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Carbonic anhydrase inhibitors. Inhibition of the β-class enzymes from the fungal pathogens *Candida albicans* and *Cryptococcus neoformans* with aliphatic and aromatic carboxylates

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ABSTRACT

The inhibition of the β-carbonic anhydrases (CAs, EC 4.2.1.1) from the pathogenic fungi *Cryptococcus neoformans* (Can2) and *Candida albicans* (Nce103) with carboxylates such as the C1–C5 aliphatic carboxylates, oxalate, malonate, maleate, malate, pyruvate, lactate, citrate and some benzoates has been investigated. The best Can2 inhibitors were acetate and maleate (K_Is of 7.3–8.7 μM), whereas formate, acetate, valerate, oxalate, maleate, citrate and 2,3,5,6-tetrafluorobenzoate showed less effective inhibition, with K_Is in the range of 42.8–88.6 μM. Propionate, butyrate, malonate, L-malate, pyruvate, L-lactate and benzoate, were weak Can2 inhibitors, with inhibition constants in the range of 225–1267 μM. Nce103 was more susceptible to inhibition with carboxylates compared to Can2, with the best inhibitors (maleate, benzoate, butyrate and malonate) showing K_Is in the range of 8.6–26.9 μM. L-Malate and pyruvate together with valerate were the less efficient Nce103 inhibitors (K_Is of 87.7–94.0 μM), while the remaining carboxylates showed a compact behavior of efficient inhibitors (K_Is in the range of 35.1–61.6 μM). Notably the inhibition profiles of the two fungal β-CAs was very different from that of the ubiquitous host enzyme hCA II (belonging to the α-CA family), with maleate showing selectivity ratios of 113.6 and 115 for Can2 and Nce103, respectively, over hCA II inhibition. Therefore, maleate is a promising starting lead molecule for the development of better, low nanomolar, selective β-CA inhibitors.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1), catalyze the interconversion of carbon dioxide and bicarbonate, and they play important roles in many physiological processes of eukaryotes including respiration, CO_2 transport, electrolyte secretion, and photosynthesis among others.[1–3] These enzymes have been thoroughly investigated in organisms from the *Eukarya* domain (where they are ubiquitous) but have received scant attention in prokaryotes from the *Bacteria* and *Archaea* domains as well as microscopic eukaryotes, such as pathogenic fungi.[1,4–7] Only recently we and others characterized two β-CAs (Nce103 and Can2) from the human fungal pathogens *Candida albicans* and *Cryptococcus neoformans*.[6–9] Carbon dioxide sensing plays an important role in fungal pathogenesis and environmental survival.[6] Indeed, concentrations of CO_2/HCO_3^- found in body fluids such as blood induce changes in fungal morphology (filamentation in *C. albicans* and capsule biosynthesis in *C. neoformans*) that are essential for pathogenesis. CO_2/HCO_3^- is thought to directly activate the fungal adenylyl cyclase.[9,10] CO_2/HCO_3^- equilibration by fungal β-CAs is essential for survival under CO_2—limiting conditions and also plays a role in fungal pathogenesis. For example an *nce103* mutant will not grow under the carbon dioxide restrictive conditions found in the atmosphere. The same strain is impaired in its ability to colonize host niches (for example skin) that do not permit build-up of sufficient CO_2 required for growth.[6–10] Thus, the link between cAMP signaling and CO_2/HCO_3^- sensing is conserved in the fungal pathogens *C. albicans* and *C. neoformans* and revealed CO_2 sensing and equilibration to be an important mediator of fungal growth metabolism and pathogenesis. Recently, we reported the first inhibition studies of these new β-CAs with inorganic anions[8] and sulfonamides,[9] whereas the first X-ray crystal structure of a fungal CA (Can2), uncomplexed and in complex with acetate has also been resolved at a very good resolution.[9] These studies provide a solid basis for the possible design of novel therapeutic agents targeting this unexplored pathway at several levels, in order to control fungal infections by means of different classes of antifungals.[1,8] Indeed, *C. albicans* and *C. neoformans* are frequent causative agents of life-threatening disease, and drug resistance to the currently used antifungals is increasing worldwide.[11,12]

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Here we report an inhibition study of Can2 and Nce103 with carboxylates incorporating aliphatic, hydroxy-/keto-aliphatic and aromatic moieties in their molecules, some of which are Krebs cycle intermediates (e.g., oxalate, malate, pyruvate and citrate). In fact, in *Saccharomyces cerevisiae*, the ortholog β-CA to the proteins investigated here (also denominated Nce103, as it is encoded by a similar gene to the one present in *C. albicans*) is required for providing sufficient bicarbonate for essential metabolic carboxylation reactions of the yeast metabolism, such as those catalyzed by pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC), carbamoyl phosphate synthase (CPSase) and phosphoribosylaminoimidazole (AIR) carboxylase.[13–15] Thus, understanding the inhibition profiles of these enzymes with carboxylates may be important, both for drug design purposes (as mentioned above), but also to better highlight the role of these enzymes in physiological processes of yeasts and fungi.

2. Results and discussion

2.1. Chemistry

The zinc(II) ion present within the β-CA active site is essential for catalysis and inhibition of these enzymes.[1–3,8,9] As shown by the recently reported X-ray crystal structure of Can2,[9] the Zn(II) ligands are two cysteine residues (Cys68 and Cys127), one histidine (His124) and a water molecule (Fig. 1A). A strong hydrogen bond between the Zn(II)-coordinated water and the carboxylate moiety of Asp70 has also been evidenced,[9] which leads to water activation for the nucleophilic attack to CO_2. This facilitates the deprotonation of water, forming the zinc-hydroxide species of the enzyme, which is the strong nucleophile promoting CO_2 hydration to bicarbonate.[1,9] Indeed, we showed earlier[8,9] that the β-CAs of pathogenic fungi show an appreciable catalytic activity for the physiologic reaction, with kinetic parameters comparable to those

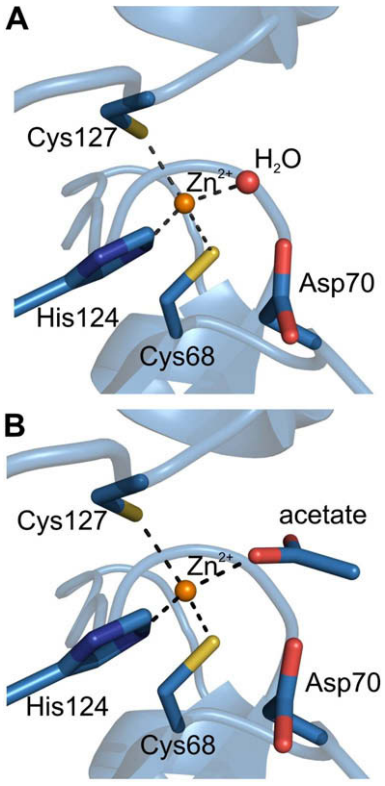

Figure 1. (A) Zn(II) coordination within the Can2 active site, and its complex with acetate (B).

Table 1
Inhibition constants of carboxylate inhibitors against isozymes hCA II (α-CA class), and β-isozymes Can2 (from *Cryptococcus neoformans*) and Nce103 (from *Candida albicans*), for the CO_2 hydration reaction, at 20 °C

No.	Inhibitor[d]	K_I[c] (μM)		
		hCA II[a]	Can2[b]	Nce103[b]
1	Formate	24,000	53.5	35.1
2	Acetate	130	7.3	36.0
3	Propionate	960	404	51.5
4	Butyrate	1032	225	26.9
5	Valerate	257	42.8	94.0
6	Oxalate	990	67.7	37.7
7	Malonate	1620	793	26.3
8	Maleate	989	8.7	8.6
9	L-Malate	1870	899	87.7
10	Pyruvate	2110	787	105.7
11	L-Lactate	3220	953	46.8
12	Citrate	2160	88.6	39.1
13	Benzoate	30	1267	24.4
14	2,3,5,6-F4-Benzoate	6	49.1	61.6

[a] From Ref. 17 at pH 7.5 (Hepes buffer).
[b] This work, at pH 8.3 (Tris buffer), as β-CAs show low catalytic activity at pH values <8.0.
[c] Errors were in the range of 3–5% of the reported values, from three different assays.
[d] As sodium salt.

of α-CA isoforms present in mammals and investigated in greater detail.[1]

In the same study,[9] we also observed an acetate ion bound to the Zn(II) ion, in the Can2-acetate adduct shown in Figure 1B. The bound acetate is monocoordinated to the Zn(II) ion, replacing the water molecule observed in the wild-type enzyme (Fig. 1A). Indeed, inorganic anions such as bicarbonate, halides, cyanide, thiocyanate, etc., are well-known inhibitors of both β- and α-CAs,[8,14,16] binding to the active site metal ion in tetrahedral or trigonal-bipyramidal geometries of Zn(II).[1] Carboxylates were on the other hand less investigated as α-CA inhibitors (one such study reported the inhibition of the human isozymes hCA I, II, IV, VA and IX with aliphatic and aromatic carboxylates)[17] and not at all investigated for their interaction with β-CAs.

Considering the strong interaction observed between the Can2 active site and acetate in the adduct mentioned above,[9] we investigated the following carboxylates for their interaction with Can2 and Nce103: the C1–C5 non-branched aliphatic carboxylates, oxalate, malonate, maleate, malate, pyruvate, lactate, citrate and some benzoates (Table 1). Some of them are obvious Krebs cycle intermediates and may be present in high enough concentrations in the fungal cell under physiologic conditions.

2.2. Can2 and Nce103 inhibition with carboxylates

Table 1 shows the inhibition data of the β-CAs from pathogenic fungi, Can2 and Nce103, with the carboxylates mentioned above. For comparison reasons, human (h) hCA II inhibition data with the same anions are also provided. Indeed, hCA II is the best studied α-CA, being ubiquitous in the human body and playing many important physiologic (or pathologic in cases of disbalances) functions.[1,3] Some of the hCA II inhibition data with these anions were reported earlier,[17] whereas the data with propionate, butyrate, valerate and maleate are new and are presented here for the first time.

The following may be observed regarding the inhibition of Can2 and Nce103 with carboxylates:[18]

(i) Can2 is weakly inhibited by the following carboxylates: propionate, butyrate, malonate, L-malate, pyruvate, L-lactate and benzoate, which showed inhibition constants in the range of 225–1267 μM. On the other hand, formate, acetate, valerate, oxalate, maleate, citrate and 2,3,5,6-tetrafluorobenzoate showed much more

effective inhibition, with K_Is in the range of 7.3–88.6 μM. The best inhibitors were acetate and maleate (K_Is of 7.3 and 8.7 μM, respectivly). This clearly explains why acetate was seen bound to the Zn(II) ion in a crystal structure of Can2 in which high amounts of acetate buffers were used for crystallization (Fig. 1B).[9] However, it is quite striking that very small structural changes in these carboxylates lead to dramatic effects on Can2 inhibition. For example, formate (one carbon atom less than acetate) or propionate (one carbon atom more) were respectively 7.3 and 55.3 times less inhibitory than acetate (K_I 7.3 μM), respectively. The variation of this property is then quite irregular considering the C4 (butyrate) and C5 (valerate) carboxylates, with the first one being an ineffective inhibitor (K_I of 225 μM) and the second one a rather effective one (inhibition constant in the the same range as that of formate), with a K_I of 42.8 μM (Table 1). The same pattern is then observed for dicarboxylates, with oxalate behaving as an effective inhibitor and malonate (possessing an extra CH_2 moiety with respect to oxalate) being 11.7 times less inhibitory than oxalate. Among the hydroxy acids investigated here, only citrate **12** behaved as an effective inhibitor (K_I of 88.6 μM). Again, a striking difference is observed between the two aromatic carboxylates **13** and **14** investigated in this study, with benzoate **13** being 25.80 times less inhibitory than its tetrafluoro-substituted analogue **14** (K_I of 49.1 μM). Thus, very small structural changes lead to very different inhibitory activities for the investigated carboxylates. This may in turn have interesting consequences for drug design, as these very sharp variations in SAR may be exploited to obtain stronger inhibitors belonging to this class. From Table 1 it may be observed that the human isoform hCA II (i.e., the host enzyme) shows a totally distinct inhibition profile compared to Can2. In fact, only the aromatic carboxylates **13** and **14** effectively inhibit hCA II (K_Is of 6-30 μM), all other carboxylates being weak or very weak inhibitors (K_Is in the range of 0.13–24 mM, see Table 1 and Ref. 17). This can be also explained from a structural point of view, since the hCA II active site is a rather opened funnel, in which bulky ligands (such as the aromatic/heterocyclic sulfonamides)[19] bind efficiently with micro-nanomolar affinity.[20] Smaller aliphatic ligands (such as the carboxylates) act as weaker inhibitors, probably because their main interaction consists in the Zn(II) coordination and possibly less favorable contacts with amino acid residues from such a large active site. On the other hand, the Can2 active site (similarly to that of many other β-CAs)[21,22] has a completely different shape,[9] being a tight channel with the Zn(II) ion at its bottom. Thus, the active site is much more restricted compared to the α-CAs, which may permit it to effectively bind aliphatic ligands such as the carboxylates investigated here. These carboxylates probably coordinate to the Zn(II) ion similarly to acetate (Fig. 1b) while their side chain participates in various interactions with amino acid residues from the channel. In fact, all aliphatic carboxylates investigated here are orders of magnitude better inhibitors for these two β-CAs than for hCA II or hCA I (data not shown)[17] which are representatives of the α-CA family.

(ii) Nce103, the *C. albicans* enzyme, shows an inhibition profile with these carboxylates that is completely different from that of the ortholog enzyme Can2 (from *C. neoformans*) discussed above. All carboxylates, except pyruvate (the weakest Nce103 inhibitor, with a K_I of 105.7 μM) showed K_Is < 100 μM. Therefore, Nce103 is much more susceptible to inhibition with this class of derivatives compared to Can2 and hCA II (Table 1). L-Malate and pyruvate together with valerate were the only other less efficient Nce103 inhibitors (K_Is of 87.7–94.0 μM). The remaining carboxylates showed a compact behavior of efficient inhibitors, with K_Is in the range of 8.6–61.6 μM. The best inhibitor was maleate (also an effective Can2 inhibitor), followed by benzoate, butyrate and malonate (K_Is of 24.4–26.9 μM). These data clearly show that both aliphatic mono- and dicarboxylates, as well as aromatic such derivatives may lead to low micromolar Nce103 inhibitors, even when screening a limited number of derivatives as those investigated in this study. SAR is again rather unpredictable, as for Can2 (discussed above). Considering the aliphatic derivatives **1–5**, the best activity was observed for the C4 carboxylate (K_I of 26.9 μM), with the C1 and C2 derivatives showing a rather similar behavior of slightly weaker inhibitors (K_Is of 35.1–36.0 μM), whereas propionate and valerate were the least inhibitory aliphatic monocarboxylates. The difference in inhibitory activity between pyruvate and L-lactate is quite important (2.25-fold), although these derivatives differ only by the absence of two hydrogen atoms in pyruvate with respect to L-lactate. Similarly, the two aromatic carboxylates **13** and **14** possess quite different Nce103 inhibitory properies, with benzoate **13** being 2.52 times a better inhibitor than the tetrafluoro derivative **14**. Just the opposite was found for the inhibition of Can2 or hCA II (Table 1).

(iii) CAs are ubiquitous enzymes being present both in the human host (with 15 different isoforms known to date)[1] and in the fungal pathogens *C. albicans* and *C. neoformans* investigated here. Thus, designing novel applications for CA inhibitors raises the important question of inhibitor selectivity for the pathogenic fungus over host enzymes.[1] However, in this specific case, the fungal CAs belong to the β-CA family, whereas mammals (including *Homo sapiens*) possess only α-CAs. As a consequence, as largely illustrated by data of Table 1 too, it may be easier to achieve a rather selective inhibition of the parasite over the host enzymes. This is for example readily observed for maleate **8**, one of the best Can2 and Nce103 inhibitors (K_Is of 8.6–8.7 μM) detected here, which is a quite weak hCA II inhibitor (K_I of 989 μM). Thus, the selectivity ratios for inhibiting the pathogenic fungal enzymes over the ubiquitous host enzyme hCA II with maleate are of 113.6 for Can2 and of 115 for Nce103. This simple carboxylate may thus be considered as a promising starting lead molecule for obtaining better, possibly low nanomolar (and selective) β-CA inhibitors.

3. Conclusions

We investigated the inhibition of the β-CAs from the fungal pathogens *C. neoformans* and *C. albicans* (Can 2 and Nce103, respectively) with a series of mono-, bi- and tri-carboxylates, some of which are Krebs cycle intermediates. The best Can2 inhibitors were acetate and maleate (K_Is of 7.3–8.7 μM), whereas formate, acetate, valerate, oxalate, maleate, citrate and 2,3,5,6-tetrafluorobenzoate showed less effective inhibition, with K_Is in the range of 42.8–88.6 μM. Propionate, butyrate, malonate, L-malate, pyruvate, L-lactate and benzoate, were weak Can2 inhibitors, with inhibition constants in the range of 225–1267 μM. Nce103 was more susceptible to inhibition with carboxylates compared to Can2, with the best inhibitors (maleate, benzoate, butyrate and malonate) showing K_Is in the range of 8.6–26.9 μM. L-Malate and pyruvate together with valerate were less efficient Nce103 inhibitors (K_Is of 87.7–94.0 μM), whereas the remaining carboxylates showed a compact behavior of efficient inhibitors, with K_Is in the range of 35.1–61.6 μM. The inhibition profiles of the two fungal β-CAs is very different from that of the ubiquitous host enzyme hCA II belonging to the α-CA family, with maleate showing a selectivity ratio of 113.6 for Can2 over hCA II, and of 115 for Nce103 over hCA II inhibition. Maleate is a promising starting lead molecule for obtaining better, possibly low nanomolar and selective β-CA inhibitors.

4. Experimental

4.1. Chemistry

Buffers and carboxylates of Table 1 (as sodium salts) were of highest purity available, and were used without further purification,

being purchased from Sigma–Aldrich (Milan, Italy). hCA II was prepared in recombinant form as described earlier,[17] whereas Can2 and Nce103 were recombinant, purified enzymes obtained as described by Mühlschlegel's and Steegborn's groups.[6–9]

4.2. Carbonic anhydrase inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[18] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 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 initial rates of 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 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 inhibitor (10 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, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[8,9] and represent the mean from at least three different determinations.

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