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Carbonic anhydrase inhibitors: Selective inhibition of the extracellular, tumor-associated isoforms IX and XII over isozymes I and II with glycosyl-thioureido-sulfonamides

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Abstract—A series of glycosyl-thioureido sulfonamides incorporating glucosamine, galactosamine, and mannosamine tails, and sulfanilamide, halogenosulfanilamide, and metanilamide heads was synthesized. Many of the new compounds showed micromolar–submicromolar affinity for the inhibition of the cytosolic isoforms I and II of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), but low nanomolar binding to the tumor-associated isozymes, CA IX and XII. The selectivity ratios for the inhibition of the tumor-associated over the cytosolic isozymes were in the range of 107–955 for the most selective such inhibitors.

In previous work from this laboratory[1,2] we showed that the 'sugar-approach', consisting in the attachment of glycosyl moieties as tails to the molecules of aromatic/heterocyclic sulfonamides/sulfamides/sulfamates, may lead to the development of very effective carbonic anhydrase (CA, EC 4.2.1.1) inhibitors targeting several isozymes of the 16 presently known in mammals. Furthermore, Poulsen's group[3] confirmed our results, reporting a large number of very potent CA I, II, IX, and XII inhibitors by means of click-tailing, sulfonamides which also incorporate simple as well as quite complex sugar tails. Some of these derivatives, of types **A**–**J** prepared by the two groups, may be used as pharmacological tools to better understand the physiology and inhibition of some CA isoforms, but may also constitute lead molecules for obtaining tighter-binding or isoform-selective compounds.[1–3] Indeed, the high resolution X-ray crystal structure of the adduct of the glucosyl-sulfanilamide compound **J** with human CA II—hCA II allowed the evidencing of precedently unknown interactions between the inhibitor and the enzyme active site, which might be useful to obtain even stronger enzyme inhibitors.[4] **J** also showed effective antiglaucoma activity in vivo, in an animal model of the disease.[1a] In fact, the presence of the hydrophilic sugar moieties in compound such as **A**–**J** is highly beneficial for various putative applications of such derivatives, due to their increased water solubility as compared to the classical sulfonamide/sulfamate inhibitors, such as acetazolamide **AZA**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and topiramate **TPM**.[5] Indeed, the CA inhibitors (CAIs) have clinical applications as antiglaucoma,[6] antiobesity,[7] antiepileptic,[8] and/or antitumor drugs/diagnostic agents.[9] It is obvious that different isozymes of the 16 mentioned above are preferentially targeted by various compounds with different pharmacological activity: antiglaucoma drugs primarily target CA II, IV, and probably XII,[6] antiobesity compounds the mitochondrial isoforms CA VA and CA VB,[7] the antiepileptic derivatives CA VII and XIV,[8] whereas the antitumor derivatives the transmembrane, tumor-associated isoforms CA IX and XII which are highly overexpressed in many types of hypoxic tumors.[9–12]

Keywords: Carbonic anhydrase; Sulfonamide; Aminosugar; Tumor-associated isoform; Isozyme-selective inhibitor.
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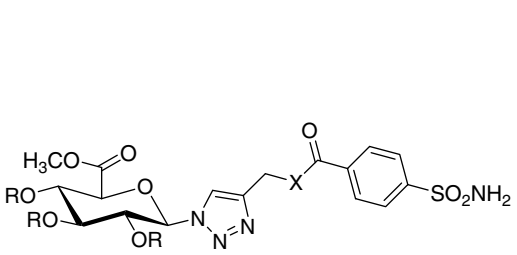
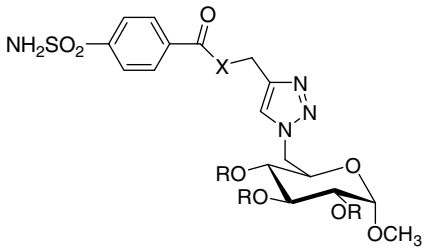
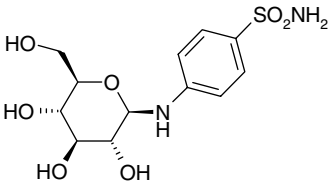


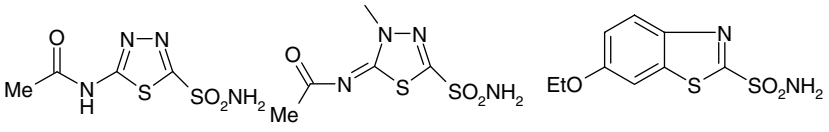
A X = NH, R = Ac, **C** X = NH, R = H
B X = O, R = Ac, **D** X = O, R = H



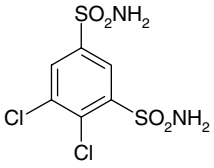
E X = NH, R = Ac, **G** X = NH, R = H
F X = O, R = Ac, **H** X = O, R = H



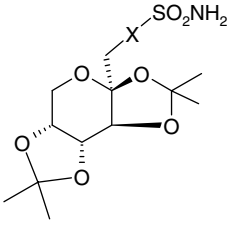
J



AZA **MZA** **EZA**



DCP



TPM: X = O
TPM_NH: X = NH

Another interesting aspect that emerged during our investigations of sugar-containing CAIs was the possibility to design compounds with reduced affinity for the ubiquitous, house-keeping isoform hCA II, which still show significant inhibitory power against other physiologically important isoforms.[1b] Thus, the topiramate sulfamide analogue **TPM_NH** is 210 times weaker hCA II inhibitor as compared to its cognate sulfamate **TPM**.[1b] This compound is a rather weak hCA I, IX, and XII inhibitor too (affinity in the micromolar range, see also Table 1) but shows nanomolar inhibitory activity against other CA isoforms, such as CA VA, VB, VII, and XIII.[1b] By resolving the high resolution X-ray crystal structure of the hCA II–TPM_NH adduct, in addition to the usual binding of all these types of inhibitors to the CA active site (Fig. 1), an important clash between the 8-methyl moiety of the inhibitor and the methyl group of Ala65 has been evidenced. Otherwise, **TPM_NH** binds in an identical manner to hCA II as **TPM**, for which this clash is absent. This was the first report showing that it is possible to obtain CAIs with diminished affinity for hCA II while still maintaining tight binding for other isoforms.[1b]

Considering the above-mentioned results of the sugar-containing sulfamide **TPM_NH**, we decided to investigate other derivatives possessing this type of scaffold in the search of inhibitors with selectivity for some medicinal chemistry targets within the CA family. Here, we report the synthesis of a series of glycosyl-thioureido sulfonamides and their inhibitory activity against four physiologically relevant isoforms, the cytosolic, ubiquitous hCA I and II, as well as the transmembrane, tumor-associated CA IX and XII (Scheme 1).

Reaction of D-glucosamine, D-galactoseamine, and D-mannoseamine **1** with isothiocyanato sulfonamides **2**[13] afforded thioureas **3**–**5** by a general procedure we have explored in detail for the preparation of various classes of CAIs (Scheme 1).[13,14] The new compounds **3**–**5** incorporated the scaffolds of sulfanilamide, 3-halogenosulfanilamides as well as metanilamide, since we showed earlier that such derivative leads generally to effective CAIs when thioureido-amino acid and/or thioureido-substituted aryl moieties are attached to them.[13]

Inhibition data with the derivatives **3**–**5** as well as standard CAIs against four isoforms, i.e., hCA I, II, IX, and

Table 1. Inhibition data of sulfonamides **3–5** reported in the present paper and standard CA inhibitors **AZA–TPM**, against isozymes I, II (cytosolic), IX, and XII (transmembrane), by a stopped-flow, CO_2 hydration assay[17]

Inhibitor	hCA I[c] (μM)	K_I[a] hCA II[c] (μM)	hCA IX[d] (μM)	hCA XII[d] (μM)
AZA	0.25	12	25	5.7
MZA	0.050	14	27	3.4
EZA	0.025	8	34	22
DCP	1.20	38	50	50
TPM	0.25	10	58	3.8
TPM_NH[b]	3.45	2130	4580	1870
3a	2.7	9700	77	7.9
3b	0.6	15,400	139	87
3c	2.8	9200	131	389
3d	0.10	8600	9.0	207
4a	3.6	7700	74	104
4b	1.3	870	13	9.6
4c	0.10	910	8.4	95
4d	4.3	940	42	14
5a	0.12	15,700	72	94
5b	0.13	18,900	57	393
5c	2.7	6800	63	9.7
5d	0.10	120	64	95

[a] Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[17]

[b] From Ref. 1b. The compound is a low nanomolar inhibitor (K_I in the range of 21–35 nM) for CA VA, CA VB, CA VII, CA XIII, and CA XIV.[1b]

[c] Human, recombinant isozymes.

[d] Catalytic domain of human, cloned isoform.[12,13]

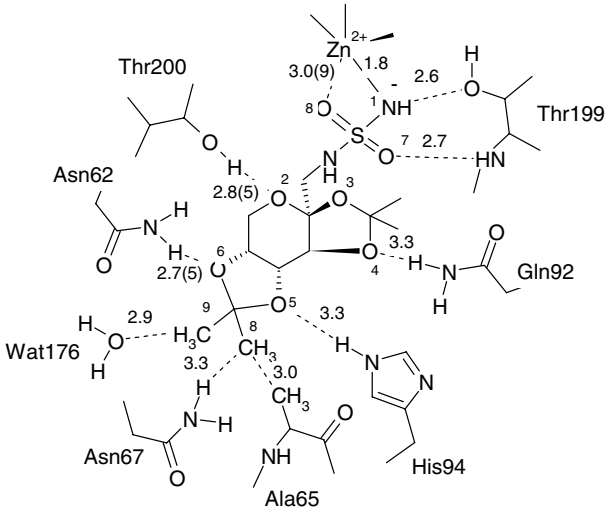
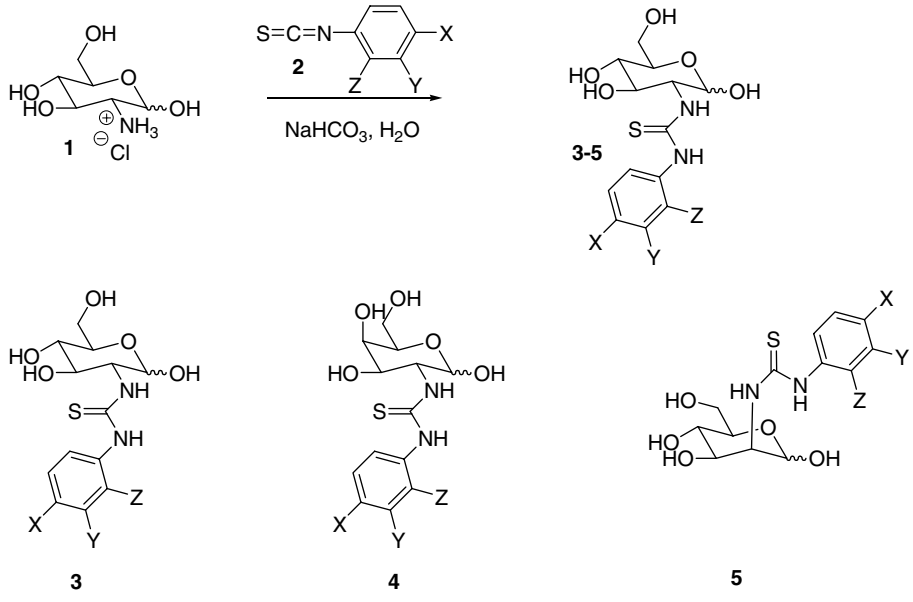


Figure 1. Schematic representation of interactions in which **TPM_NH** participates when bound to the hCA II active site (figures represent distances in Å). The clash between the C8 methyl group of the inhibitor and the methyl of Ala65 is responsible for the 210 times diminished inhibitory activity of the compound against hCA II as compared to the sulfamate analogue topiramate **TPM**. Ala65 is an amino acid residue unique to CA II.[1b]

XII, are shown in Table 1. Data of Table 1 show the following structure–activity relationship (SAR) for this series of glycosyl-thioureido sulfonamides: (i) compounds



	X	Y	Z
3a, 4a, 5a	SO_2NH_2	H	H
3b, 4b, 5b	SO_2NH_2	H	F
3c, 4c, 5c	SO_2NH_2	H	Cl
3d, 4d, 5d	H	SO_2NH_2	H

Scheme 1. Synthesis of the glycosyl-thioureido sulfonamide derivatives **3–5** described in the paper.

Table 2. Selectivity ratios for the inhibition of the tumor-associated (CA IX and XII) over the cytosolic (CA I and II) isozymes with selected CAIs

Compound	Selectivity ratio			
	hCA I/hCA IX	hCA II/hCA IX	hCA I/hCA XII	hCA II/hCA XII
AZA	10	0.48	43.8	2.10
EZA	0.73	0.23	1.13	0.36
TPM	4.31	0.17	65.7	2.63
3a	35.0	125.9	341.7	1227
3d	11.1	955.5	0.48	41.5
4b	100.0	66.9	135.4	90.6
4c	11.9	108.3	1.05	9.5
5c	42.8	107.9	278.3	701

3–**5** described here showed moderate hCA I inhibitory activity, with K_Is in the range of 0.10–4.3 μM. Usually, hCA I is an isoform with lower affinity for sulfonamide-type inhibitors as compared to hCA II,[15] as it has a more restricted active site cavity as compared to hCA II (due to the presence of the rather bulky His200 in the neighborhood of the catalytic Zn(II) ion).[16] Thus, normally, many sulfonamide/sulfamate inhibitors are inhibiting this isozyme with K_Is in the micromolar–submicromolar range, as exemplified for the clinically used derivatives **AZA**–**TPM** from Table 1 (only **EZA** is a rather potent hCA I inhibitor, with a K_I of 25 nM). The new derivatives reported here are practically in the same range as hCA I inhibitors as these clinically used compounds. It may be seen that the mannose derivatives **5** are generally better inhibitors than the corresponding glucose ones **3** which in turn are better inhibitors than the galactose derivatives **4** (however several exceptions from this rule are obvious in the data of Table 1); (ii) unexpectedly (due to reasons mentioned above regarding the active site cavity of isozymes I and II),[15,16] the hCA II inhibitory activity of these compounds was also rather weak, with inhibition constants in the range of 0.12–18.9 μM. In fact, as observed for the clinically used compounds **AZA**–**TPM**, sulfonamides and sulfamates generally show low nanomolar affinity for this isozyme.[15] Four derivatives explored here, i.e., **4b**–**4d** and **5d**, were submicromolar inhibitors (K_Is of 0.12–0.94 μM), whereas the remaining ones were much weaker inhibitors (K_Is in the range of 6.8–18.9 μM). It may be observed that generally the galactose derivatives **4** were more active than the corresponding glucose ones **3** which in turn were better hCA II inhibitors than the corresponding mannose derivatives **5** (with the notable exception of derivative **5d**). No clear-cut SAR could be drawn regarding the sulfonamide heads of these derivatives, since compounds with similar potency were obtained both for sulfanilamide/metanilamide series, as well as for the halogeno-substituted sulfanilamides. However, the best hCA II inhibitor was the thioureido-metanilamide derivative of mannose **5d**; (iii) much better inhibitory activity has been observed for the new derivatives **3**–**5** against the tumor-associated isozymes hCA IX and XII, with K_Is in the range of 8.4–139 nM against the first isoform, and 7.9–389 nM against the second one, respectively. These data clearly show that many of the new thioureido-sulfonamides reported here show a high degree of selectivity for the inhibition of the tumor-associated over the cytosolic, ubiquitous isoforms (Table 2), and this is a quite relevant result. Against hCA IX the best activity has been observed for the galactose derivatives **4** which were generally more active than the corresponding mannose ones **5** which in turn were better hCA IX inhibitors than the corresponding glucose derivative **3** (except for **3d** which was one of the best inhibitors targeting this isozyme, together with **4c**). Again the sulfonamide head seemed to be less important for obtaining effective inhibitors, since compounds possessing all the substitution patterns investigated here (sulfanilamide, metanilamide, and halogenosulfanilamide) led to inhibitors with comparable activity (Table 1). Against hCA XII the SAR data were even less sharp, since for each sugar series there was a very effective inhibitor (K_Is of 7.9–9.7 nM, i.e., **3a** for the glucose series, **4b** for the galactose one, and **5c** for the mannose derivatives) as well as a much less effective inhibitor (K_Is of 104–393 nM, i.e., **3c** for the glucose derivatives, **4a** for the galactose ones, and **5b** for the mannose ones, respectively);[17] (iv) data of Table 2 show that the presently available sulfonamides/sulfamate with clinical use do not show any selectivity for the inhibition of the tumor-associated isozyme hCA IX over the ubiquitous, cytosolic one hCA II. However, there is some degree of specificity for the inhibition of CA XII over CA I and II. However, the isoform with the greatest importance as drug target is CA IX, and the main issue is not to inhibit appreciably also CA II.[9–12] Data presented in the Table show that many of the new sulfonamides investigated here show a high degree of selectivity for the inhibition of CA IX over CA I and II, as well as the inhibition of CA XII over CA I/II. Indeed, except the clinically used compounds **AZA**, **EZA**, and **TPM** which are better CA II than CA IX inhibitors, compounds such as **3a**, **3d**, **4b**, **4c**, and **5c** among others showed 66.9–955.5 times better affinity for the inhibition of the tumor associated isoform IX over CA II (their selectivity ratio for the inhibition of C IX over CA I being also favorable). The same effect has been observed for the selective inhibition of CA XII over CA II (with these compounds being 9.5–1227 times better CA XII than CA II inhibitors and showing an acceptable selectivity profile over CA I too).

In conclusion, we report here a series of glycosyl-thioureido sulfonamides incorporating glucose, galactose, and mannose tails and sulfanilamide, halogenosulfanilamide, and metanilamide heads. Many of the new compounds showed micromolar–submicromolar affinity for the inhibition of CA I and II, but low nanomolar binding to CA IX and XII. The selectivity ratios for

the inhibition of the tumor associated over the cytosolic isozymes were in the range of 107–955 for the most selective inhibitors.

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14. To a solution of isothiocyanate **2** (1 equiv) in acetonitrile at room temperature was added the D-glycosylamine hydrochloride **1** (1 equiv) solubilized in a saturated $NaHCO_3$ aqueous solution (1 equiv). The mixture was stirred for 3 h at room temperature and then concentrated under vacuum. The residue was diluted with ether and the precipitate filtered. The residue was then washed with ethyl acetate, acetonitrile, and diethyl ether. Silicagel chromatography (eluent: methylene chloride–methanol, 9:1) yielded pure compounds **3**–**5**.
Compound **3a**: R_f (CH_2Cl_2/CH_3OH, 8:2) = 0.14; mp 89–92 °C; ^1H NMR (DMSO-d_6, 400 MHz) δ 8.6 (s, 1H), 7.7 (m, 4H); 7.3 (s, 2H), 6.9 (d, 1H, J = 8.4 Hz), 5.5 (dd, 1H, J = 2 Hz, J = 8.7 Hz), 4.9 (d, 1H, J = 6.2 Hz), 4.7 (d, 1H, J = 5.7 Hz), 4.6 (d, 1H, J = 7.5 Hz), 4.4 (m, 1H), 3.6 (m, 2H), 3–3.5 (m, 4H); MS ESI$^+$ m/z 416 $(M+H)^+$. ESI$^-$ m/z 392 $(M-H)^-$.
Compound **4a**: R_f (CH_2Cl_2/CH_3OH, 8:2) = 0.13; mp 65–70 °C;^1H NMR (DMSO-d_6, 400 MHz) δ 9.1 (s, 1H), 7.8 (m, 4H), 7.3 (s, 2H), 6.9 (d, 1H, J = 8 Hz), 5.6 (d, 1H, J = 8.4 Hz), 5.1 (m, 1H), 4.6 (d, 1H, 4.8 Hz), 4.4 (m, 2H), 4.1 (m, 1H), 3-3.6 (m, 5H); MS ESI$^+$ m/z 416 $(M+H)^+$. ESI$^-$ m/z 392 $(M-H)^-$.
Compound **5a**: R_f (CH_2Cl_2/CH_3OH, 8:2) = 0.19; mp 154–158 °C; ^1H NMR (DMSO-d_6, 400 MHz) δ 9.3 (s, 1H), 7.75 (d, 2H, J = 8.8 Hz), 7.72 (d, 2H, J = 8.8 Hz), 7.3 (s, 2H), 6 (d, 1H, J = 7.2 Hz), 5.5 (d, 1H, J = 4.4 Hz), 4.6 (m, 2H), 4.16 (m, 1H), 4.13 (d, 1H, J = 7.2 Hz), 4 (m, 1H), 3.8 (m, 1H); 3.2–3.4 (m, 4H); MS ESI$^+$ m/z 416 $(M+H)^+$. ESI$^-$ m/z 392 $(M-H)^-$.
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17. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.1 nM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier, Refs. 12 and 13, and represent means from at least three different determinations.