Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 1909–1913
Bioorganic & Medicinal Chemistry Letters

Carbonic anhydrase inhibitors. Inhibition of isozymes I, II, IV, V and IX with complex fluorides, chlorides and cyanides

Alessio Innocenti,[a] Jochen Antel,[b,*] Michael Wurl,[b] Daniela Vullo,[a] Michael A. Firnges,[b] Andrea Scozzafava[a] and Claudiu T. Supuran[a,*]

[a]*Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy*
[b]*Solvay Pharmaceuticals Research Laboratories, Hans Böckler-Allee 20, D-30173 Hannover, Germany*

Received 16 December 2004; revised 27 January 2005; accepted 31 January 2005

Abstract—The inhibition of five human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes, the cytosolic hCA I and II, the membrane-bound hCA IV, the mitochondrial hCA V and the tumour associated, transmembrane hCA IX, with complex anions incorporating fluoride, chloride and cyanide, as well as B(III), Si(IV), P(V), As(V), Al(III), Fe(II), Fe(III), Pd(II), Pt(II), Pt(IV), Cu(I), Ag(I), Au(I) and Nb(V) species has been investigated. Apparently, the most important factors influencing activity of these complexes are the nature of the central metal ion/element, and its charge. Geometry of these compounds appears to be less important, since both linear, tetrahedral, octahedral as well as pentagonal bipyramidal derivatives led to effective inhibitors. However, the five isozymes showed very different affinities for these anion inhibitors. The best hCA I inhibitors were cyanide, dicyanocuprate and dicyanoaurate (K_Is in the range of 0.5–7.7 μM), whereas the least effective were fluoride and hexafluoroarsenate. The best hCA II inhibitors were cyanide, hexafluoroferrate and tetrachloroplatinate (K_Is in the range of 0.02–0.51 mM), whereas the most ineffective ones were fluoride, hexafluoroaluminate and chloride. The best hCA IV inhibitors were dicyanocuprate (K_I of 9.8 μM) and hexacyanoferrate(II) (K_I of 10.0 μM), whereas the worst ones were tetrafluoroborate and hexafluoroaluminate (K_Is in the range of 124–126 mM). The most effective hCA V inhibitors were cyanide, heptafluoroniobate and dicyanocuprate (K_Is in the range of 0.015–0.79 mM), whereas the most ineffective ones were fluoride, chloride and tetrafluoroborate (K_Is in the range of 143–241 mM). The best hCA IX inhibitors were on the other hand cyanide, heptafluoroniobate and dicyanoargentate (K_Is in the range of 4 μM–0.33 mM), whereas the worst ones were hexacyanoferrate(III) and hexacyanoferrate(II).

1. Introduction

Anions, such as halides, pseudohalides, sulfide, act as inhibitors of many types of metalloenzymes due to their capacity of binding metal ions within the active site of such enzymes.[1,2] A particularly well-studied case is represented by the carbonic anhydrases (CAs, EC 4.2.1.1), zinc enzymes that catalyze the interconversion between carbon dioxide and bicarbonate, widely distributed all over the phylogenetic tree.[2–5] These enzymes are inhibited by inorganic, simple metal-complexing anions (such as those mentioned above) as well as by anions, which show a lower tendency to bind metal ions in solution, such as sulfate, nitrate or perchlorate, among others.[6–10]

In several previous contributions from these laboratories,[7–10] we have investigated the interaction between various α-, β- and γ-CA isozymes, such as the human isozymes hCA IV, V, IX and XIII, or the archeal isozymes Cab and Cam with a series of simple anions such as halides, pseudohalides, nitrate, sulfate, carbonate, bicarbonate, etc., or with phosphates/phosphonates.[11] Several interesting facts emerged from such studies, which allowed us, for example, to hypothesize the involvement of the newly isolated cytosolic isozyme XIII in a metabolon with anion exchangers involved in the bicarbonate/chloride transport,[10a] or to establish that the renal side effects of the antiviral drug foscarnet are probably due to its strong inhibition of the membrane-associated isozyme CA IV, highly abundant in this organ where it plays a critical role in urine formation and excretion of anions.[11] Thus, further investigations of anion inhibitors of CAs may allow a better understanding of the physiological roles of these widely

* Corresponding authors. Tel.: +49 511 8572154; fax: +49 511 8572195 (J.A.); tel.: +39 055 4573005; fax: +39 055 4573385 (C.T.S.); e-mail addresses: jochen.antel@solvay.com; claudiu.supuran@unifi.it

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.01.082

distributed enzymes, or may lead to the design of inhibitors with pharmacological applications.[2–5]

Since anions belonging to coordination compounds have not been investigated in detail up to now (only some qualitative data of CA II inhibition with ferrocyanide are mentioned by Maren,[12] whereas Scozzafava's group[1] investigated the interaction of Co(II)-substituted CA II with dicyanocuprate and dicyanoaurate, by means of electronic and NMR spectroscopy, but no precise inhibition constant measurements were provided) we report here the first inhibition study of five α-CA isozymes (i.e., the cytosolic isozymes I and II, the membrane-bound isozyme IV, the mitochondrial isozyme V as well as the transmembrane, tumour-associated isozyme IX) with complex anions containing fluoride, chloride or cyanide. A series of such complex halides/pseudohalides, among which tetrafluoroborate; hexafluorosilicate; hexafluorophosphate(V)/arsenate(V); hexafluoroaluminate; hexafluoroferrate(III); heptafluoroniobate(V); tetrachloroplatinate(II); hexachloroplatinate(IV); dicyanocuprate(I); dicyanoargentate(I); dicyanoaurate(I); tetracyanopalladate(II); hexacyanoferrate(II) and hexacyanoferrate(III) have been included in our study.

2. Chemistry

Buffers and sodium/potassium salts (tetrafluoroborate; hexafluorosilicate; hexafluorophosphate(V); hexafluoroarsenate(V); hexafluoroaluminate; hexafluoroferrate(III); heptafluoroniobate(V); tetrachloroplatinate(II); hexachloroplatinate(IV); dicyanocuprate(I); dicyanoargentate(I); dicyanoaurate(I); tetracyanopalladate(II); hexacyanoferrate(II) and hexacyanoferrate(III)) were of highest purity available, from Sigma–Aldrich (Milano, Italy) and were used without further purification. CA isozymes were prepared as previously reported by our group.[7–10]

3. CA inhibition data

Inhibition data against five CA isozymes involved in critical physiological/pathological processes, that is, hCA I, hCA II (cytosolic forms), hCA IV (membrane associated), hCA V (mitochondrial) and hCA IX (transmembrane, tumour associated),[13–17] with the above mentioned anions are shown in Table 1. Inhibition data for fluoride, chloride and cyanide (the anions from which the complexes investigated here are derived) are also provided for comparison, as they were recently reported by our groups.[7–10]

Data in Table 1 allow us to draw the following conclusions regarding CA isozyme interaction with the anions investigated here (i) against hCA I, cyanide, dicyanocuprate and dicyanoaurate act as very potent inhibitors, with inhibition constants in the range of 0.5–7.7 μM. The most potent inhibitor is just cyanide, whereas its participation in the complex anions mentioned above leads to a slightly diminished inhibitory capacity. Other complex cyanides, such as dicyanoargentate, tetracyanopalladate and the two hexacyanoferrates are on the other hand much weaker hCA I inhibitors, with K_Is in the range of 0.50–9.5 mM. Clearly, the nature of the central metal ion (and its geometry to a lower degree) seems to be the most important parameters influencing potency among these complex anions. Submillimolar hCA I inhibitors were also hexafluoroferrate(III), hepta-

Table 1. Inhibition constants of anionic inhibitors against human 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[b]	hCA V[c]	hCA IX[d]
F^-[e]	>300	>300	0.07	241	48
Cl^-[e]	6	200	0.09	156	33
CN^-[e]	0.5×10^{-3}	0.02	0.77	0.015	0.004
BF_4^-	11.6	25.5	126	143	18.3
SiF_6^{2-}	2.9	6.4	4.7	1.8	7.5
PF_6^-	11.0	17.5	19.1	>300	8.1
AsF_6^-	>300	9.4	5.5	75.6	7.9
$[AlF_6]^{3-}$	52.4	>300	124	43.6	18.5
$[FeF_6]^{3-}$	0.20	0.44	0.34	2.71	1.62
$[NbF_7]^{2-}$	0.20	0.60	0.50	0.44	0.10
$[PtCl_4]^{2-}$	0.37	0.51	27.5	41.7	35.2
$[PtCl_6]^{2-}$	13.8	6.23	17.4	10.8	3.63
$[Cu(CN)_2]^-$	6.9×10^{-3}	0.56	9.8×10^{-3}	0.79	4.77
$[Ag(CN)_2]^-$	0.50	0.76	97	1.75	0.33
$[Au(CN)_2]^-$	7.7×10^{-3}	0.58	5.36	1.91	31.3
$[Pd(CN)_4]^{2-}$	1.27	1.22	1.69	2.00	0.89
$[Fe(CN)_6]^{4-}$	5.74	9.7	0.01	3.13	87
$[Fe(CN)_6]^{3-}$	9.5	39.0	1.00	9.8	>300

[#] Errors were in the range of 3–5% of the reported values, from three different assays.
[a] Human recombinant isozymes.
[b] Truncated human isozyme lacking the first 20 amino acid residues.
[c] Full length human isozyme.
[d] Catalytic domain of human, recombinant isozyme.
[e] From Refs. 7–10.

fluoroniobate and tetrachloroplatinate(II), with K_Is in the range of 0.20–0.37 mM, whereas the other Pt(IV) derivative showed much weaker inhibitory properties (K_I of 13.8 mM, as compared to 6 mM for Cl^-). Thus, clearly it is the central metal ion/element present in these complexes that modulates the binding to the zinc ion within the enzyme active site. Fluoride is a completely inefficient hCA I inhibitor (Table 1), but some of its coordination compounds, such as hexafluorosilicate, hexafluorophosphate and tetrafluoroborate act as good inhibitors, with K_Is in the range of 2.9–11.6 mM. It is amazing on the other hand that the isosteric (with hexafluorosilicate and hexafluorophosphate) anion hexafluoroarsenate is again devoid of inhibitory activity, similarly with F^- (Table 1); (ii) against hCA II, the physiologically most relevant isozyme, the best inhibitors were cyanide, hexafluoroferrate, heptafluoroniobate, tetrachloroplatinate(II), dicyanocuprate, dicyanoargentate and dicyanoaurate, which showed inhibition constants in the range of 0.02–0.76 mM. Less effective inhibitors were hexafluorosilicate, hexafluoroarsenate, hexachloroplatinate(IV), tetracyanopalladate(II) and hexacyanoferrate(II), with K_Is in the range of 1.22–9.7 mM. Tetrafluoroborate, hexafluorophosphate and hexacyanoferrate(III) were weak inhibitors, with K_Is in the range of 17.5–39.0 mM, whereas chloride, fluoride and hexafluoroaluminate were very weak inhibitors (K_I of 200 mM for chloride) or were totally ineffective in inhibiting hCA II. Thus, SAR seem to be again dominated by the nature of the central element/metal ion present in these complexes, with all the complex cyanides less effective hCA II inhibitors as compared to cyanide, whereas all the complex fluorides (except hexafluoroaluminate) and all the complex chlorides being much more effective inhibitors as compared to F^- or Cl^-; (iii) the membrane-associated isozyme hCA IV was also inhibited by all these simple and complex anions, but with potencies very different as compared to those of the cytosolic isozymes mentioned above. Thus, fluoride and chloride were shown to be quite potent hCA IV inhibitors,[10b] with inhibition constants in the range of 70–90 μM, whereas the 'metal-poison' anion cyanide is a much weaker hCA IV inhibitor, with a K_I of only 0.77 mM.[10b] Interestingly, all the complex fluorides/chlorides investigated here showed less inhibitory effects against this isozyme as compared to the parent, uncomplexed anion fluoride/chloride, whereas for the complex cyanides the situation is more complicated, with both very effective as well as very ineffective inhibitors detected (Table 1). Thus, the best hCA IV inhibitors in this series of coordination compounds were dicyanocuprate and hexacyanoferrate(II), with inhibition constants in the range of 9.8–10.0 μM, being thus 77–78.5 times more inhibitory than CN^-, and also the best hCA IV anion inhibitors investigated here. Good inhibitors were also hexafluorosilicate, hexafluoroarsenate, hexafluoroferrate(III), heptafluoroniobate, tetracyanopalladate(II), dicyanoaurate and hexacyanoferrate(III), with K_Is in the range of 0.34–5.5 mM. It is surprising the difference in activity between the two hexacyanoferrates, with the Fe(II) derivative 100 times more active than the Fe(III) derivative. It is difficult to explain this finding, considering the fact that these two anions possess a very similar geometry. Probably the charge of the central metal ion provokes increased repulsions with the Zn(II) ion within the enzyme active site, since against all isozymes the Fe(III) derivative was less active as compared to the Fe(II) one. Still, the Nb(V) derivative NbF_7^{2-} is generally an active CA inhibitor although it possesses a formal charge of 5^+. Modest hCA IV inhibitors were hexafluorophosphate, and the two Pt(II) and Pt(IV) complexes, which showed inhibition constants in the range of 17.4–27.5 mM. The most ineffective inhibitors were tetrafluoroborate, hexafluoroaluminate and dicyanoargentate, with K_Is in the range of 97–126 mM; (iv) the mitochondrial isozyme hCA V is weakly inhibited by fluoride and chloride (similarly to hCA II, probably due to the participation of both isozymes in metabolons with anion exchangers involved in the chloride/bicarbonate transport)[19] and quite sensitive to cyanide, which shows a K_I of 15 μM.[7] The coordination compounds incorporating the first two anions were (again with one exception, hexafluorophosphate) more inhibitory than F^- or Cl^-, whereas all the complex cyanides were less inhibitory as compared to CN^-. Thus, the best hCA V inhibitors were hexafluorosilicate, hexafluoroferrate(III), heptafluoroniobate, dicyanocuprate, dicyanoargentate, dicyanoaurate, tetracyanopalladate(II) and hexacyanoferrate(II), which showed inhibition constants in the range of 0.44–3.13 mM. Rather effective inhibitors were two other anions, hexachloroplatinate(IV) and hexacyanoferrate(III), which showed K_Is in the range of 9.8–10.8 mM, whereas tetrafluoroborate, hexafluoroarsenate, hexafluoroaluminate and tetrachloroplatinate(II) were less effective, with K_Is in the range of 41.7–143 mM; (v) the tumour-associated isozyme hCA IX is moderately susceptible to inhibition by fluoride and chloride (K_Is in the range of 33–48 mM) and very sensitive to cyanide (K_Is of 4 μM).[8] The complex fluorides/chlorides were more inhibitory than F^-/Cl^- (with the usual exception, which is in this case tetrachloroplatinate), whereas all the complex cyanides were much less inhibitory than CN^- (Table 1). The best hCA IX inhibitors in the series of coordination compounds were hexafluoroferrate(III), heptafluoroniobate, hexachloroplatinate(IV), dicyanocuprate, dicyanoargentate and tetracyanopalladate(II), which showed K_Is in the range of 0.10–4.77 mM. Less inhibitory were the complex fluorides incorporating B(III), Si(IV), P(V), As(V) and Al(III) (K_Is in the range of 7.5–18.5 mM), whereas the most ineffective CA IX inhibitors were tetrachloroplatinate, dicyanoaurate and hexacyanoferrate(II), with K_Is in the range of 31.3–87 mM. Hexacyanoferrate(III) was the weakest hCA IX inhibitor in the entire series of anions investigated up to now, for reasons difficult to explain at this moment; (vi) the five isozymes investigated here showed very different affinities for these anion inhibitors; thus, the best hCA I inhibitors were cyanide, dicyanocuprate and dicyanoaurate (K_Is in the range of 0.5–7.7 μM), whereas the least effective were fluoride and hexafluoroarsenate. The best hCA II inhibitors were cyanide, hexafluoroferrate and tetrachloroplatinate (K_Is in the range of 0.02–0.51 mM), whereas the most ineffective ones were fluoride, hexafluoroaluminate and chloride. The best hCA IV inhibitors were dicyanocuprate (K_I of 9.8 μM) and

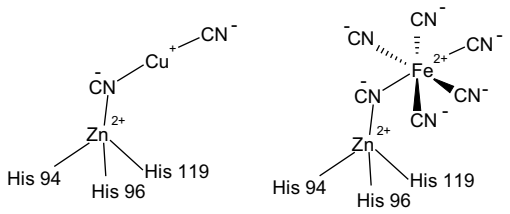

Figure 1. Schematic binding of two complex anions investigated here (dicyanocuprate(I) and hexacyanoferrate(II)) to the CA active site (the Zn(II) protein ligands—His 94, 96 and 119—are shown, as they are conserved in all isozymes investigated here). Presumably, in all complexes Zn(II) is in tetrahedral geometry, with no water bound to it, as in the complex with cyanide.[6]

hexacyanoferrate(II) (K_I of 10.0 μM), whereas the worst ones were tetrafluoroborate and hexafluoroaluminate (K_Is in the range of 124–126 mM). The most effective hCA V inhibitors were cyanide, heptafluoroniobate and dicyanocuprate (K_Is in the range of 0.015–0.79 mM), whereas the most ineffective ones were fluoride, chloride and tetrafluoroborate (K_Is in the range of 143–241 mM). The best hCA IX inhibitors were on the other hand cyanide, heptafluoroniobate and dicyanoargentate (K_Is in the range of 4 μM–0.33 mM), whereas the worst ones were hexacyanoferrate(III) and hexacyanoferrate(II).

As already demonstrated earlier by Scozzafava's group,[1] by using electronic spectroscopy and NMR in paramagnetic systems, by means of Co(II)-substituted CA II, complex anions such as dicyanocuprate/dicyanoaurate directly bind to the metal ion within the enzyme active site. It is probable that in such adducts, one of the anions present in the complex (fluoride, chloride or cyanate) acts as a bridging, bidentate ligand, substituting the zinc-bound water molecule/hydroxide ion,[6] as exemplified in Figure 1 for dicyanocuprate(I) and hexacyanoferrate(II).

4. Conclusion

Here we report a detailed inhibition study of five CA isozymes with complex anions incorporating fluoride, chloride and cyanide, as well as B(III), Si(IV), P(V), As(V), Al(III), Fe(II), Fe(III), Pd(II), Pt(II), Pt(IV), Cu(I), Ag(I), Au(I) and Nb(V) species. Apparently, the most important factors influencing activity of these compounds are the nature of the central metal ion/element, and its charge. Geometry of these compounds appears to be less important, since both linear, tetrahedral, octahedral as well as pentagonal bipyramidal such derivatives led to effective inhibitors. However, the five isozymes investigated here showed very different affinities for these anion inhibitors: thus, the best hCA I inhibitors were cyanide, dicyanocuprate and dicyanoaurate (K_Is in the range of 0.5–7.7 μM), whereas the least effective were fluoride and hexafluoroarsenate. The best hCA II inhibitors were cyanide, hexafluoroferrate and tetrachloroplatinate (K_Is in the range of 0.02–0.51 mM), whereas the most ineffective ones were fluoride, hexafluoroaluminate and chloride. The best hCA IV inhibitors were dicyanocuprate (K_I of 9.8 μM) and hexacyanoferrate(II) (K_I of 10.0 μM), whereas the worst ones were tetrafluoroborate and hexafluoroaluminate (K_Is in the range of 124–126 mM). The most effective hCA V inhibitors were cyanide, heptafluoroniobate and dicyanocuprate (K_Is in the range of 0.015–0.79 mM), whereas the most ineffective ones were fluoride, chloride and tetrafluoroborate (K_Is in the range of 143–241 mM). The best hCA IX inhibitors were on the other hand cyanide, heptafluoroniobate and dicyanoargentate (K_Is in the range of 4 μM–0.33 mM), whereas the worst ones were hexacyanoferrate(III) and hexacyanoferrate(II).

References and notes

1. (a) Scozzafava, A.; Bertini, I. In *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker: New York, 1991; Vol. 12, pp 31–74; (b) Bertini, I.; Luchinat, C.; Scozzafava, A. *Struct. Bond.* **1982**, *48*, 45–92; (c) Bertini, I.; Canti, G.; Luchinat, C.; Scozzafava, A. *J. Am. Chem. Soc.* **1978**, *100*, 4873–4877.
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) Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199–229; (b) Supuran, C. T.; Vullo, D.; Manole, G.; Casini, A.; Scozzafava, A. *Curr. Med. Chem.—Cardiovasc. Hematol. Agents* **2004**, *2*, 49–68.
4. (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; (c) Supuran, C. T.; Scozzafava, A. *Curr. Med. Chem.—Immunol. Endocrinol. Metab. Agents* **2001**, *1*, 61–97; (d) 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. 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), USA, 2004; pp 209–242.
7. Franchi, M.; Vullo, D.; Gallori, E.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2857–2861.
8. Vullo, D.; Franchi, M.; Gallori, E.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 403–406.
9. (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.
10. (a) Innocenti, A.; Lehtonen, J. M.; Parkkila, S.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5435–5439; (b) Innocenti, A.; Firnges, M. A.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5769–5773.
11. Rusconi, S.; Innocenti, A.; Vullo, D.; Mastrolorenzo, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5763–5767.
12. Maren, T. H. *Physiol. Rev.* **1967**, *47*, 595–781.

13. Pastorekova, S.; Pastorek, J. Cancer-Related Carbonic Anhydrase Isozymes and their Inhibition. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton (FL), USA, 2004; pp 253–280.
14. (a) Brown, J. M.; Wilson, W. R. *Nat. Rev. Cancer* **2004**, *4*, 437–447; (b) Höpfl, G.; Ogunshola, O.; Gassmann, M. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2004**, *286*, R608–R623.
15. Winum, J.-Y.; Vullo, D.; Casini, A.; Montero, J.-L.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2003**, *46*, 2197–2204.
16. Švastová, E.; Hulíková, A.; Rafajová, M.; Zatovicová, M.; Gibadulinová, A.; Casini, A.; Cecchi, A.; Scozzafava, A.; Supuran, C. T.; Pastorek, J.; Pastoreková, S. *FEBS Lett.* **2004**, *577*, 439–445.
17. Robertson, N.; Potter, C.; Harris, A. L. *Cancer Res.* **2004**, *64*, 6160–6165.
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.[7–10]
19. (a) 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; (b) Sterling, D.; Reithmeier, R. A.; Casey, J. R. *J. Biol. Chem.* **2001**, *276*, 47886–47894.