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SHORT COMMUNICATION

Dithiocarbamates strongly inhibit the β-class carbonic anhydrases from *Mycobacterium tuberculosis*

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Abstract

A series of *N*-mono- and *N,N*-disubstituted dithiocarbamates have been investigated as inhibitors of two β-carbonic anhydrases (CAs, EC 4.2.1.1) from the bacterial pathogen *Mycobacterium tuberculosis*, mtCA 1 (Rv1284) and mtCA 3 (Rv3273). Both enzymes were inhibited with efficacies between the subnanomolar to the micromolar one, depending on the substitution pattern at the nitrogen atom from the dithiocarbamate zinc-binding group. Aryl, arylalkyl-, heterocyclic as well as aliphatic and amino acyl such moieties led to potent mtCA 1 and 3 inhibitors in both the *N*-mono- and *N,N*-disubstituted dithiocarbamate series. This new class of β-CA inhibitors may have the potential for developing antimycobacterial agents with a diverse mechanism of action compared to the clinically used drugs for which many strains exhibit multi-drug/extensive multi-drug resistance.

Keywords: Carbonic anhydrase, dithiocarbamate, β-class enzyme, inhibitor, Mycobacterium tuberculosis

Introduction

Resistance to antibiotics belonging to several different classes is escalating and represents a worldwide problem[1–3]. Many strains of Gram-negative/positive bacteria (such as among others *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Helycobacter pylori*, *Brucella suis, Streptococcus pneumoniae*, etc.) no longer respond to various classes of antibiotics[4,5]. Cloning of the genomes of bacterial pathogens offers however the possibility to explore alternative pathways for inhibiting virulence factors or proteins essential for the pathogens life cycle. Among the many new such possible drug targets recently explored, there are a class of enzymes catalyzing a simple but physiologically essential process, i.e. carbon dioxide hydration to bicarbonate and protons[6–8]. These enzymes are the carbonic anhydrases (CAs, EC 4.2.1.1), and they belong to the metalloenzymes family of proteins. Five different genetically distinct CA families were described to date, the α-, β-, γ-, δ- and ζ-CAs[6]. Whereas α-, β- and δ-CAs use Zn(II) ions at the active site, the γ-CAs are probably Fe(II) enzymes (but they are active also with bound Zn(II) or Co(II) ions), whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis[6–10]. The metal ion from the enzyme cavity is also essential for the binding of most classes of CA inhibitors (CAIs) investigated so far[6–8].

The classical CAIs are the primary sulfonamides, RSO_2NH_2, which are in clinical use for more than 50 years as diuretics or systemically acting antiglaucoma drugs[6–8,11]. In fact there are around 30 clinically used drugs (or agents in clinical development) belonging to the sulfonamide or sulfamate class, which show significant CA inhibitory activity[6]. It has emerged in the last years that sulfonamide/sulfamate CAIs also have potential as anticonvulsant, antiobesity, anticancer, and antiinfective drugs[6–8]. All these drugs target in fact mammalian CAs, of which 16 different isoforms are known so far, except the antiinfectives which target bacterial/fungal such enzymes[6].

Except vertebrates in which they have been extensively studied for decades[6–9], as mentioned above, CAs are present in many human pathogens such as the malaria provoking protozoa *Plasmodium falciparum,*

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bacteria such as *Escherichia coli*; *H. pylori, M. tuberculosis*, *Brucella spp, S. pneumoniae*, *Salmonella enterica* and *Haemophilus influenzae* as well as pathogenic fungi (*Candida* spp., *Cryptococcus* spp., etc)[6,11,12]. Inhibition of these enzymes started to be investigated recently with sulfonamide/sulfamate inhibitors, but several other chemotypes were also explored, such as phenols, boronic acids, metal complexing anions and other similar small molecules[10]. As bacteria predominantly encode for β-class CAs, which are not present in vertebrates, these enzymes started to be seriously considered as possible drug targets for obtaining antibacterials devoid of the resistance problems mentioned above, which affect most classes of antibiotics in clinical use[11–16].

The genome of the human pathogen *M. tuberculosis* contains at least three β-CAs, mtCA 1, 2 nd 3, encoded by the genes Rv1284, Rv3588c Rv3273[17–22]. These enzymes have been cloned and their catalytic activity and inhibition profiles with sulfonamides investigated[19–22]. Although interesting, low nanomolar or subnanomolar sulfonamide inhibitors targeting these CAs have been detected[19–22], it is not yet clear whether the *in vivo* inhibition of these enzymes has an antimycobacterial effect[23]. Thus, exploring alternative chemotypes to the sulfonamides as possible inhibitors of the β-CAs from this bacterial pathogen is of great interest. Here we report that the dithiocarbamates, a class of α-CAIs recently reported by our group[24], also act as highly effective inhibitors of two of the three β-CAs from *M. tuberculosis*, i.e. mtCA 1 (Rv1284) and mtCA 3 (Rv3273).

Materials and methods

Chemistry

Dithiocarbamates **1–27** used in this work have been recently reported by our group[24]. They were prepared fom thr corresponding amine by reaction with carbon disulfide in the presence of a base[24]. Compounds **13–15** were commecially available reagents from Sigma-Aldrich (Milan, Italy).

Enzymology

mtCA1 and mtCA 3 were recombinant enzymes obtained as described earlier[19,20].

CA catalytic activity and inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity[25]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10– 20 mM Hepes (pH 7.5, for α-CAs) or TRIS (pH 8.3 for β-CAs) as buffers, and 20 mM Na_2SO_4 (for α-CAs) or 20 mM NaCl– for β-CAs (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 0.01 nM were done thereafter with distilled-deionized water. 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, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver-Burk plots, as reported earlier[19–23], and represent the mean from at least three different determinations.

Results and discussions

Dithiocarbamates (DTCs) are a well known class of compounds, which complexate metal ions due to the presence of the anionic CS_2^- moiety, which can coordinate mono- or bidentately a variety of transition metal ions[26,27]. Furthermore, many such derivatives have applications in agriculture as antifungals for the protection of crops, as well as in medicine[26,27]. Although known for at least six decades, it is amazing that they have been scarcely investigated as metalloenzyme inhibitors. Apart from being investigated as tyrosinase ihibitors (a copper enzyme which shows micromolar affinity for some of these compounds)[27] only one paper reported that *N,N*-diethyl-dithiocarbamate inhibits bovine CA (both the native and Co(II)-substituted enzymes), being proposed that the inhibitor binds to the Co(II) ion which is in a trigonal bipyramidal geometry[28].

Based on our investigations of inorganic anions as CAIs[29–31], when we have detected trithiocarbonate (CS_3^{2-}) as an interesting inhibitor of several α-CA isoforms, we have proposed that compounds possessing this new zinc-binding function found in trithiocarbonate, such as for example the dithiocarbamates (R_2N-CS_2^-), might possess better inhibitory properties compared to the simple inorganic anion mentioned above. This prediction has recently been validated by the report that DTCs of types **1–27** possess highly effective inhibitory properties against four human (h) α-CA isoforms, hCA I, hCA II, hCA IX and hCA XII as well as against the enzyme from an endangered sturgeon species[24,32].

However, this type of compounds has not been invstigated so far for its interaction with β-class CAs. Indeed, whereas in the α-CAs the catalytically crucial Zn(II) ion is coordinated by three His residues and a water molecule/hydroxide ion (which is being replaced by the inhibitor molecule[6–9]), the β-CAs have the Zn(II) coordinated by two Cys and one His residues, whereas the fourth metal ion ligand is in most cases the water molecule/hydroxide ion[33,34]. The inhibition mechanism of the α- and β-CAs (at least with zinc binders) is

however quite similar, with the inhibitor replacing the fourth, non protein zinc ligand. This is the reason why we report here he investigation of DTCs for their interaction with two β-CAs from the bacterial pathogen *M. tuberculosis*, i.e. mtCA 1 (Rv1284) and mtCA 3 (Rv3273). This is the first report on the inhibition of β-CAs with a new class of inhibitors, the DTCs.

A series of 27 DTCs possessing the general formula R^1R^2N-CSSM, **1–27**, where R^1 is H, alkyl, substituted alkyl; R^2 is alkyl, aryl, hetaryl (but R^1R^2 can also be included in a cyclic structure, such as the pyrrolidine dithiocarbamate **15**, the morpholine-dithiocarbamate **24**, or the S-prolyl-dithiocarbamate **27**, among others) and M is Na, K or triethylammonium, have been investigated for their inhibitory activity against mtCA 1 and 3 (Table 1). The inhibition of the human isoforms hCA I and II (belonging to the α-CA class) is also shown in Table 1 for comparison reasons, as these data were reported in our previous contribution[24]. The clinically used sulfonamide inhibitor acetazolamide, **AZA** (5-acetamido-1,3,4-thiadiazole-sulfonamide) has also been included among the tested compounds, as it is a standard α/β-CA inhibitor[6,7].

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

1. Against mtCA 1 the DTCs **1–27** represent a class of highly potent inhibitors, with inhibition constants in the range of 0.94–893 nM, whereas the sulfonamide **AZA** is a quite weak inhibitor, with a K_I of 481 nM (Table 1). A quite straightforward SAR may be observed for the inhibition of this enzyme with the DTCs investigated here. Thus, for compounds obtained from primary amines and carbon disulfide, of type **1–12**, a very good inhibitory activity (K_Is >10 nM) was observed for all compounds except the heterocyclic derivative **10** which was an order of magnitude less inhibitory (K_I of 89.4 nM). The effective inhibitors incorporated aromatic, aliphatic, arylalkyl, heterocyclic and amino acyl moieties. It is

Table 1. Inhibition data of the human (h) α-CA isoforms hCA I and II, and mycobacterial β-CA isoforms mtCA 1 and 3 with dithiocarbamates (R^1R^2N-CSSM) **1-27** by a stopped-flow, CO_2 hydrase assay[25].

			K_I (nM)*				
Cmpnd	R^1	R^2	hCA I	hCA II	mtCA 1	mtCA 3	M
1	H	Ph	4.8	4.5	5.6	2.5	Et_3NH
2	H	$O[(CH_2CH_2)]_2N$	4.8	3.6	6.1	2.4	K
3	H	$MeN[(CH_2CH_2)]_2N$	33.5	33.0	4.7	2.6	K
4	H	2-butyl	21.1	29.4	6.0	3.6	K
5	H	$O[(CH_2CH_2)]_2N(CH_2)_2$	31.8	36.3	7.1	2.8	K
6**	H	$N[(CH_2CH_2)N]_3$	31.9	13.5	4.2	4.0	K
7	H	$PhCH_2$	4.1	0.7	7.1	87.3	Na
8	H	4-PyridylCH_2	3.5	16.6	5.4	5.7	Et_3NH
9	H	$[(CH_2)_5N]CH_2CH_2$	4.5	20.3	9.1	8.8	K
10	H	2-thiazolyl	3.9	4.6	89.4	9.5	Et_3NH
11	H	$KOOCCH_2$	13.1	325	7.8	8.3	K
12	H	imidazol-1-yl-$(CH_2)_3$	8.6	24.7	5.3	8.7	A
13	Me	Me	699	6910	893	659	Na
14	Et	Et	790	3100	615	431	Na
15		$(CH_2)_5$	0.96	27.5	90.5	4.1	Na
16	n-Pr	n-Pr	1838	55.5	74.8	80.0	Na
17	n-Bu	n-Bu	43.1	50.9	81.7	72.8	Na
18	iso-Bu	iso-Bu	0.97	0.95	86.2	43.0	Na
19	n-Hex	n-Hex	48.0	51.3	95.4	51.7	Na
20	Et	n-Bu	157	27.8	91.6	63.5	Na
21	$HOCH_2CH_2$	$HOCH_2CH_2$	9.2	4.0	7.5	6.0	Na
22	Me	Ph	39.6	21.5	25.2	46.8	Na
23	Me	$PhCH_2$	69.9	25.4	72.0	62.5	Na
24		$O[(CH_2CH_2)]_2$	0.88	0.95	0.94	0.91	Na
25		$NaS_2CN[(CH_2CH_2)]_2$	12.6	0.92	7.7	8.0	Na
26		$(NC)(Ph)C(CH_2CH_2)_2$	48.4	40.8	93.0	61.2	N
27#		(*S*)-$[CH_2CH_2CH_2CH(COONa)]$	2.5	17.3	7.1	6.4	Na
-		AZA (acetazolamide)	250	12	481	104	–

A = imidazol-1-yl-$(CH_2)_3NH_3^+$
*Errors in the range of ± 10 % of the reported values, by a CO_2 hydrase assay method (from 3 different measurements).
**Tris-dithiocarbamate; #(*S*)-proline dithiocarbamate.

obvious that a very large range of substituents at the nitrogen atom lead to highly effective mtCA 1 inhibitors belonging to the DTC class. Both mono- as well as a tris-DTC derivative (compound **6**) behaved as very effective inhibitors of this bacterial enzyme.

For the compounds obtained from secondary amines and carbon disulfide, of type **13–27**, the SAR was more complex. Thus, the dimethyl- and diethyl-DTCs **12** and **13** were the least inhibitory compounds among the investigated derivatives, with K_Is in the range of 615–893 nM. However, an increase of the aliphatic moieties chain, such as in **16–20** (or their incorporation in a cyclic structure, such as in **15**) leads to a gradual increase of the inhibitory capacity, these compounds possessing K_Is in the range of 74.8–95.4 nM. The presence of two hydroxyethyl moieties in the DTC **21** leads on the other hand to a highly effective CAI, with a K_I of 7.5 nM (compared to the structurally related diethyl-**DTC** 13 which is a weak CAI, see above). Te compounds possessing one methyl and one aryl/arylalkyl moiety, such as **22** and **23** were again less effective CAIs compared to **21**, but they significantly inhibited this enzyme (Table 1). However, excellent inhibition has been observed with the heterocyclic compounds **24, 25** and **27** (K_Is in the range of 0.94–7.7 nM), whereas **26,** possessing a bulkier scaffold was les effective (K_I of 93 nM). It is amazing how effective the morpholine DTC **24** wass as an inhibitor of the enzyme, with a subnanomolar inhibition constant.

2. The second bacterial CA investigated here, mtCA 3, was also highly sensitive to inhibition with DTCs, the derivatives **1–27** investigated here showing K_Is in the range of 0.91–659 nM, whereas the sulfonamide compound **AZA** was a much weaker inhibitor, with a K_I of 104 nM (Table 1). The SAR was rather similar to what presented above for mtCA 1. Indeed, most of the primary DTCs **1–12** (except **7**) were effective mtCA 3 inhibitors, with inhibition constants < 10 nM (**7** had a K_I of 87.3 nM). Thus, again a wide range of aliphatic, aromatic, arylalkyl and heterocyclyl moieties lead to effective mtCA 3 inhibitors, as for the case discussed above for mtCA 1.

   For the secondary DTCs **13–27** again the simple derivatives incorporating methyl and ethyl groups (**13** and **14**) were the weakest inhibitors in the series (K_Is in the range of 431–659 nM). The increase of the aliphatic chain or its cyclization leads to more effective CAIs, with compounds **15–20** having K_Is in the range of 4.1–80 nM. The methyl-phenyl and methyl-benzyl DTCs **22** and **23** were rather effective inhibitors (K_Is of 46.8–62.5 nM), similar to the bulky DTC **26**, but the remaining derivatives (**21, 24, 25** and **27**) were the best CAIs against this isoform, with K_Is in the range of 0.91–8.0 nM. Again, mono, bis- or tris-DTCs showed highly effective CA inhibitory properties.
3. mtCA 3 was slightly more sensitive to inhibition wit DTCs compared to mtCA 1, although various cases in which a certain compounds showed a better inhibitory profile against mtCA 1 than mtCA 3 were also observed (e.g. **7, 8, 11, 1216, 22, 25**).
4. DTCs were more effective mtCA 1/3 inhibitors compared to the clinically used sulfonamide **AZA**, although this compound is a strong inhibitor of many mammalian/bacterial α- and β-CAs[6,7].
5. The DTCs showed effective inhibition of both α-class (hCA I and I) and β-class (mtCA 1 and mtCA 3) enzymes, and also the SAR for inhibiting the two types of enzymes were rather similar, as seen from data of Table 1. This is probably due to the fact that the inhibition mechanism is similar for the two categories of enzymes, with the inhibitor coordinating to the metal ion from the enzyme cavity (although the active sites of the two classes of enzymes are very different, probably the coordination interaction between the catalytic metal ion and the anion DTC inhibitor is the preponderant interaction, explaining thus the high affinity of these inhibitors to these CAs).

Conclusion

We evaluated a series of *N*-mono- and *N,N*-disubstituted dithiocarbamates as inhibitors of two β-CAs from the bacterial pathogen *M. tuberculosis*, mtCA 1 (Rv1284) and mtCA 3 (Rv3273). Both enzymes were inhibited with efficacies between the subnanomolar to the micromolar one, depending on the substitution pattern at the nitrogen atom from the dithiocarbamate zinc-binding group. Aryl, arylalkyl-, heterocyclic as well as aliphatic such moieties led to potent mtCA 1 and 3 inhibitors in both the *N*-mono- and *N,N*-disubstituted dithiocarbamate series. This new class of β-CA inhibitors may have the potential for developing antimycobacterial agents with a diverse mechanism of action compared to the clinically used drugs for which many strains exhibit multi-drug/extensive multi-drug resistance

Declaration of interest

The authors report no conflict of interest. This work was supported by an EU FP7 research grant (Metoxia project).

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