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Carbonic anhydrase inhibitors: The X-ray crystal structure of ethoxzolamide complexed to human isoform II reveals the importance of thr200 and gln92 for obtaining tight-binding inhibitors☆

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Abstract—Ethoxzolamide, an almost forgotten inhibitor of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), is the only classical inhibitor whose structure in adduct with any isoform was not reported yet. We report here the inhibition data of this molecule with the 12 catalytically active mammalian isozymes (CA I–CA XIV) and the X-ray crystal structure with the cytosolic, ubiquitous isoform CA II. These data are presumably useful for the design of novel CA inhibitors, targeting various CA isozymes, considering that ethoxzolamide was already the lead molecule to obtain the second generation inhibitors, dorzolamide and brinzolamide, clinically used antiglaucoma agents with topical action, as well as various other investigational agents.

Carbonic anhydrases (CAs, EC 4.2.1.1) are metallo-enzymes that catalyze a very simple physiological reaction, the conversion of the carbon dioxide to the bicarbonate ion and protons.[1–4] In humans 15 different α-CAs isoforms were isolated, 12 of which are catalytically active (CAs I–VA, CAVB, CAVI, CA VII, CA IX, and CAs XII–XIV), whereas the CA-related proteins (CARPs VIII, X, and XI) are devoid of catalytic activity. Among the active isozymes five are cytosolic (CA I, CA II, CA III, CA VII, and CAXIII), four are membrane-associated (CA IV, CA IX, CA XII, and CA XIV), CA VA and CA VB are mitochondrial isoforms, and CA VI is secreted in saliva.[1–10] The active site of most CAs contains a Zn^{2+} ion which is essential for catalysis. It is coordinated to three protein histidine residues, His94, His96, and His119, in a tetrahedral geometry with H_2O or OH^- as the fourth ligand. The latter is the active species, acting as a potent nucleophile.[5–7]

The CA-catalyzed reaction is involved in respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, tumorigenicity, and many other physiological and pathological processes.[8–10] As a consequence in the last years many of the CA isozymes have become important therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders.[1–10]

Two main classes of CA inhibitors (CAIs) are known: the metal-complexing anions, and the unsubstituted sulfonamides and their bioisosteres (sulfamates and sulfamides), which bind to the Zn^{2+} ion of the enzyme either by substituting the non-protein zinc ligand to

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☆ Coordinates and structure factors have been deposited with the Protein Data Bank (Accession code 3CAJ).
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generate a tetrahedral adduct or by addition to the metal coordination sphere, generating trigonal-bipyramidal species.[1–10] Acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, and dichlorophenamide **4**, as well as the more recent drugs dorzolamide **5** and brinzolamide **6** (Fig. 1) are classical sulfonamide CAIs clinically used in the therapy of different pathologies such as glaucoma, acid–base disequilibria, epilepsy and other minor neuromuscular disorders, or as diuretics.[1–5] The inhibitory effects of these molecules against the mammalian isoforms CA I–XIV (of human, hCA, or murine, mCA origin) are shown in Table 1. As can be seen from these data, a serious drawback of most of these CAIs is the lack of selectivity for inhibiting the different isozymes with various medicinal chemistry applications.[11–16]

X-ray crystallography is obviously a very useful tool for the rational drug design of more selective enzyme inhibitors, and excellent examples for its applications to CAI drug design targeting various isozymes are available.[1,2b,6–8,17–21] Thus, all sulfonamides **1**–**6** except ethoxzolamide **3** have been crystallized in adducts with various isozymes, such as CA I, II, III, IV, VA, and XII among others.[1,2b,6–8,17–21] The fact that ethoxzolamide **3** has been neglected from this point of view is rather surprising for at least two reasons: (i) ethoxzolamide is a drug (indeed not widely utilized nowadays) mainly used for the treatment of edema due to congestive heart failure, and for drug-induced edema, in addition to its applications as antiglaucoma agent;[1,2,22] (ii) ethoxzolamide was clearly the lead molecule used to design the second generation CAIs dorzolamide **5** and brinzolamide **6**. In fact, the structural similarities between the three drugs are immediately obvious. However, as seen from data of Table 1, although these compounds present a similar chemical structure, they have a rather different inhibition profile. In fact, while ethoxzolamide **3** indiscriminately inhibits all CA isozymes except CA III,[11] in the nanomolar or subnanomolar range, dorzolamide **5** and brinzolamide **6** present selectivity for the inhibition of some isozymes over the other ones. Indeed the newer agents do not inhibit appreciably CA I, CA III, and CA IV. Thus, to identify the molecular basis responsible for the different inhibition profile of these compounds, the crystal structure of ethoxzolamide **3** in complex with the main CA isozyme, hCA II, has been solved and compared with the previously reported structures of the adducts hCA II–**5**[17f] and hCA II–**6**.[17c]

The hCA II–**3** complex was prepared and crystallized using experimental conditions previously reported for other sulfonamide CA inhibitors[23] and the structure was solved using the difference Fourier method, using the structure of the native hCA II as starting model.[23–27] Following the X-ray crystallographic refinement a clear continuous feature of electron density was present in the active site. Inhibitor **3** was perfectly accommodated to the shape of this electron density (Fig. 2). The binding of ethoxzolamide **3** to the enzyme active site did not perturb the enzyme 3D structure. As a matter of fact, the superimposition of all the Cα atoms of the hCA II protein in the investigated adduct with those of the protein in the unbound form[18] yielded a rmsd value of 0.33 Å. The main protein–inhibitor interactions observed for this hCA II–sulfonamide adduct are schematically depicted in Figure 3. It may be observed that ethoxzolamide participates in various

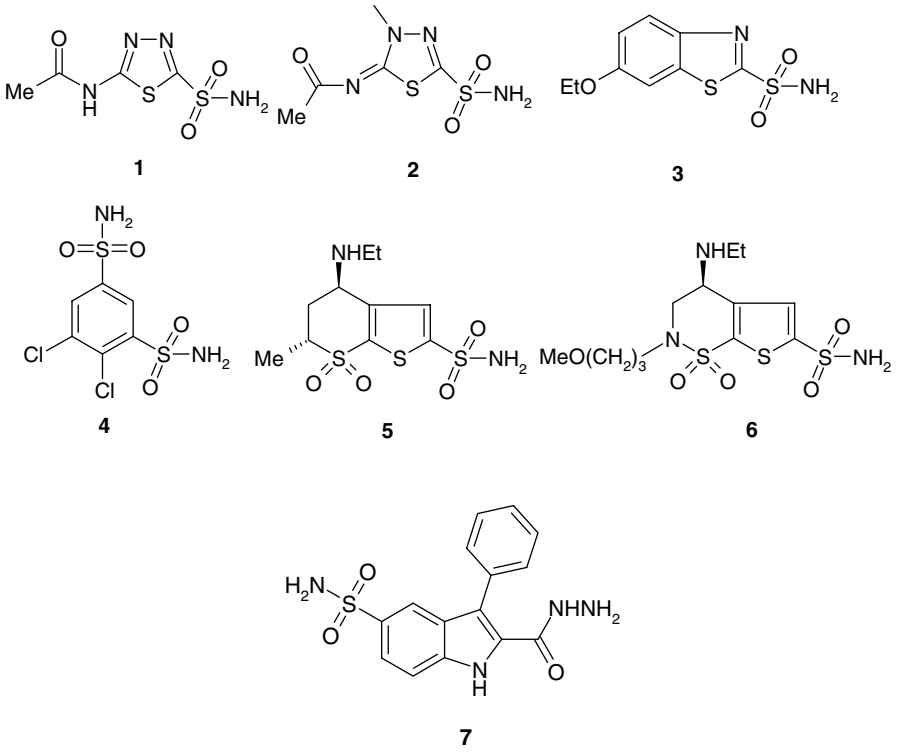

Figure 1. Chemical structure of the clinically used CAIs **1**–**6** and of the new candidate **7**.

Table 1. Inhibition data with some of the clinically used sulfonamides **1**–**7** against isozymes I–XIV (the isoforms CA VIII, X, and XI are devoid of catalytic activity and probably do not bind sulfonamides as they do not contain Zn^{2+} ions)

Isozyme[a]	K_I[b] (nM)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
hCA I[c]	250	50	25	374	50,000	45,000	7.5
hCA II[c]	12	14	8	9	9	3	7.2
hCA III[c]	2×10^5	7×10^5	1×10^6	6.3×10^5	7.7×10^5	1.1×10^5	1.4×10^6
hCA IV[c]	74	6200	93	95	8500	3950	9000
hCA VA[c]	63	65	25	81	42	50	1100
hCA VB[c]	54	62	19	91	33	30	1100
hCA VI[c]	11	10	43	134	10	0.9	2650
hCA VII[c]	2.5	2.1	0.8	6	3.5	2.8	89
hCA IX[d]	25	27	34	43	52	37	102
hCA XII[d]	5.7	3.4	22	56	3.5	3.0	110
mCA XIII[c]	17	19	50	1450	18	10	2633
hCA XIV[c]	41	43	25	1540	27	24	48

[a] h, human; m, murine isozyme.
[b] Mean from at least three different determinations by a stopped flow CO_2 hydrase assay.
[c] Full-length enzyme.
[d] Catalytic domain.

Table 2. Crystal parameters, data collection and refinement statistics

Cell parameter	
Space group	*P2*[a]
a, *b*, *c* (Å)	42.21, 41.36, 72.03
β (Å)	104.35
Data collection statistics	
Resolution (Å)	20.00–1.80
Temperature (K)	100
Total reflections	66,927
Unique reflections	21,636
Completeness (%)[a]	95.9 (80.8)
R_{sym}[a,b]	0.061 (0.134)
Mean *I*/sigma (*I*)[a]	14.9 (8.4)
Refinement statistics	
Resolution (Å)	20.00–1.80
R_{factor}[c] (%)	18.0
R_{free}[c] (%)	21.4
Rmsd from ideal geometry	
Bond lengths (Å)	0.007
Bond angles (°)	1.4
Number of protein atoms	2088
Number of inhibitor atoms	16
Number of water molecules	303
Average *B* factor (Å^b)	13.95

[a] Values in parentheses refer to the outermost data shell.
[b] $R_{sym} = \Sigma|I_i - \langle I \rangle|/\Sigma I_i$; over all reflections.
[c] $R_{factor} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$; R_{free} calculated with 5% of data withheld from refinement.

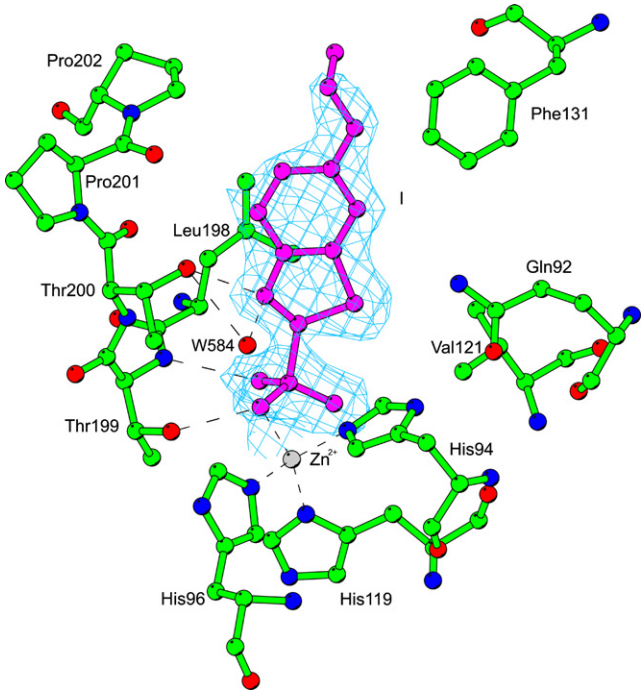

Figure 2. Active site region in the hCA II–**3** complex. The simulated annealing omit $|2F_o - F_c|$ electron density map relative to the inhibitor molecule, computed at 1.80 Å and contoured at 1.0σ, is shown.

hydrophobic and polar interactions with residues delimitating the active site cavity, when bound to hCA II. In particular, its deprotonated sulfonamide group is complexed to the metal ion present within the hCA II active site, similarly to what is observed for the same moiety in other inhibitors whose structure has been solved in complex with the enzyme (Fig. 3).[6,7] Moreover, the 6-ethoxy-benzothiazolyl scaffold was found to be located in the active site channel being involved in several van der Waals interactions with the side chains of residues Gln92, Val121, Phe131, Leu198, Thr200, Pro201, and Pro202. Finally, the endocyclic nitrogen atom of the inhibitor was observed to be hydrogen bonded to Thr200OG1 atom both directly and through a bridging water molecule (see Fig. 3).

As the second generation CAIs dorzolamide and brinzolamide clearly show a close structural similarity to ethoxzolamide, as mentioned earlier, we performed a structural overlay of ethoxzolamide **3** and dorzolamide **5**/brinzolamide **6**, when complexed to hCA II (Fig. 4A and B), in order to understand the influence of the additional substituents present in the second generation inhibitors, on the affinity for the enzyme. Figure 4 shows

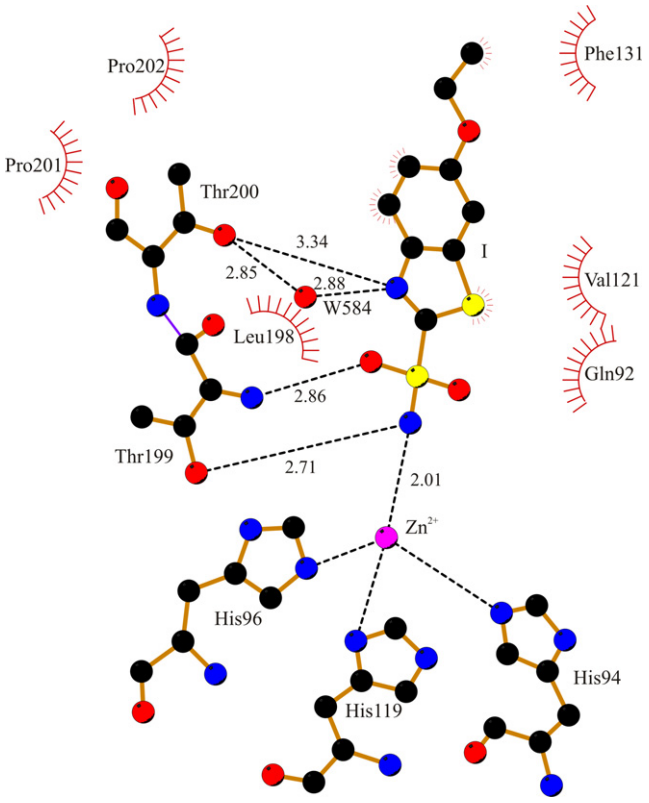

Figure 3. Schematic representation of the active site region in the hCA II–**3** complex, showing residues participating in recognition of the inhibitor molecule. Hydrogen bonds and the active site Zn^{2+} ion coordination are also shown (dotted lines).

that the binding of these compounds to the enzyme active site is rather similar. Particularly, the three heterocyclic rings of inhibitors **3**, **5**, and **6** overlap quite well, all of them establishing both polar and hydrophobic interactions with residues delimiting the enzyme active site. Worth noting is that in all the three hCA II–inhibitor adducts a key role in the stabilization of the inhibitor within the enzyme active site is played by the protein residue Thr200, which is hydrogen bonded to the endocyclic nitrogen atom in the case of hCA II–**3** adduct and to the nitrogen of the ethylamino substituent in the case of hCA II–**5** and hCA II–**6** adducts (see Fig. 4A and B). The endocyclic SO_2 moieties of dorzolamide **5** and brinzolamide **6** participate in an additional H-bond interaction with residue Gln92 (Fig. 4A and B), which with ethoxzolamide makes only a van der Waals interaction (Fig. 3). From this structural comparison it emerges that although compounds **5** and **6** present two bulky substituents on the bicyclic ring with respect to ethoxzolamide **3**, these moieties do not interfere negatively with the inhibitory properties of these molecules toward hCA II. As a consequence all three derivatives **3**, **5**, and **6** show comparable inhibition constants toward hCA II (see Table 1). On the contrary the presence of the bulky substituents may have a significant effect in the inhibition of other CA isoforms, generating the differences in the inhibition profile observed in Table 1, as probably they interact (positively or in clashes) with various amino acid residues which are different in the diverse CA isozymes. As an example the different inhibition behavior of these three molecules toward hCA I will be analyzed. Data of Table 1 show that while ethoxzolamide **3** is a good inhibitor of this isoform (K_I of 25 nM), compounds **5** and **6** do not inhibit significantly this enzyme (K_Is in the range of 45–50 μM). Several amino acid 'substitutions' are observed between hCA II and hCA I active sites, namely Phe131/Leu, Ile91/Phe, Glu69/Asn,

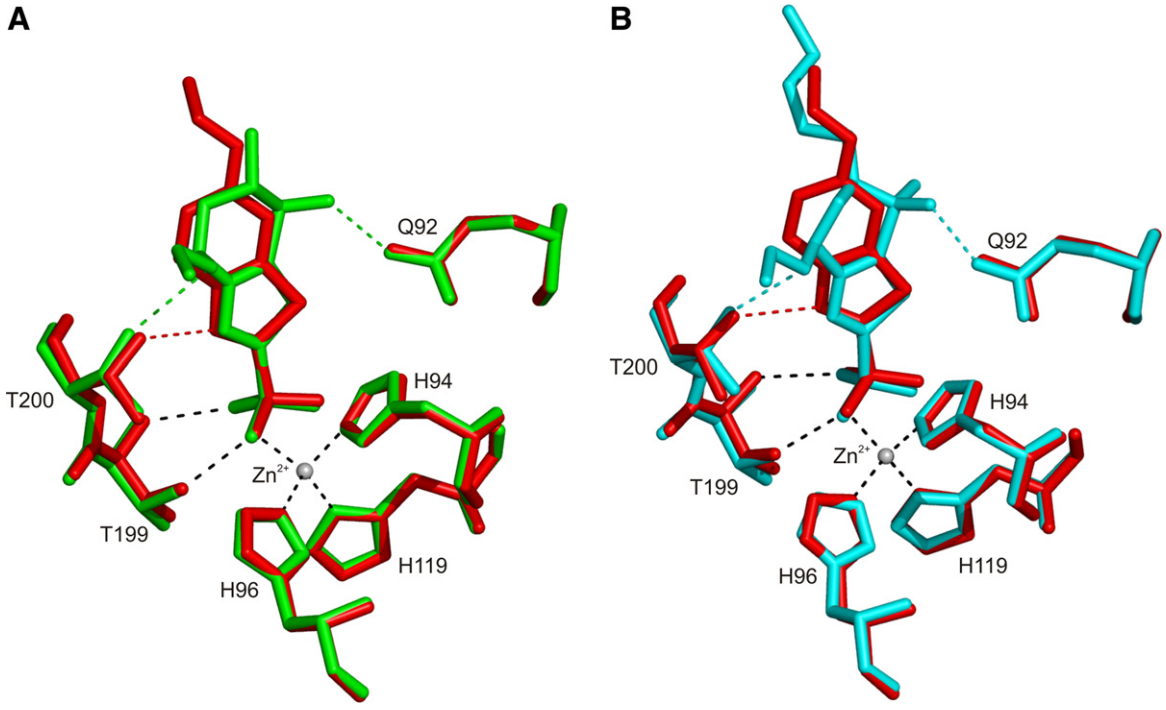

Figure 4. Superposition of hCA II–inhibitor adducts: **3** is reported in red, **5** in green (**A**) and **6** in cyan (**B**). The Zn^{2+} ion and its three catalytic histidines are also shown.

Asn67/His, Asn62/Val, Thr200/His, Val135/Ala, and Leu204/Tyr. Among them, the replacement of Thr200 in hCA II by His200 in hCA I could be responsible of the very different binding of the three studied inhibitors to the active site of these enzymes. Thus, whereas in the case of the ethoxzolamide this substitution causes only the loss of the hydrogen bond interaction between the Thr200OG1 atom and the endocyclic nitrogen atom of the inhibitor, in the case of brinzolamide and dorzolamide this amino acid substitution probably causes not only the loss of a hydrogen bond but also a significant steric hindrance between the rather bulky histidine residue and the ethylamino moieties present in inhibitors **5** and **6**. The presence of another moiety in position 6 of the bicyclic rings of **5** and **6**, that is, a methyl moiety in dorzolamide and a methoxypropyl one in brinzolamide, probably does not allow a compact rearrangement of these molecules within the hCA I active site, thus hampering a productive binding and explaining their ineffective hCA I inhibitory activity as compared to ethoxzolamide **3**.

In conclusion, the structural analysis reported here, for the complex of ethoxzolamide with the ubiquitous CA isoform II, suggests that the introduction of bulky substituents on the bicyclic ring system of CAIs may represent a powerful strategy to obtain compounds with diverse inhibition profiles and selectivity for the various mammalian CAs. Some success in this field has been recently recorded, although a lot of work still has to be performed. An interesting example may be furnished by sulfonamide **7**, recently reported by Güzel et al.[28] This compound has also been designed considering ethoxzolamide **3** as lead molecule. The main difference between **3** and **7** consists in the fact that: (i) the sulfamoyl moiety is attached on the benzene ring in **7** and not on the five-membered heterocyclic one, as in ethoxzolamide **3**; (ii) the two compounds possess rather isosteric groups in the 2 position (a sulfamoyl moiety in **3** and a carbohydrazide one in **7**), but the last compound incorporates a very bulky substituent next to it (in the 3rd position), that is, a phenyl moiety, which is absent in ethoxzolamide. Analyzing the inhibition data of this new molecule (Table 1), it may be observed that it is a very effective inhibitor of isoforms CA I and II, a weaker inhibitor of isozymes CA VII, IX, XII, and XIV (in the range of 48–110 nM), whereas it does not inhibit all other isoforms. Thus, the introduction of a very bulky substituent to the bicyclic ring system makes compound **7** a much more selective CAI as compared to all derivatives **1**–**6** discussed above (and with clinical applications).[1–5] These findings may allow important lessons for the drug design of CAIs incorporating such bicyclic rings.

References and notes

1. Supuran, C. T. *Nat. Rev. Drug Discov.* **2008**, *7*, 168.
2. (a) Supuran, C. T.; Scozzafava, A.; Conway, J. *Carbonic Anhydrase—Its Inhibitors and Activators*; CRC Press: Boca Raton, 2004, pp 1–363; (b) Köhler, K.; Hillebrecht, A.; Schulze Wischeler, J.; Innocenti, A.; Heine, A.; Supuran, C. T.; Klebe, G. *Angew. Chem., Int. Ed.* **2007**, *46*, 7697.
3. (a) Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199; (b) Supuran, C. T.; Scozzafava, A.; Casini, A. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton, 2004; pp 67–147.
4. (a) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146; (b) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Pat.* **2004**, *14*, 667; (c) Winum, J. Y.; Montero, J. L.; Scozzafava, A.; Supuran, C. T. *Mini-Rev. Med. Chem.* **2006**, *6*, 921.
5. (a) Lehtonen, J.; Shen, B.; Vihinen, M.; Casini, A.; Scozzafava, A.; Supuran, C. T.; Parkkila, A. K.; Saarnio, J.; Kivela, A. J.; Waheed, A.; Sly, W. S.; Parkkila, S. *J. Biol. Chem.* **2004**, *279*, 2719; (b) Supuran, C. T. *Curr. Top. Med. Chem.* **2007**, *7*, 825; (c) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Pat.* **2006**, *16*, 1627; (d) Supuran, C. T.; Scozzafava, A. *Bioorg. Med. Chem.* **2007**, *15*, 4336.
6. (a) Alterio, V.; Vitale, R. M.; Monti, S. M.; Pedone, C.; Scozzafava, A.; Cecchi, A.; De Simone, G.; Supuran, C. T. *J. Am. Chem. Soc.* **2006**, *128*, 8329; (b) De Simone, G.; Di Fiore, A.; Menchise, V.; Pedone, C.; Antel, J.; Casini, A.; Scozzafava, A.; Wurl, M.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2315.
7. (a) Casini, A.; Antel, J.; Abbate, F.; Scozzafava, A.; David, S.; Waldeck, H.; Schafer, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 841; (b) Abbate, F.; Casini, A.; Owa, T.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 217.
8. (a) Weber, A.; Casini, A.; Heine, A.; Kuhn, D.; Supuran, C. T.; Scozzafava, A.; Klebe, G. *J. Med. Chem.* **2004**, *47*, 550; (b) Menchise, V.; De Simone, G.; Alterio, V.; Di Fiore, A.; Pedone, C.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2005**, *48*, 5721.
9. Hilvo, M.; Rafajová, M.; Pastoreková, S.; Pastorek, J.; Parkkila, S. *J. Histochem. Cytochem.* **2004**, *52*, 1313.
10. (a) Kyllönen, M. S.; Parkkila, S.; Rajaniemi, H.; Waheed, A.; Grubb, J. H.; Shah, G. N.; Sly, W. S.; Kaunisto, K. *J. Histochem. Cytochem.* **2003**, *51*, 1217; (b) Kaunisto, K.; Parkkila, S.; Rajaniemi, H.; Waheed, A.; Grubb, J.; Sly, W. S. *Kidney Int.* **2002**, *61*, 2111.
11. Nishimori, I.; Minakuchi, T.; Onishi, S.; Vullo, D.; Cecchi, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2007**, *15*, 7229.
12. Innocenti, A.; Firnges, M. A.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1149.
13. Mincione, F.; Scozzafava, A.; Supuran, C. T. *Curr. Top. Med. Chem.* **2007**, *7*, 849.
14. (a) Thiry, A.; Dogné, J. M.; Masereel, B.; Supuran, C. T. *Trends Pharmacol. Sci.* **2006**, *27*, 566; (b) Svastova, E.; Hulikova, A.; Rafajova, M.; Zat'ovicova, M.; Gibadulinova, A.; Casini, A.; Cecchi, A.; Scozzafava, A.; Supuran, C. T.; Pastorek, J.; Pastorekova, S. *FEBS Lett.* **2004**, *577*, 439.
15. (a) Supuran, C. T. *Expert Opin. Ther. Pat.* **2003**, *13*, 1545; (b) De Simone, G.; Supuran, C. T. *Curr. Top. Med. Chem.* **2007**, *7*, 879.
16. Thiry, A.; Dognè, J.-M.; Masereel, B.; Supuran, C. T. *Curr. Top. Med. Chem.* **2007**, *7*, 855.
17. (a) Boriack-Sjodin, P. A.; Heck, R. W.; Laipis, P. J.; Silverman, D. N.; Christianson, D. W. *Proc Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10949; (b) Boriack, P. A.; Christianson, D. W.; Kingery-Wood, J.; Whitesides, G. M. *J. Med. Chem.* **1995**, *38*, 2286; (c) Stams, T.; Chen, Y.; Boriack-Sjodin, P. A.; Hurt, J. D.; Liao, J.; May, J. A.; Dean, T.;

Laipis, P.; Silverman, D. N.; Christianson, D. W. *Protein Sci.* **1998**, *7*, 556; (d) Kim, C. Y.; Chang, J. S.; Doyon, J. B.; Baird, T. T.; Fierke, C. A.; Jain, A.; Christianson, D. W. *J. Am. Chem. Soc.* **2000**, *122*, 12125; (e) Whittington, D. A.; Waheed, A.; Ulmasov, B.; Shah, G. N.; Grubb, J. H.; Sly, W. S.; Christianson, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9545; (f) Smith, G. M.; Alexander, R. S.; Christianson, D. W.; McKeever, B. M.; Ponticello, G. S.; Springer, J. P.; Randall, W. C.; Baldwin, J. J.; Habecker, C. N. *Protein Sci.* **1994**, *3*, 118.

18. (a) Eriksson, A. E.; Jones, T. A.; Liljas, A. *Proteins Struct. Funct.* **1988**, *4*, 274; (b) Vidgren, J.; Svensson, A.; Liljas, A. *Int. J. Biol. Macromol.* **1993**, *15*, 97.
19. (a) Lloyd, M. D.; Pederick, R. L.; Natesh, R.; Woo, L. W.; Purohit, A.; Reed, M. J.; Acharya, K. R.; Potter, B. V. *Biochem. J.* **2005**, *385*, 715; (b) Lloyd, M. D.; Thiyagarajan, N.; Ho, Y. T.; Woo, L. W.; Sutcliffe, O. B.; Purohit, A.; Reed, M. J.; Acharya, K. R.; Potter, B. V. *Biochemistry* **2005**, *44*, 6858.
20. Gruneberg, S.; Stubbs, M. T.; Klebe, G. *J. Med. Chem.* **2002**, *45*, 3588.
21. Alterio, V.; De Simone, G.; Monti, S. M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4201.
22. (a) Maren, T. H. *Physiol. Rev.* **1967**, *47*, 595; (b) Jackson, E. K. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics*; Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., Gilman, A. G., Eds., 9th ed.; McGraw-Hill: New York, 1996; pp 685–713; (c) Maren, T. H. In *Orphan Drugs*; Karch, F. E., Ed.; Marcel Dekker: New York and Basel, 1982; pp 89–115.
23. hCA II–**3** complex was obtained by adding a 5-M excess of inhibitor to a 10-mg/mL protein solution in 100 mM Tris–HCl, pH 8.5. Crystals of the complex were obtained using the hanging drop vapor diffusion technique. In particular 5 μL of complex solution and 5 μL of precipitant solution (2.5 M $(NH_4)_2SO_4$, 0.3 M NaCl, 100 mM Tris–HCl (pH 8.2), and 1 mM DTT) were mixed and suspended over a reservoir containing 1 mL of precipitant solution at 20 °C. Crystals grew within 3 days. X-ray diffraction data were collected at 100 K, at the Synchrotron source Elettra in Trieste, Italy, using a Mar CCD detector. Prior to cryogenic freezing, the crystals were transferred to the precipitant solution with the addition of 15% (v/v) glycerol. Data were integrated and reduced using the HKL crystallographic data reduction package (Denzo/Scalepack).[24] Diffraction data for each crystal were indexed in the $P2_1$ space group with one molecule in the asymmetric unit. Unit cell parameters and data reduction statistics are reported in Table 2. The atomic coordinates of hCA II refined at 2.0 Å resolution (PDB entry 1CA2)[18] were used as a starting model for crystallographic refinement after the deletion of non-protein atoms. An initial round of rigid body refinement followed by simulated annealing and individual *B* factor refinement was performed using the program CNS 1.1.[25] Model visualization and rebuilding were performed using the graphics program O.[26] Inhibitor molecule was identified from peaks in $|F_o|-|F_c|$ maps and was gradually built into the model over several rounds of refinement. Water molecules were built into peaks $>3\sigma$ in $|F_o|-|F_c|$ maps that demonstrated appropriate hydrogen-bonding geometry. The correctness of stereochemistry was finally checked using PROCHECK.[27] Final refinement statistics for the hCA II-inhibitor complex structure are presented in Table 2.
24. Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1997**, *276*, 307.
25. Brünger, A. T.; Adams, P. D.; Clore, G. M.; De Lano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Crystallogr. Sect. D* **1998**, *54*, 905.
26. Jones, T. A.; Zou, J. Y.; Cowan, S. W.; Kjeldgaard, M. *Acta Crystallogr. Sect. A.* **1991**, *47*, 110.
27. Laskowski, R. A.; MacArthur, M. W.; Moss, D. S.; Thornton, J. M. *J. Appl. Crystallogr.* **1993**, *26*, 283.
28. Güzel, O.; Temperini, C.; Innocenti, A.; Scozzafava, A.; Salman, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 152.