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ORIGINAL ARTICLE

Carbonic anhydrase inhibitors. Phenols incorporating 2- or 3-pyridyl-ethenylcarbonyl and tertiary amine moieties strongly inhibit *Saccharomyces cerevisiae* β-carbonic anhydrase

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Abstract

A series of phenols incorporating tertiary amine and *trans*-pyridylethenyl-carbonyl moieties were assayed as inhibitors of the β-carbonic anhydrase (CA, EC 4.2.1.1) from *Saccharomyces cerevisiae*, ScCA. One of these compounds was a low nanomolar ScCA inhibitor, whereas the remaining ones inhibited the enzyme with K_Is in the range of 23.5–95.4 nM. The off-target human (h) isoforms hCA I and hCA II were much less inhibited by these phenols, with K_Is in the range of 0.78–23.5 μM (hCA I) and 10.8–52.4 μM (hCA II). The model organism *S. cerevisiae* and this particular enzyme may be useful for detecting antifungals with a novel mechanism of action compared to the classical azole drugs to which significant drug resistance emerged.

Keywords

Antifungalscarbonic anhydrase, phenols, pyridylethenyl-carbonyl, *Saccharomyces cerevisiae*

History

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Introduction

Fungi encode carbonic anhydrases (CAs, EC 4.2.1.1) belonging to at least two families, the α- and β-class such enzymes[1–5]. Five distinct genetic families (α-, β-, γ-, δ- and ζ-CAs) encode such enzymes in organisms all over the phylogenetic tree[6–15]. CAs efficiently catalyze the simple but essential reaction between carbon dioxide and water, with generation of bicarbonate and protons, which is too slow without a catalyst at neutral pH values, by using a metal hydroxide as nucleophile, at nearly neutral pH values[10–20]. All chemical species involved in the CA-catalyzed reaction, carbon dioxide, bicarbonate and protons, are involved in many crucial physiologic processes and their perturbation may lead to dysfunctions[10–20].

CAs also play important roles in fungi/yeasts, being involved in the CO_2-sensing mechanism of these organisms and in their regulation of sexual development[1–5]. In fact physiological concentrations of CO_2/HCO_3^- induce prominent virulence attributes in *Candida albicans* (e.g. filamentation) and capsule biosynthesis in *Candida glabrata* or *Cryptococcus neoformans*, through the direct activation of the fungal adenylyl cyclase[2–6,8]. CO_2/HCO_3^- equilibration by fungal CAs, both related to CO_2 sensing and pathogenesis[2–6], were thoroughly investigated in several species. For example, the *C. albicans* enzyme CaNce103 or the orthologous *C. glabrata* one (CgNce103) – both belonging to the β-CA family – were shown to be essential for pathogenesis of these fungi in niches where CO_2 availability is limited (e.g. the skin), or essential for the growth of *C. neoformans* in its natural environment (the enzyme from this pathogen was denominated Can2)[6]. The newly discovered bZIP transcription factor CgRca1p is also required in such processes in various species such as *C. albicans*, *C. glabrata* and *S. cerevisiae*[2–6,8–13]. Thus, the link between cAMP signaling and CO_2/HCO_3^- sensing is conserved in fungi/yeasts and revealed CO_2 sensing to be an important mediator of fungal metabolism and pathogenesis[2–6,8–13].

The model organism *Saccharomyces cerevisiae* also encodes for a β-CA (the gene encoding this enzyme is denominated as Nce103), which is required for providing sufficient bicarbonate to essential metabolic carboxylation reactions for the yeast metabolism, such as those catalyzed by pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC), carbamoyl phosphate synthase (CPSase) and phosphoribosylaminoimidazole (AIR) carboxylase[11–13]. This enzyme (ScCA) has been investigated for its inhibition and activation with a range of modulators of activity such as sulfonamides and anions as inhibitors, and amines/amino acids as activators[11–13]. Another yeast which has been investigated recently for the presence of CAs was *Malassezia globosa*, the organism causing dandruff[14]. As other fungi/yeasts, *M. globosa* contains only

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one β-CA, which has been denominated as MgCA[14]. For this enzyme, a proof-of-concept study showed that inhibition of MgCA with sulfonamides has antifungal effects *in vivo*, in an animal model of dandruff[14], leading to the proposal of the fungal CAs from such pathogens as a possible antifungal target.

Apart from sulfonamides and their isosteres (sulfamates, sulfamides, etc) which represent the main class of CA inhibitors (CAIs)[16–26], CAs are also inhibited by phenols, through a different mechanism of action[7]. Indeed, whereas sulfonamides coordinate (in deprotonated state, as anions) to the metal ion from the enzyme active site, phenols (and several other classes of less investigated CAIs) anchor to the water molecule/hydroxide ion coordinated to the metal ion[7,15,21]. Phenols were investigated in some detail for the inhibition of the many mammalian CA isoforms (CA I–XV)[27–29], but not such studies for the inhibition of fungal β-CAs are available so far. Here we report the first phenol inhibition study of a fungal/yeast β-CA, more precisely, we investigated the inhibition of ScCA with a series of phenols incorporating tertiary amine and pyridylethenyl-carbonyl moieties, recently reported by one of our groups[30].

Materials and methods

Chemistry

Phenols **1**–**13** used in this study were reported earlier by one of our groups[30]. Acetazolamide used as standard was from Sigma-Aldrich (Milan, Italy). Buffers and inorganic reagents were of highest grade purity available from Sigma-Aldrich (Milan, Italy).

CA inhibition

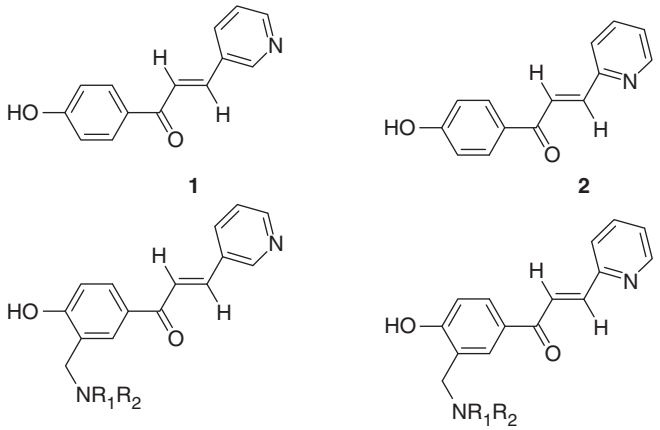
An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity[31]. Phenol red (at a concentration of 0.2 mM) has been used as an indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.4 for hCA I and II) or with 20 mM Tris (pH 8.4, for ScCA) as buffers, and 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 0.001 nM were done thereafter with the assay buffers. 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 (GraphPad Software, La Jolla, CA), as reported earlier[11–13], and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained as reported earlier in our laboratories[11–13,32,33]. The concentration of the enzymes in the assays was of 8.0 nM for hCA I, of 5.9 nM for hCA II and of 12.5 nM for ScCA, respectively.

Results and discussion

Inhibition data for two human CA isoforms (hCA I and II) and ScCA with compounds **1**–**13** and acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, as standard inhibitor) are shown in Table 1.

Figure 1 shows an alignment of the amino acid sequences of selected β-CAs from fungal, bacterial, plant and algal species, whereas Figure 2 present a phylogenetic analysis of many such enzymes from a large variety of bacteria, fungi/yeasts, algae and plants.

Table 1. Human (h) hCA I, II and yeast (*S. cerevisiae*) ScCA inhibition data with phenols **1–13** by a stopped flow CO_2 hydrase assay[31].



Compound	NR_1R_2	Ki (μM)* hCA I	Ki (μM)* hCA II	Ki (nM)* ScCA
1	–	0.78	12.5	76.5
2	–	1.77	10.8	65.8
3	Me_2N	23.5	29.8	23.5
4	Et_2N	7.76	26.9	77.1
5	$(CH_2)_4N$	5.40	34.7	4.9
6	$(CH_2)_5N$	17.9	41.5	65.0
7	$MeN(CH_2CH_2)N$	3.35	14.7	76.5
8	Me_2N	9.01	34.9	90.1
9	Et_2N	4.05	43.1	81.9
10	$(CH_2)_4N$	2.36	45.8	85.8
11	$(CH_2)_5N$	1.88	48.0	79.1
12	$MeN(CH_2CH_2)N$	2.01	47.7	79.5
13	$O(CH_2CH_2)N$	0.91	52.4	95.4
AAZ	–	0.25	0.012	82.6

*Mean from three different assays (errors in the range of ±10% of the reported values, data not shown).

Data from Table 1 show that the human (off-target) isoforms are poorly inhibited by phenols **1**–**13**, investigated here, which typically had inhibition constants in the micromolar range. For example, against hCA I the K_Is were in the range of 0.78–23.5 μM, whereas against hCA II in the range of 10.8–2.4 μM. The unsubstituted phenols **1** and **2** were among the most effective hCA I inhibitors (K_Is were in the range of 0.78–1.77 μM), and the introduction of tertiary amine moieties in *ortho* generally leads them to a strong diminution of the inhibitory activity. This is even more evident in the case of hCA II, for which the simple phenols **1** and **2** were again the best inhibitors (K_Is were in the range of 10.8–12.5 μM), whereas the tertiary amines **3**–**13** were generally much weaker inhibitors (Table 1). It should be observed that the sulfonamide AAZ is a much more potent inhibitor of both these α-CAs, with K_Is in the range of 12–250 nM.

Interestingly, the phenols **1**–**13** investigated here showed very good ScCA inhibitory activity, with K_Is in the range of 4.9–95.4 nM (Table 1). This is an unexpected result due to the fact that phenols were generally weaker inhibitors against most investigated CAs[3,4], but it should be mentioned, as noted in the ''Introduction'' section, that phenols were not yet investigated as ScCA inhibitors till the present work. The two parent compounds **1** and **2** were medium potency ScCA inhibitors, with inhibition constants in the range of 65.8–76.5 nM, similar to the sulfonamide



Figure 1. Alignment of the amino acid sequences of selected β-CAs from four species. The *S. cerevisiae* numbering system was used. Zinc ligands (amino acids: 57, 112 and 115) are indicated in red whereas the catalytic dyad (amino acids 59 and 61) typical of β-CAs in blue. The asterisk (*) indicates identity at a position; the symbol (:) designates conserved substitutions, while (·) indicates semi-conserved substitutions. Multiple alignment was performed with the program Clustal W, version 2.1 (http://www.ebi.ac.uk/services/proteins). Sequence accession numbers are indicated in Figure 2.

AAZ (K_I of 82.6 nM, investigated earlier by these groups)[11]. However, many of the derivatized phenols **3**–**13** showed enhanced ScCA inhibitory action compared to the parent phenols **1** and **2**. For example, compound **5**, incorporating the 3-pyridiyl moiety and the pyrrolidine ring in *ortho* to the phenol moiety, was a low nanomolar (K_I of 4.9 nM) inhibitor of the yeast enzyme. Interestingly, its homolog **6**, with an extra CH_2 moiety (piperidine instead of pyrrolidine ring) was 13.2 times a weaker ScCA inhibitor compared to **5**. Thus, small structural changes of the scaffold lead to dramatic changes of the ScCA inhibitory action in this class of compounds. Structure–activity relationship (SAR) is in fact rather complicated. The nature of the R_1 and R_2 groups substituting the amine functionality seem to be the major factor influencing enzyme inhibitory activity. However, this influence is rather complicated. For example, for the 3-pyridyl-substituted compounds, activity is increasing with the introduction of the Me_2N moiety in the lead **1** (compound **3**, K_I of 23.5 nM) but then decrease again for the diethylamino derivative **4** (K_I of 77.1 nM). As mentioned above, the cyclic derivative **5** is the most potent ScCA inhibitor, but then its homolog **6** and the similar compound **7** showed activities comparable to that of the diethylamino derivative **4**. For the 2-pyridyl-substituted series activity of the tertiary amines **8**–**13** was lower compared to that of the lead **2**, making this subseries less interesting.

But how can these results be rationalized, considering than no X-ray crystal structure of ScCA in adducts with inhibitors is available? In order to explain these data, we aligned the amino acid sequences of the ScCA enzyme with those of other β-CAs investigated earlier, from bacterial plant or algae (Figure 1).

As seen from Figures 1 and 2, ScCA shares many common feature with the corresponding β-CAs from other fungi/yeasts, bacteria or plants/algae. As seen from the alignment of Figure 1, all these enzymes possess the typical β-CA zinc-coordinating ligands, i.e. two cysteine and one histidine residue, in the specific case Cys57, His112 and Cys115 (ScCA numbering system is used throughout this paper[11–13]). The fourth zinc ligand is a water molecule/hydroxide ion which acts as nucleophile in the catalytic cycle, attacking the CO_2 molecule bound in a hydrophobic pocket nearby. However, unlike the highly investigated α-class CAs, in which the zinc-bound hydroxide is easily generated (and it participates in a hydrogen bond network with Thr199 and Asp106), for the β-CAs a different catalytic dyad participates in the activation of the zinc-coordinated water molecule, constituted by Asp59 and Arg61 (Figure 1), which is also present in ScCA and conserved in all β-CAs investigated so far[11–13].

Furthermore, the phylogenetic tree from Figure 2, in which all fungal/yeast β-CAs for which the sequence is available in data bases were included, shows these enzymes to be related with each other, all of them clustering on very nearby branches of the tree. It is interesting to note that the enzyme more phylogenetically similar to ScCA is the one from *Dekkera bruxellensis* (DbrCA). These enzymes cluster on a branch, which is intermediate bewteen the bacterial β-CAs and such plant/algal enzymes. However, the yeast *S. pombe* did not cluster with the other yeast but on a separate branch together with some bacterial enzymes (from *B. thailandensis* and *B. suis*) (Figure 2).

Conclusions

In conclusion, we report here a series of phenols incorporating tertiary amine and *trans*-pyridylethenyl-carbonyl moieties, which were assayed as inhibitors of the β-carbonic anhydrase (CA, EC

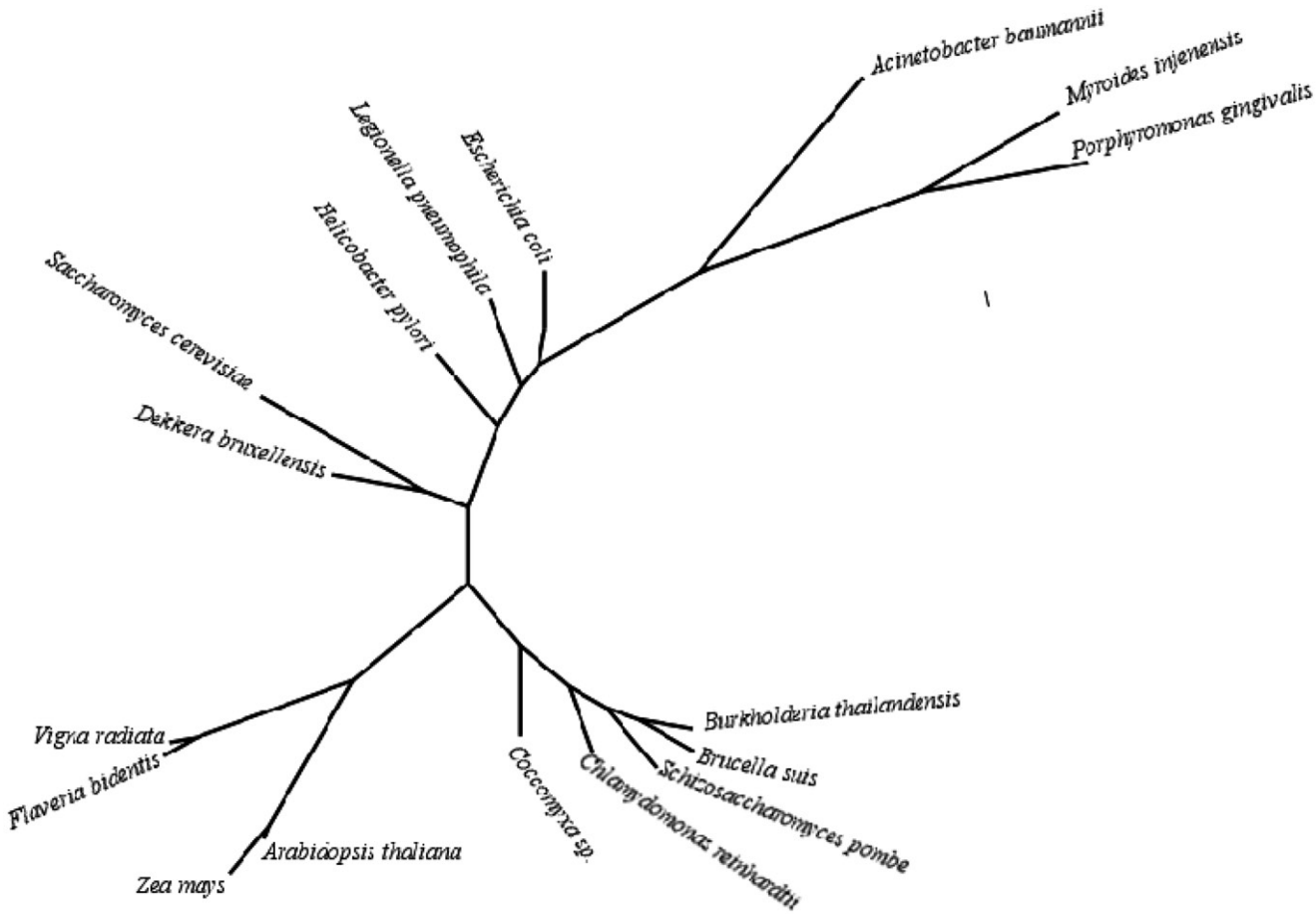


Figure 2. Phylogenetic tree of the beta CAs from selected eukaryotic and prokaryotic species. The tree was constructed using the program PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/). Organisms and sequence accession numbers are as follows: *Schizosaccharomyces pombe* (CAA21790); *Brucella suis 1330* (NP_699962.19); *Burkholderia thailandensis Bt4* (ZP_02386321); *Coccomyxa* sp. (AAC33484.1); *Chlamydomonas reinhardtii* (XP_001699151.1); *Acinetobacter baumannii (*YP_002326524); *Porphyromonas gingivalis* ATCC 33277 (YP_001929649.1); *Myroides injenensis* (ZP_10784819); *Zea mays* (NP_001147846.1); *Vigna radiata* (AAD27876); *Flaveria bidentis*, isoform I (AAA86939.2); *Arabidopsis thaliana* (AAA50156); *Helicobacter pylori* (BAF34127.1); *Legionella pneumophila 2300/99* (YP_003619232); *Escherichia coli* (ACI70660); *Saccharomyces cerevisiae* (GAA26059); *Dekkera bruxellensis AWRI1499* (EIF49256).

4.2.1.1) from *S. cerevisiae*, ScCA. One of these compounds was a low nanomolar ScCA inhibitor (K_I of 4.9 nM), whereas the remaining ones inhibited the enzyme with K_Is in the range of 23.5–95.4 nM. The off-target human (h) isoforms hCA I and hCA II were much less inhibited by these phenols, with K_Is in the range of 0.78–23.5 μM (hCA I) and 10.8–52.4 μM (hCA II). The model organism *S. cerevisiae* and this particular enzyme may be useful for detecting antifungals with a novel mechanism of action compared to the classical azole drugs to which significant drug resistance emerged, also considering the fact that we have recently shown that some potent sulfonamide ScCA inhibitors also inhibit the growth of the yeast *in vivo*[34].

Declaration of interest

The authors declare no conflict of interest.

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