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Bioorganic & Medicinal Chemistry Letters 15 (2005) 1149–1154
Bioorganic & Medicinal Chemistry Letters

Carbonic anhydrase inhibitors. Inhibition of the membrane-bound human and bovine isozymes IV with sulfonamides

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Received 4 November 2004; revised 1 December 2004; accepted 2 December 2004

Abstract—An inhibition study of the human and bovine membrane-associated isozymes of carbonic anhydrase (CA, EC 4.2.1.1), hCA IV and bCA IV, with a series of sulfonamides and sulfamates, some of which are widely clinically used, such as acetazolamide, methazolamide, ethoxzolamide, topiramate, dorzolamide, dichlorophenamide, celecoxib, and valdecoxib among others, is reported. In contrast to bCA IV, which is generally strongly inhibited by most of these derivatives, hCA IV has a rather different inhibition profile. Several of these compounds such as acetazolamide, ethoxzolamide, and bromosulfanilamide are potent hCA IV inhibitors (K_i's of 74–93 nM), others, such as celecoxib and some halogenated sulfanilamides are medium potency inhibitors (K_i's of 450–880 nM) whereas most of them are weak hCA IV inhibitors (methazolamide: 6.2 μM; dorzolamide 8.5 μM; topiramate 4.9 μM; dichlorophenamide: 15.3 μM). The hCA IV/bCA IV inhibition ratios for all the investigated compounds ranged between 1.05 (for acetazolamide) and 198.37 (for dorzolamide). Based on these results, we doubt that hCA IV is indeed one of the main contributors to the intraocular pressure (IOP) lowering effects of sulfonamide CA inhibitors, in addition to hCA II, as hypothesized earlier by Maren et al. (*Mol. Pharmacol.* **1993**, *44*, 901–906). Indeed, both the very good hCA IV inhibitors (acetazolamide and ethoxzolamide) as well as the quite weak hCA IV inhibitors (methazolamide, dorzolamide, or dichlorophanamide) are effective in lowering IOP when administered either systemically or topically. The membrane-associated isozyme which probably is critical for aqueous humor secretion is hCA XII and not hCA IV.

1. Introduction

The involvement of the metalloenzyme carbonic anhydrase CA (EC 4.2.1.1) in various physiological processes has been recognized for a long period, being shown that the deregulated expression and/or abnormal performance of the 15 presently known isozymes may have important pathological consequences.[1,2] In fact, there are several human diseases whose pathopysiological characteristics include disbalance in the conversion between carbon dioxide and bicarbonate (the two substrates of these enzymes), resulting in perturbed ion transport, shift in pH, abnormal fluid secretion, etc.[1,2] Therefore, it seems plausible that modulation of CA activity to normal levels either by inhibition or activation offers interesting therapeutic options.[1–5] Clinical testing and/or use of the first CA inhibitors (CAIs) dates back several decades, to a period before the recognition of the diversity of isoforms within the CA family and their differential distribution in various human tissues and organs.[1,2] Traditionally, the use of inhibitors was based on histochemical, biochemical, and functional evidences for the presence of active CAs, potentially contributing to disease, and consequences of the treatment were evaluated mainly symptomatically.[1,2] Because of their favorable outcomes, sulfonamides became widely accepted drugs in the treatment of several CA-based diseases, especially as antiglaucoma agents, diuretics, and antiulcer agents among others.[1,2] However, systemic and even topically administered CA inhibitors regularly showed serious side effects.[1–4] It is now understood that these undesired effects are due to the existence of at least 12 different active CA isoforms (together with the three inactive isozymes, the CA related proteins, CARPs VIII, X, and XI),[1–4] that are indiscriminately inhibited irrespective of whether they play a real role in disease

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doi:10.1016/j.bmcl.2004.12.009

or are just coexpressed in the same tissue and elsewhere in the body. Moreover, certain drugs directed primarily against different CA unrelated targets may also inhibit activity of CAs. This may be exemplified by the antiinflammatory cyclooxygenase-2-selective drugs celecoxib and valdecoxib that show nanomolar affinity to several CA isoforms, but are generally well tolerated and give clinical responses in several disorders.[5] Thus, it is critically important to thoroughly characterize the affinity of different isozymes for sulfonamide CAIs, due to the wide range of applications of such drugs, and also to better understand the side effects due to inhibition of isozymes, which do not constitute the main target for a certain disease/application.[1–4]

The membrane-associated isoform CA IV was the first membrane-type CA to be isolated,[6] being originally purified from bovine lung as a 52 kDa glycoprotein with high CO_2-hydrase activity.[6a] It was subsequently shown that the human isozyme, hCA IV, has many similar properties with the bovine enzyme (bCA IV), but in contrast to this, hCA IV is smaller (around 35 kDa) and contains no carbohydrate residues in its molecule.[6b] Furthermore, CA IV is anchored to membranes in a unique way among the membrane-type CAs, by means of glycosylphosphatidylinositol moieties, from which it may be released by treatment with phosphatidylinositol-specific phospholipase C.[6,7] hCA IV is quite abundant in a multitude of tissues, such as nasal mucosa, esophageal epithelium, kidneys, pancreas, salivary glands, heart muscle (endothelial and muscle cells), eyes, lungs, brain capillaries, and colon, playing important physiological functions related to the nasal chemosensitivity to CO_2, the antireflux defense, bicarbonate reabsorption, NH_4^+ output, pH regulation, production of ocular fluid, gas exchange, etc., only to mention the most important ones.[1,2]

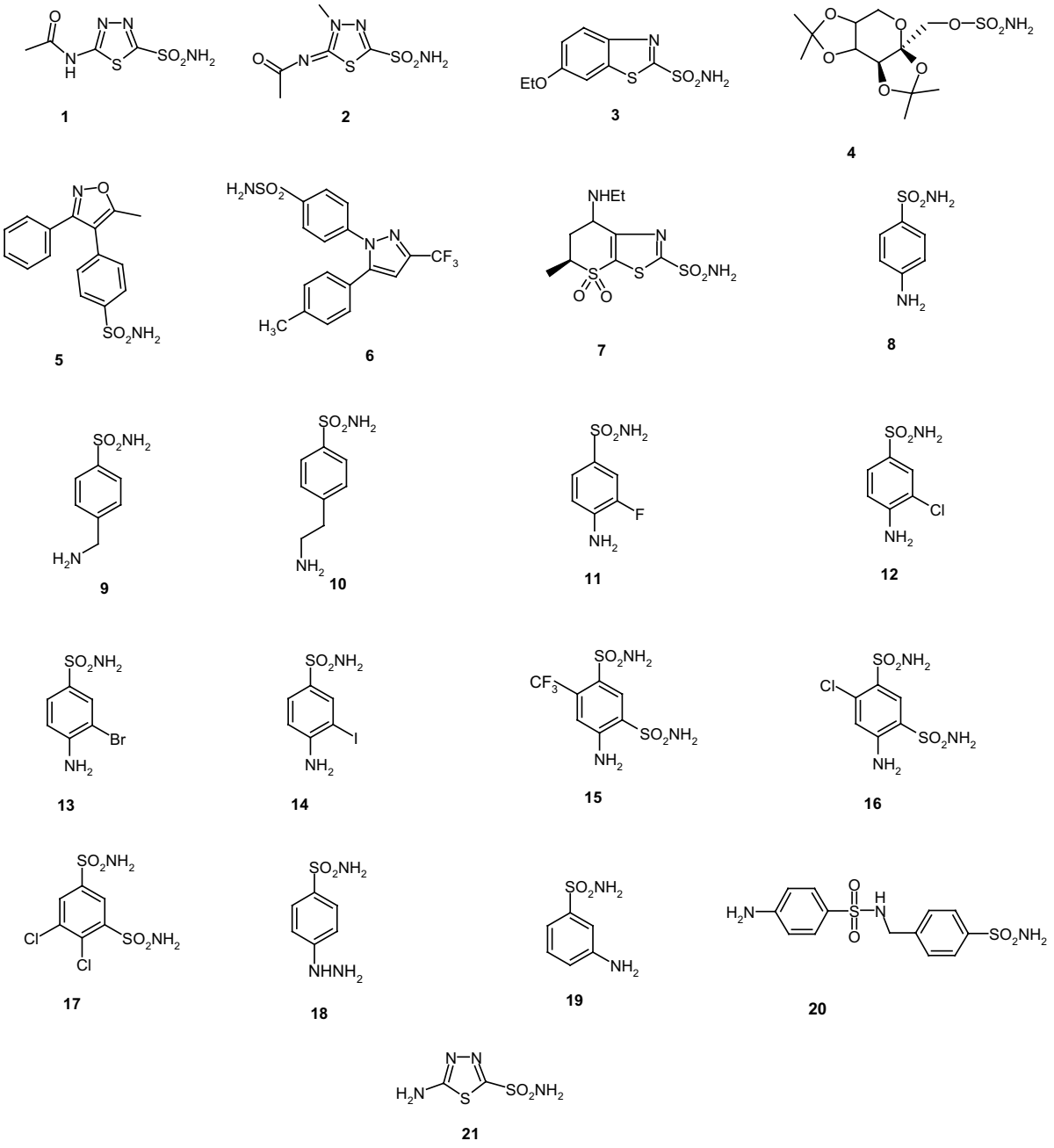
The catalytic properties of hCA IV have been studied in detail by Baird et al.[8] who showed that this is a high activity isoform both for the CO_2 hydration as well as the bicarbonate dehydration reactions. Furthermore, for the last reaction, hCA IV is even more active than hCA II, one of the best studied CAs, and also the isozyme known to play a host of critical physiological and physiopathological functions.[1–3,6,7] The X-ray crystal structure of the C-terminal deletion mutant of recombinant hCA IV (G267X CA IV) has been determined by Christianson's group.[9] This truncation converts the normally glycosylphosphatidylinositol-anchored enzyme into a soluble secretory form possessing the same catalytic properties as the membrane-associated enzyme purified from human tissues.[9] The three-dimensional structure of this secretory form revealed that the zinc binding site and the hydrophobic substrate binding pocket of hCA IV are generally similar to those of other mammalian isozymes, the unique structural differences being found elsewhere in the active site: two disulfide linkages, Cys 6-Cys 11G and Cys 23-Cys 203, were shown to stabilize the conformation of the N-terminal domain. The latter disulfide additionally stabilizes an active site loop containing a *cis*-peptide linkage between Pro 201 and Thr 202. On the opposite side of the active site, the substrate binding pocket comprising the Val 131-Asp 136 segment was shown to adopt an extended loop conformation instead of an α-helix conformation found in other isozymes. The C-terminus part of hCA IV is surrounded by a substantial electropositive surface potential, which is likely to stabilize the interaction of the enzyme with negatively charged phospholipid headgroups of the membrane.[9] The same group also reported the X-ray crystal structure of the murine isozyme IV, mCA IV, which is quite similar to the human corresponding isozyme.[10] Although hCA IV is a rather well characterized enzyme, paradoxically, very few sulfonamide inhibition studies have been reported with it. Except the recent paper by Christianson's group[11] on the binding of brinzolamide-like sulfonamides to hCA II and hCA IV (a crystallographic study where the K_i of four brinzolamide derivatives against hCA IV are also provided), all reported CA IV inhibition studies have been done with the bovine isozyme, both by Maren's[12] as well as our group,[13–16] probably due to the relative ease of isolation and abundance of bCA IV in bovine lungs.[12] The interesting bCA IV inhibitory properties of many sulfonamides with clinical applications (such as acetazolamide **1**, methazolamide **2**, or ethoxzolamide **3** among others) against bCA IV led Maren et al.[12] to propose an important role of this isozyme in the secretion of aqueous humor within the eye, its inhibition being considered critical for the antiglaucoma activity of the clinically used sulfonamides such as **1**–**3** mentioned above, dichlorophanamide **17** (used systemically) or the topically acting antiglaucoma drug dorzolamide **7**. Here we report the first detailed inhibition study of hCA IV with sulfonamide and sulfamate derivatives of types **1**–**21**, some of which are important clinically used drugs. In addition to the compounds mentioned above, topiramate **4** is an antiepileptic,[16d] whereas valdecoxib **5** and celecoxib **6** are cyclooxygenase-2 (COX-2) specific inhibitors also showing significant CA inhibitory properties.[5] To our greatest surprise, hCA IV has a much lower affinity for these inhibitors as compared to bCA IV.

2. Chemistry

Buffers and sulfonamide/sulfamates **1**–**21** are of the highest purity available, and were either commercially available from Sigma–Aldrich (derivatives **1**–**3**, **8**–**10**, **15**–**17**), J&J (topiramate **4**), Merck (dorzolamide **7**), or Pfizer (valdecoxib **5** and celecoxib **6**), or prepared by literature procedures as reported earlier (derivatives **11**–**14** and **18**–**21**).[13–16] Recombinant human isozymes hCA I, II were used for comparison in the inhibition studies, being obtained as reported earlier,[17–21] whereas hCA IV has been prepared by a new procedure described here.[22] bCA IV was isolated from bovine lungs as described in Ref. 12.

3. CA inhibition

Inhibition data against four CA isozymes, that is, hCA I, II, IV, and bCA IV, with sulfonamides/sulfamates **1**–**21** are shown in Table 1.



As seen from the data of Table 1, the membrane-associated isozyme hCA IV is inhibited by all the investigated sulfonamides/sulfamates **1**–**21**, but its affinity for these inhibitors is rather different as compared to that of the bovine isozyme bCA IV. The following SAR should be noted: (i) good hCA IV inhibitory properties were shown by acetazolamide **1**, ethoxzolamide **3**, 3-bromosulfanilamide **13**, and sulfanilyl-homosulfanilamide **20**, which showed K_i's in the range of 74–96 nM. These compounds also showed good bCA IV inhibitory properties (K_i's in the range of 13–70 nM), and except ethoxzolamide, case in which a 7-fold difference of K_i's between the human and bovine enzymes is observed, for the other three derivatives, affinity of the two enzymes are rather uniform (variation in the range of 1.05–1.68 times); (ii) another group of sulfonamides, including derivatives **6**, **11**, **12**, **14**, **16**, and **21**, showed K_i's in the range of 380–940 nM. All these compounds except **21** (deacetylated acetazolamide) are benzenesulfonamide or benzene-1,3-disulfonamide derivatives. These derivatives are generally 1.40–7.36 less inhibitory against hCA IV as compared to bCA IV, enzyme for which they showed K_i's in the range of 125–540 nM; (iii) four other derivatives, including topiramate **4**, valdecoxib **5**, **10**, and **15**, showed K_i's in the range of 1.34–4.9 μM, whereas their bCA IV inhibitory power was in the range of 54–2450 nM. Thus, the hCA IV/bCA IV inhibition ratio was in these cases in the range of 1.45–90.74, with topiramate showing a dramatic difference of affinity for the two isozymes (90 times less inhibitory for the human than the bovine isozyme); (iv) the remaining derivatives, that is, methazolamide

Table 1. Inhibition constants of sulfonamide/sulfamate inhibitors against the human isozymes hCA I, II, IV, and bovine isozyme bCA IV, for the CO_2 hydration reaction, at 20 °C[23]

Inhibitor	K_i[a]				Ratio
	hCA I[b] (μM)	hCA II[b] (nM)	bCA IV[c] (nM)	hCA IV[d] (nM)	hCA IV/bCA IV
1 (acetazolamide)	0.25	12	70	74	1.05
2 (methazolamide)	0.05	14	36	6200	172.22
3 (ethoxzolamide)	0.025	8	13	93	7.15
4 (topiramate)	0.25	5	54	4900	90.74
5 (valdecoxib)	54	43	340	1340	3.94
6 (celecoxib)	50	21	290	880	3.03
7 (dorzolamide)	50,000	9	43	8530	198.37
8 (sulfanilamide)	28	300	3000	9760	3.25
9	25	170	2800	8600	3.07
10	21	160	2450	3570	1.45
11	8.3	60	180	910	5.05
12	9.8	110	320	450	1.40
13	6.5	40	66	96	1.45
14	6.0	70	125	920	7.36
15	5.8	63	240	4830	20.12
16	8.4	75	160	380	2.37
17 (dichlorophenamide)	1.2	38	380	15,330	40.34
18	78.5	320	3200	7700	2.40
19	25	240	2200	7750	3.52
20	0.10	33	50	84	1.68
21	8.6	60	540	940	1.74

[a] Errors were in the range of 3–5% of the reported values, from three different assays.
[b] Human cloned isozyme.
[c] Human truncated (−20 aminoterminal amino acids) isozyme.
[d] Bovine isozyme isolated from lung microsomes as described in Ref. 12.

2, dorzolamide **7**, sulfanilamide **8**, homosulfanilamide **9**, dichlorophenamide 17 as well as compounds **18** and **19**, showed K_i's in the range of 6.2–15.33 μM, being the weakest hCA IV inhibitors in this series of derivatives, whereas their hCA IV/bCA IV inhibition ratio was in the range of 2.40–198.37. It is noteworthy the tremendous difference of affinity of methazolamide and dorzolamide for hCA IV as compared to bCA IV. The first compound is 172 times less potent hCA IV inhibitor, whereas dorzolamide almost 200 times less effective as a hCA IV over bCA IV inhibitor. In the case of the other clinically used antiglaucoma drug dichlorophenamide, **17**, this ratio is around 40. These data throw an important doubt whether the isozyme CA IV is indeed one of the main contributors to the intraocular pressure (IOP) lowering effects of sulfonamide CAIs, together with hCA II (whose critical role is well established),[24] as hypothesized earlier by Maren et al.[12] Indeed, both the very good hCA IV inhibitors (such as acetazolamide **1** and ethoxzolamide **3**) as well as the quite weak hCA IV inhibitors (such as methazolamide **2**, dorzolamide **7**, or dichlorophanamide **17**) are effective in lowering IOP when administered either systemically or topically,[24,25] and this has been considered up to now to be due to the inhibition of isozymes II and IV. Since some of these compounds do not significantly inhibit hCAIV (but they do inhibit bCA IV) it is obvious that this isozyme plays a marginal role, or not a role at all, in the secretion of aqueous humor in the human eye. In line with these findings, one must mention that recently, Lerman's group[26] reported the presence of high amounts of another membrane-bound CA within the human eye, that is, CA XII, which is frequently overexpressed in the eyes of glaucoma patients. This may be indeed the membrane-associated isozyme (in addition to the cytosolic one CA II) playing a role in aqueous humor secretion, and not hCA IV, whose role has been proposed[12] based on sulfonamide inhibition studies done with the bovine isozyme; (v) the general trend of affinity of these compounds for the diverse CA isozymes investigated here was: hCA II > bCA IV > hCA IV > hCA I.

It is rather difficult to rationalize at this moment, from a structural point of view, these results, but work is in progress in our laboratory to obtain X-ray crystal structures of some of these adducts. It is thus clear that hCA IV-selective inhibitors may have important clinical applications (but not as antiglaucoma drugs) since this isozyme is highly active and involved in many physiological processes, among which for example the formation of metabolons with sodium bicarbonate cotransporters (NBCs).[27]

4. Conclusions

We investigated the inhibition of the human and bovine membrane-associated isozymes hCA IV and bCA IV with a series of sulfonamides and sulfamates, some of which widely clinically used, such as acetazolamide, methazolamide, ethoxzolamide, topiramate, dorzolamide, dichlorophenamide, celecoxib, and valdecoxib among others. In contrast to the bovine isozyme, which generally is strongly inhibited by most of these derivatives, hCA IV has a rather different inhibition profile. Several of these compounds, such as acetazolamide, ethoxzolamide, and bromosulfanilamide, are potent

hCA IV inhibitors (K_i's of 74–93 nM); others, such as celecoxib and several halogenated sulfanilamides are medium potency inhibitors (K_i's of 450–880 nM), whereas most of them are rather weak hCA IV inhibitors (methazolamide: 6.2 μM; dorzolamide 8.5 μM; topiramate 4.9 μM; dichlorophenamide: 15.3 μM). The hCA IV/bCA IV inhibition ratio for all the investigated compounds ranged between 1.05 (for acetazolamide) and 198.37 (for dorzolamide). Based on these results, we doubt that hCA IV is indeed one of the main contributors to the intraocular pressure lowering effects of sulfonamide CAIs, together with hCA II, as hypothesized earlier by Maren et al.[12] Indeed, both the very good hCA IV inhibitors (such as acetazolamide and ethoxzolamide) as well as the quite weak hCA IV inhibitors (such as methazolamide, dorzolamide, or dichlorophanamide) are effective in lowering IOP when administered either systemically or topically. The membrane-associated isozyme which probably is critical for aqueous humor secretion is hCA XII and not hCA IV.

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22. Construction of bacterial expression vector containing hCAIV: truncated human carbonic anhydrase 4 (M83670, Genbank) was isolated from a human lung cDNA library by PCR using oligonucleotides F1 and R1 (F1, 5′-TGCAGAGTCACACTGGTGCT-3′; R1, 5′-TCATGC-CTAAAGTCCCACCT-3′). The purified PCR product was used for the cloning of DNA encoding the mature protein (102-899bp) by PCR with oligonucleotides MP-F and MP-R (MP-F, 5′-GGGAATTCCATATGGCAGA-GTCACAC-3′; MP-R, 5′-CCGCTCGAGGGACTT-TATCACCGTGCG-3′). The resulting product was cut with *Nde*I and *Xho*I and ligated into the bacterial expression vector pET24 (Novagen) using the *Nde*I and *Xho*I cloning sites to create pET24-hCAIV. Expression and purification of recombinant human CAIV: *Escherichia coli*, strain BL21-CodonPlus (DE3)-RIL (Stratagene), transformed with pET24-hCAIV was grown at 37 °C in 50 mL LB medium containing 25 μg/mL kanamycin. The overnight cultures were diluted 50 times into 0.5–1l LB medium containing 25 μg/mL kanamycin and

grown at 37 °C to OD_{600} 0.5–0.7. The expression of hCA IV was induced by adding 0.5 mM IPTG and the cells were grown at 37 °C for another 4 h. The bacterial cells were recovered by centrifugation at 5000*g* for 20 min at 4 °C and stored overnight at −80 °C. For purification the cells were thawed on ice for 15–30 min and resuspended in 5 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) per gram of cells containing 200 μg/mL lysozyme. The suspension was incubated on ice for 30 min and treated with a sonicator (six times for 10 s with 10 s cooling time in between). After centrifugation at 10,000*g* for 30 min at 4 °C the cleared lysate was incubated with Ni-NTA suspension (Qiagen). The expressed protein was purified according to the manufacturer's protocol. The isolated hCA IV was further purified by Sephadex G25 chromatography (Amersham). The purified enzyme had a catalytic activity very similar to that described in Ref. 8.

23. An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA I, II, IV 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, 10 mM Na_2SO_4, following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10–50 mM (in the assay buffer) and dilutions up to 0.01 nM done with the assay buffer mentioned above. Enzyme concentrations were 0.09 μM for hCA I, 0.06 μM for hCA II, 1 μM for hCA IV and bCA IV. Inhibition constants were calculated as described by Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573.
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