carbonic anhydrases in cancer. Br J Cancer. 2003;89(1):2-7.

[25] Thiry A, Dogné J-M, Masereel B, Supuran CT. Targeting tumor-associated carbonic anhydrase IX in cancer therapy. Trends Pharmacol Sci. 2006;27(11):566-73.

[26] Wilkinson BL, Bornaghi LF, Houston TA, Innocenti A, Supuran CT, Poulsen S-A. A Novel Class of Carbonic Anhydrase Inhibitors: Glycoconjugate Benzene Sulfonamides Prepared by "Click-Tailing". J Med Chem. 2006;49(22):6539-48.

[27] Wilkinson BL, Bornaghi LF, Houston TA, Innocenti A, Vullo D, Supuran CT, et al. Carbonic Anhydrase Inhibitors: Inhibition of Isozymes I, II, and IX with Triazole-Linked O-Glycosides of Benzene Sulfonamides. J Med Chem. 2007;50(7):1651-7.

[28] Wilkinson BL, Bornaghi LF, Houston TA, Innocenti A, Vullo D, Supuran CT, et al. Inhibition of membrane-associated carbonic anhydrase isozymes IX, XII and XIV with a library of glycoconjugate benzenesulfonamides. Bioorg Med Chem Lett. 2007;17(4):987-92.

[29] Wilkinson BL, Innocenti A, Vullo D, Supuran CT, Poulsen S-A. Inhibition of Carbonic Anhydrases with Glycosyltriazole Benzene Sulfonamides. J Med Chem. 2008 02/29/;51(6):1945-53.

[30] Lopez M, Drillaud N, Bornaghi LF, Poulsen S-A. Synthesis of S-Glycosyl Primary Sulfonamides. J Org Chem. 2009;74(7):2811-6.

[31] Lopez M, Paul B, Hofmann A, Morizzi J, Wu QK, Charman SA, et al. S-Glycosyl Primary Sulfonamides - A New Structural Class for Selective Inhibition of Cancer-Associated Carbonic Anhydrases. J Med Chem. 2009;52(20):6421-32.

[32] Singer M, Lopez M, Bornaghi LF, Innocenti A, Vullo D, Supuran CT, et al. Inhibition of carbonic anhydrase isozymes with benzene sulfonamides incorporating thio, sulfinyl and sulfonyl glycoside moieties. Bioorg Med Chem Lett. 2009;19(8):2273-6.

[33] Lopez M, Bornaghi LF, Innocenti A, Vullo D, Charman SA, Supuran CT, et al. Sulfonamide Linked Neoglycoconjugates - A New Class of Inhibitors for Cancer-Associated Carbonic Anhydrases. J Med Chem. 2010;53(7):2913-26.

[34] Kerns EH, Di L. Permeability Methods. In: Kerns EH, Di L, eds. *Drug-like Properties: Concepts, Structure Design and Methods: from ADME to Toxicity Optimization*. London: Academic Press 2008:287-98.

[35] Vullo D, Franchi M, Gallori E, Pastorek J, Scozzafava A, Pastorekova S, et al. Carbonic anhydrase inhibitors: inhibition of the tumor-associated isozyme IX with aromatic and heterocyclic sulfonamides. Bioorg Med Chem Lett. 2003;13(6):1005-9.

[36] Vullo D, Innocenti A, Nishimori I, Pastorek J, Scozzafava A, Pastoreková S, et al. Carbonic anhydrase inhibitors. Inhibition of the transmembrane isozyme XII with sulfonamides--a new target for the design of antitumor and antiglaucoma drugs? Bioorg Med Chem Lett. 2005;15(4):963-9.

[37] Wilkinson BL, Bornaghi LF, Poulsen S-A, Houston TA. Synthetic utility of glycosyl triazoles in carbohydrate chemistry. Tetrahedron. 2006;62(34):8115-25.

[38] Kerns EH, Di L. Lipophilicity Methods. In: Kerns EH, Di L, eds. *Drug-like Properties: Concepts, Structure Design and Methods:*
from ADME to Toxicity Optimization. London: Academic Press 2008:260-4.

[39] Kansy M, Senner F, Gubernator K. Physicochemical high throughput screening: Parallel artificial membrane permeation assay in the description of passive absorption processes. J Med Chem. 1998;41:1007–10.

[40] Zhu C, Jiang L, Chen T-M, Hwang K-K. A comparative study of artificial membrane permeability assay for high throughput profiling of drug absorption potential. Eur J Med Chem. 2002;37:399–407.

[41] FDA Guidance for Industry 2000 “Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System”. 2000.

[42] Borras J, Alzuet G, Ferrer S, Supuran CT. Metal complexes of heterocyclic sulfonamides as carbonic anhydrase inhibitors. In: Supuran CT, Scozzafava A, Conway J, eds. *Carbonic Anhydrase: Its Inhibitors and Activators*. Florida: CRC Press 2004:183-208.

[43] Braun C, Towle J. N1-Silver1 Derivatives of Sulfanilamide and Some Related Compounds. J Am Chem Soc. 1941;63(12):3523-.

[44] Dyson P, Sava G. Metal-based antitumour drugs in the post genomic era. Dalton Trans. 2006:1929-33.

[45] Van Staveren D, Metzler-Nolte N. Bioorganometallic Chemistry of Ferrocene. Chem Rev. 2004;104(12):5931-85.

[46] Biot C, Glorian G, Maciejewski LA, Brocard JS. Synthesis and Antimalarial Activity in Vitro and in Vivo of a New Ferrocene−Chloroquine Analogue. J Med Chem. 1997;40:3715-8.

[47] Top S, Tang J, Vessières A, Carrez D, Provot C, Jaouen G. Ferrocenyl hydroxytamoxifen: a prototype for a new range of oestradiol receptor site-directed cytotoxics. Chem Commun. 1996:955-6.

[48] Salmon AJ, Williams ML, Innocenti A, Vullo D, Supuran CT, Poulsen S-A. Inhibition of carbonic anhydrase isozymes I, II and IX with benzenesulfonamides containing an organometallic moiety. Bioorg Med Chem Lett. 2007;17(18):5032-5.

[49] Siemens Medical Solutions USA Inc, inventor The development of molecular imaging probes for carbonic anhydrase-IX using click chemistry. WO 2008/124703, October 16, 2008.

[50] Conroy CW, Schwam H, Maren TH. The nonenzymatic displacement of the sulfamoyl group from different classes of aromatic compounds by glutathione and cysteine. Drug Metabolism and Disposition. 1984 September 1984;12(5):614-8.

[51] Poulsen S-A, Vu H. In-situ fragment-based medicinal chemistry: screening by mass spectrometry. In: Zartler ER, Shapiro MJ, eds. *Fragment-based drug discovery A practical Approach*. Chichester: John Wiley & sons, Ltd 2008:159-98.

[52] Lewis WG, Green LG, Grynszpan F, Radic Z, Carlier PR, Taylor P, et al. Click Chemistry In Situ: Acetylcholinesterase as a Reaction Vessel for the Selective Assembly of a Femtomolar Inhibitor from an Array of Building Blocks. Angew Chem Int Ed. 2002;41(6):1053-7.

[53] Mocharla VP, Colasson B, Lee LV, Röper S, Sharpless KB, Wong C-H, et al. In Situ Click Chemistry: Enzyme-Generated Inhibitors of Carbonic Anhydrase II. Angew Chem Int Ed. 2005;44(1):116-20.

[54] Sharpless KB, Manetsch R. In situ click chemistry: a powerful means for lead discovery Expert Opin Drug Discovery. 2006;1(6):525-38.

[55] Wang J, Sui G, Mocharla VP, Lin RJ, Phelps ME, Kolb HC, et al. Integrated Microfluidics for Parallel Screening of an In Situ Click Chemistry Library. Angew Chem Int Ed. 2006;45(32):5276-81.