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Carbonic anhydrase inhibitors. Interaction of isozymes I, II, IV, V, and IX with carboxylates

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Abstract—A detailed inhibition study of five carbonic anhydrase (CA, EC 4.2.1.1) isozymes with carboxylates including aliphatic (formate, acetate), dicarboxylic (oxalate, malonate), hydroxy/keto acids (L-lactate, L-malate, pyruvate), tricarboxylic (citrate), or aromatic (benzoate, tetrafluorobenzoate) representatives, some of which are important intermediates in the Krebs cycle, is presented. The cytosolic isozyme hCA I was strongly activated by acetate, oxalate, pyruvate, L-lactate, and citrate (K_A around 0.1 μM), whereas formate, malonate, malate, and benzoate were weaker activators (K_A in the range 0.1–1 mM). The cytosolic isozyme hCA II was weakly inhibited by all the investigated anions, with inhibition constants in the range of 0.03–24 mM. The membrane-associated isozyme hCA IV was the most sensitive to inhibition by carboxylates, showing a K_I of 99 nM for citrate and oxalate, of 2.8 μM for malonate and of 14.5 μM for pyruvate among others. The mitochondrial isozyme hCA V was weakly inhibited by all these carboxylates (K_Is in the range of 1.67–25.9 mM), with the best inhibitor being citrate (K_I of 1.67 mM), whereas this is the most resistant CA isozyme to pyruvate inhibition (K_I of 5.5 mM), which may be another proof that CA V is the isozyme involved in the transfer of acetyl groups from the mitochondrion to the cytosol for the provision of substrate(s) for de novo lipogenesis. Furthermore, the relative resistance of CA V to inhibition by pyruvate may be an evolutionary adaptation of this mitochondrial isozyme to the presence of high concentrations of this anion within this organelle. The transmembrane, tumor-associated isozyme hCA IX was similar to isozyme II in its slight inhibition by all these anions (K_I in the range of 1.12–7.42 mM), except acetate, lactate, and benzoate, which showed a $K_I > 150$ mM. The lactate insensitivity of CA IX also represents an interesting finding, since it is presumed that this isozyme evolved in such a way as to show a high catalytic activity in hypoxic tumors rich in lactate, and suggests a possible metabolon in which CA IX participates together with the monocarboxylate/H^+ co-transporter in dealing with the high amounts of lactate/H^+ present in tumors.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1), metalloenzymes widespread all over the phylogenetic tree, were until recently considered only as catalysts involved in a fundamental physiological reaction, the interconversion between carbon dioxide and bicarbonate, with generation of a proton.[1–5] Indeed, these three simple chemical entities, CO_2, HCO_3^-, and H^+ are essential in a host of physiological and pathological processes, such as among others: pH regulation and homeostasis, gas exchange, ion transport and secretion, electrolyte secretion in many tissues/organs, metabolic pathways involving biosynthetic reactions of many essential cell components, cell proliferation, and differentiation, etc.[1–6] In recent years it has become increasingly evident that the high catalytic efficiency of the many CA isozymes described in higher vertebrates (14 are presently known in humans),[1–5] is crucial for other physiologically relevant proteins/enzymes. CA isoforms form metabolons with companion proteins, such as, for example, Cl^-/HCO_3^- anion exchangers (AEs) and the sodium bicarbonate cotransporter proteins NBC1 and NBC3 (which interact with the cytosolic isozyme CA II or the membrane bound isozyme CA IV),[6–9] or gluconeogenic enzymes such as malate dehydrogenase and pyruvate

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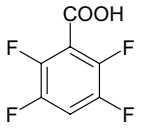
carboxylase, which interact with the mitochondrial isozyme CA V.[10] For example, a physical interaction has been identified between hCA II, and the erythrocyte membrane Cl^-/HCO_3^- anion exchanger, AE1, mediated by an acidic motif in the AE1 carboxy-terminus.[6–9] The presence of hCA II attached to AE1 accelerates AE1 bicarbonate transport activity, as AE1 moves bicarbonate either into or out of the cell.[6–9] Functional and physical interactions were also shown to occur between CA II or CA IV and Na^+/HCO_3^- co-transporter isoforms NBC1 and NBC3 by the same group.[6–9] Recently, a CA II-sulfate/anion exchanger metabolon has been described too,[11] that was shown to speed both the secretory and reabsorptive processes in the renal proximal tubule of vertebrates. Thus, the interaction between different anions and diverse CA isozymes is of critical importance to understanding some fundamental biochemical/physiological processes.

Inhibition of CAs by anions is important from the physiological point of view, considering the high concentrations of some anions (bicarbonate, chloride, phosphate, sulfate, etc.) in different tissues. Indeed, anions represent an important class of inhibitors of these metalloenzymes,[12] as they directly bind to the Zn(II) ion within the enzyme active site, assuring a tetrahedral or trigonal bipyramidal coordination sphere (depending on whether only the anion is bound as the fourth Zn(II) ligand, in addition to the protein ligands, His 94, 96, and 119, or whether a water molecule is coordinated too).[1–5] In previous work from this laboratory we investigated in detail the inhibition of different CAs belonging both to the α- as well as β- or γ-CA families with inorganic anions such as, for example, the halogenides, pseudohalogenides (cyanate, cyanide, azide), hydrogen sulfide, nitrate, nitrite, sulfate, sulfamate, sulfamide as well as with phenylboronic and phenylarsonic acids among others.[13–16] Still, no other organic anions (such as, e.g., carboxylates) have been investigated in detail up to now, although some CA II inhibition data with acetate and formate have been reported in the literature (but no exact measurements of the inhibition constants are available; the interaction of these anions with the enzyme has been monitored spectroscopically using the Co(II)-substituted CAs).[17] Here we report a detailed study of the five CA isozymes, i.e., cytosolic hCA I and II, membrane-bound hCA IV, mitochondrial isozyme hCA V, as well as the tumor associated, transmembrane hCA IX, together with the effects of the diverse carboxylates, aliphatic monocarboxylates formate and acetate, the dicarboxylates oxalate and malonate, or the aromatic derivatives benzoate and 2,3,5,6-tetrafluorobenzoate. Several carboxylate salts of hydroxy-, keto- or di/tricarboxylic acids (pyruvate, lactate, malate, and citrate) involved in fundamental biochemical processes, such as the Krebs cycle, were also included in the present study.

2. Chemistry

Buffers and sodium salts of acetic, formic, oxalic, malonic, pyruvic, L-lactic, L-malic, citric, benzoic, and 2,3,5,6-tetrafluorobenzoic acid were of highest purity available, from Sigma–Aldrich (Milano, Italy) and were used without further purification.



2,3,5,6-tetrafluorobenzoic acid

3. CA inhibition data

Inhibition data against five CA isozymes, that is, hCA I, hCA II (cytosolic forms), hCA IV (membrane-associated), hCA V (mitochondrial), and hCA IX (transmembrane),[18] with the above-mentioned anions are shown in Table 1. Inhibition data for bicarbonate (one of the CA substrates) are also provided for comparison, as they were recently reported by this group.[16]

Data of Table 1 allow us to draw the following conclusions regarding the interaction of CA isozymes with carboxylates: (i) isozyme hCA I is inhibited by bicarbonate (with a K_I of 12 mM), but activated by all carboxylates investigated here. CA activators have been described from many classes of compounds, such as amines, amino acids, peptides, azoles and anions,[19,20] and the X-ray crystal structure of two adducts of activators with isozyme hCA II have been reported by this group.[21,22] Thus, it is generally accepted that activators bind at a site distinct from that of the inhibitors within the enzyme active cavity[21,22] and that activators participate in the rate-limiting step of the CA catalytic cycle, a proton transfer reaction between the active site and the reaction medium, shuttling protons by means of groups possessing an appropriate pK_a, among which the carboxylate groups are also candidates.[22] Indeed, acetate, oxalate, pyruvate, lactate, and citrate behave as potent hCA I activators, showing activation constants (K_A) in the range of 0.09–1 μM, whereas malonate, malate, and benzoate are weaker activators, showing K_A values in the range of 0.1–2.1 mM. The worst activator was formate (K_A of 5 mM). On the other hand, 2,3,5,6-tetrafluorobenzoate was a very potent hCA I activator, showing an activation constant of 23 nM, the lowest among simple compounds ever reported[21,22] (Table 1). It must be mentioned that although hCA I is a very abundant isozyme, isolated more than four decades ago, its precise physiological function is unknown, and details of the catalytic mechanism, such as the nature of the proton shuttling moiety (which in most isozymes, such as CA II, IV, VII, and IX is His 64), are also rather controversial.[1–5]

Our data may furnish some insights regarding both these aspects mentioned above: considering the catalytic mechanism and the propensity of hCA I to be activated by carboxylates, it is likely that the proton shuttling moiety of hCA I is a carboxylate present within the active site of this isozyme, and not a histidine as in all

Table 1. Inhibition constants of carboxylates and bicarbonate against isozymes hCA I, II, IV, V, and IX, for the CO_2 hydration reaction, at 20 °C[18]

Inhibitor*	K_I [mM]#				
	hCA I[a]	hCA II[a]	hCA IV[a]	hCA V[b]	hCA IX[c]
HCO_3^-**	12	85	6.6	82	13
Formate	A	24.0	1.25	9.97	1.22
Acetate	A	0.13	0.55	25.9	>150
Oxalate	A	0.99	9.9×10^{-5}	2.23	3.28
Malonate	A	1.62	2.8×10^{-3}	6.00	1.27
Pyruvate	A	2.11	14.5×10^{-3}	5.50	1.12
L-Lactate	A	3.22	1.53	3.37	>150
L-Malate	A	1.87	53.7×10^{-3}	2.49	7.42
Citrate	A	2.16	9.9×10^{-5}	1.67	4.93
Benzoate	A	0.03	74.7×10^{-3}	7.01	>150
2,3,5,6-F_4-benzoate***	A	0.006	74.5×10^{-3}	1.62	0.77

A = activator: K_A (formate) = 5 mM; K_A (acetate) = K_A (oxalate) = 0.1 μM; K_A (malonate) = 0.1 mM; K_A (pyruvate) = 0.09 μM; K_A (lactate) = 0.1 μM; K_A (malate) = 0.1 mM; K_A (citrate) = 1 μM; K_A (benzoate) = 2.1 mM; K_A (tetrafluorobenzoate) = 23 nM (K_A = activation constant).
[a] Human cloned isozymes.
[b] Recombinant, full-length form of hCA V.
[c] Catalytic domain of the human, recombinant isozyme.
* As sodium salts.
** From Ref. 16.
*** See structure in the text.
# Errors were in the range of 3–5% of the reported values, from three different assays.

other investigated α-CAs. This situation has already been reported for a β-CA isolated from the archaeon *Methanobacterium thermoautotrophicum* (Cab) by Ferry's group,[23] in which the carboxylate residue of Asp 34 is actively involved in the transfer of protons from the active site to the environment. On the other hand, the potent activation of hCA I by many carboxylates belonging to the Krebs cycle (such as pyruvate, lactate, or citrate) may be indicative of a putative role of this isozyme in metabolic processes involving these anions. All these hypotheses should attentively be checked in order to elucidate the real physiological role of this highly abundant protein in many vertebrates;[5] (ii) against hCA II, one of the physiologically most important isozymes, all the anions investigated here show inhibitory properties (Table 1). Thus, the best hCA II inhibitors were tetrafluorobenzoate and benzoate, acetate, and oxalate (K_I in the range of 0.006–0.99 mM), whereas malonate, pyruvate, L-lactate, L-malate, and citrate were weaker anionic inhibitors (K_Is in the range of 1.62–3.22 mM), being much more effective than formate and bicarbonate, which showed inhibition constants in the range of 24–85 mM; (iii) the most susceptible isozyme to inhibition by carboxylates seems to be hCA IV (Table 1). Thus, oxalate and citrate act as very potent hCA IV inhibitors, with inhibition constants of 99 nM, being among the best anion inhibitors ever reported for any CA isozyme.[16] Malonate and pyruvate are also effective hCA IV inhibitors, with inhibition constants in the range of 2.8–14.5 μM, whereas L-malate, benzoate, and tetrafluorobenzoate were weaker inhibitors (K_Is in the range of 53.7–74.7 μM). Weak CA IV inhibitors were bicarbonate, formate, acetate, and L-lactate, with inhibition constants in the range of 0.55–6.6 mM. It is rather difficult to explain this very different behavior of isozyme hCA IV towards these carboxylates, and work is in progress in this laboratory to determine the X-ray crystal structure of adducts of these anions with hCA IV, in order to rationalize this behavior. From a physiological standpoint the sensitivity of CA IV to the carboxylates is not unreasonable, since the extracellular-localized CA IV active site would not normally encounter high concentrations of carboxylates; (iv) the mitochondrial isozyme hCA V is also inhibited by all the investigated carboxylates (Table 1). The best inhibitors were tetrafluorobenzoate, citrate, oxalate, and L-malate, which showed inhibition constants in the range of 1.62–2.23 mM. Less inhibitory were L-lactate, pyruvate, malonate, and benzoate, with K_Is in the range of 3.37–7.01 mM, whereas formate and acetate were the weakest inhibitors (K_Is of 9.97–25.9 mM) together with bicarbonate (which is a very weak inhibitor, K_I of 82 mM).

At this point it should be mentioned that in several important biosynthetic processes involving pyruvate carboxylase (PC), acetyl CoA carboxylase (ACC), and carbamoyl phosphate synthetases I and II, bicarbonate, not carbon dioxide is the real substrate of these carboxylating enzymes, and the provision of enough bicarbonate is assured mainly by the catalysis involving just the mitochondrial isozyme CA V (probably assisted by the high activity cytosolic isozyme CA II, see Fig. 1).[3b,24,25] Mitochondrial PC is needed for the efflux of acetyl groups from the mitochondria to the cytosol where the fatty acid biosynthesis takes place.[3b,24,25] Practically, pyruvate is carboxylated to oxaloacetate in the presence of bicarbonate and PC. The bicarbonate needed for this process is generated catalytically by mitochondrial CA V (and this may explain the insensitivity of CA V to bicarbonate inhibition, see Table 1). The mitochondrial membrane is impermeable to acetyl-CoA, which condenses with oxaloacetate to form citrate. Citrate is thereafter translocated to the cytoplasm by means of the tricarboxylic acid transporter. In the cytosol, the citrate is cleaved and regenerates acetyl-CoA and oxaloacetate. As oxaloacetate also cannot cross the mitochondrial membrane, its decarboxylation regenerates pyruvate, which can be then transported into the

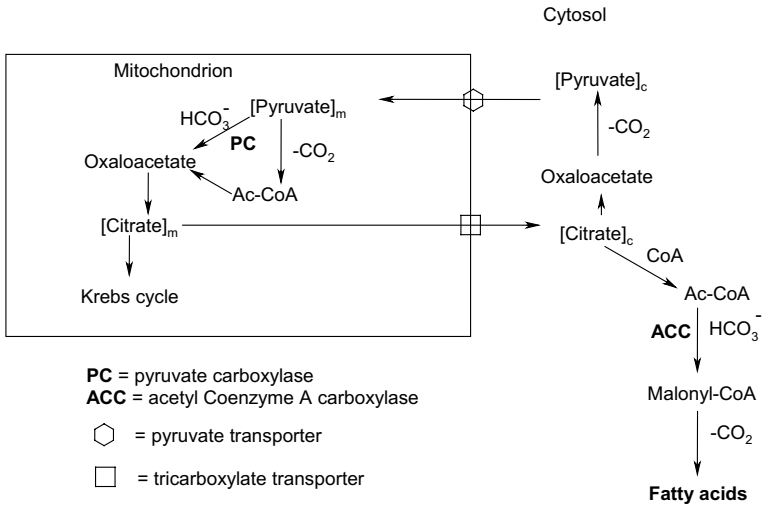


Figure 1. The transfer of acetyl groups from the mitochondrion to the cytosol (as citrate) for the provision of substrate for de novo lipogenesis.[25] All steps involving bicarbonate also need the presence of at least two CA isozymes: CA V in the mitochondrion and CA II in the cytosol (see Discussion in the text) (reproduced with permission from Ref. 25, copyright ACS, 2004).

mitochondria by means of the pyruvate transporter (Fig. 1). The acetyl-Co A thus generated in the cytosol is in fact used for the de novo lipogenesis, by carboxylation in the presence of ACC and bicarbonate, with formation of malonyl-Co A. The bicarbonate needed in this process is furnished by the CA II catalyzed conversion of CO_2 to bicarbonate. Subsequent steps involving the sequential transfer of acetyl groups lead to longer chain fatty acids.[3b,24,25] As a whole, two CA isozymes are critical to the entire process of fatty acid biosynthesis: CA V within the mitochondria (to provide enough substrate to PC), and CA II within the cytosol (for providing sufficient substrate to ACC). In fact, inhibition of CAs (and principally CA V) by sulfonamides acting as low nanomolar inhibitors, decreases lipogenesis in cultured adipocytes.[3b,24,25]

Returning to the data of Table 1 in this context, we may observe that CA V is the most resistant isozyme to inhibition by pyruvate as compared to isozymes I, II, IV, and IX, and this may constitute an evolutionary adaptation of the mitochondrial isozyme to the presence of high concentrations of this anion within mitochondria. This is also consistent with the relative sensitivity of CA V to inhibition by citrate, which must be extruded from the mitochondria to the cytosol in order to allow the fatty acid biosynthesis (Fig. 1); (v) the tumor-associated, transmembrane isozyme hCA IX is also inhibited by most of the anions investigated here. The best CA IX inhibitors were tetrafluorobenzoate, formate, malonate, and pyruvate (K_Is in the range of 0.77–1.27 mM), followed by oxalate, citrate, and L-malate (K_Is in the range of 3.28–7.42 mM). Bicarbonate shows an inhibition constant of 13 mM against hCA IX. But the most intriguing observation is that acetate, benzoate, and L-lactate do not inhibit CA IX significantly even at 150 mM concentrations of inhibitor (in fact it was impossible to determine the precise K_Is of these carboxylates due to the fact that at very high concentrations of inhibitor (i.e., >200 mM) the ionic strength in the assay system does not remain constant, and important deviations from the Michaelis–Menten behavior are observed). The tremendous difference of activity between tetrafluorobenzoate (a good CA IX inhibitor) and benzoate, which practically does not inhibit this isozyme at all should also be noted. It is rather difficult to explain these results at this point, without the X-ray crystal structure of this isozyme available. The lactate data presented here are the most important, and a tentative explanation of CA IX insensitivity to this anion is proposed below.

Thus, it must be stressed that the transport of L-lactate across the plasma membrane is of considerable importance to almost all mammalian cells.[26] In most cells a specific H^+-monocarboxylate co-transporter (MCT) is largely responsible for this process, and the capacity of this carrier is usually very high, to support the high rates of production or utilization of this metabolite. The best characterized MCT transports L-lactate and a wide range of other aliphatic monocarboxylates, including pyruvate and the ketone bodies acetoacetate and β-hydroxybutyrate across the erythrocyte membrane.[26] This carrier has been identified as a protein of 35–50 kDa on the basis of purification and specific labeling experiments. Other cells possess similar MCTs, but in some cases there are significant differences in the properties of these systems, sufficient to suggest the existence of a family of such carriers. In particular, cardiac muscle and tumor cells have transporters that differ in their K_m values for certain substrates (including stereoselectivity for L- over D-lactate) and in their sensitivity to inhibitors. Mitochondria, bacteria, and yeast also possess MCTs that share some properties in common with those in the mammalian plasma membrane but are adapted to their specific roles. However, there are distinct Na^+-monocarboxylate cotransporters on the luminal surface of intestinal and kidney epithelia, which enable active uptake of lactate, pyruvate, and ketone bodies in these tissues.[26] MCT and sodium-bicarbonate cotransporters (NBCs) mentioned earlier in this paper transport acid/base equivalents and coexist in many

epithelial and glial cells.[27] In the nervous systems, electroneutral MCT1 cotransports lactate and other monocarboxylates with H^+, and is believed to be involved in the shuttling of energy-rich substrates between astrocytes and neurons.[27] Recently facilitation of MCT1 transport activity has been found in the presence of NBC, resulting from the increase in the apparent buffer capacity contributed by NBC, which suppresses the build-up of intracellular H^+ during the influx of lactate/H^+ (which would reduce MCT1 activity).[27] Hence these membrane transporters functionally cooperate and are able to increase ion/metabolite transport activity, probably participating in a metabolon similarly to the CA-NBC/AE metabolons. Diverse MCTs have been isolated from tissues very rich in CA isozymes, such as the blood red cells,[26] retinal pigment epithelium,[28] the ruminal epithelium,[29] or the mammalian medullary thick ascending limb.[30]

Tumors that overexpress CA IX are hypoxic and more acidic than the non-tumoral surrounding tissue.[31–33] Moreover, lactic acid levels are elevated in these tumors. CA IX, is a high activity isoform, which catalyses CO_2 hydration to bicarbonate in the presence of high amounts of lactic acid/lactate. Importantly, we found that CA IX has low sensitivity to inhibition by lactate (Table 1). This phenomenon may represent an evolutionary adaptation of CA IX to working in this harsh environment, characterized by the presence of high extracellular concentrations of lactate/lactic acid, enhanced acidity and reducing conditions (hypoxia). It is also interesting to note that the oxidized analogue of L-lactate, pyruvate, is a CA IX inhibitor (K_I of 1.12 mM) but in the reducing conditions present in hypoxic tumors, it is probable that all pyruvate is reduced to lactate. Thus, although rather similar to CA II from the point of view of its catalytic properties,[1–5] CA IX is evolved to assure an efficient CO_2 hydration activity in tumors characterized by a lower pH than the physiological one, high concentrations of lactate/lactic acid and hypoxia (reducing environment). MCT-mediated efflux of the anaerobic metabolic waste product, lactate, in hypoxic tumors will be facilitated by the presence of CAIX, with its extracellular catalytic site. The H+ cotransported by MCT would be rapidly converted to CO_2 by CA IX, thus enhancing the driving force for lactate efflux. It is also probable that CA IX participates in metabolons involving AEs, NBCs, or MCTs, proteins present in high amounts in tumor cells, and involved in the transport of acid/base equivalents and thus pH regulation.

4. Conclusion

We report here the first detailed inhibition study of five CA isozymes with carboxylates including aliphatic (formate, acetate), dicarboxylic (oxalate, malonate), hydroxy/keto acids (L-lactate, L-malate, pyruvate), tricarboxylic (citrate), or aromatic (benzoate and tetrafluorobenzoate) representatives, some of which are important intermediates in the Krebs cycle. The cytosolic isozyme hCA I was strongly activated by acetate, oxalate, pyruvate, L-lactate, and citrate (K_A around 0.1 μM), whereas formate, malonate, malate, and benzoate were weaker activators (K_A in the range 0.1–1 mM). The cytosolic isozyme hCA II was weakly inhibited by all the investigated anions, with inhibition constants in the range of 0.03–24 mM. The membrane-associated isozyme hCA IV was the most sensitive to inhibition by carboxylates, showing a K_I of 99 nM for citrate and oxalate, of 2.8 μM for malonate and of 14.5 μM for pyruvate among others. The mitochondrial isozyme hCA V was weakly inhibited by all these carboxylates (K_Is in the range of 1.67–25.9 mM), with the best inhibitor being citrate (K_I of 1.67 mM), whereas this is the most resistant CA isozyme to pyruvate inhibition (K_I of 5.5 mM), which may be another proof that CA V is the isozyme involved in the transfer of acetyl groups from the mitochondrion to the cytosol for the provision of substrate(s) for de novo lipogenesis. Furthermore, the relative resistance of CA V to inhibition by pyruvate may be an evolutionary adaptation of this mitochondrial isozyme to the presence of high concentrations of this anion within this organelle. The transmembrane, tumor-associated isozyme hCA IX was similarly to isozyme II slightly inhibited by all these anions (K_I in the range of 1.12–7.42 mM) except acetate, lactate, and benzoate, which showed a $K_I > 150$ mM. The lactate insensitivity of CA IX also represents an interesting finding, since it is presumed that this isozyme evolved in such a way as to show a high catalytic activity in hypoxic tumors rich in lactate, and suggests a possible metabolon in which CA IX participates together with the monocarboxylate/H^+ co-transporter in dealing with the high amounts of lactate/H^+ present in tumors.

Acknowledgements

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References and notes

1. Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enz. Inhib. Med. Chem.* **2004**, *19*, 199–229.
2. *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton, FL, 2004; pp 1–363, and references cited therein.
3. (a) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Pat.* **2004**, *14*, 667–702; (b) Supuran, C. T. *Expert Opin. Ther. Pat.* **2003**, *13*, 1545–1550.
4. (a) Supuran, C. T.; Scozzafava, A. *Curr. Med. Chem.—Imm., Endoc. Metab. Agents* **2001**, *1*, 61–97; (b) Supuran, C. T.; Scozzafava, A. *Expert Opin. Ther. Pat.* **2000**, *10*, 575–600.
5. (a) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146–189; (b) Supuran, C. T.; Scozzafava, A. *Expert Opin. Ther. Pat.* **2002**, *12*, 217–242.

6. McMurtrie, H. L.; Alvarez, B. V.; Loiselle, F. B.; Sterling, D.; Morgan, P. E.; Cleary, H. J.; Johnson, D. E.; Casey, J. R. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 231–236.
7. Sterling, D.; Reithmeier, R. A.; Casey, J. R. *J. Biol. Chem.* **2001**, *276*, 47886–47894.
8. (a) Sterling, D.; Casey, J. R. *Biochem. Cell Biol.* **2002**, *80*, 483–497; (b) Sterling, D.; Alvarez, B. V.; Casey, J. R. *J. Biol. Chem.* **2002**, *277*, 25239–25246.
9. (a) Sterling, D.; Brown, N. J. D.; Supuran, C. T.; Casey, J. R. *Am. J. Physiol.—Cell Physiol.* **2002**, *283*, C1522–C1529; (b) Alvarez, B.; Loiselle, F. B.; Supuran, C. T.; Schwartz, G. J.; Casey, J. R. *Biochemistry* **2003**, *42*, 12321–12329.
10. Sly, W. S.; Waheed, A.; Shah, G.; Zhou, X. Y. In Sixth International Conference on the Carbonic Anhydrases, 20–25 June 2003, Smolenice, Slovakia, Abstract Book, p 23.
11. Pelis, R. M.; Renfro, J. L. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2004**, *287*, 491–501.
12. Ilies, M. A.; Banciu, M. D. Nonsulfonamide carbonic anhydrase inhibitors. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton, FL, 2004; pp 209–242.
13. Franchi, M.; Vullo, D.; Gallori, E.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2857–2861.
14. Vullo, D.; Franchi, M.; Gallori, E.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *J. Enz. Inhib. Med. Chem.* **2003**, *18*, 403–406.
15. (a) Innocenti, A.; Zimmerman, S.; Ferry, J. G.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3327–3331; (b) Innocenti, A.; Zimmerman, S.; Ferry, J. G.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4563–4567.
16. Innocenti, A.; Lehtonen, J. M.; Parkkila, S.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5435–5439.
17. (a) Bertini, I.; Luchinat, C.; Scozzafava, A. *Struct. Bond.* **1982**, *48*, 45–92; (b) Bertini, I.; Canti, G.; Luchinat, C.; Scozzafava, A. *J. Am. Chem. Soc.* **1978**, *100*, 4873–4877.
18. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573. An SX.18MV-R Applied Photophysics stopped-flow instrument has been used. 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. 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 water) and dilutions up to 0.1 nM done with the assay buffer mentioned above. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The recombinant CA isozymes have been obtained as previously reported.[16]
19. Supuran, C. T.; Vullo, D.; Manole, G.; Casini, A.; Scozzafava, A. *Curr. Med. Chem.—Cardiovasc. Hematol. Agents* **2004**, *2*, 49–68.
20. (a) Ilies, M.; Scozzafava, A.; Supuran, C. T. Carbonic Anhydrase Activators. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton, FL, 2004; pp 317–352; (b) Supuran, C. T.; Scozzafava, A. Activation of Carbonic Anhydrase Isozymes. In *The Carbonic Anhydrases—New Horizons*; Chegwidden, W. R., Carter, N., Edwards, Y., Eds.; Birkhäuser: Basel, Switzerland, 2000; pp 197–219.
21. Briganti, F.; Mangani, S.; Orioli, P.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T. *Biochemistry* **1997**, *36*, 10384–10392.
22. Briganti, F.; Iaconi, V.; Mangani, S.; Orioli, P.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T. *Inorg. Chim. Acta* **1998**, *275–276*, 295–300.
23. Smith, K. S.; Ingram-Smith, C.; Ferry, J. G. *J. Bacteriol.* **2002**, *184*, 4240–4245.
24. Chegwidden, W. R.; Dodgson, S. J.; Spencer, I. M. The Roles of Carbonic Anhydrase in Metabolism, Cell Growth and Cancer in Animals. In *The Carbonic Anhydrases—New Horizons*; Chegwidden, W. R., Edwards, Y., Carter, N., Eds.; Birkhäuser: Basel, 2000; pp 343–363.
25. Vullo, D.; Franchi, M.; Gallori, E.; Antel, J.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2004**, *47*, 1272–1279.
26. Poole, R. C.; Halestrap, A. P. *Am. J. Physiol.* **1993**, *264*, C761–C782.
27. Becker, H. M.; Broer, S.; Deitmer, J. W. *Biophys. J.* **2004**, *86*, 235–247.
28. Hamann, S.; Kiilgaard, J. F.; la Cour, M.; Prause, J. U.; Zeuthen, T. *Exp. Eye Res.* **2003**, *76*, 493–504.
29. Gabel, G.; Aschenbach, J. R.; Muller, F. *Anim. Health Res. Rev.* **2002**, *3*, 15–30.
30. Eladari, D.; Chambrey, R.; Irinopoulou, T.; Leviel, F.; Pezy, F.; Bruneval, P.; Paillard, M.; Podevin, R. A. *J. Biol. Chem.* **1999**, *274*, 28420–28426.
31. Pastorekova, S.; Pastorek, J. Cancer-related Carbonic Anhydrase Isozymes. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton, FL, 2004; pp 253–280.
32. (a) Brown, J. M.; Wilson, W. R. *Nature Rev. Cancer* **2004**, *4*, 437–447; (b) Höpfl, G.; Ogunshola, O.; Gassmann, M. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2004**, *286*, R608–R623.
33. Swinson, D. E.; Jones, J. L.; Cox, G.; Richardson, D.; Harris, A. L.; O'Byrne, K. J. *Int. J. Cancer* **2004**, *111*, 43–50.