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Dithiocarbamates with potent inhibitory activity against the Saccharomyces cerevisiae \( \beta \)-carbonic anhydrase

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Abstract

Dithiocarbamates (DTCs) prepared from primary or secondary amines, which incorporated amino/hydroxyl-alkyl, mono/bicyclic aliphatic/heterocyclic rings based on the quinuclidine, piperidine, hydroxy-carboxy/amino-substituted piperidine, morpholine and piperazine scaffolds, were investigated for the inhibition of \( \alpha \)- and \( \beta \)-carbonic anhydrases (CAs, EC 4.2.1.1) of pharmacologic relevance, such as the human (h) isoform hCA I and II, as well as the Saccharomyces cerevisiae \( \beta \)-CA, scCA. The yeast and its \( \beta \)-CA were shown earlier to be useful models of pathogenic fungal infections. The DTCs investigated here were medium potency hCA I inhibitors (\( K_\text{I} \)s of 66.5–910 nM), more effective as hCA II inhibitors (\( K_\text{I} \)s of 8.9–107 nM) and some of them showed excellent, low nanomolar activity against the yeast enzyme, with inhibition constants ranging between 6.4 and 259 nM. The detailed structure activity relationship for inhibition of the yeast and human enzymes is discussed. Several of the investigated DTCs showed excellent selectivity ratios for inhibiting the yeast over the human cytosolic CA isoforms.

Introduction

The primary sulfonamides possessing the general formula \( \text{RSO}_2\text{NH}_2 \) (where \( R \) may be an aromatic, heterocyclic or aliphatic moiety, but also an even smaller groups such as OH and NH\(_2\)) dominated the landscape of carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) drug design for 70 years, since the discovery that such compounds inhibit the enzyme, in the 1940s\(^8\)–\(^15\). However, recently, new important chemotypes with such properties emerged, by combining structure-based drug discovery strategies (X-ray crystallography and fragment-based drug design) with massive campaigns of screening vast library of compounds for detecting such an activity\(^16\)–\(^38\). The interest in CAIs is mainly motivated by their pharmacological properties and clinical use as diuretics, antiglaucoma, antiobesity or antiepileptic agents, as well as anticancer agents/diagnostic tools\(^1\)–\(^3\),\(^29\)–\(^39\). Among the new chemotypes with CA inhibitory properties ultimately reported, the dithiocarbamates (DTCs) are undoubtedly among the most interesting ones\(^40\)–\(^44\).

These compounds have been rationally discovered as CAIs after our report of trithiocarbonate (CS\(_3^2\)-, TTC), an inorganic anion similar to carbonate, as an interesting (milli–micromolar) CAI\(^45\). In the X-ray crystal structure of this anion bound to hCA II, a monodentate coordination of the inhibitor through one sulfur atom to the zinc ion from the enzyme active site has been observed. Furthermore, a hydrogen bond in which another sulfur of TTC and the OH of Thr199 (a crucial residue for the catalytic cycle of \( \alpha \)-CAs\(^1\)–\(^3\)) were involved, also stabilized the anion when bound within the enzyme active site. Thus, the CS\(_2\) moiety present in TTC was detected as a new zinc-binding group (ZBG) for CA inhibition. As DTCs incorporate this ZBG, a first series of such compounds was prepared and evaluated for their inhibitory activity against several mammalian, fungal and bacterial CAs by this group\(^40\)–\(^44\). Several low nanomolar and subnanomolar CAIs were thus detected against all these enzymes. X-ray crystal structures were also reported for three DTCs complexed to hCA II, i.e. compounds A–C (Figure 1)\(^40\),\(^41\). DTCs A–C inhibited hCA II with \( K_\text{I} \)s of 25, 41 and 0.95 nM, respectively, and hCA IX (an isomer involved in cancerogenesis)\(^1,3\) with \( K_\text{I} \)s of 53, 757 and 6.2 nM, respectively\(^40\),\(^41\). As seen from Figure 1, the binding mode of the new ZBG present in these compounds was identical to that of TTC, with one sulfur coordinated to the metal ion, but the organic scaffold present in the DTC was observed to make extensive contacts with many amino acid residues from the active site. This explains the wide range of inhibitory power of these derivatives, from the subnanomolar to the micromolar range, for the entire series of around 30 DTC reported so far\(^40\)–\(^44\).

Interestingly, these compounds are highly water soluble (being salts), and the DTC C was also effective \textit{in vivo} as an...
antiglaucoma agent when administered topically directly to the eye of hypertensive rabbits, a widely used animal model of this disease\(^1\). However, for the moment only one series of DTCs was reported and investigated as CAIs, and this is the reason why we extend here the earlier studies\(^40,41\), reporting the CA inhibitory properties of a new series of such compounds against another CA of interest, the \(\beta\)-class enzyme from the yeast, \textit{Saccharomyces cerevisiae} scCA\(^46–48\), a model organism of great relevance when investigating fungal CAs as drug targets\(^49–51\).

Materials and methods

Chemistry

The DTCs 1b–21b investigated as scCA inhibitors were prepared by reaction of the amines 1a–21a with CS\(_2\) in the presence of bases, such as NaOH or KOH\(^52\).

CA inhibition

A stopped-flow instrument (SX.18MV-R Applied Photophysics model) was used for assaying the CA-catalyzed \(\text{CO}_2\) hydration activity\(^53\). Inhibitor and enzyme were preincubated for 15 min for allowing the complete formation of the enzyme-inhibitor adduct. IC\(_{50}\) values were obtained from dose response curves working at seven different concentrations of test compound (from 0.1 nM to 50 \(\mu\)M), by fitting the curves using PRISM (www.graphpad.com) and non-linear least squares methods, the obtained values representing the mean of at least three different determinations\(^54\). The inhibition constants (\(K_i\)) were derived from the IC\(_{50}\) values by using the Cheng–Prusoff equation, as follows: 

\[
K_i = IC_{50}/(1 + [S]/K_m),
\]

where \([S]\) represents the \(\text{CO}_2\) concentration at which the measurement was carried out and \(K_m\) the concentration of substrate at which the enzyme activity is at half maximal. All enzymes used were recombinant, produced in \textit{Escherichia coli} as reported earlier\(^13,47\). The concentrations of enzymes used in the assay were: hCA I, 12.0 nM; hCA II, 9.2 nM and scCA, 15.3 nM.

Results and discussion

In the present article, we extended the series of DTCs investigated earlier\(^40–44\), including both primary as well as secondary derivatives, which were obtained in such a way as to explore novel chemical space. The starting amines (1a–21a) used to synthesize DTCs 1b–21b reported here (Scheme 1) included \(N,N\)-dimethylaminomethylenediamine 1a, aminoaacohols with three to five carbon atoms in their molecule 2a–4a, the bicyclic quinuclidine-3-amine (both the racemate as well as the R- and S-enantiomeric DTCs 5b–7b incorporating this scaffold were obtained), piperidine 8a and several of its derivatives with hydroxyl-, carboxy-, acetamido- and boc-amido functionalities in various positions of the heterocyclic ring, of types 9a–16a; morpholine and piperazine derivatives 17a–19a, as well as phenethylamines 20a and its sulfamoylated derivative, 4-aminoethylbenzenesulfonamide 21a, a well known CAI which binds to the enzyme through the sulfamoyl moiety\(^1,3\) (Scheme 1). The choice of these scaffolds was motivated by the fact that the structure–activity relationship (SAR) for the inhibition of CAs with the DTCs reported earlier\(^7,8\) was primarily influenced by the organic scaffold of the inhibitor. In fact, important differences of activity were observed between primary and secondary DTCs and between aliphatic or aromatic/heterocyclic derivatives, respectively\(^40–44\). Here we investigated compounds belonging to these DTCs types, with a variety of substitution patterns, based on several scaffolds mentioned earlier (Table 1) among which primary, aliphatic aminoketyl- or hydroxalkyl derivatives (1b–4b); primary, cyclic, bulky DTCs (5b–7b), with various stereochromies; secondary, piperidine, morpholine and piperazine-based DTCs, of types 8b–19b, for which the lead compound was the morpholine-DTC (compound C) reported earlier\(^40\). Indeed, the largest number of the new DTCs investigated here belongs to this subgroup.

We investigated the CA inhibitory properties of DTCs 1b–21b, as well as the sulfonamide CAI acetazolamide (AAZ) as standard, against three relevant CAs, the human (h) cystolic CA isoforms, hCA I and II (widespread, off-target enzymes when non-mammalian CAs should be inhibited) as well as the yeast scCA, an enzyme belonging to the \(\beta\)-class, which we showed earlier to be a good model for studying fungal enzymes belonging to this class\(^5\). Indeed, several pathogenic fungi, such as \textit{Cryptococcus neoformans}, \textit{Candida albicans} and \textit{C. glabrata}, encode \(\beta\)-CAs homologous to scCA\(^49–51\).

The inhibition data of compounds 1b–21b are shown in Table 1, and the following SAR can be delineated:

(i) The slow isoform hCA I was poorly inhibited by most of the DTCs investigated here. Few compounds, i.e. 1b, 4b, 18b and 21b showed inhibition constants ranging between 66.5 and 97.5 nM, being rather efficient hCA I inhibitors. The remaining derivatives were weaker inhibitors, with inhibition constants ranging between 109 and 910 nM, and were much less efficient compared to the clinically used...
The SAR for the inhibition of this isoform is straightforward: primary DTCs were the most effective CAIs detected here (e.g. 1b, 4b and 21b), whereas among the secondary DTCs only the 3-morpholine-carboxylic acid derivative 18b was a good inhibitor. Among the hydroxyalkyl-substituted DTCs 2b–4b, it may be observed that hCA I inhibitory power was very weak for the compound with three carbon atoms (2b), increased considerably for its homolog with four carbon atoms (3b) whereas for the 5-aminopentanol derivative 4b it reached a good level, with a $K_I$ of 66.5 nM

(a $>$ 10 times increase of the inhibitory power with an increase of the aliphatic chain from 3 to 5 CH$_2$ groups).

(ii) The physiologically dominant cytosolic isofrom hCA II was more sensitive to inhibition by the compounds investigated here compared to hCA I. DTCs 1b–21b showed inhibition constants ranging between 8.9 and 107 nM (AAZ has a $K_I$ of 12 nM against this isofrom, Table 1). Most of the new DTCs were medium potency hCA II inhibitors, with $K_I$s ranging between 30.1 and 80.5 nM. They include the following DTCs: 1b, 2b, 5b, 7b–12b, 14b, 15b, 17b–19b and 21b. The most ineffective hCA II inhibitor was the phenethyl derivative 20b ($K_I$ of 107 nM), whereas the sulfamoylated analog 21b showed an improved efficacy, with a $K_I$ of 48.1 nM. The best hCA II inhibitors ($K_I$s in the range of 8.9–24.3 nM), were 3b, 4b, 6b, 13b and 16b (Table 1). These effective inhibitors belong to both classes of DTCs, primary (3b, 4b and 6b) and secondary ones (13b and 16b), respectively. They incorporate the hydroxyalkyl moieties, and as for hCA I, the inhibition efficiency increases with the length of the aliphatic chain from 3 to 5. Another interesting case was observed for the quinuclidine derivatives. The racemate 5b was a medium potency inhibitor ($K_I$ of 48.7 nM) whereas the R-stereoisomer was 2.57 times a more effective inhibitor than the racemic compound, with a $K_I$ of 18.9 nM. On the contrary, the S-stereoisomer was the least effective hCA II inhibitor among these derivatives, with a $K_I$ of 65.9 nM (3.5 times less effective compared to its antipode 6b). The same behavior was observed for the other racemate and the two enantiomers investigated here, the derivatives of nipecontic acid 11b–13b. In this case the S-enantiomer 13b was 9 times a better hCA II inhibitor compared to its antipode 12b, whereas the racemate 11b had an intermediate behavior between the two diastereoisomers, with a $K_I$ of 45.4 nM (Table 1). The position of the other functional group on the heterocyclic ring on which the CS moiety was attached was also an important factor influencing the activity of the heterocyclic DTCs investigated here.

The regioselect DTCs derived from picpolic, nipecontic and isopecontic acids (10b, 11b and 14b, respectively) had quite different activities: the nipecontic acid derivative 14b was roughly twice times more effective as a hCA II inhibitor compared to its isomers 10b and 11b. The two amine derivatives 15b and 16b were also different in their affinity for hCA II, with the bulkier, Boc derivative 16b being a potent inhibitor ($K_I$ of 13.2 nM), whereas the acetamido one 15b having a weaker activity ($K_I$ of 47.9 nM). All these data show that rather small differences in the scaffold of these DTCs lead to quite different inhibitory activities, probably due to the fact that as explained earlier, the scaffold of the inhibitor participates in many interactions with amino acid residues from the CA active site, which may stabilizing or in case of clashes, weaken the inhibitory power of the corresponding compound.

(iii) scCA was also effectively inhibited by DTCs 1b–21b, which showed inhibition constants in the range of 6.4–278 nM. The SAR for the inhibition of the yeast enzyme is more complicated compared to the ones outlines above for the inhibition of the two human cytosolic isofoms. Thus, two DTCs, the quinuclidine derivatives 5b–7b and the bulkier derivative 19b, behaved as weak scCA inhibitors, with $K_I$s of 181–278 nM. It may be observed that all of them are rather bulky, sterically hindered compounds which probably cannot easily accommodate within the scCA active site which is a long and not very wide channel$^{38–50}$. Another group of DTCs, among which 1b–3b, 15b, and 20b, 21b, similarly to AAZ ($K_I$ of 82.6 nM) showed medium potency

### Table 1. hCA I, II and scCA inhibition data with DTCs 1b–21b and acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide) as standard drug, by a stopped-flow CO$_2$ hydrase assay.

<table>
<thead>
<tr>
<th>No</th>
<th>$R$</th>
<th>$R'$ hCA I</th>
<th>$R'$ hCA II</th>
<th>$R'$ scCA</th>
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</thead>
<tbody>
<tr>
<td>1b</td>
<td>Me$_2$N(CH$_3$)$_2$</td>
<td>H</td>
<td>85.9</td>
<td>35.8</td>
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<tr>
<td>2b</td>
<td>HO(CH$_2$)$_3$</td>
<td>H</td>
<td>706</td>
<td>41.7</td>
</tr>
<tr>
<td>3b</td>
<td>HO(CH$_2$)$_4$</td>
<td>H</td>
<td>295</td>
<td>24.3</td>
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<tr>
<td>4b</td>
<td>HO(CH$_2$)$_5$</td>
<td>H</td>
<td>66.5</td>
<td>17.3</td>
</tr>
<tr>
<td>5b</td>
<td></td>
<td>H</td>
<td>494</td>
<td>48.7</td>
</tr>
<tr>
<td>6b (R)</td>
<td></td>
<td>H</td>
<td>240</td>
<td>18.9</td>
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<tr>
<td>7b (S)</td>
<td></td>
<td>H</td>
<td>615</td>
<td>65.9</td>
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<tr>
<td>8b</td>
<td>–(CH$_2$)$_3$</td>
<td></td>
<td>252</td>
<td>30.1</td>
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<tr>
<td>9b</td>
<td>–(CH$_2$)$_3$–CH(OH)CH$_2$</td>
<td></td>
<td>428</td>
<td>60.7</td>
</tr>
<tr>
<td>10b</td>
<td>–(CH$_2$)$_3$–CH(COONa)–</td>
<td></td>
<td>485</td>
<td>80.1</td>
</tr>
<tr>
<td>11b</td>
<td>–(CH$_2$)$_3$–CH(COONa)–CH$_2$–</td>
<td></td>
<td>290</td>
<td>45.4</td>
</tr>
<tr>
<td>12b (R)</td>
<td>–(CH$_2$)$_3$–CH(COONa)–CH$_2$–</td>
<td></td>
<td>496</td>
<td>80.5</td>
</tr>
<tr>
<td>13b (S)</td>
<td>–(CH$_2$)$_3$–CH(COONa)–CH$_2$–</td>
<td></td>
<td>109</td>
<td>8.9</td>
</tr>
<tr>
<td>14b</td>
<td>–(CH$_2$)$_2$–CH(COONa)–(CH$_2$)$_2$–</td>
<td></td>
<td>337</td>
<td>78.7</td>
</tr>
<tr>
<td>15b</td>
<td>–(CH$_2$)$_3$–CH(NHAc)–CH$_2$–</td>
<td></td>
<td>910</td>
<td>47.9</td>
</tr>
<tr>
<td>16b</td>
<td>–(CH$_2$)$_3$–CH(NH)boc–CH$_2$–</td>
<td></td>
<td>683</td>
<td>13.2</td>
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<tr>
<td>17b</td>
<td>–(CH(Me)CH$_2$–O–(CH$_2$)$_2$–</td>
<td></td>
<td>434</td>
<td>60.2</td>
</tr>
<tr>
<td>18b</td>
<td>–CH(COONa)–CH$_2$–O–(CH$_2$)$_2$–</td>
<td></td>
<td>84.7</td>
<td>78.5</td>
</tr>
<tr>
<td>19b</td>
<td>–(CH$_2$)$_3$(CH$_2$CONHCH$_2$H)$_1$–(CH$_2$)$_2$–</td>
<td></td>
<td>415</td>
<td>67.2</td>
</tr>
<tr>
<td>20b</td>
<td>Ph(CH$_2$)$_2$</td>
<td>H</td>
<td>425</td>
<td>107</td>
</tr>
<tr>
<td>21b</td>
<td>H$_2$NO$_2$SC$_2$H$_4$(CH$_2$)$_2$–</td>
<td>H</td>
<td>97.5</td>
<td>48.1</td>
</tr>
<tr>
<td>AAZ</td>
<td></td>
<td>–</td>
<td>250</td>
<td>12</td>
</tr>
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</table>

*Mean from three different assays. Errors were in the range of ±5–10% of the reported values (data not shown).
activity against scCA, with $K_i$ ranging between 41.1 and 82.4 nM (Table 1). These compounds incorporate the amino-hydroxylalkyl chains with two to four carbon atoms (1b–3b), arylalkyl chains (as in 20b and 21b) or the acetamido-piperidine scaffold present in 15b (which is in fact an outlier, as all other piperidine, and morpholine DTCs investigated here showed a better inhibition profile against scCA compared to 15b. Indeed, the most effective scCA inhibitors were just these derivatives, i.e. 8b–14b, 16b–18b ($K_i$s of 6.4–24.1 nM) together with the hydroxyl-pentyl DTC 4b ($K_i$ of 20.5 nM). Thus, for the primary aliphatic derivatives 2b–4b, scCA inhibitory activity increased with the length of the carbon atoms chain. For the piperidine derivatives activity was better when another functional group (such as OH, COOH, BocNH) was attached on the ring (compared to the parent derivative of the series, 8b), but the substitution pattern and the stereochemistry were factors which influenced the activity considerably. For example the nipecotic acid 11b–13b are again of interest to analyze: the R-enantiomer 12b was highly effective ($K_i$ of 9.1 nM, the same potency as the hydroxyl-substituted piperidine 10b), whereas the S-enantiomer 13b had a 2.5 times lower activity ($K_i$ of 23.8 nM). The best scCA inhibitor was however the isonippecotic acid derivative 14b, which had a $K_i$ as low as 6.4 nM, being the best scCA inhibitor reported to date. The morpholine DTCs 17b and 18b also had a good scCA inhibition profile, with $K_i$s of 16.5–18.4 nM. It should be noted that many DTCs investigated here were several times better scCA inhibitors compared to AAZ, a clinically used sulfonamide inhibitor, but a moderate scCA inhibitor. Another important feature, some of the DTCs investigated here showed selectivity for the inhibition of the yeast over the human enzymes. Examples of such derivatives are 8b–12b, 14b, 17b and 20b. In the case of 14b, the selectivity ratio for inhibiting scCA over hCA I was of 52.6 and of inhibiting scCA over hCA II of 12.3, making this derivative one of the most selective inhibitor for the yeast over the human CA isoforms.

Conclusion

We investigated the inhibition of two human, off-target CA isoforms and the yeast β-class enzyme scCA with a series of DTCs prepared both from primary and secondary amines and carbon disulfide, in the presence of strong bases. These DTCs incorporated amino/hydroxyl-alkyl, mono-/bicyclic aliphatic/heterocyclic rings based on the quinuclidine, piperidine, hydroxy-/carboxy-/amino-substituted piperidine, morpholine and piperazine scaffolds. The investigated compounds were medium potency hCA I inhibitors ($K_i$s of 66.5–910 nM), they were more effective as hCA II inhibitors ($K_i$s of 8.9–107 nM), and some of them showed excellent, low nanomolar activity against the yeast enzyme, with inhibition constants ranging between 6.4 and 259 nM. The detailed structure activity relationship for inhibition of the yeast and human enzymes is discussed. Several of the investigated DTCs showed excellent selectivity ratios for inhibiting the yeast over the human cytosolic CA isoforms.

Declaration of interest

This research was financed in part by two EU projects (Dynamo and Metoxia). The authors declare no conflict of interest.

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