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RESEARCH ARTICLE

Dithiocarbamates with potent inhibitory activity against the *Saccharomyces cerevisiae* β-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 α- and β-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* β-CA, scCA. The yeast and its β-CA were shown earlier to be useful models of pathogenic fungal infections. The DTCs investigated here were medium potency hCA I inhibitors (K_Is of 66.5–910 nM), were more effective as hCA II inhibitors (K_Is 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.

Keywords

β-Class enzyme, carbonic anhydrase, dithiocarbamate, inhibitor, *Saccharomyces cerevisiae*

History

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Introduction

The primary sulfonamides possessing the general formula RSO_2NH_2 (where R may be an aromatic, heterocyclic or aliphatic moiety, but also an even smaller groups such as OH and NH_2)[1–7] 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 α-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_Is of 25, 41 and 0.95 nM, respectively, and hCA IX (an isoform involved in cancerogenesis)[1,3,5] with K_Is 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 *in vivo* as an

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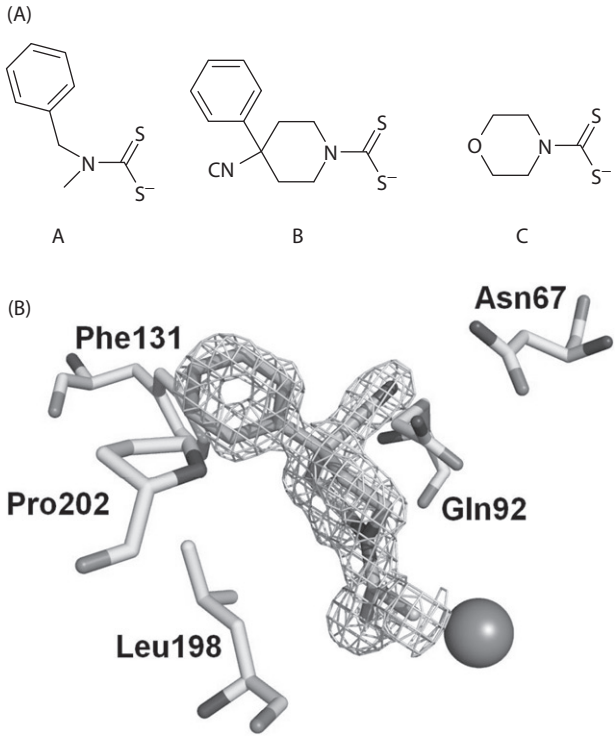


Figure 1. (A) Structure of DTCs **A**–**C** used as lead molecules in the present study. (B) Electronic density for the adduct of dithiocarbamate **B** bound within the active site of hCA II[40,41]. The zinc ion is shown as the central sphere and the amino acid residues involved in the binding are evidenced and numbered (hCA I numbering system)[40]. The zinc ligands (except the sulfur from the DTC compound), His94, 96 and 119, are not shown for the sake of simplicity.

antiglaucoma agent when administered topically directly to the eye of hypertensive rabbits, a widely used animal model of this disease[41]. 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 β-class enzyme from the yeast, *Saccharomyces cerevisiae* scCA[46–48], a model organism of great relevance when investigating fungal CAs as drug targets[49–51].

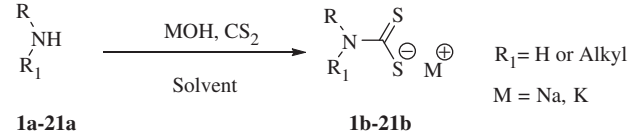
Materials and methods

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 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 μM), 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 CO_2 concentration at which the measurement was carried out and K_m the concentration of substrate at which



Scheme 1. Preparation of DTCs **1b–21b**, by reaction of amines **1a–21a** with carbon disulfide in the presence of sodium/potassium hydroxide.

the enzyme activity is at half maximal. All enzymes used were recombinant, produced in *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

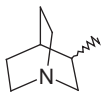
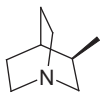
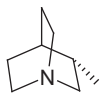
In the present article, we extended the series of DTCs investigated earlier[40–44], including both primary as well as secondary derivatives, which were obtained in such a way as to explore novel chemical space. The starting amines (**1a**–**21a**) used to synthesize DTCs **1b**–**21b** reported here (Scheme 1) included *N,N*-dimethylaminoethylenediamine **1a**, aminoalcohols 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 phenethylamine **20a** and its sulfamoylated derivative, 4-aminoethylbenzenesulfonamide **21a**, a well know 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 aminoalkyl- or hydroxyalkyl derivatives (**1b**–**4b**); primary, bicyclic, bulky DTCs (**5b**–**7b**), with various stereochemistries; 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) cytosolic 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 β-class, which we showed earlier to be a good model for studying fungal enzymes belonging to this class[13]. Indeed, several pathogenic fungi, such as *Cryptococcus neoformans*, *Candida albicans* and *C. glabrata*, encode β-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:

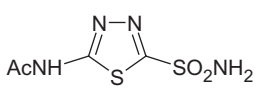
(i) The slow isoform hCA I was poorly inhibited by most of the DTCs investigated here. Few compounds, i.e. **1b**, **4b**, **18b** and **21b** showed inhibition constants ranging between 66.5 and 97.5 nM, being rather efficient hCA I inhibitors. The remaining derivatives were weaker inhibitors, with inhibition constants ranging between 109 and 910 nM, and were much less efficient compared to the clinically used

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.

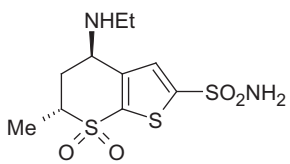
			K_I (nM)*		
No	*R*	R^1	hCA I	hCA II	scCA
1b	$Me_2N(CH_2)_2$	H	85.9	35.8	82.4
2b	$HO(CH_2)_3$	H	706	41.7	72.5
3b	$HO(CH_2)_4$	H	295	24.3	41.4
4b	$HO(CH_2)_5$	H	66.5	17.3	20.5
5b		H	494	48.7	216
6b (R)		H	240	18.9	278
7b (S)		H	615	65.9	181
8b	$-(CH_2)_5-$		252	30.1	13.8
9b	$-(CH_2)_3-CH(OH)CH_2-$		428	60.7	19.1
10b	$-(CH_2)_4-CH(COONa)-$		485	80.1	10.5
11b	$-(CH_2)_3-CH(COONa)CH_2-$		290	45.4	18.7
12b (R)	$-(CH_2)_3-CH(COONa)CH_2-$		496	80.5	9.1
13b (S)	$-(CH_2)_3-CH(COONa)CH_2-$		109	8.9	23.8
14b	$-(CH_2)_2-CH(COONa)(CH_2)_2-$		337	78.7	6.4
15b	$-(CH_2)_3-CH(NHAc)CH_2-$		910	47.9	45.9
16b	$-(CH_2)_3-CH(NHBoc)CH_2-$		683	13.2	24.1
17b	$-CH(Me)CH_2-O-(CH_2)_2-$		434	60.2	16.5
18b	$-CH(COONa)CH_2-O-(CH_2)_2-$		84.7	78.5	18.4
19b	$-(CH_2)_2N(CH_2CONHC_6H_{11})(CH_2)_2-$		415	67.2	259
20b	$Ph(CH_2)_2$	H	425	107	51.5
21b	$H_2NO_2SC_6H_4(CH_2)_2$	H	97.5	48.1	78.9
AAZ	–	–	250	12	82.6

*Mean from three different assays. Errors were in the range of ±5–10% of the reported values (data not shown).

sulfonamide acetazolamide (AAZ, K_I of 250 nM), Table 1. This is a positive feature for a CAI, since hCA I inhibition is associated with side effects of the systemically or topically acting sulfonamides, such as **AAZ** or dorzolamide **DZA**[1,3,5].



AAZ



DZA

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 isoform 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 isoform, Table 1). Most of the new DTCs were medium potency hCA II inhibitors, with K_Is 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_Is 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 nipecotic 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_2^- moiety was attached was also an important factor influencing the activity of the heterocyclic DTCs investigated here. The regiomer DTCs derived from pipecolic, nipecotic and isonipecotic acids (**10b**, **11b** and **14b**, respectively) had quite different activities: the nipecotic acid derivative **14b** was roughly two 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 isoforms. Thus, two DTCs, the quinuclidine derivatives **5b–7b** and the bulkier derivative **19b**, behaved as weak scCA inhibitors, with K_Is 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[48–50]. Another group of DTCs, among which **1b–3b**, **15b**, and **20b**, **21b**, similarly to AAZ (K_I of 82.6 nM) showed medium potency

activity against scCA, with K_Is ranging between 41.1 and 82.4 nM (Table 1). These compounds incorporate the amino/hydroxyalkyl 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_Is 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 isonipecotic 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_Is 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_Is of 66.5–910 nM), they were more effective as hCA II inhibitors (K_Is 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 (Dynano and Metoxia). The authors declare no conflict of interest.

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