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Carbonic anhydrase activators: Activation of the β-carbonic anhydrase from the pathogenic yeast *Candida glabrata* with amines and amino acids

Alessio Innocenti [a], Worraanong Leewattanapasuk [b], Gheorghe Manole [c], Andrea Scozzafava [a], Fritz A. Mühlschlegel [b], 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] *School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom*
[c] *Colentina Hospital, Department of Internal Medicine, Sos. Colentina 120, Bucharest, Romania*

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ABSTRACT

The protein encoded by the *NCE103* gene of *Candida glabrata*, a β-carbonic anhydrase (CA, EC 4.2.1.1) designated as CgCA, was investigated for its activation with amines and amino acids. CgCA was weakly activated by amino acids such as L-/D-His, L-Phe, L-DOPA, and L-Trp and by histamine or dopamine (K_As of 21.2–37 μM) but more effectively activated by D-Phe, D-DOPA, D-Trp as well as serotonin, pyridyl-alkyl-amines, aminoethyl-piperazine/morpholine (K_As of 10.1–16.7 μM). The best activators were L-/D-Tyr, with activation constants of 7.1–9.5 μM. This study may bring a better understanding of the catalytic/activation mechanisms of β-CAs from pathogenic fungi.

The recent characterization of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) in fungi[1,2] led us to initiate a systematic search for modulators of these enzymes activities, that is, inhibitors and activators, which might show therapeutic potential.[3–5] Indeed, among the five independently-evolved (α, β, γ, δ, and ζ) classes of CAs reported up to date, the α-class from mammalian sources has been studied to a far greater extent than the other four classes.[3] Yet, CAs other than those belonging to the α-class are widely distributed in nature, with the β-CAs being the most widespread such catalysts for the interconversion between carbon dioxide and the bicarbonate ions.[1–5] Recent work has shown that various CAs are present in metabolically diverse species, from both the *Archaea* and *Bacteria* but also in microscopic eukaryotes, such as yeast and other pathogenic fungi, indicating that these enzymes have a more extensive and fundamental role than originally recognized.[1,3–8] It is now known that the genomes of basidiomycetes and hemiascomycetouos yeasts contain only β-CAs whereas the filamentous ascomycetes also possess α-class CAs in addition to the β-CAs.[1] Muhlschlegel's group showed[2a] that inhibition (with ethoxzolamide, a sulfonamide CA inhibitor) of the fungal β-CAs from pathogenic species such as *Candida albicans* or *Cryptococcus neoformans* lead to the inhibition of the growth of the fungus, in certain conditions.

Candida glabrata is a pathogenic fungus which, similar to *C. albicans*, possesses a recently characterised β-CA isoform encoded by the *NCE103* gene.[9] *C. glabrata* is a common cause of mucosal and invasive, systemic infections, accounting for 15% of *Candida* infections in the general patient population.[10,11] Furthermore, this pathogen is innately resistant to azole antifungal agents and it is frequently reported to be resistant or less susceptible to other clinically used antifungal agentsl.[12] Recently, we have shown that[9] this β-CA has a significant enzymatic activity for the hydration of carbon dioxide to bicarbonate and protons, and, that similar to β-CAs from other fungal species (e.g., *C. albicans, C. neoformans* and *Saccharomyce cerevisiae*), is inhibited by the major classes of CA inhibitors (CAIs): the inorganic anions and the sulfonamides and their bioisosteres.[13–16]

Inhibition of CAs, mainly the mammalian α-CAs, was investigated in great detail[3,17,18] with several clinically used CA inhibitors (CAIs) having applications as diuretics, antiglaucoma, antiobesity or anticancer agents/diagnostic tools.[3] CA activators (CAAs), however, received less attention, and this class of enzyme modulators started to be investigated systematically for their interaction with mammalian α-CAs only in the past 10 years.[19] Indeed, our groups have reported several kinetic and X-ray crystallographic studies regarding the interaction between all mammalian isoforms (CA I–XV) with amino acid and amine activators, also unravelling the activation mechanism of these enzymes.[19–22] Furthermore, very recently we have extended the CA activation studies to the β-class

* Corresponding author. Tel.: +39 055 4573005; fax: +39 055 4573835.
E-mail address: claudiu.supuran@unifi.it (C.T. Supuran).

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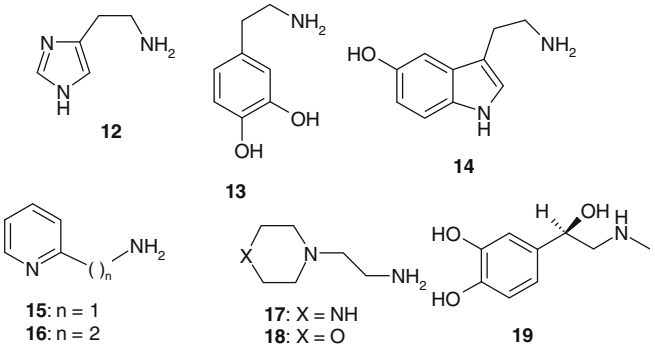
enzymes. We have reported the first CA activation studies of Cab, a β-CA from the archaeon *Methanobacterium thermoautotrophicum*[23] as well as the *S. cerevisiae* β-class enzyme Nce103p,[24] encoded by the *NCE103* gene, which in fact can be found in all basidiomycetes and hemiascomycetous yeasts.[1,2] Here, we extend our CA activation studies of β-CAs to *C. glabrata* CA (CgCA), an homologous enzyme to *S. cerevisiae* Nce103p, which has been scantly investigated to date.

The rate-determining step of the CA catalytic cycle for the hydration of CO_2 to bicarbonate is the formation of the zinc hydroxide species of the enzyme,[3] which occurs via the transfer of a proton from the Zn(II)-coordinated water molecule to the environment. This process can be assisted by amino acid residues from the enzyme active site or by the binding of an activator molecule within the enzyme cavity.[10–14] Such phenomena that are well understood for the α-CAs (with many X-ray crystal structures of enzyme-activator adducts available),[19–22] only recently started to be investigated for the β- and γ-classes enzymes.[23,24] However, from these few studies that have been published to date, there is evidence that activation mechanisms of the α-, β- and γ-CAs are similar. For example, the binding of the CA activator to the enzyme active site, forming an enzyme–activator complex, which facilitates the shuttling of protons between the Zn(II) ion-coordinated water molecule and the environment, in order to generate the nucleophilic zinc hydroxide, catalytically active species of the enzymes, occurs in all α-, β- and γ-CAs.[3,23,24]

Here we report the first activation study of CgCA with a series of amines and amino acids (of types **1–19**), which have been investigated earlier[10–14] for their interactions with mammalian α-CAs as well as very recently[23,24] with the β- and γ-class enzymes from the *Archaea* domain and from the yeast *S. cerevisiae*, respectively. This study may help a better understanding of the β-CA catalytic/activation mechanism (the natural proton shuttling residue in this class of enzymes has not been yet identified).



Data of Table 1[25] show histamine to be an activator of CgCA as well as Cab and hCA II.[23] At a concentration of 10 μM, histamine enhances k_{cat} values for all the enzymes mentioned above, whereas K_M values remain unchanged. According to previous studies, histamine is a millimolar activator for the α-class enzyme (hCA II), with K_A of 125 μM,[19] whereas it is a more effective activator for the archaeal one Cab (K_A of 76 μM) and the yeast ScCA investigated earlier (K_A of 20.4 μM). The results from the present study show that histamine has high affinity against CgCA with a K_A of 27.4 μM (see discussion later in the text). It is thus obvious that the activation mechanisms of the α- and β-CAs are likely to be similar, that is, the CA activator enhances k_{cat} but does not influence on K_M, facilitating the release of the proton from water coordinated to the catalytic zinc ion.

Data of Table 2 show that all amino acids and amines **1–19,** which have been investigated in this study, act as CAAs against the fungal enzyme CgCA. However, the activation profiles of these

Table 1
Kinetic parameters for the activation of human (hCA) isozyme II (α-class) and β-class enzymes Cab, ScCA and CgCA with histamine (Hst), measured at 25 °C, pH 8.3 in 20 mM Tris buffer and 20 mM $NaClO_4$, for the CO_2 hydration reaction[25]

Isozyme	k_{cat}* (s^{-1})	K_M* (mM)	$(k_{cat})_{Hst}$** (s^{-1})	K_A*** (μM) Hst
hCA II[a]	1.4×10^6	9.3	2.0×10^6	125
Cab[b]	3.1×10^4	1.7	4.5×10^4	76
ScCA[c]	9.4×10^5	9.5	19.6×10^5	20.4
CgCA[c]	$(3.8 \pm 0.1) \times 10^5$	7.9 ± 0.05	$(10.7 \pm 0.2) \times 10^5$	27.4 ± 1.1

* Observed catalytic rate without activator. K_M values in the presence and the absence of activators were the same for the various CAs (data not shown).
** Observed catalytic rate in the presence of 10 μM activator.
*** The activation constant (K_A) for each enzyme was obtained by fitting the observed catalytic enhancements as a function of the activator concentration.[25] Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[25] Standard errors were in the range of 5–10% of the reported values.
[a] Human recombinant enzyme, data from Ref. 10.
[b] Archaeal recombinant enzyme, data from Ref. 15.
[c] Yeast/fungal recombinant enzymes. Mean ± standard error (from three different assays).

Table 2
Activation constants of hCA II (cytosolic α-isozyme), Cab (archaeal β-CA) yeast β-CAs from *S. cerevisiae* (ScCA) and *C. glabrata* (CgCA) with amino acids and amines **1–19**

No.	Compound	K_A* (μM)			
		hCA II[a]	Cab[b]	ScCA[c]	CgCA[d]
1	L-His	10.9	69	82	37.0 ± 1.61
2	D-His	43	57	85	21.2 ± 0.93
3	L-Phe	0.013	70	86	24.1 ± 0.89
4	D-Phe	0.035	10.3	86	15.7 ± 0.41
5	L-DOPA	11.4	11.4	90	23.3 ± 0.99
6	D-DOPA	7.8	15.6	89	15.1 ± 0.62
7	L-Trp	27	16.9	91	22.8 ± 1.13
8	D-Trp	12	41	90	12.1 ± 0.40
9	L-Tyr	0.011	10.5	85	9.5 ± 0.37
10	D-Tyr	0.058	19.2	84	7.1 ± 0.18
11	4-H_2N-L-Phe	0.15	89	21.3	31.6 ± 1.55
12	Histamine	125	76	20.4	27.4 ± 1.10
13	Dopamine	9.2	51	13.1	27.6 ± 0.84
14	Serotonin	50	62	15.0	16.7 ± 0.75
15	2-Pyridyl-methylamine	34	18.7	16.2	15.0 ± 0.42
16	2-(2-Aminoethyl)pyridine	15	40	11.2	16.3 ± 0.37
17	1-(2-Aminoethyl)-piperazine	2.3	13.8	9.3	14.9 ± 0.29
18	4-(2-Aminoethyl)-morpholine	0.19	18.5	10.2	10.1 ± 0.48
19	L-Adrenaline	96	11.5	0.95	10.8 ± 0.51

Activation data of hCA II, Cab and ScCA with these compounds are retrieved from Refs. 23,24.
* Mean from three determinations by a stopped-flow, CO_2 hydrase method.[25] Standard errors were in the range of 5–10% of the reported values for the activation of hCA II, Cab and ScCA.[21,23,24]
[a] Human recombinant isozyme, from Ref. 21.
[b] Recombinant archaeal enzyme, from Ref. 23.
[c] Recombinant yeast enzyme, from Ref. 24.
[d] Recombinant yeast enzyme, this study. Mean ± standard error (from three different assays).

CAAs against CgCA are different from other recently investigated α- and β-class enzymes, including hCA II, Cab and ScCA,[19,23,24] which are included in Table 2 for comparison. The following structure–activity relationship (SAR) can be observed for the activation of CgCA with compounds **1–19**:[25]

(i) Several amino acids, such as L-/D-His, L-Phe, L-DOPA, L-Trp, 4-amino-L-phenylalanine, as well as histamine **12** and dopamine **13**, showed activation constants in the range of 21.2–37.0 μM, therefore they are considered as moderate CgCA activators. However, it may be observed that most of these derivatives (except histamine and dopamine) are much

weaker activators of the yeast enzyme ScCA. Cab, on the other hand has completely different activation profiles with these derivatives, whereas hCA II is very effectively activated by most amino acids, as well as by dopamine, but is weakly activated by histamine.

(ii) A second group of derivatives, including D-Phe, D-DOPA, D-Trp, as well as arylalkylamines **14-19**, act as more effective CgCA activators when compared to the compounds discussed above, with the activation constants in the range of 10.1–16.7 μM. Thus, it may be observed that all D-amino acids were more effective activators when compared to the corresponding L-enantiomers. The nature of the heterocyclic/aromatic ring to which the aminoalkyl chain is linked, also influences on the CA activating properties, as the morpholine derivative **18** and L-adrenaline **18**, are shown to be the best CAAs in this subseries. Similar to the results from the first group of derivatives, the activation profiles of these compounds against CgCA are very different from other α- and β-class enzymes which have been investigated earlier (Table 2). For example, L-adrenaline, which is considered as an ineffective hCA II activator (K_A of 96 μM), shows similar activation effects against Cab and CgCA (K_As of 10.8–11.5 μM), whereas it is shown to be a submicromolar activator of the ScCA (K_A of 0.95 μM).

(iii) The most potent activators of CgCA were L- and D-Tyr, with an activation constant in the range of 7.1–9.5 μM. Again the D-enantiomer was a better activator compared to the L-one, as for the amino acids mentioned above. It may be observed that, similar to L-adrenaline discussed above, Cab and CgCA show similar affinities for L-Tyr, with K_As of 9.5–10.5 μM, but rather different affinities towards D-Tyr. In contrary to CgCA, L-Tyr is a very weak CAA against ScCA with a K_A of 85 μM, whereas it is shown to be an extremely potent hCA II activator (K_A of 11 nM).

Some other SAR data are obvious: for example, small structural changes, such as the introduction of an amino (compound **11**) or OH (L-Tyr, compound **9**) moiety in the *para*-position of the phenyl ring in the molecule of a weak CAA (against CgCA), L-Phe (K_A of 24.1 μM), lead to important differences of activity. Indeed, L-Tyr is 2.5 times a better CgCA activator when compared to L-Phe and a 3.3 times better activator when compared to 4-amino-L-phenylalanine. Thus, the activation profile of CgCA is very different from that of other α- or β-CAs which have been investigated earlier.

Another striking difference between the enzyme investigated here and other fungal β-CAs is outlined below. For ScCA there is a striking difference in the K_As of the investigated amino acids versus the amines, with 4-H_2N-Phe **11** behaving as an amine and not as an amino acid. Indeed, the 'amine type' CAAs showed K_As in the range of 0.95–21.4 μM, whereas the 'amino acid type' CAAs in the range of 82–91 μM (Table 2). In the case of CgCA, this net difference of activity between amines and amino acids has not been observed at all, with efficient activators detected among both amines (e.g., **18** and **19**) and amino acids (e.g., **8**–**10**).

A possible activation mechanism of the *C. glabrata* β-CAs by L-Tyr **9** is depicted schematically in Figure 1. As for other fungal β-CAs, the catalytic Zn(II) ion in the CgCA active site is coordinated to residues Cys106, His161 and Cys164 (Nce103p of *C. albicans* numbering system).[4,24] A second pair of conserved amino acid residues in all sequenced β-CAs known to date,[2,4,24] is constituted by the dyad Asp108-Arg110 (Nce103 of *C. albicans* numbering, Fig. 1). These amino acids are close[2,3] to the zinc-bound water molecule, which is the fourth Zn(II) ligand in this type of open active site β-CAs,[2,3] and participate in a network of hydrogen bonds, which probably assist the water deprotonation and the formation of the nucleophilic, zinc hydroxide species of the enzyme. The active site

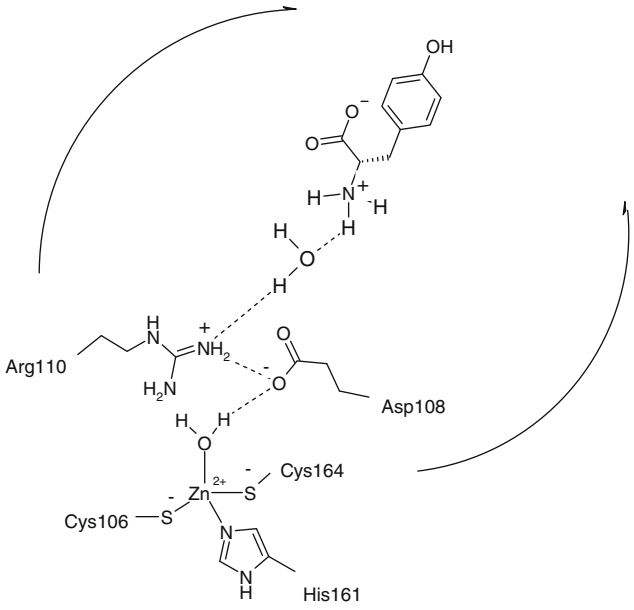


Figure 1. Proposed schematic interactions between an activator (L-Tyr **9**) and the CgCA active site. The Zn(II) ion is coordinated by two Cys and one His residue, and by a water molecule (*C. albicans* numbering of amino acid residues).[4] Both the amino and carboxylate moieties of the activator **9** can participate in hydrogen bonds with amino acid residues in the neighborhood of the metal ion center and facilitate the transfer of the proton from the zinc-bound water to the environment with generation of the zinc hydroxide species of the enzyme. A bridge of one water molecule between the activator and the Arg110 residue has been hypothesized here, but the real number of such water molecules is unknown at this time. In hCA II – activator crystal structures it has been shown to be between 1 and 3.[22]

channel of β-CAs (as exemplified by the recently determined X-ray crystal structure of the *C. neoformans* enzyme Can2p)[2b] is a channel which can accommodate elongated molecules such as the aromatic amino acids/amines which have been investigated here. Thus, we hypothesize that the activator (such as L-Tyr **9**) may bind closely to the pocket defined by Asp108/Arg110, establishing supplementary hydrogen bonds with the polar moieties of these amino acids or with the zinc-bound water molecule (directly or through a relay of several other water molecules, as demonstrated for the interaction of α-CAs with this type of activator)[19] and thus assist the water deprotonation and facilitate the catalytic turnover. Indeed, both the amino or carboxyl moieties of the activators can establish hydrogen bonds with these structural elements, due to the presence of many heteroatoms in their molecules. Figure 1 shows schematically a putative binding mode of L-Tyr **9** within the active site of CgCA. This hypothesis should be confirmed by X-ray crystallography, however the structure of CgCA has not yet been resolved.

In conclusion, we report the first activation study of the β-CA from the fungal pathogen *C. glabrata* with amines and amino acids. CgCA was weakly activated by amino acids such as L-/D-His, L-Phe, L-DOPA, and L-Trp and by histamine or dopamine (K_As of 21.2–37 μM). It was more effectively activated by D-Phe, D-DOPA, D-Trp as well as serotonin, pyridyl-alkylamines, and aminoethyl-piperazine/morpholine (K_As of 10.1–16.7 μM). The best activators of CgCA were L-and D-Tyr, with activation constant of 7.1–9.5 μM. This study may bring a better understanding of the catalytic/activation mechanisms of β-CAs and lead to the designation of modulators for the activity of CAs from pathogenic fungi.

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References and notes

1. Elleuche, S.; Poggeler, S. *Curr. Genet.* **2009**, *55*, 211.
2. (a) Hall, R. A.; Mühlschlegel, F. A. Fungal and Nematode Carbonic Anhydrases: Their Inhibition in Drug Design. In *Drug Design of Zinc-Enzyme Inhibitors: Functional, Structural, and Disease Applications*; Supuran, C. T., Winum, J. Y., Eds.; John Wiley & Sons: Hoboken, 2009; pp 301–322; (b) Schlicker, C.; Hall, R. A.; Vullo, D.; Middelhaufe, S.; Gertz, M.; Supuran, C. T.; Muhlschlegel, F. A.; Steegborn, C. *J. Mol. Biol.* **2009**, *385*, 1207.
3. Supuran, C. T. *Nat. Rev. Drug Disc.* **2008**, *7*, 168.
4. (a) Klengel, T.; Liang, W. J.; Chaloupka, J.; Ruoff, C.; Schropel, K.; Naglik, J. R.; Eckert, S. E.; Mogensen, E. G.; Haynes, K.; Tuite, M. F.; Levin, L. R.; Buck, J.; Mühlschlegel, F. A. *Curr. Biol.* **2005**, *15*, 2021; (b) Bahn, Y. S.; Cox, G. M.; Perfect, J. R.; Heitman, J. *Curr. Biol.* **2005**, *15*, 2013.
5. (a) Mogensen, E. G.; Janbon, G.; Chaloupka, J.; Steegborn, C.; Fu, M. S.; Moyrand, F.; Klengel, T.; Pearson, D. S.; Geeves, M. A.; Buck, J.; Levin, L. R.; Mühlschlegel, F. A. *Eukaryot. Cell* **2006**, *5*, 103; (b) Bahn, Y. S.; Mühlschlegel, F. A. *Curr. Opin. Microbiol.* **2006**, *9*, 572.
6. (a) Tripp, B. C.; Smith, K. S.; Ferry, J. G. *J. Biol. Chem.* **2001**, *276*, 48615; (b) Smith, K. S.; Ferry, J. G. *FEMS Microbiol. Rev.* **2000**, *24*, 335; (c) Zimmerman, S. A.; Ferry, J. G.; Supuran, C. T. *Curr. Top. Med. Chem.* **2007**, *7*, 901.
7. (a) Götz, R.; Gnann, A.; Zimmermann, F. K. *Yeast* **1999**, *15*, 855; (b) Amoroso, G.; Morell-Avrahov, L.; Muller, D.; Klug, K.; Sultemeyer, D. *Mol. Microbiol.* **2005**, *56*, 549.
8. (a) Aguilera, J.; Van Dijken, J. P.; De Winde, J. H.; Pronk, J. T. *Biochem. J.* **2005**, *391*, 311; (b) Aguilera, J.; Petit, T.; de Winde, J.; Pronk, J. T. *FEMS Yeast Res.* **2005**, *5*, 579.
9. Innocenti, A.; Leewattanapasuk, W.; Mühlschlegel, F. A.; Mastrolorenzo, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4802.
10. (a) Fidel, P. L., Jr.; Vazquez, J. A.; Sobel, J. D. *Clin. Microbiol. Rev.* **1999**, *12*, 80; (b) Kaur, R.; Domergue, R.; Zupancic, M. L.; Cormack, B. P. *Curr. Opin. Microbiol.* **2005**, *8*, 378; (c) Klevay, M. J.; Horn, D. L.; Neofytos, D.; Pfaller, M. A.; Diekema, D. J. *Diagn. Microbiol. Infect. Dis* **2009**, *64*, 152.
11. (a) Pai, M.; Turpin, R.; Garey, K. *Antimicrob. Agents Chemother.* **2007**, *51*, 35; (b) Krcmery, V.; Barnes, A. J. *J. Hosp. Infect.* **2002**, *50*, 243.
12. (a) Bennett, J. E.; Izumikawa, K.; Marr, K. A. *Antimicrob. Agents Chemother.* **2004**, *48*, 1773; (b) Hitchcock, C. A.; Pye, G. W.; Troke, P. F.; Johnson, E. M.; Warnock, D. W. *Antimicrob. Agents Chemother.* **1993**, *37*, 1962; (c) Fadda, M. E.; Podda, G. S.; Pisano, M. B.; Deplano, M.; Cosentino, S. *J. Prev. Med. Hyg.* **2008**, *49*, 69.
13. Innocenti, A.; Mühlschlegel, F. A.; Hall, R. A.; Steegborn, C.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5066.
14. Innocenti, A.; Hall, R. A.; Schlicker, C.; Mühlschlegel, F. A.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 2654.
15. Innocenti, A.; Hall, R. A.; Schlicker, C.; Scozzafava, A.; Steegborn, C.; Mühlschlegel, F. A.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 4503.
16. Innocenti, A.; Winum, J.-Y.; Hall, R. A.; Mühlschlegel, F. A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2642.
17. (a) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146; (b)*Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton (FL), USA, 2004; pp 1–364; (c) Supuran, C. T.; Scozzafava, A. *Expert Opin. Ther. Patent* **2002**, *12*, 217; (d) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Patent* **2004**, *14*, 667.
18. (a) Supuran, C. T.; Scozzafava, A. *Bioorg. Med. Chem.* **2007**, *15*, 4336; (b) Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 603.
19. (a) Temperini, C.; Scozzafava, A.; Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 708; (b) Briganti, F.; Mangani, S.; Orioli, P.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T. *Biochemistry* **1997**, *36*, 10384; (c) 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 Press: Boca Raton (FL), USA, 2004; pp 317–352; (d) Supuran, C. T.; Scozzafava, A. Activation of Carbonic Anhydrase Isozymes. In *The Carbonic Anhydrases—New Horizons*; Chegwidden, W. R., Carter, N., Edwards, Y., Eds.; Birkhauser Verlag: Basel, Switzerland, 2000; pp 197–219; (e) Supuran, C. T. *Therapy* **2007**, *4*, 355.
20. (a) Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2002**, *45*, 284; (b) Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1177; (c) Ilies, M.; Banciu, M. D.; Ilies, M. A.; Scozzafava, A.; Caproiu, M. T.; Supuran, C. T. *J. Med. Chem.* **2002**, *45*, 504; (d) Scozzafava, A.; Iorga, B.; Supuran, C. T. *J. Enzyme Inhib.* **2000**, *15*, 139; (e) Temperini, C.; Scozzafava, A.; Vullo, D.; Supuran, C. T. *J. Med. Chem.* **2006**, *49*, 3019.
21. (a) Parkkila, S.; Vullo, D.; Puccetti, L.; Parkkila, A. K.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3955; (b) Vullo, D.; Nishimori, I.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1336.
22. (a) Temperini, C.; Innocenti, A.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 628; (b) Vullo, D.; Nishimori, I.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1336; (c) Vullo, D.; Innocenti, A.; Nishimori, I.; Scozzafava, A.; Kaila, K.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4107; (d) Pastorekova, S.; Vullo, D.; Nishimori, I.; Scozzafava, A.; Pastorek, J.; Supuran, C. T. *Bioorg. Med. Chem.* **2008**, *16*, 3530.
23. Innocenti, A.; Zimmerman, S. A.; Scozzafava, A.; Ferry, J. G.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6194.
24. Isik, S.; Kockar, F.; Aydin, M.; Arslan, O.; Ozensoy Guler, O.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1662.
25. Khalifah, R.G. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped-flow instrument was used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, 10–20 mM Hepes (pH 7.5) or Tris (pH 8.3) as buffers, and 20 mM Na_2SO_4 or 20 mM $NaClO_4$ (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 °C. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators **1**–**19** (10 mM) were prepared in distilled-deionized water and dilutions up to 0.001 μM were done thereafter with distilled-deionized water. Activator 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–A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I,[20–22] can be obtained by considering the classical Michaelis–Menten equation (Eq. 1), which has been fitted by non-linear least squeares by using PRISM 3:

$$v = v_{max}/\{1 + K_M/[S] \times (1 + [A]_f/K_A)\} \quad (1)$$

where $[A]_f$ is the free concentration of activator.
Working at substrate concentrations considerably lower than K_M ($[S] \ll K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. 2:[20–22]

$$v = v_0 \cdot K_A/\{K_A + ([A]_t - 0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t \cdot [E]t)^{1/2}\}\} \quad (2)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[20–22]