Inhibition survey with phenolic compounds against the \( \delta \)- and \( \eta \)-class carbonic anhydrases from the marine diatom *thalassiosira weissflogii* and protozoan *Plasmodium falciparum*

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**ABSTRACT**

The inhibition of \( \delta \)- and \( \eta \)-class carbonic anhydrases (CAs; EC 4.2.1.1) was poorly investigated so far. Only one \( \delta \)-CA, TweCA from the diatom *Thalassiosira weissflogii*, and one \( \eta \)-CA, PICA, from *Plasmodium falciparum*, have been cloned and characterised to date. To enrich \( \delta \)- and \( \eta \)-CAs inhibition profiles, a panel of 22 phenols was investigated for TweCA and PICA inhibition. Some derivatives showed effective, sub-micromolar inhibition of TweCA (K\( \text{I}_\text{S} \) 0.81–65.4 \( \mu \text{M} \)) and PICA (K\( \text{I}_\text{S} \) 0.62–78.7 \( \mu \text{M} \)). A subset of compounds demonstrated a significant selectivity for the target CAs over the human physiologically relevant ones. This study promotes the identification of new potent and selective inhibitors of TweCA and PICA, which could be considered as leads for finding molecular probes in the study of carbon fixation processes (in which TweCA and orthologue enzymes are involved) or drug candidates in the treatment of malaria.

1. Introduction

Carbonic anhydrases (CAs; EC 4.2.1.1) compose a superfamily of metalloenzymes that owe the role of speeding up the carbon dioxide hydration to bicarbonate and proton. Crucial biological processes in most organisms of tree of life are related to such a reversible reaction: respiration, photosynthesis, pH regulation, CO\(_2\) and HCO\(_3\)\(^-\) transport, biosynthetic processes, production of body fluids, bone resorption, etc. Eight evolutionarily unrelated CA classes have been identified to date, which are named as \( x \)-, \( \beta \)-, \( \gamma \)-, \( \delta \)-, \( \zeta \)-, \( \eta \)-, \( \theta \)- and \( i \)-CAs. The \( x \)-CAs are present in vertebrates, protozoa, algae, corals, bacteria and cytoplasm of green plants. Human, in particular, encode only for \( x \)-class isozymes. The \( \beta \)-CAs have been identified in bacteria, fungi, Archaea, algae and chloroplasts of both mono- and dicotyledons. The \( \gamma \)-CAs are encoded in Archaea, bacteria and plants. \( \delta \)-CAs have been discovered in marine phytoplankton, such as haptophytes, dinoflagellates, diatoms and chlorophyptic prasinophytes, while \( \zeta \)-CAs appear to be present only in marine diatoms. A unique \( \eta \)-CA has been identified to date in the protozoa *Plasmodium falciparum*. \( \theta \)-CAs have been recently discovered in the marine diatom *Phaeodactylum triacanthum*. A first specimen of \( i \)-CAs was recently labelled from the marine diatom *Thalassiosira pseudo-nanum*. A unique \( \delta \)-CA, TweCA, from the diatom *Thalassiosira weissflogii* was cloned and characterised in detail to date, though orthologues of this enzyme have been identified in most diatoms from natural phytoplankton assemblages and are responsible (along with other CAs) for CO\(_2\) fixation by marine organisms. TweCA is upregulated by low pCO\(_2\) and, under Zn-limited conditions, the zinc ion at the active site can be substituted by Co(II) in vivo. TweCA is a protein of 281 amino acid residues. A subunit molecular mass of 32.0 kDa was estimated by SDS-PAGE, while the molecular mass of 32.2 kDa was calculated from the amino acid sequence. TweCA does not share any sequence homology to any other known CAs. The alignment of the amino acid sequence of TweCA with the polypeptide chain of the bovine \( x \)-CA (isoform bCA II) shows the low degree of identity with the mammalian \( x \)-CA. Nonetheless, it was shown that the active site of TweCA is similar to that of mammalian \( x \)-CA, with the metal coordination pattern formed by three histidines as found in \( x \) and \( \gamma \)-CAs (Figure 1). Unfortunately, no structural data are available on \( \delta \)-CAs. A phylogenetic analysis carried out using \( x \), \( \gamma \) and \( \delta \)-CAs from different prokaryotic and eukaryotic organisms showed that the \( x \)-CAs appear closely related to the \( \delta \)-CAs, but clustered in a branch distinct from that of \( \gamma \)-CAs. CA inhibitors, such as sulphonamides, inorganic anions, mono- and dithiocarbamates were screened as TweCA inhibitors with the aim to uncover molecular probes to investigate the role of this enzyme in the carbon fixation processes in marine diatoms that are responsible for removing large amounts of CO\(_2\) from the atmosphere.

The \( \eta \)-class of CAs was firstly described in 2015 by analysis of the amino acid sequences of CAs from *Plasmodia*, parasitic protozoa responsible of malaria in humans and other animals. The first and unique member of the family to be characterised in *in vitro* to
date was PfCA, a protein of 600 amino acid residues, identified in Plasmodium falciparum, one of the five species causing malaria in humans. Interestingly, PfCA was initially described as an α-CA enzyme, due to significant similarities with members of this class, but was subsequently reclassified into a new CA class, the η, due to some peculiar features. In particular, the zinc coordination pattern of PfCA is formed by two histidines and one glutamate, distinctly from α-CAs, and many insertions and deletions in the protozoan enzyme were identified with respect to common α-CAs: insertions were observed in PfCA at the N-terminus and in the middle of the protein (69 additional residues after residue 152 of hCA II, chosen as reference protein). A three-dimensional model of PfCA was built by homology using the structure of Thermovibrio ammonificans CA (TaCA) as template. Because of low sequence homology only 267 residues (198–327 and 397–535) out of the 600 of the full-length protein could be modelled. A folding similar to that of α-CAs was found with the active site located in a large cavity with the zinc ion on the bottom (Figure 1), coordinated by His299, His301 and Gln320. The 69 residues insertion was located at the edge of the active site cleft, being presumably implicated in the catalysis.

A significant interest is being dedicated to PfCA, because the enzyme has been recognised as possible target for the development of antimalarial drugs based on innovative mechanism of action. Indeed, a crucial role was suggested for PfCA in the Plasmodium parasites, being involved in the production of HCO₃⁻/CO₂-bisynthetic pathway. Its targeting to block this pathway could thus represent an efficient strategy for the development of new pharmacological agents against malaria. In 1998, Sein and Aikawa showed that addition of carbonic anhydrase inhibitors (CAIs) to a culture of P. falciparum provoked a remarkable reduction in parasitemia. Successive reports illustrated that specific CA inhibition in P. falciparum and in the rodent parasite P. berghei produced the death of the parasite in in vitro cultures. Starting from these data, the search of new PfCA inhibitors has started with sulphonamides and inorganic anions, and, though encouraging results have been obtained, more efforts are still necessary to obtain candidate drug molecules.

Here, a series of phenolic derivatives (1–22, Figure 1) was assessed for the inhibition of TweCA and PfCA to extend such isoforms inhibition profiles, in search of novel leads for drug candidates or molecular probes which show the selective modulation of CAs from diatoms and protozoa over human isozymes.

2. Methods
2.1. Chemistry
Phenols 1–22 were commercially available from Sigma-Aldrich (Milan, Italy) and were used without further purification (purity >95%). All other reagents, salts, buffers and solvents were the highest purity available ones from Sigma-Aldrich (Milan, Italy).
many of which are clinically used as diuretics, antiglaucoma, antiepileptic or in clinical trials for the management of advanced, hypoxic solid tumors. In fact, whether sulphamides directly coordinate the Zn(II) ion from the CA active site replacing the coordinated water molecule/hydroxide ion by a hydrogen bond network. Up to now, phenolic derivatives, among which compounds 1–22 investigated here (Figure 2), were assayed as inhibitors of the human CA I, II, IX and XII, of β-CAs, from the fungi Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans and Malassezia Globoza, or from the bacteria Mycobacterium tuberculosis and Pseudomonas gingivalis, Vibrio cholerae and from the Antarctic bacteria Pseudodatheromonas haloplanktis and Colwellia psychrerythraea.

As the δ- and η-CAs active sites are narrower than those of α-CAs, only phenyl derivatives, and not complex natural polyphenols, were considered. A large variety of electron donating and electron withdrawing groups were investigated as substituents on the phenolic scaffold to uncover on the role of acidity of the electron withdrawing groups were investigated as substituents on the phenolic scaffold to uncover on the role of acidity of the phenol scaffold led to light worsening of inhibitory action of the anchoring group in the inhibitory activity (Table 2).

### 3.2. δ- and η-class carbonic anhydrases inhibition

Phenols 1–22 were assayed as inhibitors of the unique δ- and η-class CAs identified to date, specifically from the marine diatom T. weissflogii and protozoan P. falciparum, respectively. A stopped flow CO2 hydrase assay was used including acetazolamide (AAZ) as standard inhibitor. The inhibition profiles against the human ubiquitous CAs I and II are displayed for comparison. The following structure–activity relationships (SAR) can be drawn up from the inhibition data reported in Table 2.

As a general trend, it can be stated that phenolic compounds are able to interfere with the CO2 hydrase activity of δ- and η-class CAs in the micromolar range. Inhibition constants (KIs) spanned, in fact, between 0.81 and 65.4 μM against TweCA and 0.62 and 78.7 μM against PICA, while compounds 13 and 14 did not show inhibition below 100 μM.

It is fair to immediately stress that even carboxylic acids can act as CAIs, and can do that by two distinct mechanisms of action: coordination of the metal(II) ion or anchorage to the zinc-bound nucleophile. As a result, one cannot exclude that compounds 10, 11, 18–22, which bear both phenolic and carboxylic groups, produce CA inhibition by the COOH function in place of the OH group.

Most substitutions at the phenol 1 scaffold produce enhancement in the inhibition of both TweCA and PICA, with the exception of m-substituents of the amine type (6 and 13) and an o-chlorine atom (14), that presumably induce significant steric hindrance for the binding in the active site. Also a p-CN group at the phenol scaffold led to light worsening of inhibitory action of 9 against TweCA in comparison to the lead 1 (Ks of 52.3 and 56.9 μM, respectively).

As for TweCA, a consistent subset of derivatives showed Ks lower than 10 μM (Ks in the range 0.81–7.9 μM). In particular, 1,2-diols 1 and 5 exhibited the most potent TweCA inhibition (Ks of 4.5 and 2.0 μM) among those compounds possessing solely OH and not COOH groups. On the other hand, swapping the second aromatic OH group to the m- or p-position did not produce a consistent increase of TweCA inhibition which settled for 3 and 4 at 48.2 and 34.9 μM. The substitution of hydrogens with fluorine atoms on the phenol scaffold increased the inhibition of TweCA by 15–17 (Ks in the range 13.8–30.7 μM) with respect to the

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**Figure 2.** Structures of phenolic compounds 1–22.
lead 1. In contrast, all benzoic derivatives reported lower to submicromolar activity. Precisely, the 2-hydroxy-benzoic acids 10 and 20 resulted to be the best TweCA inhibitors with submicromolar K_i of 0.95 and 0.81 μM. The presence of COOH group of the cinnamic acid type, such as in 21 or 22 did not elicit the same inhibition increased observed with benzoic acids, though the presence of a 1,2-diol portion in 22 drove its K_i against TweCA below 10 μM. None of the assayed compounds provoked as inhibitory effect as the reference AAZ (K_i of 83 nM).

As anticipated above, a superimposable inhibitory trend was measured for PICA with phenols 1–22 (Table 2). The 1,2,4-trioli 5 showed inhibition of the plasmoidal CA in the submicromolar range (K_i of 0.83 μM) reaching almost the same efficacy of benzoic acid derivatives with respect to those observed for TweCA. As sole exceptions, carboxylates 18, 20 and 21 showed a significant selectivity of action with SI settling between 5 and 10 over both CA I and II. Also the trifluorophenol 17 displayed an interesting selectivity against TweCA over human CA isozymes (SI of 7).

Even higher SI were calculated against PICA over both hCAs I and II (Table 3). 1,2-Diols 2 and 5 showed the most selective and promising inhibition of the target PICA with respect to human CAs. While the SI of 2 settled at 70 and 4 over hCA I and II, respectively, those of 5 were even higher than 100 in both cases. The hCAs/PICA SI values were also increased with most benzoic acid derivatives with respect to those observed for TweCA. As sole exceptions, carboxylates 18, 20 and 21 should be cited, since reported specificity of action for the human isozyme over the target ones (SI < 1). Analogue selectivity trend was observed for most other phenols (not showing COOH groups), such as 13 and 14, which were particularly selective against hCAs over the plasmoidal and diatom isozymes.

### Table 2: Inhibition data of TweCa and PICA with phenols 1–22 and the standard sulphamamide inhibitor acetazolamide (AAZ) by a stopped flow CO2 hydrase assay.

| Cmpd | R       | TweCa      | PICA       | CA IA | CA IB |
|------|---------|------------|------------|-------|-------|
| 1    | H       | 52.3       | 68.1       | 10.2  | 5.5   |
| 2    | 2-OH    | 4.5        | 1.4        | >100  | 5.5   |
| 3    | 3-OH    | 48.2       | 26.9       | >100  | 9.4   |
| 4    | 4-OH    | 34.9       | 21.0       | 10.7  | 0.1   |
| 5    | 2,4-diOH| 2.0        | 0.83       | >100  | >100  |
| 6    | 3-NH2   | 65.4       | 78.7       | 4.9   | 4.7   |
| 7    | 4-NH2   | 17.6       | 33.8       | >100  | >100  |
| 8    | 4-NHCOCH3| 7.9       | 26.4       | 10.0  | 6.2   |
| 9    | 4-CN    | 56.9       | 36.9       | >100  | 0.1   |
| 10   | 2-COOH  | 0.95       | 0.72       | 9.9   | 7.1   |
| 11   | 4-COOH  | 1.4        | 0.90       | 9.8   | 10.6  |
| 12   | 4-CH2OH | 35.9       | 47.1       | 68.9  | 95.3  |
| 13   | 3-NH2-4-Cl| >100      | >100       | 6.3   | 4.9   |
| 14   | 4-NH2-2-Cl| >100     | >100       | 57.8  | 57.5  |
| 15   | 2,5-diF | 30.7       | 41.3       | >100  | >100  |
| 16   | 3,5-diF | 21.0       | 32.7       | 38.8  | 33.9  |
| 17   | 2,4,6-triF| 13.8      | 22.8       | >100  | >100  |
| 18   | 4-COOH-2-OH| 4.9      | 1.6        | 1.1   | 0.5   |
| 19   | 2-COOH-3-OH| 2.7      | 2.5        | 5.7   | 5.2   |
| 20   | 2-COOH-4-OH| 0.81     | 0.62       | 4.2   | 4.1   |
| 21   | 4-(CH2COOH)| 14.5    | 5.7        | 1.1   | 1.3   |
| 22   | 2-OH-4-(CH2COOH)| 5.9  | 11.2       | 2.4   | 1.6   |
| AAZ  | –       | 0.08       | 0.36       | 0.25  | 0.01  |

aMean from 3 different assays, by a stopped flow technique (errors were in the range of ±5–10% of the reported values)
bData from ref.33

Table 3. Selectivity index (SI) for target CA over the off-target hCA I and II.

| Cmpd | R       | I/TweCA | II/TweCA | PICA/I | PICA/II |
|------|---------|---------|----------|--------|--------|
| 1    | H       | 0.2     | 0.1      | 0.1    | 0.08   |
| 2    | 2-OH    | >22.2   | 1.2      | 71.4   | 3.9    |
| 3    | 3-OH    | >2.0    | 0.2      | >3.7   | 0.3    |
| 4    | 4-OH    | 0.3     | 0.01     | 0.5    | <0.01  |
| 5    | 2,4-diOH| >50     | >50      | >100   | >100   |
| 6    | 3-NH2   | 0.07    | 0.06     | 0.05   |        |
| 7    | 4-NH2   | >5.6    | >5.6     | >2.9   | >0.6   |
| 8    | 4-NHCOCH3| 1.2     | 0.8      | 0.4    | 0.2    |
| 9    | 4-CN    | >1.7    | <0.01    | >2.7   | >0.01  |
| 10   | 2-COOH  | 10.4    | 7.4      | 13.7   | 9.8    |
| 11   | 4-COOH  | 7.0     | 7.5      | 10.9   | 11.8   |
| 12   | 4-CH2OH | 1.9     | 2.6      | 1.4    | 2.0    |
| 13   | 3-NH2-4-Cl| <0.06   | <0.05    | <0.06  |        |
| 14   | 4-NH2-2-Cl| <0.6   | <0.5     | <0.6   | <0.6   |
| 15   | 2,5-diF | >3.2    | >3.2     | >2.4   | >2.4   |
| 16   | 3,5-diF | 1.8     | 1.6      | 1.1    | 1.0    |
| 17   | 2,4,6-triF| >7.2    | >7.2     | >4.3   | >4.3   |
| 18   | 4-COOH-2-OH| 0.2   | 0.1      | 0.6    | 0.3    |
| 19   | 2-COOH-3-OH| 2.1    | 1.9      | 2.3    | 2.1    |
| 20   | 2-COOH-4-OH| 5.2    | 5.0      | 6.8    | 6.2    |
| 21   | 4-(CH2COOH)| 0.07   | 0.09     | 0.2    | 0.2    |
| 22   | 2-OH-4-(CH2COOH)| 0.4  | 0.3      | 0.2    | 0.1    |
| AAZ  | –       | 3.1     | 0.1      | 0.7    | 0.03   |

4. Conclusions
CAs of α- and γ-classes have not been extensively characterised from the inhibitory standpoint in comparison to α- and β-class isozymes. A unique α-CA, TweCA, from the diatom Thalassiosira weissflogii was cloned and characterised in detail to date, though orthologues of this enzyme have been identified in most diatoms from natural phytoplankton assemblages and are responsible,
along with other CAs for CO₂ fixation by marine organisms. The identification of selective inhibitors of these isoforms is of significant importance to uncover molecular probes to investigate the role of this enzyme in the carbon fixation processes of marine diatoms that are responsible for removing large amounts of CO₂ from the atmosphere.

Meanwhile a significant interest has been dedicated to PfCA, the unique specimen of η-CA, which was identified in Plasmodium falciparum, one of the five species causing malaria in humans. The research of PfCA inhibitors has started with sulphonamides and inorganic anions, and, though encouraging results have been obtained, more efforts are still necessary to obtain candidate drug molecules.

To extend TweCA and PfCA inhibition profiles, in search of novel leads for drug candidates or molecular probes selectively modulating these CAs over human isoforms, a panel of 22 phenols was investigated for these isoforms’ inhibition. The exploration of the chemical space around the main functional group led to the discovery of a number of such derivatives showing effective, sometimes sub-micromolar, inhibition against TweCA (Kᵢₛ 0.81 and 65.4 μM) and PfCA (Kᵢₛ 0.62 and 78.7 μM). A subset of compounds even demonstrated a significant selectivity for the target CAs over the human physiologically relevant isoforms CA I and II. This study improves the knowledge on the modulation of CAs belonging to uncommon classes such as δ and η. As a result, it promotes the identification of new potent and selective inhibitors against diatom and plasmodial isoforms over human off-target CAs, which could be adopted as leads for finding molecular probes in the study of carbon fixation processes or drug candidates in the treatment of malaria.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was funded by the Deanship of Scientific Research at Princess Nourah bint Abdulrahman University, through the Research Groups Programme Grant no. [RGP-1440–0024].

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