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Inhibition of membrane-associated carbonic anhydrase isozymes IX, XII and XIV with a library of glycoconjugate benzenesulfonamides

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Abstract—A library of glycoconjugate benzenesulfonamides that contain diverse carbohydrate-triazole tails were investigated for their ability to inhibit the enzymatic activity of the three human transmembrane carbonic anhydrase (CA) isozymes hCA IX, hCA XII and hCA XIV. These isozymes have their CA domains located extracellularly, unlike the physiologically dominant hCA II, and are of immense current interest as druggable targets. Elevated expression of isozymes IX and XII is a marker for a broad spectrum of hypoxic tumors—this physiology may facilitate a novel approach to discriminate between healthy cells and cancerous cells. Many of these glycoconjugates were potent inhibitors (low nM), but importantly exhibited different isozyme selectivity profiles. The most potent hCA IX inhibitor was the glucuronic acid derivative **20** (K_i = 23 nM). This compound was uniquely hCA IX selective cf. all other isozymes (16.4-, 16.8- and 4.6-fold selective against hCA II, XII, and XIV, respectively). At hCA XII there were many inhibitors with K_is < 10 nM that also demonstrated excellent selectivity (up to 344-fold) against other isozymes. Potent hCA XIV inhibitors were also identified, several with K_is ~ 10 nM, however no hCA XIV-selective derivatives were evidenced from this library. The sugar tails of this study have shown promise as a valuable approach to both solubilize the aromatic sulfonamide CA recognition pharmacophore and to deliver potent inhibition and isozyme differentiation of the transmembrane CAs.

The carbonic anhydrases (CA, EC 4.2.1.1) are abundant zinc metalloenzymes found in a diversity of organisms including higher vertebrates, green plants, algae, bacteria and archaea.[1] In humans 15 different CA isozymes (designated hCA) belonging to the α-CA class are presently known; they exhibit variable enzyme kinetics, tissue distribution, expression levels and subcellular locations.[1–4] Many of these isozymes are quite recent discoveries cf. the physiologically abundant and widely distributed isozymes hCA I and hCA II (known since the 1930s, with isozymes acknowledged in the 1960s) and many have shown promise as druggable targets through inhibition of their catalytic activity, which may elicit desirable, yet variable physiological responses.[1–4] The key challenge that confronts medicinal chemists in the quest to deliver CA based therapies or diagnostics has thus widened from designing potent CA inhibitors (now relatively 'simple' owing to the prolific efforts of researchers over several decades[5])—to include the broader challenge of discovering isozyme selective inhibitors—either by drug design and/or drug delivery mechanisms. The classical recognition motif for small molecules to bind the active site of CA is an aromatic sulfonamide moiety—$ArSO_2NH_2$.[1–6] The deprotonated sulfonamide moiety ($ArSO_2NH^-$) coordinates to the CA active site Zn^{2+} and so inhibits the catalytic ability of the enzyme. This CA recognition moiety exhibits remarkable reliability in anchoring the inhibitor molecule within the CA active site such that tethering groups to this CA recognition pharmacophore has made for an effective means to incorporate and also optimize desirable chemical properties of sulfonamide CA inhibitors.[5]

Transmembrane hCAs—isozymes IX, XII and XIV[7–9]—like other hCAs regulate pH and carbon

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dioxide (CO_2):bicarbonate anion (HCO_3^-) homeostasis, through catalysis of the reversible hydration of CO_2 to give HCO_3^- and a proton (H^+). An additional feature that distinguishes the transmembrane isozymes from most other CAs is that their CA catalytic domain is located in the extracellular (rather than intracellular) space. The expression level of isozymes hCA IX and XII is elevated in response to hypoxia and research on the involvement of these isozymes in cancer has progressed considerably in recent years, particularly for hCA IX.[9–13] It has been confirmed that hCA IX is a high activity CA isozyme responsible for the extracellular acidification (pH_e) of the tumor microenvironment. Multiple downstream effects of this reduced pH_e are associated with tumor progression and poor prognosis.[11,12] Aromatic sulfonamide compounds have been shown to reverse the effect of tumor acidification; to inhibit the growth of cancer cells and to suppress tumor invasion mediated by these CAs.[9–13] Thus, the data from these many physiological studies appear to have identified a CA mediated, hypoxic tumor-specific pathway. This provides firm grounds for exploring the effects of this class of compounds as a novel approach to discriminate between healthy cells and cancerous cells, specifically targeting hypoxic tissues—an attractive attribute that is lacking in many existing cancer therapies.[14,15] In addition to a potential role in cancer, it was recently determined that hCA XII is highly expressed in the eyes of glaucoma patients.[16] Current antiglaucoma drugs were thought to target hCA II and IV, but hCA XII may in part be responsible for the intraocular pressure effects of clinically used sulfonamides and further research on the role of isozyme XII in glaucoma therapies is necessary to verify.

hCA XIV is a low activity CA isozyme[17] that shares the general topology of isozymes IX and XII (i.e. an extracellular CA domain, a single span transmembrane domain, and a short cytoplasmic domain) but, unlike CA IX and XII, is not associated with tumor cells. This isozyme shows abundant expression in brain, liver, and spinal cord, with weaker expression in kidney, colon, small intestine, and urinary bladder.[8,18–21] The downstream physiological effects of hCA XIV mediated pH regulation and CO_2/HCO_3^- ion transport are still yet to be fully revealed so that the potential for therapeutic applications associated with this isozyme is not yet entirely apparent, however it is likely that a role for therapeutic intervention with this recently discovered isozyme, as for other CA isozymes, will indeed arise.

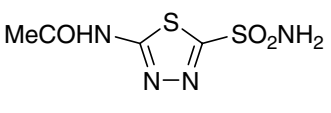
The CA inhibition data against all three of these membrane-associated CA isozymes have so far been reported for only a small number of sulfonamide derivatives, all of which are quite simple with respect to 'tail' groups.[17] The inhibition trends are variable, rather than clustered, this indicating that the selective inhibition of these isozymes may indeed be possible. It is however clearly apparent that a more exhaustive investigation of this CA-subclass with a larger number of inhibitors of varying tails will be required to deliver such an outcome.

The physiological roles for the transmembrane-associated CA isozymes in cancer and possibly other disease states provide the impetus to investigate the inhibition of these isozymes to develop compounds that can discriminate them from the physiologically dominant, cytosolic hCA I and II. The preparation of aromatic sulfonamide inhibitors with an impaired ability to diffuse through lipid membranes is one possible means by which to target these isozymes. It is noteworthy that attention from the pharmaceutical industry for carbohydrate-based drugs is not prevalent in comparison with more traditional small organic molecules. The degree of oral bioavailability of glycoconjugates is compromised (they violate Lipinksi's 'Rule of 5'[22]) by the poor ability of sugars to diffuse across cell membranes, however oral bioavailability is not always a primary consideration as there are other routes for drug administration (e.g. intravenous), drug transporting and specifically for transmembrane CAs an extracellular location of the drug target. We have recently demonstrated the versatility of the 1,3-dipolar cycloaddition reaction 'click-tailing' synthetic methodology to readily generate 1,4-disubstituted-1,2,3-triazole glycoconjugates **3**–**30** from azido sugars **a**–**g** and sulfonamide-alkyne scaffolds **1** and **2** (Scheme 1).[23,24] All compounds were synthesized as reported earlier by this group.[23] Compounds **3**–**30** (each with a sugar tail) were very effective inhibitors of the high activity transmembrane isozyme hCA IX, and importantly for some compounds selectivity for CA IX over I and II was observed.[23] Our results with hCA IX have encouraged us to further investigate the inhibition properties of these glycoconjugates at the remaining two transmembrane hCA isozymes XII and XIV, these results are reported here.

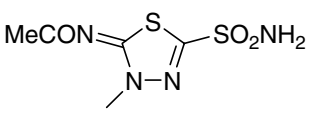
hCA II IX, XII and XIV enzyme inhibition data for parent scaffolds **1** and **2**, glycoconjugate compound library **3**–**30** and standard CA inhibitors are reported in Table 1, data for hCA XII and XIV being reported for the first time here. CA inhibition against these isozymes was determined by assaying the CA-catalyzed hydration of CO_2.[25] Inhibition data against the other physiologically dominant isozyme hCA I have been reported previously[23] and are not included in Table 1 for clarification of presentation—however for reference **1**–**30** are typically micromolar inhibitors at this isozyme such that selectivity of transmembrane isozymes against this isozyme is for most compounds 2–3 orders of magnitude (the only exceptions are **14** and **22**, with nanomolar hCA I K_is of 7.7 and 9.6 nM, respectively).

Clinically used aromatic sulfonamides that are CA inhibitors include acetazolamide (AZA), methazolamide (MZA), ethoxazolamide (EZA), brinzolamide (BRZ), dorzolamide (DRZ) and dichlorophenamide (DCP).[1–4] The assembly of CA inhibition data for these sulfonamides against hCA IX, XII and XIV was recently completed[17] and demonstrates they are quite effective inhibitors (mid-low nM) against these isozymes, generally with slightly higher inhibition at hCA XII compared to hCA IX and XIV (Table 1).

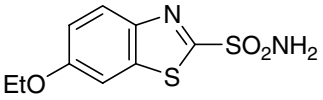
The parent scaffolds lacking the sugar tails: *N*-propynyl amide benzenesulfonamide **1** and *O*-propynyl ester ben-



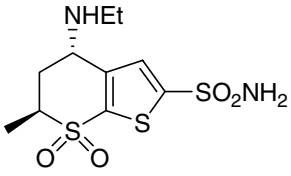
acetazolamide (AZA)



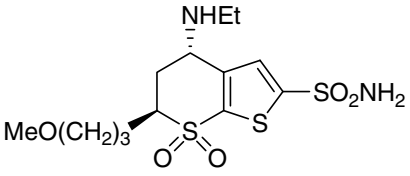
methazolamide (MZA)



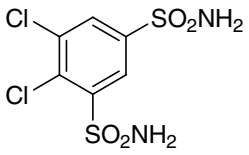
ethoxazolamide (EZA)



dorzolamide (DRZ)



brinzolamide (BRZ)



dichlorphenamide (DCP)

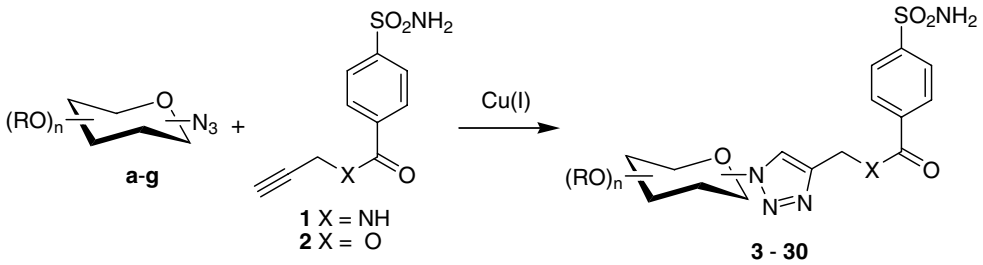
zenesulfonamide **2** exhibited greatest efficacy at hCA XII (K_is of 1.0 and 8.3 nM, respectively), approximately 10-fold weaker inhibition at hCA XIV (K_is of 11.3 and 83 nM, respectively) and an order of magnitude again weaker inhibition at hCA IX for the amide **1** (K_i of 113 nM) or just slightly weaker for ester **2** (K_i of 104 nM).

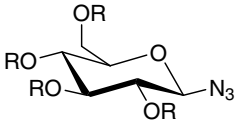
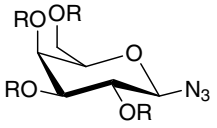
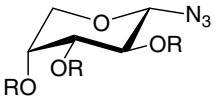
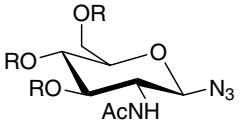
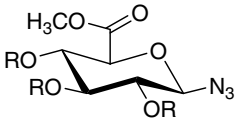
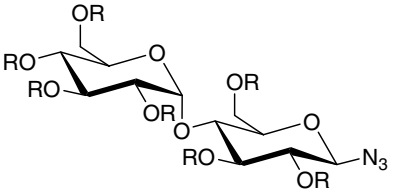
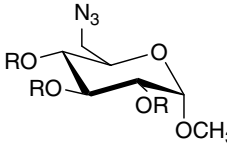
Isozyme hCA IX.[23] At hCA IX the parent compounds **1** and **2** had K_is of 113 and 104 nM, respectively, weaker inhibition than that observed at the other isozymes. With five of the sugar tails (**b**, **c**, **e**, **f** and **g**) there was at least one compound of the grouping (amide, ester, sugar-OAc or sugar-OH) that exhibited improved hCA IX inhibition over the parent scaffolds. The exceptions were the glucose tail **a** and the *N*-acetyl glucosamine tail **d** in which the sugar triazole tail always leads to reduced inhibition when compared to the non-glycoconjugate parent scaffolds. For each sugar tail (except the glucuronic acid derivatives **19**–**22**) the amide sugar-OAc derivatives were more potent than the amide sugar-OH derivatives, while in the ester series this trend was reversed, with sugar-OH derivatives more potent than sugar-OAc derivatives. The strongest hCA IX inhibitor was the amide-linked deprotected glucuronic acid derivative **20** (K_i = 23 nM). This compound was uniquely hCA IX selective cf. all other isozymes (16.4-, 16.8- and 4.6-fold selective against hCA II, XII and XIV, respectively). There were several other compounds with mild hCA IX selectivity cf. hCA II, of note is the amide-linked galactose-OAc derivative **7** with 6.2-fold selectivity (76 nM vs 470 nM).

Isozyme hCA XII. At hCA XII the parent compounds **1** and **2** had low nanomolar K_is of 1.0 and 8.3 nM, respectively. These parent compounds were selective over hCA II (47- and 5.4-fold, respectively); hCA IX (113- and 12.5-fold, respectively) and hCA XIV (11.3- and 10-fold, respectively). For the glycoconjugates **3**–**30** K_is ranged from 1.0 to 388 nM, however there is a single outlier within this group—the amide-linked deprotected glucuronic acid derivative **20** (K_i = 388 nM). When excluding this compound the K_i range for the remaining 27 glycoconjugates was far more clustered (1.0–19.7 nM). All of the ester derivatives had hCA XII K_is <10 nM, and in addition were all selective cf. hCA IX and XIV however some of them were non-selective cf. hCA II. Compounds of note are the glucose-OAc derivative **3**, with a hCA XII K_i of 4.3 nM it is 89-fold selective cf. hCA II and 100-fold selective cf. hCA IX; the *N*-acetyl glucosamine-OAc derivative **17**, with a hCA XII K_i of 3.9 nM it is 56-fold selective cf. hCA II and 307-fold selective cf. hCA IX, and the maltose-OH derivative **24**, with a hCA XII K_i of 3.2 nM it is 83-fold selective cf. hCA II and 344-fold selective cf. hCA IX. All glycoconjugates also had some selectivity for hCA XII compared to XIV—the only exception being compound **4** which was ~equipotent at these isozymes.

Isozyme hCA XIV. At hCA XIV the parent compounds **1** and **2** had K_is of 11.3 and 83 nM, respectively. Compound **1** was mildly selective over hCA II (4.2-fold), while compound **2** was slightly selective for hCA II over hCA XIV. Thus, the replacement of the amide *NH* with the ester *O* reverses the selectivity profile of the parent compounds—this reversal in trend has not been observed against the other four isozymes (I, II, IX and XII) for which inhibition data have so far been determined and so may highlight a region in the hCA XIV active site that is worthy of further investigation for isozyme selectivity. Glycoconjugates **3**, **4**, **14**, **22**, **26** and **30** each had tightly clustered potent K_is of 10.0–11.0 nM. Esters **14**, **22**, **26** and **30** have free sugar tails, a result that demonstrates the polar sugar tails delivered approximately 8-fold stronger hCA XIV inhibition than the non-glycoconjugate parent ester **2**. The remaining glycoconjugates were generally weaker inhibitors than the amide parent **1**, but had similar or very slightly reduced inhibition cf. the non-glycoconjugate ester parent **2**. A compound to highlight in terms of selectivity against hCA II is the glucose-OAc amide derivative **3**, with a K_i of 11.0 nM it is 35-fold selective cf. hCA II and 39-fold selective cf. hCA IX.

Conservation of active site structure and topology within the CA enzyme family has made it challeng-



sugar-N_3		X = NH	X = O
a		**3** R = Ac **4** R = H	**5** R = Ac **6** R = H
b		**7** R = Ac **8** R = H	**9** R = Ac **10** R = H
c		**11** R = Ac **12** R = H	**13** R = Ac **14** R = H
d		**15** R = Ac **16** R = H	**17** R = Ac **18** R = H
e		**19** R = Ac **20** R = H	**21** R = Ac **22** R = H
f		**23** R = Ac **24** R = H	**25** R = Ac **26** R = H
g		**27** R = Ac **28** R = H	**29** R = Ac **30** R = H

Scheme 1. Preparation of glycoconjugate sulfonamide library by 1,3-dipolar cycloaddition reaction of azido sugars with sulfonamide-alkynes.[23]

ing to target subtle isozyme differences, however to maximize the benefits of any future therapies involving CA inhibition it is essential to develop isozyme-specific compounds. Here we have explored a library of benzenesulfonamides with triazole-tethered carbohydrate tails against the transmembrane subclass of CAs—hCA IX, XII and XIV. The qualitative structure–activity relationship demonstrated that the stereochemical diversity within the carbohydrate tails effectively interrogated the CA active site topology, generating many potent inhibitors with a range of isozyme selectivity profiles—an important outcome in the quest for potential cancer therapy applications. Sugar tails have therefore shown promise as a valuable approach to solubilize the aromatic sulfonamide pharmacophore and target it to extracellular CA domains as well as to deliver isozyme differentiation.

Table 1. Carbonic anhydrase inhibition data for sulfonamides **1**, **2**, glycoconjugates **3**–**30** and standard inhibitors against human isozymes hCA II, IX, XII and XIV

Compound	K_i (nM)[a]			
	hCA II[b]	hCA IX[b]	hCA XII[c]	hCA XIV[d]
AZA	12	25	5.7	41
MZA	14	27	3.4	43
EZA	8	34	22	25
BRZ	3	37	3.0	24
DRZ	9	52	3.5	27
DCP	38	50	50	345
1	47	113	1.0	11.3
2	45	104	8.3	83
3	384	430	4.3	11
4	8.2	442	11.4	10
5	119	1238	7.7	123
6	7.0	183	7.1	58
7	470	76	14.7	87
8	7.4	360	14.5	55
9	6.8	132	7.5	64
10	8.1	65	7.5	95
11	279	103	11.7	71
12	128	420	11.5	101
13	7.3	114	7.6	67
14	5.8	96	1.1	10.9
15	45	124	13.1	78
16	265	238	19.7	103
17	218	1200	3.9	71
18	7.3	108	7.4	75
19	7.6	471	8.8	94
20	378	23	388	105
21	7.0	125	6.1	155
22	7.2	241	7.4	10.8
23	7.5	221	7.9	134
24	267	1100	3.2	64
25	423	130	7.7	73
26	7.3	39	1.0	10.8
27	44	135	8.0	76
28	90	204	3.9	78
28	50	54	8.1	74
30	8.6	67	7.3	10.6

[a] Errors in the range of ±5–10% of the reported value, from three determinations, by the CO_2 hydration method.[25]
[b] Data for hCA II and hCA IX were previously published.[23]
[c] Catalytic domain of human (cloned) isozymes.[25]
[d] Full-length human (cloned) isozyme.[17]

Acknowledgments

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References and notes

1. Supuran, C. T. Carbonic anhydrases: catalytic and inhibition mechanism, distribution and physiological roles. In *Carbonic Anhydrase: Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Florida, 2004; p 1.
2. Scozzafava, A.; Supuran, C. T. *Curr. Med. Chem.: Imm., Endoc. Metab. Agents.* **2001**, *1*, 61.
3. Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146.
4. Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enz. Inhib. Med. Chem.* **2004**, *19*, 199.
5. Reviewed in: Supuran, C. T.; Casini, A.; Scozzafava, A. Development of sulfonamide carbonic anhydrase inhibitors. In *Carbonic Anhydrase: Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Florida, 2004; p 67.
6. Erlanson, D. A.; McDowell, R. S.; O'Brien, T. *J. Med. Chem.* **2004**, *47*, 3463.
7. Opavský, R.; Pastoreková, S.; Zelník, V.; Gibadulinová, A.; Stanbridge, E. J.; Závada, J.; Kettmann, R.; Pastorek, J. *Genomics* **1999**, *33*, 480.
8. Fujikawa-Adachi, K.; Nishimori, I.; Taguchi, T.; Onishi, S. *Genomics* **1999**, *61*, 74.
9. Türeci, O.; Sahin, U.; Vollmar, E.; Siemer, S.; Göttert, E.; Seitz, G.; Parkkila, A. K.; Shah, G. N.; Grubb, J. H.; Pfreundschuh, M.; Sly, W. S. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 7608.

10. Wykoff, C. C.; Beasley, N. J.; Watson, P. H.; Turner, K. J.; Pastorek, J.; Sibtain, A.; Wilson, G. D.; Turley, H.; Talks, K. L.; Maxwell, P. H.; Pugh, C. W.; Ratcliffe, P. J.; Harris, A. L. *Cancer Res.* **2000**, *60*, 7075.
11. Parkkila, S.; Rajaniemi, H.; Parkkila, A. K.; Kivelä, J.; Waheed, A.; Pastoreková, S.; Pastorek, J.; Sly, W. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2220.
12. Svastova, E.; Hulikova, A.; Rafajova, M.; Zatovicova, M.; Gibadulinova, A.; Casini, A.; Cecchi, A.; Scozzafava, A.; Supuran, C. T.; Pastorek, J.; Pastorekova, S. *FEBS Lett.* **2004**, *577*, 439.
13. Cecchi, A.; Hulikova, A.; Pastorek, J.; Pastorekova, S.; Scozzafava, A.; Winum, J.-Y.; Montero, J.-L.; Supuran, C. T. *J. Med. Chem.* **2005**, *48*, 4834.
14. Michinton, A. I.; Tannock, I. F. *Nat. Rev.: Cancer* **2006**, *6*, 583.
15. Kamb, A. *Nat. Rev.: Drug Discovery* **2005**, *4*, 161.
16. Liao, S. Y.; Ivanov, S.; Ivanova, A.; Ghosh, S.; Cote, M. A.; Keefe, K.; Coca-Prados, M.; Stanbridge, E. J.; Lerman, M. I. *J. Med. Genet.* **2003**, *40*, 257.
17. Nishimori, I.; Vullo, D.; Innocenti, A.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3828.
18. Parkkila, S.; Parkkila, A. K.; Rajaniemi, H.; Shah, G. N.; Grubb, J. H.; Waheed, A.; Sly, W. S. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 1918.
19. Parkkila, S.; Kivelä, A. J.; Kaunisto, K.; Parkkila, A. K.; Hakkola, J.; Rajaniemi, H.; Waheed, A.; Grubb, J.; Sly, W. S. *BMC Gastroenterol.* **2002**, *2*, 13.
20. Kivelä, A. J.; Kivelä, J.; Saarnio, J.; Parkkila, S. *World J. Gastrenterol.* **2005**, *11*, 155.
21. Kaunisto, K.; Parkkila, S.; Rajaniemi, H.; Waheed, A.; Grubb, J.; Sly, W. S. *Kidney Internat.* **2002**, *61*, 2111.
22. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Del. Rev.* **2001**, *46*, 3.
23. Wilkinson, B. L.; Bornaghi, L. F.; Houston, T. A.; Innocenti, A.; Supuran, C. T.; Poulsen, S.-A. *J. Med. Chem.* **2006**, *49*, 6539.
24. Wilkinson, B. L.; Bornaghi, L. F.; Poulsen, S.-A.; Houston, T. A. *Tetrahedron* **2006**, *62*, 8115.
25. Khalifah, R. G. T. *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), at 25 °C, following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s (the uncatalyzed reaction needs around 60–100 s in the assay conditions, whereas the catalyzed ones are of around 6–10 s). The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of kinetic parameters. For each inhibitor, tested in the concentration range between 0.01 nM and 100 μM, 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 (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 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 and catalytic activity (in the absence of inhibitors) of these enzymes were calculated as reported earlier, and represent the mean from at least three different determinations.[17,23,26] Enzyme concentrations in the assay system were: 0.11 μM for hCA XII and 0.13 μM for hCA XIV. Enzymes used here were recombinant ones, prepared and purified as described earlier.[17,26]
26. Vullo, D.; Innocenti, A.; Nishimori, I.; Pastorek, J.; Scozzafava, A.; Pastoreková, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 963.