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Carbonic anhydrase inhibitors. Inhibition of the β-class enzyme from the pathogenic yeast *Candida glabrata* with anions

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ABSTRACT

A β-carbonic anhydrase (CA, EC 4.2.1.1), the protein encoded by the *NCE103* gene of *Candida glabrata* which also present in *Candida albicans* and *Saccharomyces cerevisiae*, was cloned, purified, characterized kinetically and investigated for its inhibition by a series simple, inorganic anions such as halogenides, pseudohalogenides, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, perchlorate, sulfate and some isosteric species. The enzyme showed significant CO_2 hydrase activity, with a k_{cat} of $3.8 \times 10^5\ s^{-1}$ and k_{cat}/K_M of $4.8 \times 10^7\ M^{-1}\ s^{-1}$. The Cà glabrata CA (CgCA) was moderately inhibited by metal poisons (cyanide, azide, cyanate, thiocyanate, K_Is of 0.60–1.12 mM) but strongly inhibited by bicarbonate, nitrate, nitrite and phenylarsonic acid (K_Is of 86–98 μM). The other anions investigated showed inhibition constants in the low millimolar range, with the exception of bromide and iodide (K_Is of 27–42 mM).

In previous Letters from these groups[1] we have reported the cloning, purification, kinetic properties and inhibition by simple anions of several β-carbonic anhydrases (CAs, EC 4.2.1.1) from fungi, including the fungal pathogens *Candida albicans* (denominated Nce103p)[1a] and *Cryptococcus neoformans* (denominated Can2p)[1a] as well as the more benign fungi *Saccharomyces cerevisiae* (ScCA).[1b] Among the five independently-evolved (α, β, γ, δ, and ζ) classes of CAs reported to date,[3–5] the β-CAs are the most abundant catalysts for the interconversion between carbon dioxide and bicarbonate ion in many organisms which are plants, algae, bacteria, archaea and fungi.[3–7] None of β-CAs have yet been detected in higher eukaryotes, including vertebrates. Therefore, the β-CAs are a family of enzymes present in many pathogenic organisms and not the mammalian host. The lack of β-CAs in the mammalian host makes them attractive and can be considered as novel possible drug targets.[3] Recently, we have explored the druggability of several proteins including the β-CAs from *Helicobacter pylori*,[8] *Mycobacterium tuberculosis*,[9] *C. albicans*[10] and *C. neoformans*.[11] These enzymes are shown to be inhibited, in the micro—nanomolar range, by several classes of compounds, such as simple inorganic anions, carboxylates, boronic acids and sulfonamides/sulfamates.[3,8–11]

Candida glabrata is a pathogenic fungi which, similar to *C. albicans*, possesses a recently characterized β-CA isoform encoded by the *NCE103* gene.[2d,12] *C. glabrata* is a common cause of mucosal and invasive, systemic infections, accounting for 15% of *Candida* infections in the general patient population.[13,14] Furthermore, this pathogen is innately resistant to azole antifungal agents and is less susceptible to commonly used in clinical practice antifungals and resistance to these agents in *C. glabrata* has been frequently reported as well.[15] Thus, finding new therapeutic agents for inhibiting the growth of *C. glabrata* may lead to the development of more efficient therapies for the treatment of *C. glabrata* infections. Recently we have shown that *C. glabrata* has evolved additional signaling mechanisms to permit it to respond to elevated CO_2 concentrations, compared to *C. albicans* and *C. neoformans*.[12] In the same study it has been shown that CA expression in *C. glabrata* is tightly controlled in accordance with the availability of bicarbonate, which is essential as a carbon source for the intermediate metabolism.[12]

In this Letter we have cloned, purified and characterized *C. glabrata* β-CA (denominated CgCA)[12,16] to enable the investigation of its kinetic properties for the physiological reaction (i.e., CO_2 hydration to bicarbonate and protons), as well as its inhibition screening by anions known to interact with most metal centers of metalloenzymes.[1,3] The aim of this study is thus to understand the catalytic efficiency of CgCA, an enzyme essential for the growth of

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Table 1
Kinetic parameters for the CO_2 hydration reaction catalyzed by the human cytosolic isozymes hCA I and II (α-class CAs) at 20 °C and pH 7.5 in 10 mM HEPES buffer and 20 mM Na_2SO_4, and the β-CAs Can2p, Nce103p (from *C. neoformans* and *C. albicans*, respectively), ScCA (from *S. cerevisiae*) and CgCA (from *C. glabrata*) measured at 20 °C, pH 8.3 in 20 mM TRIS buffer and 20 mM $NaClO_4$[18]

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I[a]	Moderate	2.0×10^5	5.0×10^7	250
hCA II[a]	Very high	1.4×10^6	1.5×10^8	12
Can2p[a]	Moderate	3.9×10^5	4.3×10^7	10.5
Nce103p[a]	High	8.0×10^5	9.7×10^7	132
ScCA[a]	High	9.4×10^5	9.8×10^7	82
CgCA[b]	Moderate	3.8×10^5	4.8×10^7	11

Inhibition data with the clinically-used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided.
[a] Data from Ref. 1.
[b] This work.

Table 2
Inhibition constants of anionic inhibitors against isozymes hCA II (α-CA class), and the β-CAs Nce103p (from *C. albicans*),[1a] ScCA (from *S. cerevisiae*),[1b] and CgCA (from *C. glabrata*) for the CO_2 hydration reaction, at 20 °C[18]

Inhibitor	K_I (mM)[b]			
	hCA II	Nce103p (*C. albicans*)	ScCA	CgCA
F^-	>300	0.69	2.85	0.36
Cl^-	200	0.85	0.85	0.58
Br^-	63	0.94	0.0108	27.0
I^-	26	1.40	0.0103	42.4
CNO^-	0.03	1.18	31.7	0.60
SCN^-	1.6	0.65	55.6	0.73
CN^-	0.02	0.011	16.8	1.12
N_3^-	1.5	0.52	27.9	1.03
HCO_3^-	85	0.62	0.78	0.086
CO_3^{2-}	73	0.010	0.76	0.31
NO_3^-	35	0.69	13.9	0.097
NO_2^-	63	0.53	0.46	0.088
HS^-	0.04	0.37	0.33	0.10
HSO_3^-	89	0.54	0.33	0.10
SO_4^{2-}	>200	14.15	0.58	0.58
ClO_4^-	>200	>200	>200	>200
$H_2NSO_2NH_2$	1.13	0.30	0.0087	0.42
H_2NSO_3H[a]	0.39	0.70	0.84	0.11
Ph-$B(OH)_2$	23.1	30.85	38.2	0.10
Ph-AsO_3H_2[§]	49.2	30.84	0.40	0.098

[a] As sodium salt.
[b] Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[18]

C. glabrata in ambient CO_2 concentration, and to find CA inhibitors that may lead to the development of novel antifungal agents with an alternative therapeutic mechanism compared to azoles and related drugs.[2d]

Several simple chemical species are fundamental in many physiological processes and are found in relevant concentrations in many eukaryotic cell compartments (e.g., bicarbonate, chloride, sulfate, etc). Here, we investigated the simplest class of CA inhibitors (CAIs), that is, inorganic anions[3] and 'metal poisons' (CN^-, N_3^-, SCN^-, etc), for their interaction with CgCA in order to develop novel CAIs with potential biomedical or environmental applications.

We kinetically characterized the purified CgCA and its kinetic parameters (k_{cat} and k_{cat}/K_M) was compared with the thoroughly investigated CAs, such as the cytosolic, ubiquitous human isozymes hCA I and II, which are α-class CAs, as well as the recently investigated fungi β-CAs, *C. neoformans* Can2p, *C. albicans* Nce103p and *S. cerevisiae* CA (ScCA).[1] The latter two enzymes are encoded by the orthologous *NCE103* gene found in *C. glabrata* (Table 1).

It is also evident from Table 1 show that CgCA, similar to other recently investigated α- and β-CAs, possessed appreciable CO_2 hydrase activity, with a k_{cat} of 3.8×10^5 s^{-1}, and k_{cat}/K_M of 4.8×10^7 M^{-1} s^{-1}. The CgCA catalytic efficiency observed in this study is similar to the *C. neoformans* Can2p, but slightly lower than Nce103p from both *C. albicans* and *S. cerevisiae*. Data from Table 1 also show that CgCA was inhibited appreciably by the clinically-used sulfonamide, acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), with an inhibition constant of 11 nM, which is the similar range as observed in the ubiquitous human isoform hCA II and *C. neoformans* Can2p.

Table 2 shows the CgCA inhibition screening data with anionic physiological species (such as chloride, bicarbonate, sulfate, etc) as well a other non-physiological anions.[17,18] Here, we also include recently reported inhibition data of hCA II, Nce103p and ScCA[1] in order to compare the newly generated data with those of the better investigated CAs. The following observations are notable regarding the CA inhibition data of Table 2:

(i) CgCA, like other α- and β-CAs investigated so far, was not inhibited by perchlorate, the anion which showed an inhibition constant of >200 mM.

(ii) Bromide and iodide inhibited the ScCA and the *C. albicans* Nce103p in a low millimolar range, however, these two anions are much weaker hCA II and CgCA inhibitors with K_Is of 26–63 mM and 27.0–42.4 mM, respectively.

(iii) Most of the investigated anions, including fluoride, chloride, cyanate, thiocyanate, cyanide, azide, carbonate and sulfamide, showed low inhibition constants in the range of 0.31–1.12 mM against CgCA. It was observed that the majority of these anions are also effective *C. albicans* Nce103p inhibitors, suggesting that CAs from these two *Candida* species are rather similar. However, the notable differences in inhibition screening data between these two CAs were also observed. For example, cyanide's inhibition constant against Nce103p was 102 times lower than CgCA; carbonate's inhibition constant against Nce103p was 31 times lower than CgCA. On the other hand, sulfate's inhibition constant against CgCA was 24 times lower than Nce103p. The differences of these anions inhibition screening data between ScCA and CgCA were, on the other hand, quite diverse, suggesting that these two CAs possess very distinct inhibition profiles with this class of CAIs. The same observation was found when compared the anions inhibition profiles between hCA II and CgCA. According to their different CA families, which are α- and β-CA classes, respectively, this observation was not unexpected.

(iv) A group of anions/compounds, including bicarbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, sulfamic acid, phenylboronic acid and phenylarsonic acid showed even better CgCA inhibitory activities, with inhibition constants in the range of 86–110 μM. It is interesting that the best CgCA inhibitor detected so far was bicarbonate, which was recently shown by us[12] to tightly control the expression of this enzyme.[6,7] In high CO_2 concentration, such as physiological CO_2, the conversion of CO_2 to bicarbonate, in addition to being catalyzed by CA, can also spontaneously occur and presumably produces higher level of bicarbonate compared to ambient CO_2 concentration. It is possible that, in this condition, bicarbonate may act as feed-back inhibitor and inhibit the expression of CA encoding gene. The high affinity of the enzyme for bicarbonate, the product formed through its catalytic activity, may represent an additional regulation mechanism for the expression of the CA encoding gene in various conditions. It is also interesting to note that the orthologous enzymes from *C. albicans* or *S. cerevisiae* were 7–9 times less susceptible to be inhibited by bicarbonate compared to CgCA, whereas, the affinity of hCA II for this anion is indeed very low (Table 2). Anions which highly similar geometrically and electronically with bicarbonate, such as nitrate, nitrite and bisulfite, showed very similar inhibitory activity against CgCA. Hydrogen sulfide, on the other hand, was the only 'metal poison'

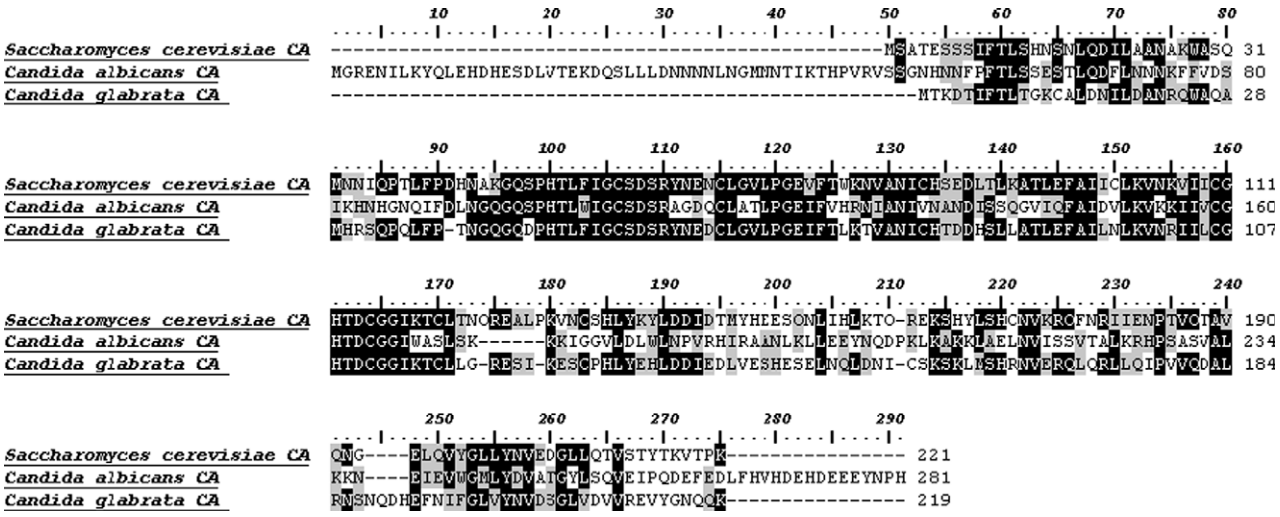

Figure 1. Alignment of CgCA (*C. glabrata*), Nce103p (*C. albicans*), and ScCA amino acid sequences. The three zinc ligands are conserved in all these enzymes (Cys106, His161 and Cys164) whereas the other conserved/semiconserved amino acid residues between the three β-CAs are evidenced by black boxes. The two residues Asp108, Arg110, thought to be involved in the β-CA catalytic cycle[1] are also conserved in the three enzymes (the numbering system used here corresponds to the Nce103p of *C. albicans* amino acid sequence).[6,7]

showing efficient inhibition of CgCA, unlike cyanide and related anions, which behaved as weak inhibitors (see discussion above). Sulfamic acid, phenylboronic acid and phenylarsonic acid also showed efficient CgCA inhibitory activities (K_Is of 98–110 μM) and are therefore interesting leads for the development of even stronger CAIs targeting this enzyme. In fact, it has been shown earlier that scaffolds incorporating the sulfamate or boronic acid zinc-binding groups may lead to low micromolar–nanomolar inhibitors of several β-CAs from various pathogenic organisms.[9–11]

Data from Figure 1 show an alignment of the β-CAs from three fungal species (*C. glabrata, C. albicans* and *S. cerevisiae*). It was observed that the putative zinc ligands of these fungal β-CAs are, corresponding to residues Cys106, His161 and Cys164 (Nce103p of *C. albicans* numbering system, see Fig. 1) all conserved.[6,7,10b] A second pair of amino acid residues conserved in all known sequenced β-CAs,[1,2,11] consists of the dyad Asp108—Arg110 (Nce103p of *C. albicans* numbering, Fig. 1). These amino acids are close[1] to the zinc-bound water molecule, which is the fourth zinc ligand in this type of open active site, and participate in a network of hydrogen bonds which probably assists water deprotonation and formation of the nucleophilic, zinc hydroxide species of the enzyme. Indeed in β-CAs, unlike the α-class enzymes, the formal zinc charge is zero (the two cysteinates ligands 'neutralize' the +2 charge of the zinc ion), and as a consequence the activation of the zinc-coordinated water molecule (for the hydration of CO_2 to bicarbonate) requires the assistance of additional amino acids.[1,2,11] Thus, the catalytic water molecule is activated both by the metal ion (as in metalloproteases[19] and α-CAs[1–3]), but also by an aspartic acid residue, as in aspartic proteases.[20] This particular mechanism makes the β-CAs, including CgCA, very different compared to all other known enzyme classes involved in hydrolytic or hydration processes.

In conclusion, the β-CA encoded by the *NCE103* gene from *C. glabrata* was cloned, purified, characterized kinetically and investigated for its inhibition by a series of simple inorganic anions such as halogenides, pseudohalogenides, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, perchlorate, sulfate and some isosteric species. The enzyme showed significant CO_2 hydrase activity, with a k_{cat} of $3.8 \times 10^5\ s^{-1}$, and k_{cat}/K_M of $4.8 \times 10^7\ M^{-1}\ s^{-1}$. CgCA was moderately inhibited by metal poisons (cyanide, azide, cyanate, thiocyanate, K_Is of 0.60–1.12 mM) but strongly inhibited by bicarbonate, nitrate, nitrite and phenylarsonic acid (K_Is of 86–98 μM). The other investigated anions showed inhibition constants in the low millimolar range, with the exception of bromide and iodide (K_Is of 27–42 mM).

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16. Full length *CgNCE103* was amplified by PCR from genomic DNA and ligated into the pGex-6P-2 vector and expressed in BL21 *Escherichia coli* as previously described.[1a,12] Recombinant protein was purified using Fast Flow glutathione S-sepharose (Amersham) as per the manufacturer's recommendations. To prevent protein degradation 1 mM PMSF and 25 μl proteinase cocktail (Roche Complete mini EDTA-free, 1 tablet dissolved in 2 ml PBS) were added to samples prior to lysis. Elution fractions were combined and dialzsed against 10 mM HEPES pH 7.5. Protein was size-fractionated on denaturing 12% SDS Tris–HCl gels (BioRad) with 1× MOPS buffer (BioRad) at 150 V.
17. Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, hydrogen sulfite, sulfate and perchlorate) were of highest purity available, and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid and phenylarsonic acid were from Sigma–Aldrich.
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