Synthesis of glycoconjugate carbonic anhydrase inhibitors by ruthenium-catalysed azide-alkyne 1,3-dipolar cycloaddition

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[¥]Nonstandard abbreviations: CA, carbonic anhydrase; HIF, hypoxia-inducible transcription factor; pH_o, extracellular pH; pH_i, intracellular pH; SAR, structure-activity relationships; SPR, structure-property relationships; 1,3-DCR, 1,3-dipolar cycloaddition reaction; CuAAC, copper-catalysed azide-alkyne cycloaddition; RuAAC, ruthenium-catalysed azide-alkyne cycloaddition; Cp*, pentamethylcyclopentadienyl; cod, cyclooctadiene; AZA, acetazolamide.

Key words: carbonic anhydrase, click chemistry, triazole, RuAAC, carbohydrate, inhibitor

Abstract

Carbonic anhydrase IX (CA IX) is a recently validated target for the development of new cancer therapies. In this paper we describe the synthesis and CA inhibition of a novel series of carbohydrate-based 1,5-disubstituted-1,2,3-triazole benzenesulfonamides. The key step of our synthesis is the regioselective Huisgen's 1,3-dipolar cycloaddition reaction (1,3-DCR) from carbohydrate azide substrates and 4-ethynylbenzenesulfonamide using a ruthenium-catalysed azide-alkyne cycloaddition (RuAAC). Our findings identified a number of triazole inhibitors (compounds **18**, **19**, **21-23** and **26**) that block CA IX activity with inhibition constants less than 10 nM. One inhibitor (compound **17**) possessed very good selectivity for CA IX over off-target CAs. These CA inhibitors have developmental potential to selectively target cancer cells, leading to cell death.

Carbonic anhydrase enzymes (CA, EC 4.2.1.1) catalyse the reversible hydration of carbon dioxide (CO_2) to generate a bicarbonate anion (HCO_3^-) and a proton (H^+).^{1, 2} Recent advances in our knowledge of the physiological impact of this simple reaction have validated CA IX as a target for cancer therapy intervention. CA IX expression is upregulated in a wide variety of hypoxic tumors, while expression is typically absent in healthy cells (with the exception of cells in the stomach and gastrointestinal tract).³ CA IX underpins a robust pH-regulating system that allows tumor cells growing in a hypoxic and acidic microenvironment to survive and proliferate.⁵ The topology of CA IX comprises an extracellular catalytic domain, this differs to the ubiquitously expressed, off-target CA II which is a soluble, cytosolic enzyme.² CA IX inhibitor development requires balancing potency, selectivity and physicochemical properties that are needed to be useful *in vivo*, allowing the inhibitor to find and interact with CA IX to elicit the desired pharmacological response. The tumor-selective expression of CA IX together with the extracellular localisation of its active site enhances the druggability prospects for CA IX as a molecular target for small molecule inhibitors. The role of medicinal chemistry in the CA field is to develop small molecule inhibitors within this framework. The CA active site comprises a tetrahedral Zn^{2+} cation that plays both a structural and a catalytic role and is the implied target for drug design. Almost all reported small molecule CA inhibitors comprise a zinc binding group (ZBG) and a 'tail' group.² The primary sulfonamide moiety ($-\text{SO}_2\text{NH}_2$) is the foremost example of a ZBG for CA inhibitors. The 'tail' approach for CA inhibitor development was first described by Supuran and co-workers.⁶ Appending tail groups to an ArSO_2NH_2 ZBG has proven effective in enhancing physicochemical properties such as lipophilicity, solubility, permeability, stability and cytotoxicity for a large number of CA inhibitors.^{7, 8, 9} CA inhibitors that incorporate a hydrophilic moiety such as a cationic pyridinium^{10, 11} and 'Cp*Ru(II)' salts,¹² metal-chelating agents,¹³⁻¹⁵ or carbohydrate moieties¹⁶⁻²¹ have been developed. These compounds have limited membrane permeability and so may better target CA IX over CA II. The attachment of the tail moiety to the $\text{Ar-SO}_2\text{NH}_2$ CA anchor has been achieved using straightforward chemistry to form covalent links such as esters, amides, imines, ureas and thioureas.

Our research group has demonstrated the versatility of the 1,3-dipolar cycloaddition reaction (1,3-DCR) of organic azides (R-N_3) with alkynes ($\text{R}'\text{-C}\equiv\text{CH}$), *i.e.* the Huisgen reaction,²² to form 1,2,3-triazole glycoconjugate CA inhibitors.⁹ Both

Meldal²³ and Sharpless²⁴ discovered that catalytic Cu(I) leads to a dramatic rate enhancement (up to 10⁷-fold) and synthesis of the 1,4-disubstituted-1,2,3-triazole product. The reaction is now referred to as the copper-catalysed azide-alkyne cycloaddition (CuAAC) and has rapidly become the premier click chemistry reaction.^{25, 26} The regioselective synthesis of the other possible product from (1,3-DCR), the 1,5-disubstituted-1,2,3-triazole, has been achieved more recently by catalysis with ruthenium complexes such as [Cp*RuCl(PPh₃)₂] under relatively mild conditions.²⁷ The 1,2,3-triazole mimics a peptide bond but is chemically robust *in vivo*, hence the adoption of this moiety into medicinal chemistry research programs.^{26, 28} The triazole moiety can 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.²⁶ While applications of CuAAC in medicinal chemistry abound,^{25, 28-30} to date there remains relatively few applications of RuAAC.³¹⁻³⁹

Our group has previously developed membrane impermeable carbohydrate-based CA inhibitors from azido sugars **2-6** (Figure 1a) and *p*-ethynylbenzenesulfonamide **1** using CuAAC to generate 1,4-disubstituted-1,2,3-triazole benzenesulfonamides **7-16**, Figure 2b.¹⁹ In another study we reacted organometallic ferrocenyl and ruthenocenyl alkynes with *p*-azidobenzenesulfonamide using both CuAAC and RuAAC to generate 1,4- and 1,5-disubstituted 1,2,3-triazole benzenesulfonamide compounds, respectively, to the best of our knowledge the first medicinal chemistry application utilising the regioselective RuAAC, Figure 2c.³⁹ We found that the differing orientation of the metallocene moiety relative to the benzenesulfonamide moiety impacted on both potency and CA isozyme selectivity. In these compounds the 1,4-disubstituted triazole regioisomer had better CA IX selectivity than the 1,5-disubstituted triazole regioisomer.

Figure 1. *intended for color reproduction on the Web and in print*

a)

b)

c)

In the present study our intention is to build upon SAR generated from carbohydrate-based triazole CA inhibitors prepared with CuAAC¹⁹ by applying the ‘reverse regioselectivity’ RuAAC reaction conditions to generate a corresponding library of 1-(β -D-glycosyl)-5-benzenesulfonamide-1,2,3-triazole compounds **17-26**, Figure 2. To commence, a panel of per-*O*-acetyl- β -D-glycopyranosyl azides (**2-6**, Figure 1a) were first synthesised from the corresponding α -glycosylbromide or α -glycosylchloride and NaN_3 by phase transfer nucleophilic displacement of the halide. The per-*O*-acetyl- β -D-glycopyranosyl azide library included derivatives of D-glucose (**2**), D-galactose (**3**), D-glucuronic acid methyl ester (**4**), D-arabinose (**5**) and D-maltose (**6**). Optimisation of the RuAAC reaction conditions was performed by studying the synthesis of the 1,5-disubstituted-1,2,3-triazole **17** from azide **2** and *p*-ethynylbenzenesulfonamide **1**. Reaction conditions were adapted from those reported by Zhang and co-workers using $[\text{Cp}^*\text{RuCl}(\text{PPh}_3)_2]$ as catalyst.²⁷ We observed that an increase in catalyst loading and/or reaction time results in an improved reaction yield (Table 1, entries 1-3), however yields are low. Decomposition of the catalyst to a tetraazadiene may in part account for the low yields.⁴⁰ In addition triphenylphosphine and triphenylphosphine oxide (formed from oxidation of triphenylphosphine ligands) proved difficult to separate from the target

triazole compound during purification. Boren and co-workers reported a study of $[\text{Cp}^*\text{RuCl}(\text{PPh}_3)_2]$ and $[\text{Cp}^*\text{RuCl}(\text{cod})]$ catalysts in the RuAAC synthesis of 1,5-disubstituted-1,2,3-triazoles.⁴⁰ These catalysts share the same mechanism, however the cod (cyclooctadiene) ligand of $[\text{Cp}^*\text{RuCl}(\text{cod})]$ is more labile than the triphenylphosphine ligand of $[\text{Cp}^*\text{RuCl}(\text{PPh}_3)_2]$, and this resulted in higher reactivity and reaction yields.⁴⁰ We explored the use of $[\text{Cp}^*\text{RuCl}(\text{cod})]$ to synthesise **17** as an alternate ‘ Cp^*RuCl ’ catalyst. Our findings were consistent with those reported, a comparison of Table 1, entries 2 and 4 shows the yield improves from 25% to 42% when $[\text{Cp}^*\text{RuCl}(\text{cod})]$ is used as RuAAC catalyst in place of $[\text{Cp}^*\text{RuCl}(\text{PPh}_3)_2]$. The cod ligand impurity was separated from the target triazole product in a straightforward manner, in contrast to the difficult removal of triphenylphosphine and triphenylphosphine oxide by-products when $[\text{Cp}^*\text{RuCl}(\text{PPh}_3)_2]$ was used as catalyst.

Figure 2.

Table 1.

Entry	Catalyst ^a	Catalyst loading (mol %)	Reaction time (h)	Yield (%)
1	$[\text{Cp}^*\text{RuCl}(\text{PPh}_3)_2]$	5	4.5	20
2	$[\text{Cp}^*\text{RuCl}(\text{PPh}_3)_2]$	5	18	25
3	$[\text{Cp}^*\text{RuCl}(\text{PPh}_3)_2]$	20	18	34
4	$[\text{Cp}^*\text{RuCl}(\text{cod})]$	5	18	42

^aReagents and Conditions: azide **2** (0.2 M), alkyne **6** (1 equiv.), 'Cp*RuCl' catalyst, toluene, N₂, 100 °C.

The regioselective synthesis of 1,5-disubstituted-1,2,3-triazoles **17-21** was achieved by RuAAC between azides **2-6** and *p*-ethynylbenzenesulfonamide **1** using [Cp*RuCl(cod)], Scheme 1. Compounds **17-21** were prepared in moderate yield (21-63 %). Deacetylation of the sugar moieties of glycoconjugate compounds was conducted using the standard Zemplén conditions of 0.05 M sodium methoxide in methanol,⁴¹ to afford compounds **22, 23, 25** and **26** in good yields (65-99 %).⁴¹ The characteristic singlet resonance in the ¹H NMR spectrum attributable to the H-5 proton of the triazole moiety is evidence for the formation of the desired regioselectivity. The H-4 proton of the 1,4-disubstituted triazoles resonates at δ 9.87 and 8.68 ppm,¹⁹ while the H-5 proton of **17-26** resonates at δ 8.12 and 8.04 ppm, around 1 ppm upfield of the 1,4-disubstituted regioisomer. ¹³C NMR spectroscopy revealed the resonance expected for the triazole CH carbon. For the 1,4-disubstituted triazoles this carbon resonates at δ 122.7 – 122.4,¹⁹ while for the 1,5-disubstituted triazole this carbon resonates at δ 134.9 – 133.5. These chemical shifts are fully consistent with those reported previously for 1,4- and 1,5-disubstituted 1,2,3-triazoles.^{42, 43} Deprotection of the glucuronic acid methyl ester analogue **19** to give **24** resulted in saponification of the methyl ester, this was an oily residue that could not be purified to the purity level desired for use in downstream enzyme inhibition studies.

Scheme 1. *intended for color reproduction on the Web and in print*

^aReagents and Conditions: (i) azide **2-6** (0.2 M), alkyne **6** (1.0 – 1.5 equiv.), 5 mol% [Cp*RuCl(cod)], toluene, N₂, 100 °C, 18 h, (21 – 63 %); (ii) NaOMe in MeOH (0.05 M, pH 12), anhydrous MeOH, 0 °C–rt, 30 min-16 h, (65 – 99 %).

The enzyme inhibition data for **1**, 1,4-disubstituted-1,2,3-triazole benzenesulfonamide

glycoconjugates **7-11** and their deprotected analogues **12-16**,¹⁹ and the 1,5-disubstituted-1,2,3-triazole benzenesulfonamide glycoconjugates of this study **17-26** is presented in Table 2. Data is acquired at the off-target CA I and II, and tumor-associated CA IX. Data for acetazolamide (**AZA**), a clinically used CA inhibitor, is also included.

Table 2. *intended for color reproduction on the Web and in print*

Compound	K_{iS} (nM) ^{a,b}			K_i Selectivity Ratios ^c	
	CA I	CA II	CA IX	CA I/ CA IX	CA II/ CA IX
AZA	250	12	25	0.48	0.48
1	1080	5.1	8.1	133.3	0.63
7	4400	9.1	120	36.7	0.08
8	4300	8.7	110	39.1	0.08
9	48	13	9.9	4.84	1.3
10	10.5	8.2	134	0.09	0.06
11	2500	235	120	20.8	1.96
12	9.4	380	89	0.11	4.3
13	9.3	8.8	120	0.08	0.07
14	560	13	8.4	50.8	1.6
15	2.5	2.3	28	0.09	0.08
16	5000	432	130	38.5	3.32
17	1360	163	32.6	42.7	5
18	71	2.4	7.3	9.7	0.33
19	3.5	3.1	7.7	0.45	0.40

20	4390	2.9	68.3	64	0.04
21	6.7	6.3	7.4	0.91	0.85
22	74	7.5	7.0	11	1.1
23	79	13.9	8.4	9.4	1.7
24	n/a	n/a	n/a	n/a	n/a
25	83	8.9	48.3	1.7	0.18
26	4.9	4.6	8.7	0.56	0.53

^aErrors in the range of $\pm 5\%$ of the reported value, from three determinations. ^cObtained using a stopped flow assay that monitors the physiological reaction (CA catalysed hydration of CO₂). ^eThe K_i ratios are indicative of isozyme selectivity for CA IX.

The parent alkyne **1**, lacking a carbohydrate tail, displays no selectivity for CA IX (K_i 8.1 nM) with respect to CA II (K_i 5.1 nM) but is 133-fold stronger inhibitor of CA IX than CA I (K_i 1080 nM). At CA I the 1,4-disubstituted triazoles displayed a range of inhibition constants (K_i s 2.5 – 5000 nM), similarly the 1,5-disubstituted triazoles inhibited CA I over a large range (K_i s 3.5 – 4390 nM). Most of the 1,5-disubstituted triazoles were significantly more potent than the parent alkyne **1** (K_i = 1080 nM) with K_i s 3.5 – 83 nM, there are only two exceptions, the per-*O*-acetyl arabinosyl (**20**: K_i 4390 nM) and glucosyl (**17**: K_i 1360 nM) derivatives. At CA II, most triazoles were potent inhibitors, many with K_i s below 10 nM, and selectivity for CA II over CA I, similarly to the parent alkyne **1** (K_i 5.1 nM, 211-fold selective for CA II over CA I). The most noteworthy selectivity compared to CA I was with the 1,5-disubstituted per-*O*-acetyl arabinosyl triazole derivative **20** (CA II K_i = 2.9 nM) which was 1514-fold selective for CA II compared to CA I. This differs to the corresponding 1,4-disubstituted per-*O*-acetyl arabinosyl triazole derivative **10** which had no selectivity (CA I K_i = 10.5 nM and CA II K_i = 8.2 nM). At CA IX triazoles displayed a range of inhibition constants (K_i s 7.0 – 134 nM) compared to the parent alkyne **1** (K_i 8.1 nM) and with activity similar to CA II. The 1,5-disubstituted triazoles had good CA IX inhibition (K_i s 7.0 – 68.3 nM), nearly all were better CA IX inhibitors than their 1,4-disubstituted counterparts, for example the 1,4- per-*O*-acetyl galactosyl derivative **8** (K_i 110 nM) is 15-fold less active than the corresponding 1,5- galactose derivative **18** (K_i 7.3 nM). The per-*O*-acetyl glucosyl analogue **17** (CA IX K_i = 32.6 nM) was the best triazole (1,4- or 1,5-disubstituted) with regard to selectivity for CA IX over off-target CA I and II, it was 42-fold and 5-fold selective, respectively. This selectivity is

opposite to that observed with the hydrophobic metallocene tail moieties.³⁹ In these compounds the 1,4-disubstituted triazole regioisomer had better CA IX selectivity than the 1,5-disubstituted triazole regioisomer.³⁹ As the CA active site encompasses two distinct regions to which the CA inhibitor tail can potentially interact, one lined by hydrophobic amino acids (consisting of Phe₁₃₀, Val₁₃₄, Leu₂₀₃ and Pro₂₀₁) and the other located directly opposite and lined by hydrophilic amino acids (primarily Asn₆₂, Asn₆₇ and Gln₉₂),² it is logical that hydrophobic versus hydrophilic tail groups may lead to differential isozyme selectivity.

In summary the new compounds of this study comprise a sugar moiety tethered to an aromatic sulfonamide CA pharmacophore through an intervening triazole ring. Compounds **22**, **23**, **25** and **26** each contain a free sugar moiety and so provide a selection of likely membrane impermeable small molecule CA inhibitors with selectivity for the extracellular active site CA IX over other (intracellular) CA isozymes. The key step of our synthesis is the regioselective Huisgen's 1,3-DCR using RuAAC with [Cp*RuCl(cod)] as catalyst. Our findings identified a number of 1,5-disubstituted triazole inhibitors (compounds **18**, **19**, **21-23** and **26**) that block CA IX with inhibition constants less than 10 nM. One inhibitor (compound **17**) possessed very good selectivity for CA IX over off-target CAs. Our results have demonstrated that subtle structural differences in the sugar tail can discriminate the CA isozyme active site topology to influence enzyme inhibition characteristics. A comparison of the CA IX inhibition of the new 1,5-disubstituted triazoles (K_i s 7.0 – 68.3 nM) with their 1,4-disubstituted counterparts showed that nearly all were better CA IX inhibitors, a significant step towards further building SAR with carbohydrate-based CA inhibitors. The enzyme inhibition attributes of these compounds are an important result for potential future biological studies or therapeutic applications of carbohydrate-based primary sulfonamide compounds.

Supplementary Data. Supplementary data comprises synthetic procedures; compound characterisation data and the CA assay procedure for new compounds **17-26**.

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Figure/Scheme/Table Captions

Figure 1. a) Glycosyl azide building blocks **2-6**. b) Glycoconjugate 1,4-disubstituted-1,2,3-triazole benzenesulfonamides **7-16** synthesised by CuAAC. c) Organometallic 1,4- and 1,5-disubstituted 1,2,3-triazoles benzenesulfonamide compounds synthesised by CuAAC and RuAAC, respectively. (intended for color reproduction on the Web and in print)

Figure 2. Target carbohydrate-based 1,5-disubstituted-1,2,3-triazole benzenesulfonamides.

Table 1. Reaction optimisation for RuAAC from azide **2** and alkyne **6** to give 1,5-disubstituted-1,2,3-triazole **17**.

Scheme 1. Synthesis of target 1,5-disubstituted-1,2,3-triazoles **17-26**. (intended for color reproduction on the Web and in print)

Table 2. Inhibition and isozyme selectivity ratio data of CA isozymes I, II, and IX with triazoles **7-26**, compound **1**, and the established drug and CA inhibitor, **AZA**. (intended for color reproduction on the Web and in print)

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