Bioorganic & Medicinal Chemistry Letters 19 (2009) 650–653

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Carbonic anhydrase inhibitors: Inhibition studies of a coral secretory isoform with inorganic anions

Anthony Bertucci [a], Alessio Innocenti [b], Didier Zoccola [a,*], Andrea Scozzafava [b], Denis Allemand [a], Sylvie Tambutté [a], Claudiu T. Supuran [b,*]

[a] Centre Scientifique de Monaco, Avenue Saint-Martin, MC-98000 Principality of Monaco, Monaco
[b] University of Florence, Dipartimento di Chimica, Via della Lastruccia, 3, Rm. 188, Polo Scientifico, 50019 Sesto Fiorentino (Firenze), Italy

ARTICLE INFO

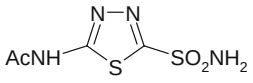
Article history:
Received 18 November 2008
Revised 11 December 2008
Accepted 12 December 2008
Available online 24 December 2008

Keywords:
Carbonic anhydrase
Coral
Enzyme inhibitor
Inorganic anion
Carbonate
Iodide
Biomineralization

ABSTRACT

The inhibition of a coral carbonic anhydrase (CA, EC 4.2.1.1) has been investigated with a series of inorganic anions such as halogenides, pseudohalogenides, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, perchlorate, sulfate. The full-length scleractinian coral *Stylophora pistillata* CA, STPCA, has a significant catalytic activity for the physiological reaction of CO_2 hydration to bicarbonate, similarly to the ubiquitous human isoforms hCA I (cytosolic) and hCA VI (secreted). The best STPCA anion inhibitors were bromide, iodide, carbonate, and sulfamate, with inhibition constants of 9.0–10.0 μM.

Carbonic anhydrases (CA, EC 4.2.1.1) are ubiquitous metalloenzymes that catalyze the reversible hydration of carbon dioxide into bicarbonate and protons: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. There are at least five classes of CAs with polyphyletic origin[1–6]: the α-class (in vertebrates, invertebrates, bacteria and some chlorophytes), the β-class (in eubacteria and chlorophytes), the γ-class (archea and some eubacteria), and the δ- and ζ-family (present only in marine diatoms). Among their roles in physiological processes, CAs are involved in biomineralization both in invertebrates and vertebrates, although the ensuing mechanisms are poorly understood at this moment.[7–11] Scleractinian corals are major biomineralizing organisms which precipitate an aragonitic calcium carbonate ($CaCO_3$) skeleton. As early as 1959, Goreau[7] showed the first evidence of the involvement of CA in coral calcification. Indeed, he demonstrated that the sulfonamide CA inhibitor acetazolamide reduces calcification rates, and he suggested that the enzyme has a role in removing carbonic acid from the skeletogenic sites during the calcification process.[7] Since then, numerous authors have observed that CA inhibitors decrease calcification rates, being thus suggested that CAs are involved in the inorganic carbon supply for calcification and/or the regulation of pH at the calcification sites.[12–15] All subsequent studies have then confirmed that sulfonamides (acetazolamide **AZA** or ethoxzolamide) inhibit calcification[12–16] with an inhibition up to 73%.



AZA

We have recently cloned, sequenced and localized an α-CA from the coral *Stylophora pistillata*, named STPCA.[17] We have demonstrated that STPCA is a secreted isoform and due to its specific secretion by the calicoblastic calcifying ectoderm, it was proposed that this enzyme plays a direct role in biomineralization. Many pharmacological studies employing ethoxzolamide or **AZA** have been performed on living corals to determine the potential role of their CAs in calcification.[12–16] However, inhibition studies on purified such enzymes were never reported up to now. The present work is aimed at determining the catalytic and inhibition profile of recombinant STPCA with simple inorganic anions, which are a well-known class of CA inhibitors.[18–26]

CA activity and the inhibition assay with acetazolamide and inorganic anions were performed on coral recombinant STPCA obtained as described earlier.[17] The kinetic parameters for the CO_2

* Corresponding authors. Tel.: +377 97770873; fax: +377 92167981 (D. Zoccola), tel.: +39 055 4573005; fax: +39 055 4573385 (C.T. Supuran).
E-mail addresses: zoccola@centrescientifique.mc (D. Zoccola), claudiu.supuran@unifi.it (C.T. Supuran).

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.12.056

hydration reaction by STPCA, as well as for the human isoforms, hCA I, hCA II and hCA VI are shown in Table 1. The results show that STPCA has a moderate activity level, in the same order of magnitude as the cytosolic isoform hCA I and the human secreted isoform hCA VI, but lower than the cytosolic isoform hCA II. Moreover, STPCA is highly inhibited by acetazolamide, similarly to hCA II and hCA VI. Relative to the kinetic parameters and the inhibition by acetazolamide, it can be concluded that STPCA shows characteristics similar to the secreted isoform hCA VI, that is, STPCA is moderately active as a CO_2 hydration catalyst but is highly inhibited by acetazolamide.

Apart from the sulfonamides such as acetazolamide,[2] anions constitute the second major class of CA inhibitors.[18–26] Indeed, being zinc-containing enzymes, CAs bind both metal-complexing as well as metal non-complexing anions with different affinities.[2,18–26] All 13 catalytically active human CA isoforms have been investigated for their interaction with anions, such as the typical metal poisons cyanide, (thio)cyanate, hydrogen sulfide, azide, etc., but also for their interaction with anions which show less propensity to complex metal ions in solution (such as sulfate, nitrate, perchlorate, etc.).[2,18–26]

In this study, buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, and sulfate) were of highest purity available, and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid were also commercially available compounds.

Table 1
Kinetic parameters for the CO_2 hydration reaction[30] catalyzed by the human cytosolic α-hCA isozymes I, II, the secreted isoform hCA VI and STPCA at 20 °C and pH 7.5 in 10 mM Hepes buffer, and their inhibition data with acetazolamide **AZA** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide),[31] a clinically used drug

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I	Moderate	2.0×10^5	5.0×10^7	250
hCA II	Very high	1.4×10^6	1.5×10^8	12
hCA VI	Moderate	3.4×10^5	4.9×10^7	11
STPCA	Moderate	3.1×10^5	4.6×10^7	16

Table 2
Inhibition constants of anionic inhibitors against isozymes hCA I, II and VI (human, enzymes), and the CA from the coral *Stylophora pistillata*, STPCA, for the CO_2 hydration reaction, at 20 °C[30]

Inhibitor	K_I[#] [mM]			
	hCA I[a]	hCA II[a]	hCA VI[a]	STPCA[b]
F^-	> 300	>300	0.60	0.62
Cl^-	6	200	0.72	0.50
Br^-	4	63	0.73	0.0097
I^-	0.3	26	0.81	0.0090
CNO^-	0.0007	0.03	0.69	0.59
SCN^-	0.2	1.6	0.89	0.68
CN^-	0.0005	0.02	0.07	0.58
N_3^-	0.0012	1.5	0.07	0.52
HCO_3^-	12	85	0.80	0.45
CO_3^{2-}	15	73	0.69	0.010
NO_3^-	7	35	0.76	0.56
NO_2^-	8.4	63	0.82	0.77
HS^-	0.0006	0.04	0.71	0.58
HSO_3^-	18	89	14.2	0.41
SO_4^{2-}	63	>200	9.9	0.91
$H_2NSO_2NH_2$	0.31	1.13	0.07	0.010
H_2NSO_3H	0.021	0.39	0.09	0.81
Ph-$B(OH)_2$	58.6	23.1	0.82	0.68
Ph-AsO_3H_2	31.7	49.2	13.9	0.78

[#] Mean from three different assays.
[a] Human recombinant isozyme, data from Ref. 31.
[b] This work.

The data in Table 2 show the inhibition of STPCA with a set of physiological and non-physiological anions (literature inhibition data of human isozymes I, II, and VI are also included in the table for the sake of comparison, since they are useful for a better understanding of the significance of STPCA inhibition data).[18–26] The following can be observed:

(i) A large number of physiologically relevant, as well as non-physiological anions, such as the halides (fluoride and chloride) and pseudohalides (cyanate, thiocyanate, isocyanide, azide), bicarbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, sulfate, sulfamic acid (as sulfamate anion), phenylboronic acid and phenylarsonic acid, showed a very similar inhibition behavior, with K_Is in the range of 0.41–0.91 mM. The least effective STPCA anion inhibitor was sulfate which showed a K_I of 0.91 mM.

(ii) The most effective STPCA inhibitors were bromide, iodide, carbonate and sulfamide, with K_Is in the range of 9.0–10.0 μM. Thus, STPCA has an anion inhibition profile which is completely different from the cytosolic isozymes (hCA I and hCA II). Indeed, hCA I and hCA II generally have a much lower affinity for most of these anions compared to hCA VI and STPCA. On the other hand, STPCA shows similar catalytic activity and anion inhibition characteristics with its human counterpart, hCA VI (more than half, that is, 10 of 18 investigated anions, have the same range of inhibition for both secretory isoforms, that is, hCA VI and STPCA). However, there are also some differences between these two enzymes. Indeed, the inhibition constants of the two isozymes for fluoride and chloride show a K_I in the same range whereas STPCA show a higher affinity for bromide and iodide than hCA VI (around 100-fold). Similarly, carbonate, bisulfite, sulfate and phenylarsonic acid are more effective as STPCA inhibitors than as hCA VI inhibitors (i.e., 69-, 34-, 10-, and 17-fold, respectively). Finally cyanide, azide, sulfamide are the most effective inhibitors for hCA VI whereas bromide, iodide, carbonate, and sulfamide are the most effective inhibitors for STPCA. It is quite interesting to speculate on the very strong inhibition observed with carbonate against STPCA (K_I of 10 μM). The high susceptibility to this anion (which at pH values >8 is in equilibrium with one of the enzyme substrates, bicarbonate) may be an indication that STPCA evolved as an enzyme mostly active to work as an efficient catalyst for CO_2 hydration to bicarbonate in neutral or slightly acidic conditions, and that the activity of this enzyme may be seriously compromised by the substrate when the pH arrives in the (slightly) alkaline domain. Indeed, bicarbonate is a 45-times weaker STPCA inhibitor as compared to carbonate (Table 2). This raises the problem of the functioning of this CA within the extracellular medium, which likely possess an alkaline pH.[14]

These inhibition data may give some information on metabolic pathways involving CAs. Indeed, in metabolically active tissues, and especially in situations requiring efficient ion transport, many CA isoforms interact with bicarbonate transporters or biosynthetic enzymes to form spatially and functionally orchestrated protein complexes called metabolons.[27] For example, CAs can improve the transmembrane movement of bicarbonate or other membrane-impermeable anions which are transported by integral membrane proteins including the Cl^-/HCO_3^- anion exchangers, Na^+-coupled HCO_3^- co-transporters, and SLC26A transporters.[27,28] Thus, one of the role of CAs (which physically interact with these anion transporters) in these metabolons is to increase the local availability of bicarbonate (or other anions, such as sulfate or carboxylates) and thereby accelerate their transmembrane flux. Via this mechanism, various CAs contribute to the modulation of pH at both sides of plasma membranes. It is thus clear that in addition to their catalytic activity, most CAs are also involved in much more complex processes in which they interact with anions present in

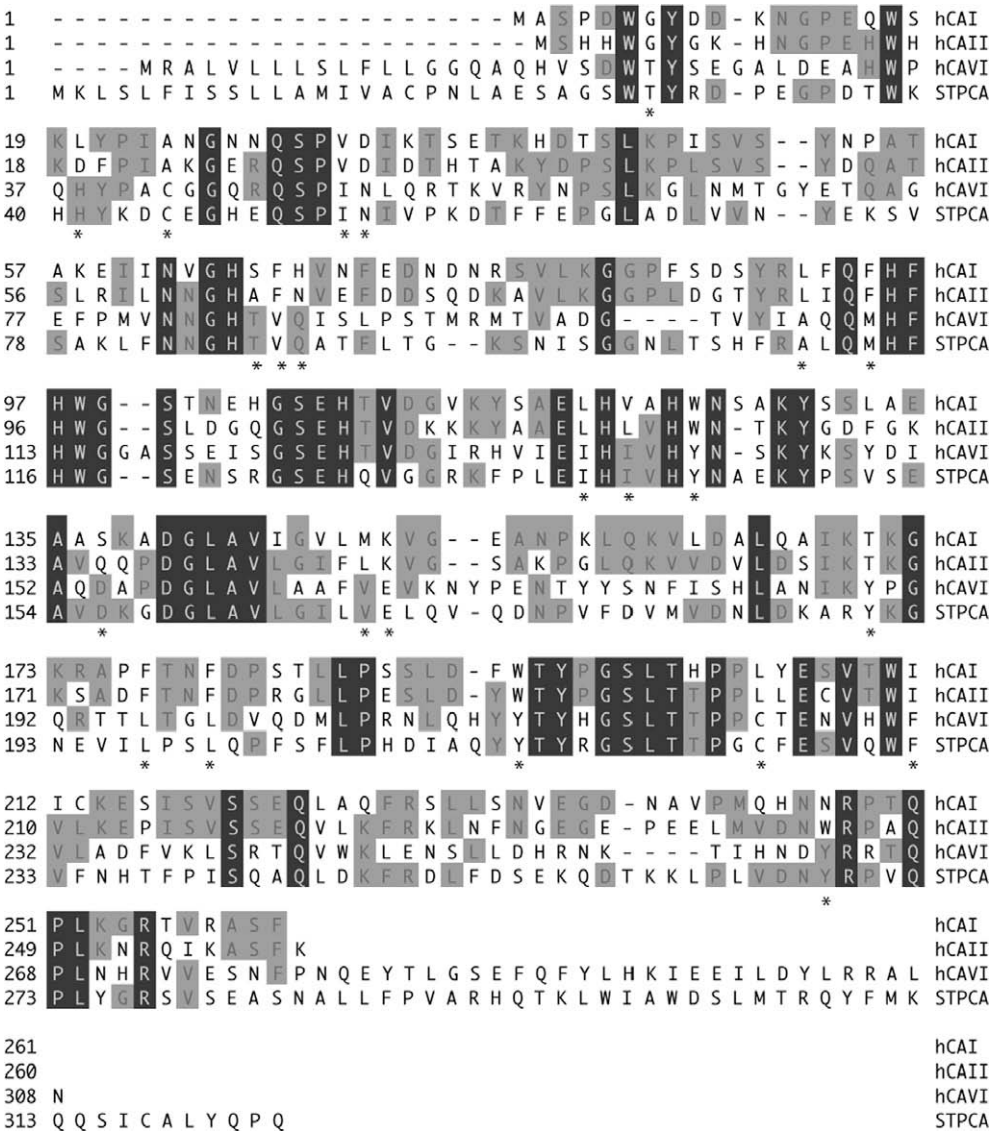


Figure 1. Sequence alignment of the CA domain of human isozymes hCA I, II, VI, and the coral enzyme STPCA. Conserved amino acids (AA) are in black, AA identical in at least 50% of these enzymes are in light grey. Asterisk marks represent common shared residues between STPCA and hCA VI, which are not present in the cytosolic isoforms.

rather high concentrations in various tissues, as well as with their transporters. It is also interesting to note that as the global warming processes continue or even accelerate, in parallel with an enhancement of CO_2 concentration in the atmosphere and sea water, such processes may lead to disequilibria of biomineralization, in which corals play a crucial function. Thus, understanding better how the activity of this enzyme is influenced by inorganic anions, as those investigated here, may a crucial point. Indeed, the seawater contains high concentrations of some of these anions, such as chloride (546 mM); sulfate (28 mM); bromide (0.84 mM); and fluoride (0.068 mM).[29a] It can be observed from data of Table 2, that the chloride, bromide and sulfate present in the sea water may virtually completely inhibit the catalytic activity of STPCA. However, the chemical composition of the sub-calicoblastic extracellular calcifying medium, where the mineralization occurs, is not known but it is estimated to be different from that of the seawater.[29b] In any case, the coral probably adopted strategies to protect the enzyme from the anions present in the seawater, in order to use it for the calcification processes. Further studies are warranted for resolving this problem.

Similarly to all human CAs, STPCA has the conserved three histidine residues acting as Zn(II) ligands, that is, His114, His116, and His127 (corresponding to His94, His96, and His119 of hCA II). The proton shuttle residue (His64, hCA II numbering) is also identical in the human and coral enzymes (residue His86 in STPCA). Identical are also the gate-keeper residues of the two enzymes (Glu106–Thr199, hCA II numbering, corresponding to Glu126–Thr221 in STPCA), which orient the substrate (CO_2) for the nucleophilic attack by the Zn(II) hydroxide species of the enzyme. However, the comparison between the different human CAs used in this study and STPCA shows that, beyond the identical residues important for the catalytic action of all α-CAs, STPCA and hCAVI share some more residues which are not present in the cytosolic isoforms (23 residues indicated by an * in Fig. 1). Obviously, there are also many differences of sequence between the human and coral secretory CAs (Fig. 1), which may be responsible for the different inhibition profiles with some of the investigated anions.

In conclusion, the present work is the first study on the inhibition of an invertebrate CA performed at the molecular level. By comparing k_{cat}/K_M values of Table 1, it can be observed that STPCA shows around 33% of the enzymatic activity of hCA II for the CO_2 hydration reaction. Since hCA II is a very efficient catalyst for this crucial physiological reaction, it can be stated that STPCA shows moderate catalytic activity for this reaction. At the site of calcification in corals,

bicarbonate and CO_2 are the two potential forms of inorganic carbon which can be transported by the calcifying cells.[16] The hydration of CO_2 is thus one major limiting step in which STPCA plays a crucial role in this reaction. In the previous studies, Tambutté et al.[12,16] have shown that cyanide and ethoxzolamide are effective inhibitors of calcification and from the study of Moya et al.[17] and the present one, we can suggest that one of their target is STPCA. The transport of bicarbonate can also be affected by STPCA since, as mentioned above, the role of CAs in metabolons is to increase the local availability of bicarbonate. It is also noteworthy that among the anionic inhibitors, STPCA is very sensitive to carbonate. As the final form of inorganic carbon used for the calcification reaction is carbonate, we can speculate that carbonate plays a feedback inhibitory role on STPCA, thus regulating the inorganic carbon available for calcification. These results, combined to pharmacological experiments performed on living corals, will help to better understand the mechanism of calcification and especially the role CA plays in such processes, and surely warrant further studies.

Acknowledgments

This research was financed in part by a grant of the 6th Framework Programme of the European Union (DeZnIT project), by an Italian FIRB project (MIUR/FIRB RBNE03PX83_001) and by the Centre Scientifique de Monaco Research Program, which is supported by the Government of Principality of Monaco.

References and notes

1. (a) Hewett-Emmett, D.; Tashian, R. E. *Mol. Phylogenet. Evol.* **1996**, *5*, 50; (b) Tripp, B. C.; Smith, K.; Ferry, J. G. *J. Biol. Chem.* **2001**, *276*, 48615.
2. Supuran, C. T. *Nat. Rev. Drug Discov.* **2008**, *7*, 168.
3. So, A. K.; Espie, G. S.; Williams, E. B.; Shively, J. M.; Heinhorst, S.; Cannon, G. C. *J. Bacteriol.* **2004**, *186*, 623.
4. Lane, T. W.; Saito, M. A.; George, G. N.; Pickering, I. J.; Prince, R. C.; Morel, F. M. *Nature* **2005**, *435*, 42.
5. Supuran, C. T.; Scozzafava, A. *Bioorg. Med. Chem.* **2007**, *15*, 4336.
6. Xu, Y.; Feng, L.; Jeffrey, P. D.; Shi, Y.; Morel, F. M. *Nature* **2008**, *452*, 56.
7. Goreau, T. F. *Biol. Bull.* **1959**, *116*, 59.
8. Mitsunaga, K.; Akasaka, K.; Shimada, H.; Fujino, Y.; Yasumasu, I.; Numandi, H. *Cell Differ.* **1986**, *18*, 257.
9. Kakei, M.; Nakahara, H. *Biochim. Biophys. Acta* **1996**, *1289*, 226.
10. Miyamoto, H.; Miyoshi, F.; Kohno, J. *Zool. Sci.* **2005**, *22*, 311.
11. Tohse, H.; Murayama, E.; Ohira, T.; Takagi, Y.; Nagasawa, H. *Comp. Biochem. Physiol.* **2006**, *145B*, 257.
12. Tambutté, E.; Allemand, D.; Mueller, E.; Jaubert, J. *J. Exp. Biol.* **1996**, *199*, 1029.
13. Furla, P.; Galgani, I.; Durand, I.; Allemand, D. *J. Exp. Biol.* **2000**, *203*, 3445.
14. Al-Horani, F. A.; Al-Moghrabi, S. M.; de Beer, D. *Mar. Biol.* **2003**, *142*, 419.
15. Marshall, A. T.; Clode, P. L. *Comp. Biochem. Physiol.* **2003**, *136A*, 417.
16. Tambutté, S.; Tambutté, E.; Zoccola, D.; Caminiti, N.; Lotto, S.; Moya, A.; Allemand, D.; Adkins, J. *Mar. Biol.* **2007**, *161*, 71.
17. Moya, A.; Tambutte, S.; Bertucci, A.; Tambutte, E.; Lotto, S.; Vullo, D.; Supuran, C. T.; Allemand, D.; Zoccola, D. *J. Biol. Chem.* **2008**, *283*, 25475.
18. (a) Vullo, D.; Franchi, M.; Gallori, E.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 403; (b) Innocenti, A.; Lehtonen, J. M.; Parkkila, S.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5435.
19. Winum, J. Y.; Innocenti, A.; Gagnard, V.; Montero, J. L.; Scozzafava, A.; Vullo, D.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1683.
20. (a) Innocenti, A.; Firnges, M. A.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5769; (b) Nishimori, I.; Innocenti, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1037; (c) Innocenti, A.; Vullo, D.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Nishimori, I.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1532.
21. Lehtonen, J. M.; Parkkila, S.; Vullo, D.; Casini, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3757.
22. Innocenti, A.; Vullo, D.; Scozzafava, A.; Casey, J. R.; Supuran, C. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 573.
23. Franchi, M.; Vullo, D.; Gallori, E.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2857.
24. (a) Innocenti, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 567; (b) Casini, A.; Scozzafava, A.; Mincione, F.; Menabuoni, L.; Ilies, M. A.; Supuran, C. T. *J. Med. Chem.* **2000**, *43*, 4884.
25. (a) Innocenti, A.; Antel, J.; Wurl, M.; Vullo, D.; Firnges, M. A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1909; (b) Clare, B. W.; Supuran, C. T. *Eur. J. Med. Chem.* **1999**, *34*, 463.
26. Vullo, D.; Ruusuvuori, E.; Kaila, K.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3139.
27. Sterling, D.; Reithmeier, R. A. F.; Casey, J. R. *J. Biol. Chem.* **2001**, *276*, 47886.
28. McMurtrie, H. L.; Cleary, H. J.; Alvarez, B. V.; Loiselle, F. B.; Sterling, D.; Morgan, P. E.; Johnson, D. E.; Casey, J. R. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 231.
29. (a) Pinet, P. R. *Invitation to Oceanography*; St. Paul: West Publishing Company, 1996. pp 134–135; (b) Allemand, D.; Ferrier-Pagès, C.; Furla, P.; Houlbrèque, F.; Puverel, S.; Reynaud, S.; Tambutté, É.; Tambutté, S.; Zoccola, D. *C. R. Palevol.* **2004**, *3*, 453.
30. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor concentration (ranging from 0.01 μM to 90 mM, with 6–7 different inhibitor concentrations being used to obtain the inhibition curve) at least six traces of the initial 5–10% of the reaction have been used for measuring the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10–100 mM) were prepared in distilled-deionized water and dilutions up to 0.01 μM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[18–26] and represent the mean from at least three different determinations.
31. Supuran, C. T.; Popescu, A.; Ilisiu, M.; Costandache, A.; Banciu, M. D. *Eur. J. Med. Chem.* **1996**, *31*, 439.