**ABSTRACT**

The inhibition of the δ-class carbonic anhydrase (CAs, EC 4.2.1.1) from the diatom *Thalassiosira weissflogii*, TweCAδ, was investigated using a panel of 36 mono- and di-thiocarbamates that have recently been shown to inhibit mammalian and pathogenic CAs belonging to the α- and β-classes. TweCAδ was not significantly inhibited by most of such compounds (Kᵢ values above 20 μM). However, some aliphatic, heterocyclic, and aromatic mono and di-thiocarbamates inhibited TweCAδ in the low micromolar range. For some compounds incorporating the piperazine ring, TweCAδ was effectively inhibited (Kᵢs from 129 to 791 nM). The most effective inhibitors identified in this study were 3,4-dimethoxyphenyl-ethyl-monothiocarbamate (Kᵢ of 67.7 nM) and the R-enantiomer of the nipecotic acid di-thiocarbamate (Kᵢ of 93.6 nM). Given that the activity and inhibition of this class of enzyme have received limited attention until now, this study provides new molecular probes and information for investigating the role of δ-CAs in the carbon fixation processes in diatoms, which are responsible for significant amounts of CO₂ taken from the atmosphere by these marine organisms.

**KEYWORDS**

Carbonic anhydrase; metalloenzymes; mono-thiocarbamate; di-thiocarbamate; *Thalassiosira weissflogii*

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enzyme, TweCAδ, which was cloned and characterised from the marine diatom *T. weissflogii*.

**Materials and methods**

**Materials**

MTCs 1–15 and DTCs 16–36 were reported earlier by our group. Reagents/buffers of the highest available purity were obtained from Sigma-Aldrich, Milan, Italy. TweCAδ was a recombinant protein produced as reported earlier by our group. CA enzyme inhibition assay

An Sx.18Mv-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic activity of various CA isozymes for CO₂ hydration reaction. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, and 0.1 M Na₂SO₄ (for maintaining constant ionic strength, which is not inhibitory against TweCAδ), following the CA-catalysed CO₂ hydration reaction for a period of 10 s at 25°C. The CO₂ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial rate. The uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitors (10 mM) were prepared in distilled-deionised diluted to 1 nM using the assay buffer. Inhibitor and enzyme solutions were pre-incubated together for 15 min (standard assay at room temperature) prior to assay, in order to allow for the formation of the enzyme inhibitor complex. The inhibition constant (KI), was obtained by considering the classical Michaelis–Menten equation and the Cheng-Prusoff algorithm by using non-linear least squares fitting as reported earlier.

**Results and discussion**

TweCAδ is the only CA belonging to the δ-class for which anion and sulphonamide inhibition studies were reported so far. Here, we investigated the inhibition of this enzyme with the panel of MTCs and DTCs of the types 1–36 shown in Figures 1 and 2. The results are shown in Table 1, where for comparison reasons,
the inhibition of the human dominant isoforms hCA I and II with the same compounds are reported\(^1,2,4\).

The following structure-activity relationship (SAR) can be obtained from the data of Table 1:

(i) A number of MTCs, including 4–6, 10 and the DTCs 20, 21, 23–25, 32, and 33, did not inhibit TweCA\(_d\) up to 20 \(\mu\)M, although many of these compounds were rather effective inhibitors of hCA I and/or hCA II (Table 1). Such MTCs/DTCs inhibitors are classified as aliphatic, heterocyclic, aromatic, or polycyclic types. Given the structural diversity of such compounds and high inhibition constants, it is challenging to delineate the SAR.

(ii) The MTCs/DTCs 3, 13–19, 22, 26, 29, and 31 were relatively ineffective inhibitors of TweCA\(_d\) with inhibition constants in the micromolar range (\(K_s\) ranged between 1142 and 9239 nM; Table 1). These compounds are also highly heterogeneous. The main observation of these data is that the identity of the zinc-binding group, ZBG (MTC or DTC), does not significantly impact the activity of TweCA\(_d\).

(iii) The MTC/DTCs 1, 2, 7–9, 28, 30, and 34–36 were relatively effective inhibitors of TweCA\(_d\), with inhibition constants in the range of 129–997 nM (Table 1). Some of the MTC and DTCs incorporate the piperazine ring (7–9, 34). In addition, MTC 9 and DTC 34 have the same scaffold but a different ZBG. In this particular case, MTC 9 inhibited TweCA\(_d\) 6.1-times more efficiently than DTC 34. Interestingly, for the \(\beta\)-CAs, the MTCs were usually much weaker inhibitors compared to the structurally similar DTCs\(^4\). In addition, the sulphonamide-containing DTC 36 (which contains two potential ZBGs, the sulphonamide and the DTC), there are no net

Figure 2. Dithiocarbamates (DTCs) 16–36 investigated as CA inhibitors\(^1,2,4\).
Table 1. TweCAα, hCA I, and hCA II Inhibition Data with MTCs 1–15, DTCs 16–36, and acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulphonamide) as standard drug, by a stopped-flow CO2 hydrase assay.

| No. | R       | R1                  | TweCAα | hCA I  | hCA II |
|-----|---------|---------------------|--------|--------|--------|
| 1   | n-Pr    | n-Pr                | 806.7  | > 2000 | 46.7   |
| 2   | Et      | n-Pr                | 783.3  | 700    | > 2000 |
| 3   | n-Bu    | n-Bu                | 1142   | 909    | > 2000 |
| 4   | i-Bu    | i-Bu                | > 2000 | 681    | 43.0   |
| 5   | Me      | CH3COEt             | > 2000 | 827    | 44.5   |
| 6   | H       | –(CH2CH2)2O(CH2CH2)– | > 2000 | 569    | > 2000 |
| 7   | H       | –(CH2CH2)N(CH3CH2CH2)– | 487    | > 2000 | 35.0   |
| 8   | H       | –(CH2CH2)N(CH2CH2)– | 483    | 876    | 22.4   |
| 9   | Me      | –(CH2CH2)N(CH2CONHCH3)– | 129    | 949    | 45.9   |
| 10  | Me      | CH2Ph               | > 2000 | > 2000 | > 2000 |
| 11  | H       | CH2CH2Ph            | 997    | > 2000 | 43.7   |
| 12  | H       | HCH2CH2(3,4-diMeO-C6H4) | 67.7   | 891    | 26.7   |
| 13  | H       | –(CH2CH2)N(3-Cl-C6H4)– | 1505   | 686    | > 2000 |
| 14  | H       | –(CH2CH2)N(4-F-C6H4)– | 1498   | 895    | 46.8   |
| 15  | H       | –(CH2CH2)N(4-CF3-C6H4)– | 1152   | > 2000 | 43.6   |
| 16  | Me       | N(CH2)2             | 8406   | 85.9   | 35.8   |
| 17  | HO(CH2)3 | H                   | 8691   | 706    | 41.7   |
| 18  | HO(CH2)3 | H                   | 7168   | 295    | 24.3   |
| 19  | HO(CH2)3 | H                   | 8597   | 66.5   | 17.3   |
| 20  | H       | > 2000              | 494    | 48.7   |        |
| 21  |      |                     | > 2000 | 240    | 18.9   |
| 22  |      |                     | 7995   | 615    | 65.9   |
| 23  |      | –(CH2)3             | > 2000 | 252    | 30.1   |
| 24  |      | –(CH2)3–CH(OH)CH2– | > 2000 | 428    | 60.7   |
| 25  |      | –(CH2)3–CH(COONa)–  | > 2000 | 485    | 80.1   |
| 26  |      | –(CH2)3–CH(COONa)CH2– | 8429   | 290    | 45.4   |
| 27  |      | (R)–(CH2)3–CH(COONa)CH2– | 93.6   | 496    | 80.5   |
| 28  |      | (S)–(CH2)3–CH(COONa)CH2– | 556    | 109    | 8.9    |
| 29  |      | –(CH2)3–CH(COONa)(CH2)2– | 8980   | 337    | 78.7   |
| 30  |      | –(CH2)3–CH(NHAc)CH2– | 783    | 910    | 47.9   |
| 31  |      | –(CH2)3–CH(NHAc)CH2– | 9239   | 683    | 13.2   |
| 32  |      | –CH(Me)CH2–O(–CH2)2– | > 2000 | 434    | 60.2   |
| 33  |      | –CH(COONa)CH2–O(–CH2)2– | > 2000 | 84.7   | 78.5   |
| 34  |      | –(CH2)3N(CH2CONHCH3)– | 791    | 415    | 67.2   |
| 35  |      | Ph(CH2)2            | 897    | 425    | 107    |
| 36  |      | HNO2SC6H5(CH2)2H    | 704    | 97.5   | 48.1   |
| AAZ |      |                     | 83     | 250    | 12.1   |

*Mean ± standard error (from three different assays), by a stopped-flow technique (errors were in the range of ±5–10% of the reported values).

The differences of TweCAα inhibitory activity compared to the structurally similar derivatives (e.g. 35) which probably is due to the fact that the DTC in 36 is primarily binding to the metal ion in the enzyme active site, and not the sulphonamide moiety. However, the heterocyclic sulphonamide acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulphonamide), a clinically used drug, is a much more potent inhibitor (K<sub>i</sub> of 83 nM) of TweCAα compared to the structurally similar derivatives (e.g. 35). The R-enantiomer 27 was on the other hand 5.9 times more effective inhibitor compared to the S-enantiomer 28. All these data show that small changes in the structure or the stereochemistry of a DTC/MTC lead too dramatic changes of affinity for the target enzyme.

(v) With a few exceptions, TweCAα was less sensitive to this class of CAIs compared to the α-CAs hCA I and II (Table 1). There are several X-ray crystal structures that demonstrate that the DTCs (and presumably also the MTCs) bind to the metal ion in the α-CAs to the active site by substituting the hydroxide nucleophile that is responsible for the catalytic activity of the enzyme<sup>1-8</sup>. Most probably, this is also the inhibition mechanism by which DTCs and MTCs interact with δ-CAs. However, this enzyme class is the least studied of the 7 CA genetic families, and there are no X-ray crystal structures or even homology models available for any δ-CAs.

We try to rationalise the obtained inhibition data based on the amino acid sequence of TweCAα, which has been aligned with that of α-CAs for which the X-ray crystal structure is known, of bacterial (HpylCA, α-CAs from Helicobacter pylori, SspCA, α-CAs from Sulphuricoglobin yellowstonensis) or human origin (hCA I and II) (Figure 3). Data of Figure 3 show that for the α-CAs, the zinc
ligands are three His residues (His94, 96, and 119, hCA I numbering system), which align well for the bacterial and human enzymes, whereas the putative zinc ligands of TweCA do not align at all with those of the α-class enzyme. The same is true for other amino acid residues from the α-CAs, such as the proton shuttle ligands of the α-CAs and the putative zinc ligands of TweCA are evidenced in red, whereas amino acid residues involved in the catalytic inhibition/mecchanism (e.g. His64 and Asp106, hCA I numbering) are shown in green and blue, respectively.

**Figure 3.** Multialignment of the TweCA α amino acid sequence with those of bacterial (HpylCA, α-CA from *Helicobacter pylori*, SspCA, α-CA from *Sulfurihydrogenibium yellowstonensis*) and human (hCA I and II) α-class enzymes. The zinc ligands of the α-CAs and the putative zinc ligands of TweCA are evidenced in red, whereas amino acid residues involved in the catalytic inhibition/mechanism (e.g. His64 and Asp106, hCA I numbering) are shown in green and blue, respectively.

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**Conclusions**

The first inhibition study of a δ-CA with mono- and di-thiocarbamates, classes of CAIs recently discovered, was reported. TweCAδ from the marine diatom *T. weissflogii* was not particularly sensitive to inhibition by these classes of compounds. Many of the mono- and di-thiocarbamates did not show inhibitory action up to 20 μM, whereas some aliphatic, heterocyclic, and aromatic inhibited this enzyme in the low micromolar range. Several MTCs/DTCs incorporating the piperazine ring effectively inhibited TweCAδ with Kᵢ values in the range of 129–791 nM. The most effective inhibitors identified were 3,4-dimethoxyphenyl-ethyl-mono-thiocarbamate (Kᵢ of 93.6 nM). Such inhibitors can now be used as molecular probes to investigate the role of this enzyme in the carbon fixation processes in diatom marine organisms that are responsible for removing large amounts of CO₂ from the atmosphere.

**Disclosure statement**

The authors do not declare any conflict of interest.

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