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Synthesis of a new series of dithiocarbamates with effective human carbonic anhydrase inhibitory activity and antiglaucoma action

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ABSTRACT

A new series of dithiocarbamates (DTCs) was prepared from primary/secondary amines incorporating amino/hydroxyl-alkyl, mono- and bicyclic aliphatic ring systems based on the quinuclidine, piperidine, hydroxy-/carboxy-/amino-substituted piperidine, morpholine and piperazine scaffolds, and carbon disulfide. The compounds were investigated for the inhibition of four mammalian α-carbonic anhydrases (CAs, EC 4.2.1.1) of pharmacologic relevance, that is, the human (h) hCA I, II, IX and XII, drug targets for antiglaucoma (hCA II and XII) or antitumor (hCA IX/XII) agents. The compounds were moderate or inefficient hCA I inhibitors (off-target isoform for both applications), efficiently inhibited hCA II, whereas some of them were low nanomolar/subnanomolar hCA IX/XII inhibitors. One DTC showed excellent intraocular pressure (IOP) lowering properties in an animal model of glaucoma, with a two times better efficiency compared to the clinically used sulfonamide dorzolamide.

1. Introduction

There are several classes of inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), among which: (i) metal ion binders (inorganic anions,[1,2] sulfonamides, sulfamates and sulfamides;[1–6] dithiocarbamates and xanthates;[7,8] aromatic/heterocyclic thiols[9] and hydroxamates[10]); (ii) compounds anchoring to the zinc-coordinated water molecule/hydroxide ion from the enzyme active site (e.g., phenols,[11] carboxylates,[12] polyamines,[13] esters[14] and sulfocoumarins[15]); (iii) coumarins/lactones and related compounds (thiocoumarins, dithiocoumarins, etc.), which bind even further away from the metal ion, towards the exit of the active site cavity, in hydrolyzed form as hydroxycinnamic acid derivatives.[16,17] These inhibitors are classified according to their distance and/or direct interaction with the metal ion from the enzyme active site cavity. The zinc binders were the first CA inhibitors (CAIs) to be investigated in detail, with some of these compounds (sulfonamides and sulfamates) clinically used as antiglaucoma agents, diuretics, antiepileptics, antiobesity drugs and more recently, as theragnostics for hypoxic tumors.[1–6,18,19]

The dithiocarbamates were recently discovered to act as efficient CAIs of α- and β-class CAs from various organisms.[7,8] In fact there are six genetic families (α-, β-, γ-, δ-, ζ- and η-CAs)[20] encoding such enzymes in organisms widely distributed over the phylogenetic tree. This is due to the fact that CAs catalyze (usually very efficiently)[21] the interconversion between CO_2 and bicarbonate, which otherwise is a slow process at physiologic pH values. This reaction also leads to the formation of species involved in acid-base equilibria and buffering (e.g., bicarbonate and H^+ ions), which further pleas for a crucial role played by CAs in a host of physiologic and pathologic states, in very diverse organisms.[1–6,18–20] It has been demonstrated for a long time that CA inhibition elicits pharmacologic effects which was exploited by using sulfonamide or sulfamate CAIs for the treatment and prevention of diseases as those mentioned above.[1–6,18,19]

The dithiocarbamates (DTCs) have been rationally discovered as CAIs after our report of the inorganic ion trithiocarbonate (CS_3^{2-}) as an interesting but inefficient (milli-micromolar) CAI.[22] In the X-ray crystal structure of this anion bound to the widespread and physiologically dominant cytosolic human (h) isoform CA II, hCA II, it has been observed a monodentate coordination of the inhibitor via one sulfur atom to the zinc ion from the enzyme active site. Another hydrogen bond between another sulfur atom of trithiocarbonate and the OH of Thr199 was also evidenced, which further stabilized

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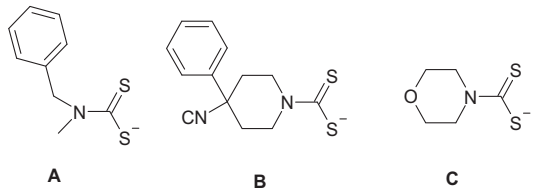
the enzyme-inhibitor adduct.[22] Thr199 is an amino acid residue crucial for the catalytic cycle of α-CAs but also for the binding of inhibitors, being conserved in all α-CAs.[1–3] Thus, with the help of this inorganic anion, the CS_2^- moiety was detected as a new zinc binding group (ZBG) in the design of CAIs. As DTCs incorporate this new ZBG, a first series of such compounds was shortly thereafter prepared and evaluated for their inhibitory activity against several mammalian, fungal and bacterial CAs.[7,8] Several low nanomolar/subnanomolar CAIs were thus detected against all these isoforms.[7,8]



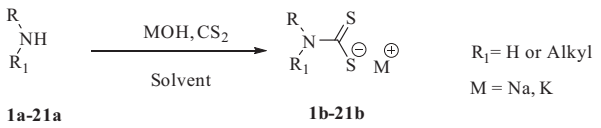
X-ray crystal structures were also reported for several of these DTCs complexed to hCA II, such as for example compounds **A**–**C**. These DTCs (**A**–**C**) effectively inhibited various human isoforms, such as hCA II and hCA IX, with inhibition constants in the nanomolar range.[7a] Their binding mode was rather identical to that of trithiocarbonate, regarding the ZBG, with one sulfur coordinated to the metal ion and the second one interacting with Thr199, but the organic scaffold present in the DTC was observed to make extensive contacts with amino acid residues/water molecules from the active site, which explained the wide range of inhibitory power of these derivatives (from the subnanomolar to the micromolar, for the entire series of around 30 DTCs reported earlier).[7,8] Interestingly, the highly water soluble compound **C** was also effective in vivo as an antiglaucoma agent when administered topically, directly into the eye of hypertensive rabbits, a widely used animal model of glaucoma.[7b]

However only one series of such CAIs of the DTC type was reported so far, by our group.[7,8] Here we extend the earlier investigations and report new DTCs, possessing diverse scaffolds compared to the first series of such compounds, investigating their inhibitory action against CA isoforms involved in severe pathologies, such as glaucoma (CA II and XII) and cancer (CA IX and XII). One of the new compounds also showed effective antiglaucoma activity in an animal model of the disease.

2. Results and discussion

2.1. Chemistry

In the previous work[7,8] on DTCs as CAIs, we investigated both primary and secondary derivatives which incorporated simple alkyl, aralkyl, aryl and hetaryl moieties, as well as two amino acid scaffolds (the Gly and Pro DTC derivatives). In the present paper we extended the series of DTCs, including again both primary as well as secondary derivatives, which were obtained in such a way as to explore novel chemical space. Indeed, the starting amines (**1a**–**21a**) used to synthesize DTCs **1b**–**21b** reported here (Scheme 1) included *N,N*-dimethylaminoethylenediamine **1a**, aminoalcohols with 3-5 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 peiparazine 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] (Scheme 1). The choice of these scaffolds was motivated by the fact that the structure–activity relationship (SAR) for the inhibition of the various CA isoforms with the DTCs reported earlier[7,8] was primarily influenced by the organic scaffold present in the inhibitor molecule. Furthermore, important differences of activity were observed between the primary and secondary DTCs (i.e., the compounds prepared from primary or secondary amines, respectively) and, between the aliphatic derivatives and the compounds incorporating aromatic/heterocyclic groups in their molecules.[7,8] Thus, we report here compounds belonging to the two DTCs type, with a variety of substitution patterns, based on several general scaffolds, among which: (i) primary, aliphatic aminoalkyl- or hydroxyalkyl derivatives (**1b**–**4b**); (ii) primary, bicyclic, bulky DTCs (**5b**–**7b**), with various stereochemistries at the C-3 of the quinuclidine ring; (iii) secondary, piperidine, morpholine and piperazine-based DTCs, of types **8b**–**19b**, for which the lead compound was the morpholine-DTC (compound **C**) reported earlier,[7] which showed excellent in vitro CA inhibitory properties and also antiglaucoma activity in an animal model of this disease. Indeed, the largest number of the new DTCs investigated here belongs to this subgroup. We also explored whether the presence and position of various functionalities on the six-membered heterocyclic amine scaffold (such as OH, COOH, AcNH, BocNH, etc.) is beneficial or not for the inhibitory properties of the new DTCs. Thus the DTC derivatives of 3-hydroxy-piperidine **9b**, pipecolic acid **10b**, nipecotic acid **11b** as well as isonipecotic acid **14b** (together with other structurally related such compounds) were synthesized (Scheme 1, Table 1). For the nipecotic acid **11b**, both the racemic as well as the two stereoisomeric DTCs **12b** and **13b** were prepared in order to investigate possible sterochemical requirements for effective CA inhibition; and (iv) primary, phenethylamine-based DTCs, **20b** and **21b**. In the earlier communications[7] we observed that the benzylamine-DTC, $PhCH_2NHCS_2Na$ (and the corresponding xanthate, $PhCH_2OCS_2Na$)[8d] were highly effective CAIs. As the homolog with one more carbon atoms was not reported in the first DTC series, we prepared here this compound, **20b**.[23] Unfortunately, due to lack of reactivity of the aniline, the phenyl-substituted compound $PhNHCS_2Na$ could not be obtained. As the sulfamoylated derivative of phenethylamine, **21a**, is a widely used CAI,[1] we also performed the reaction of its amino moiety with CS_2 and report the corresponding DTC, compound **21b**. The interest for this compound possessing two different ZBGs, the sulfonamide and the dithiocarbamate ones, was just the possible competition between them for binding to the metal ion within the CA active site, and whether such effects may lead to more effective CAIs.

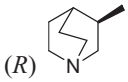
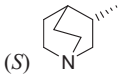


Scheme 1. Synthesis of DTCs **1b**–**21b** from amines **1a**–**21a** and carbon disulfide.

2.2. CA inhibition

We investigated the CA inhibitory properties[24] of compounds **1b**–**21b** reported here, as well as the sulfonamide CAI acetazolamide (**AAZ**) as standard inhibitor, against four physiologically relevant human (h) CA isoforms, the cytosolic, hCA I and II, as well as the transmembrane, tumor-associated hCA IX and XII (the last isoform is however also present in normal tissues, being up-regulated in the eyes of glaucoma patients).[25] The reasons why we investigated these isoforms are the following: hCA II and XII are targets

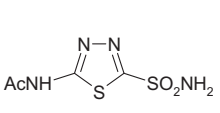
Table 1
hCA I, II, IX and XII inhibition data with DTCs **1b**–**21b** and acetazolamide (**AAZ**, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide) as standard drug, by a stopped-flow CO_2 hydrase assay[24]

No	R	R^1	K_1^* (nM)			
			hCA I	hCA II	hCA IX	hCA XII
1b	$Me_2N(CH_2)_2$	H	85.9 ± 2.3	35.8 ± 1.9	37.9 ± 2.0	9.2 ± 0.8
2b	$HO(CH_2)_3$	H	706 ± 56	41.7 ± 3.9	0.91 ± 0.08	6.5 ± 0.52
3b	$HO(CH_2)_4$	H	295 ± 17	24.3 ± 0.6	0.97 ± 0.09	8.3 ± 0.61
4b	$HO(CH_2)_5$	H	66.5 ± 3.7	17.3 ± 1.0	2.6 ± 0.13	4.7 ± 0.34
5b		H	494 ± 28	48.7 ± 3.1	93.5 ± 6.4	91.0 ± 7.2
6b (*R*)		H	240 ± 16	18.9 ± 0.95	71.8 ± 5.9	90.1 ± 6.8
7b (*S*)		H	615 ± 47	65.9 ± 4.0	82.4 ± 7.3	93.8 ± 3.2
8b	$-(CH_2)_5-$		252 ± 15	30.1 ± 1.5	0.92 ± 0.05	53.1 ± 3.9
9b	$-(CH_2)_3-CH(OH)CH_2-$		428 ± 39	60.7 ± 3.3	7.6 ± 0.62	45.9 ± 3.9
10b	$-(CH_2)_4-CH(COONa)-$		485 ± 44	80.1 ± 5.0	72.0 ± 3.7	50.8 ± 1.9
11b	$-(CH_2)_3-CH(COONa)CH_2-$		290 ± 14	45.4 ± 2.4	67.1 ± 6.0	33.3 ± 2.1
12b	(R) $-(CH_2)_3-CH(COONa)CH_2-$		496 ± 28	80.5 ± 3.9	41.3 ± 2.5	66.2 ± 1.8
13b	(S) $-(CH_2)_3-CH(COONa)CH_2-$		109 ± 8.6	8.9 ± 0.65	86.2 ± 4.0	24.5 ± 1.3
14b	$-(CH_2)_2-CH(COONa)(CH_2)_2-$		337 ± 25	78.7 ± 2.2	77.9 ± 4.7	35.8 ± 1.6
15b	$-(CH_2)_3-CH(NHAc)CH_2-$		910 ± 63	47.9 ± 4.1	9.5 ± 0.82	8.4 ± 0.66
16b	$-(CH_2)_3-CH(NHBoc)CH_2-$		683 ± 54	13.2 ± 0.93	0.97 ± 0.02	0.76 ± 0.01
17b	$-CH(Me)CH_2-O-(CH_2)_2-$		434 ± 42	60.2 ± 5.9	55.1 ± 3.7	91.3 ± 7.0
18b	$-CH(COONa)CH_2-O-(CH_2)_2-$		84.7 ± 7.9	78.5 ± 5.0	6.7 ± 0.43	8.0 ± 0.60
19b	$-(CH_2)_2N(CH_2CONHC_6H_{11})(CH_2)_2-(CH_2)_2-$		415 ± 39	67.2 ± 5.6	79.9 ± 4.3	414 ± 33
20b	$Ph(CH_2)_2$	H	425 ± 24	107 ± 6.8	25.1 ± 1.8	15.3 ± 1.0
21b	$H_2NO_2SC_6H_4(CH_2)_2$	H	97.5 ± 7.9	48.1 ± 2.6	0.91 ± 0.02	3.9 ± 0.24
AAZ	—	—	250 ± 13	12.1 ± 0.90	25.0 ± 1.4	5.7 ± 0.31

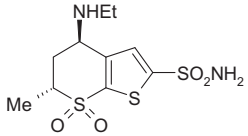
* Mean ± standard error (from three different assays).

for antiglaucoma drugs, and no new agents based on CAIs were launched for clinical use in the last 15 years.[1,3d] hCA IX and XII on the other hand are targets for obtaining antitumor agents or diagnostic tools with a novel mechanism of action.[2a,3a] hCA I is an abundant protein in the blood and the gastro-intestinal tract, being one of the main off-target isoforms when considering both antiglaucoma or anticancer CAIs in drug design.[26,27] The inhibition data of compounds **1b**–**21b** are shown in Table 1, and the following SAR can be delineated:

(i) The off-target isoform hCA I was poorly inhibited by most of the DTCs reported here. Four compounds, **1b**, **4b**, **18b** and **21b** showed K_Is <100 nM (inhibition constants ranging between 66.5 and 97.5 nM) being the most efficient hCA I inhibitors in the series. The remaining 17 derivatives were weaker inhibitors, with inhibition constants ranging between 109 nM and 910 nM, and are much less efficient compared to the clinically used 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]



AAZ



DZA

The SAR for the inhibition of this isoform is thus rather straightforward: some of the primary DTCs were the most effective CAIs detected here (e.g., **1b**, **4b** and **21b**), whereas among the secondary DTCs only one compound, 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 short compound with 3 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 10.6-times increase of the inhibitory power with an increase of the aliphatic chain from 3 to 5 CH_2 groups).

(ii) hCA II was much more sensitive to inhibition by the DTCs reported here compared to hCA I, as compounds **1b**–**21b** had inhibition constants ranging between 8.9 and 107 nM (**AAZ** has a K_I of 12 nM against this isoform, Table 1). 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. 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**, **13b**, **15b**, **17b**–**19b** and **21b**. The best hCA II inhibitors (inhibition constants in the range of 8.9–24.3 nM, the same as **AAZ**), were **3b**, **4b**, **6b**, **13b** and **16b** (Table 1). These highly 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 again, as for hCA I, the inhibition efficiency increases with the length of the aliphatic chain from 3 to 5. Another interesting case is 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 three 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. 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 very small differences in the scaffold of these DTCs lead to different inhibitory activities, probably due to the fact that as explained above, the scaffold of the inhibitor participates in many interactions with amino acid residues from the CA active site (stabilizing ones or clashes, which weaken the inhibitory power).

(iii) The transmembrane isoform hCA IX was very efficiently inhibited by the DTCs investigated here, with K_Is ranging between 0.91 nM and 93.5 nM (Table 1). For comparison, the clinically used agent **AAZ**, which shows interesting antitumor effects in animal models of the disease, alone or in combination with other anticancer agents (e.g., doxorubicin), has a K_I of 25 nM.[27] Several subnanomolar hCA IX inhibitors were detected among the investigated DTCs, such as **2b**, **3b** (aliphatic, primary DTCs with hydroxyalkyl moieties), the piperidine DTC **8b**, its 3-Boc-amide derivative **16b**, as well as the sulfonamide DTC **21b** (K_Is in the range of 0.91–0.97 nM, Table 1). These data demonstrate that highly effective CA IX inhibitors belonging to the DTC class can be obtained with a variety of scaffolds: aliphatic, cyclic aliphatic as well as arylalkyl ones. Several other compounds (**4b**, **9b**, **15b** and **18b**) had K_Is <10 nM whereas the remaining ones were less effective inhibitors, with inhibition constants ranging between 25.1 and 93.5 nM. The SAR was different from what discussed above for the inhibition of hCA I and II. For example among the hydroxyalkyl-substituted DTCs, the best hCA IX inhibitor was the shortest compound **2b** and the least effective one the longer molecule compound **4b** (although all of them are highly effective low nanomolar or subnanomolar CAIs). For the nipecotic acid derivatives, the *R*-stereoisomer was a better hCA IX inhibitor compared to the *S*-enantiomer or the racemic. These differences are probably due to the different amino acid residues from the enzyme active site with which the DTCs interact, for this transmembrane isoform compared to the cytosolic ones hCA I and II.

(iv) hCA XII was also effectively inhibited by the DTCs **1b**–**21b** investigated here, with K_Is ranging between 0.76 nM and 414 nM (Table 1). One compound was a highly effective inhibitor (**16b**, with the K_I of 0.76 nM, incorporating the boc-amide-piperidine scaffold), whereas one derivative was highly inefficient as hCA XII inhibitor (**19b**, K_I of 414 nM, incorporating the bulky acetamido-cyclohexyl functionality attached at the second piperazine nitrogen atom—the first nitrogen has the DTC functionality). Among the remaining derivatives, **1b**–**4b** (all aliphatic, primary DTCs) and **18b**, **20b** and **21b** showed quite effective hCA XII inhibitory properties, with K_Is in the range of 3.9–15.3 nM. The remaining compounds, although effective hCA XII inhibitors, show K_Is higher than acetazolamide, in the range of 24.5–93.8 nM.

These data show that the four CA isoforms possess a very diverse inhibition profile with the DTCs reported here, which is highly relevant for the design of isoform-specific inhibitors with various applications.

2.3. IOP lowering activity

An animal model of glaucoma, that is, rabbits with slightly elevated IOP, induced by the injection of 0.1 mL hypertonic saline solution (5% in distilled water) into the vitreous of both eyes (denominated 'normotensive' rabbits)[28] has been employed for assessing the potential use of some of new compounds reported here for the management of glaucoma. Although systemically acting sulfonamides such as acetazolamide **AAZ** do show effective antiglaucoma action, leading to a 25–30% drop of the IOP in patients with open-angle glaucoma,[3d] the side effects due to inhibition of CAs present in other tissues than the eyes leads to many undesired side effects and poor compliance of the patients with this drug. The topically acting drug dorzolamide **DZA** (administered as 2% solution of the hydrochloride salt) is more effective than **AAZ**, and does not show the systemic side effects of the systemic drug, but it is administered as a rather acidic solution (of pH 5.5) and leads to ocular side effects such as stinging and eye reddening, blurred vision, pruritus, etc.[3d] In addition, the drug is not long acting (the peak IOP lowering is at 2 h post-administration) and also its efficacy is rather limited, with maximal values of 4–5 mm Hg IOP drop being the most common effect, both in humans and experimental animals.[3d] In fact one of the main hurdles to obtain efficient antiglaucoma CAIs is the low water solubility of the sulfonamoides, in addition to a potent inhibitory action against the CA isoforms present in the ciliary processes (CA II and XII), some of which were also shown to be overexpressed in the eyes of patients with glaucoma (e.g., CA XII).[25] We designed the DTC compounds reported here in such a way as to possess an enhanced water solubility, which is mainly due to the salt-like character of these compounds. Indeed, all DTCs reported here were possible to be formulated as water solutions at concentrations of 1–2%, the normal concentration of CAIs in ophthalmologic eye drops. Among the 21 derivatives reported here we chose **16b** as our candidate for in vivo studies due to its good water solubility as well as excellent inhibition profile (Table 1). In fact **16b** does not significantly inhibits the off-target isoform hCA I (K_I of 683 nM) but is a very efficient inhibitor of CA II and XII, isoforms involved in aqueous humor secretion and established antiglaucoma targets (K_I of 13.2 nM against hCA II, and K_I of 0.76 nM against hCA XII). The IOP drop after topical administration of **16b** formulated as 2% eye drops, in rabbits with slightly enhanced IOP (see Experimental for details) is shown in Figure 1, with **DZA** as standard drug. It may be seen that the vehicle had no influence on the IOP lowering over the three hours course of the experiment. **DZA** was effective, with a maximal IOP drop of 4 mm Hg at 1 h post-administration, which was reduced at 3 mm Hg after another hour. Overall, the efficacy of **DZA** is of around 2.5–3 h. DTC **16b** showed a more than double IOP lowering compared to the standard drug at 1 h (of 8.6 mm Hg) which was maintained (IOP lowering of 8.0 mm Hg) at 2 h post-administration, whereas after 3 h it still had an effect superior to **DZA** (at its peak value), of 5.8 mm Hg (Fig. 1).

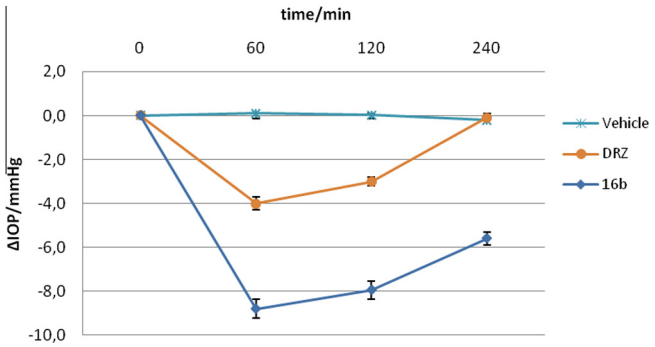


Figure 1. Drop of intraocular pressure (ΔIOP, mm Hg) versus time (min), in normotensive rabbit eyes treated with DTC **16b** at a concentration of 2%, dorzolamide DZA as standard drug at 2%, and vehicle. Errors were in the range of 5–10% of the reported IOP values (from three different measurements for each of the four animals in the study group) and were statistically significant ($p = 0.045$, by Student's t test).

2.4. Molecular modelling

In order to explain the good in vitro and in vivo activity of some of the new DTCs reported, both enantiomers of compound **16b** have been docked into the crystal structure of hCA II. The docking poses for both stereoisomers are comparable (see Fig. 2 for representative poses for the (*S*)-enantiomer). The negatively charged zinc-binding function (the carbodithioate moiety) interacts with the Zn^{2+} ion and forms hydrogen bonds with both the side-chain (OH group) and the backbone NH of Thr199, an amino acid residue crucial for the binding of inhibitors. Several docked poses show also hydrogen bonds with Thr200 (side-chain and backbone). The substituted piperidine ring of the compound was capable of participating to hydrophobic interactions with the side-chains of His94, Val121 and Leu198. The ligand carbonyl group forms hydrogen bonds with Asn62 and/or Asn67 and/or Gln92. Finally, *tert*-butyl group of the ligand can participate in hydrophobic interactions with the side chains of His64 and His94. Similar poses for both stereoisomers have been obtained due to the flexibility of the ligand, which may explain their very good inhibitory properties and affinity for the target isoform.

3. Conclusions

A series of dithiocarbamates (DTCs) was prepared from primary/secondary amines incorporating amino/hydroxyl-alkyl, mono- and bicyclic aliphatic ring systems based on the quinuclidine, piperidine, hydroxy-/carboxy-/amino-substituted piperidine, morpholine and piperazine scaffolds, and carbon disulfide. The compounds were investigated for the inhibition of four α-carbonic anhydrases (CAs, EC 4.2.1.1) of pharmacologic relevance, that is, CA I, II, IX and XII, drug targets for antiglaucoma (CA II and XII) or antitumor (CA IX/XII) agents. The compounds were moderate or inefficient CA I inhibitors (off-target isoform for both applications), efficiently inhibited CA II, whereas some of them were low nanomolar/subnanomolar CA IX/XII inhibitors. One DTC showed excellent intraocular pressure (IOP) lowering properties in an animal model of glaucoma, with a two times better efficiency compared to the clinically used sulfonamide dorzolamide. As the main drawback of the clinically used CAIs such as **DZA** consists in the limited duration of their efficacy, compounds such as the DTC **16b** investigated here seem to be quite promising antiglaucoma agents due to their simple chemical structure and ease of

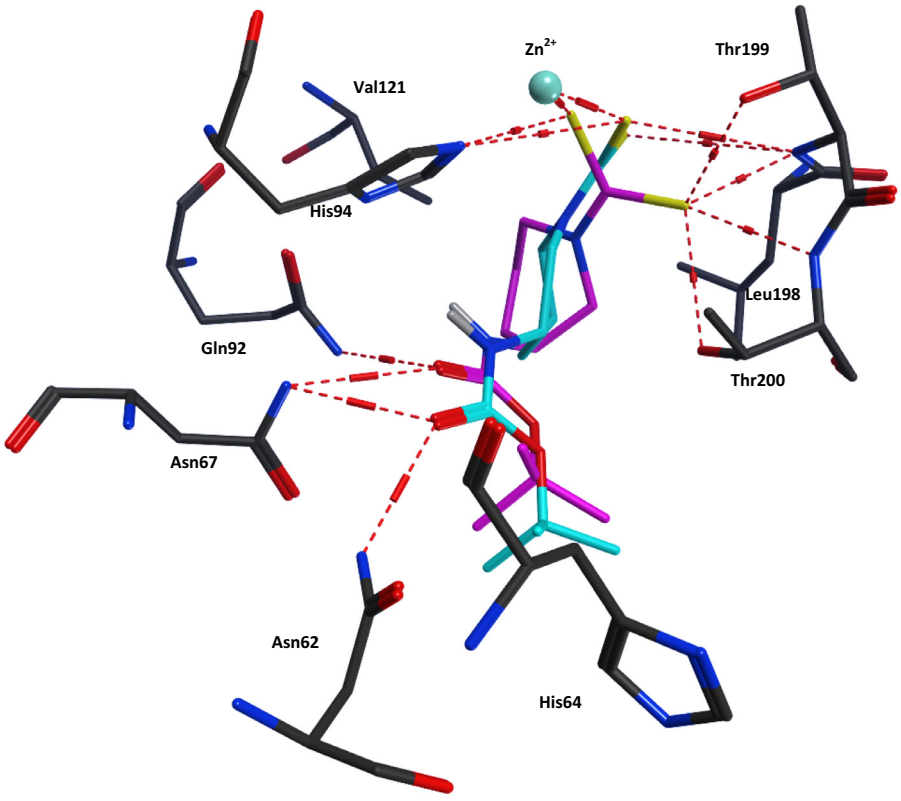


Figure 2. Representative docked poses of compound **16b** in two different conformations (turqoise and purple) in the binding pocket of hCA II (PDB code: 3B4F). The ligand is able to form interactions with the zinc ion, Thr199 and Thr200 via its dithioate moiety and with Asn62, Asn67 and Gln92 via its carbonyl group. In addition, hydrophobic interactions with His94, Val121 and Leu198 were evidenced. The ligand depicted is the (*S*)-enantiomer; hydrogen bonds and the interactions with the zinc ion are depicted as red dashed lines. For the sake of clarity, the interactions between the three histidine residues (His94, 96 and 119) and the zinc ion are not shown.

preparation, as well as increased efficacy both in terms of the maximal IOP lowering and duration of action.

4. Experimental protocols

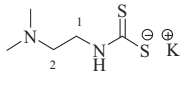
4.1. General

^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC spectra were recorded using a Bruker Avance III 400 MHz spectrometer. The chemical shifts are reported in parts per million (ppm) and the coupling constants (*J*) are expressed in Hertz (Hz). For all new compounds DEPT, COSY, HMQC and HMBC were routinely used to definitely assign the signals of ^{1}H and ^{13}C. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} aluminium backed plates. Elution of the plates was carried out using ethyl acetate/*n*-hexane or MeOH/DCM systems. Visualization was achieved with UV light at 254 nm, by dipping into a 0.5% aqueous potassium permanganate solution, by Hanessian's Stain solution and heating with a hot air gun or by exposure to iodine.Optical rotation experiments were performed at 589 nM and 20 °C on a Perkin Elmer polarimeter 343 at a concentration of 50 mg/5 ml in H_2O and 10 cm length tube. All other solvents and chemicals were used as supplied from Aldrich Chemical Co., Acros, Fisher, Alfa Aesar or Lancaster Synthesis (Milan, Italy).

4.2. General procedure for the synthesis of compounds 1b–21b[7]

Amines **1a**–**21a** (1.0 equiv) were treated with NaOH or KOH in MeOH, Et_2O, H_2O or THF, then carbon disulfide was added dropwise and the mixture was stirred at rt until the starting materials were consumed (TLC monitoring). The solvents were removed under vacuo at rt and the residues obtained were purified by recrystallization from MeOH or trituration from diethyl ether to afford compounds **1b**–**21b**.

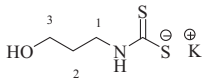
2-(Dimethylamino)ethylcarbamodithioate **1b**



1b

2-(Dimethylamino)ethylcarbamodithioate ***1b***: 54% yield; mp 152–153 °C; δ_H (400 MHz, DMSO-d_6+ D_2O) 2.39 (6H, s, 2× CH_3), 2.77 (2H, t, *J* 6.4, 1-H_2), 3.57 (2H, t, *J* 6.4, 2-H_2); δ_C (100 MHz, DMSO-d_6) 44.5, 45.5, 58.1, 215.6 (*C*=S), *m*/*z* (ESI positive) 202.91 $[M+H]^+$.

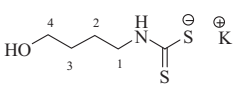
Potassium 3-hydroxypropylcarbamodithioate **2b**



2b

Potassium 3-hydroxypropylcarbamodithiate ***2b***: 80% yield; mp 165–166 °C (dec); δ_H (400 MHz, DMSO-d_6) 1.58 (2H, pent, *J* 6.0, 2-H_2), 3.40 (4H, m, 1-H_2, 3-H_2), 4.55 (1H, t, *J* 8.0, exchange with D_2O, O*H*), 8.07 (1H, brt, *J* 8.0, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 33.0, 44.5, 59.4, 215.4 (*C*=S), *m*/*z* (ESI negative) 149.91 $[M-K]^-$. Experimental data in agreement with reported data.[29a]

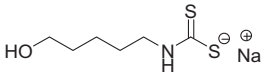
Potassium 4-hydroxybutylcarbamodithioate **3b**.



3b

Potassium 4-hydroxybutylcarbamodithioate ***3b***: 78% yield; mp 174–175 °C (dec); δ_H (400 MHz, DMSO-d_6) 1.49 (4H, m, 2-H_2, 3-H_2), 3.40 (4H, m, 1-H_2, 4-H_2), 4.38 (1H, t, *J* 8.0, exchange with D_2O, O*H*), 7.95 (1H, brt, *J* 8.0, exchange with D_2O, N-*H*); δ_C (100 MHz, DMSO-d_6) 26.2, 31.1, 47.2, 61.6, 215.4 (*C*=S), *m*/*z* (ESI negative) 163.91 $[M-K]^-$.

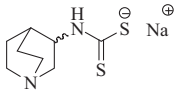
Sodium 5-hydroxypentylcarbamodithioate **4b**



4b

Sodium 5-hydroxypentylcarbamodithioate ***4b***: 37% yield; mp 115–116 °C; δ_H (400 MHz, D_2O) 1.32 (2H, pent, *J* 7.2), 1.54 (4H, m), 3.46 (2H, t, *J* 6.6), 3.55 (2H, t, *J* 6.6); δ_C (100 MHz, D_2O) 23.1, 28.1, 31.6, 48.6, 62.3, 210.6 (*C*=S), *m*/*z*(ESI negative)178.10 $[M-Na]^-$.

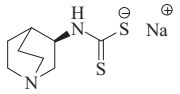
Sodium quinuclidin-3-ylcarbamodithioate **5b**



5b

Sodium quinuclidin-3-ylcarbamodithioate ***5b***: 62% yield; mp 185–186 °C (dec); δ_H (400 MHz, D_2O) 1.74 (4H, m), 2.07 (1H, m), 2.60 (5H, m), 3.25 (1H, m), 4.32 (1H, m); δ_C (100 MHz, D_2O) 19.9, 25.1, 25.3, 46.0, 46.5, 53.3, 55.3, 211.5 (*C*=S); *m*/*z* (ESI negative) 201.00 $[M-Na]^-$.

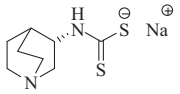
Sodium (*R*)-(+)-quinuclidine-3-yl carbamodithioate **6b**



6b

Sodium (R)-(+)- quinuclidine-3-yl carbamodithioate ***6b***: 83% yield; mp 187–188 °C (dec); δ_H (400 MHz, D_2O) 1.70 (4H, m), 2.1 (1H, m), 2.80 (5H, m), 3.25 (1H, m), 4.30 (1H, m); δ_C (100 MHz, D_2O) 19.9, 25.1, 25.3, 46.0, 46.6, 53.3, 55.2, 211.6(C=S); *m*/*z* (ESI negative) 201.00 $[M-Na]^-$; $[\alpha]_D$ +13.6.

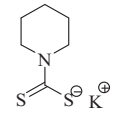
Sodium (*S*)-(−)-quinuclidine-3-yl carbamodithioate **7b**



7b

Sodium (S)-(−)-quinuclidine-3-yl carbamodithioate ***7b***: 89% yield; mp 182–183 °C (dec); δ_H (400 MHz, D_2O) 1.70 (4H, m), 2.11 (1H, m), 2.70 (5H, m), 3.32 (1H, m), 4.34 (1H, m); δ_C (100 MHz, D_2O) 19.9, 25.1, 25.3, 46.0, 46.5, 53.3, 55.2, 211.5 (*C*=S); *m*/*z* (ESI positive) 225.00 $[M+H]^+$; $[\alpha]_D$ −14.4.

Potassium piperidine-1-carbodithioate **8b**

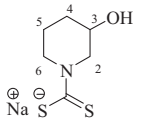


8b

Potassium piperidine-1-carbodithioate ***8b***: 84% yield; mp 75–76 °C (dec); δ_H (400 MHz, DMSO-d_6) 1.43 (4H, m), 1.57 (2H, m), 4.32 (4H, t, *J* 5.5); δ_C (100 MHz, DMSO-d_6) 25.4, 26.6, 51.0, 213.8

(*C*=S); *m*/*z* (ESI negative) 160.0 [M−K]$^-$. Experimental data in agreement with reported data.[5]

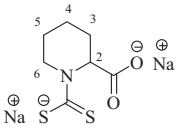
Sodium 3-hydroxypiperidine-1-carbodithioate **9b**



9b

Sodium 3-hydroxypiperidine-1-carbodithioate ***9b***: 66% yield; mp 80–81 °C (dec); δ_H (400 MHz, DMSO-d_6) 1.28 (2H, m), 1.56 (1H, m), 1.89 (1H, m), 2.66 (2H, m), 2.79 (1H, t, *J* 6.0), 4.78 (1H, d, *J* 4.0, exchange with D_2O, O*H*), 5.64 (1H, d, *J* 6.0), 5.8 (1H, d, *J* 8.0); δ_C (100 MHz, DMSO-d_6) 24.1, 34.7, 50.3, 57.5, 66.6, 214.3 (*C*=S); *m*/*z* (ESI negative) 175.91 [M−Na]$^-$.

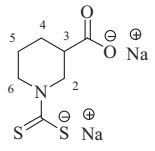
Disodium 1-dithiocarboxylatopiperidine-2-carboxylate **10b**



10b

Disodium 1-dithiocarboxylatopiperidine-2-carboxylate ***10b***: 62% yield; mp 121–122 °C; δ_H (400 MHz, DMSO-d_6) 1.40 (2H, m), 1.60 (3H, m), 2.18 (1H, d, *J* 8.0), 3.25 (1H, t, *J* 12.0), 5.81 (1H, d, *J* 8.0), 6.53 (1H, s); δ_C (100 MHz, DMSO-d_6) 22.5, 26.6, 29.2, 47.5, 62.8, 176.1 (*C*=O), 212.6 (*C*=S); *m*/*z* (ESI positive) 272.00 [M+Na]$^+$.

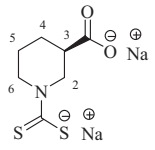
Disodium 1-dithiocarboxylatopiperidine-3-carboxylate **11b**



11b

Disodium 1-dithiocarboxylatopiperidine-3-carboxylate ***11b***: 90% yield; mp 124–125 °C; δ_H (400 MHz, DMSO-d_6) 1.56 (4H, m), 1.99 (2H, m), 2.56 (1H, m), 5.85 (2H, m); δ_C (100 MHz, DMSO-d_6) 26.4, 30.0, 46.0, 51.1 54.4, 179.6 (*C*=O), 213.0 (*C*=S);*m*/*z* (ESI positive) 272.00 [M+Na]$^+$.

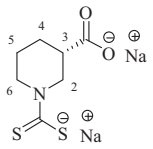
Disodium (*R*)-(−)-1-dithiocarboxylatopiperidine-3-carboxylate **12b**



12b

Disodium (R)-(−)-1-dithiocarboxylatopiperidine-3-carboxylate ***12b***: 94% yield; mp 105–106 °C (dec); δ_H (400 MHz, DMSO-d_6) 1.42 (4H, m), 1.90 (2H, m), 2.60 (1H, m), 6.0 (2H, m); δ_C (100 MHz, DMSO-d_6) 26.4, 30.1, 46.0, 51.0, 54.4, 179.5 (*C*=O), 213.1 (*C*=S); *m*/*z* (ESI negative) 203.91 [M−2Na]$^{2-}$; $[\alpha]_D^{20}$ −39.8.

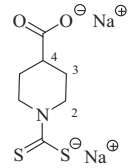
Disodium (*S*)-(+)-1-dithiocarboxylatopiperidine-3-carboxylate **13b**



13b

Disodium (S)-(+)-1-dithiocarboxylatopiperidine-3-carboxylate ***13b***: With 91% yield; mp 82–83 °C (dec); δ_H (400 MHz, DMSO-d_6) 1.40 (4H, m), 1.92 (2H, m), 2.55 (1H, m), 5.99 (2H, m); δ_C (100 MHz, DMSO-d_6) 26.4, 30.0, 46.0, 51.1, 54.4, 179.6 (*C*=O), 213.0 (*C*=S); $[\alpha]_D^{20}$ +71.5.

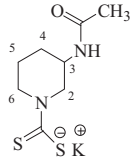
Disodium 1-dithiocarboxylatopiperidine-4-carboxylate **14b**



14b

Disodium 1-dithiocarboxylatopiperidine-4-carboxylate ***14b***: 47% yield, mp 125–126 °C; δ_H (400 MHz, D_2O) 1.54 (2H, m), 1.85 (2H, m), 2.43 (1H, m), 3.18 (2H, dt, *J* 12.7 2.3), 5.32 (2H, d, *J* 12.7); δ_C (100 MHz, D_2O) 29.6, 44.7, 52.0, 184.7 (*C*=O), 206.9 (*C*=S); *m*/*z* (ESI positive) 272.06 [M+Na]$^+$.Experimental data in agreement with reported data.[29b]

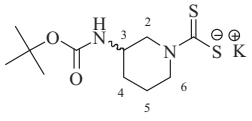
Potassium 3-acetylaminopiperidine-1-carbodithioate **15b**



15b

Potassium 3-acetylaminopiperidine-1-carbodithioate ***15b***: 51% yield; mp 110–111 °C; δ_H (400 MHz, D_2O): 1.54 (2H, m), 1.71 (1H, m), 1.90 (3H, s, CH_3), 3.80 (1H, m), 3.93 (2H, m), 4.48 (1H, m), 4.70 (2H, m); δ_C (100 MHz, D_2O) 22.6, 22.9, 29.5, 46.9, 52.3, 55.2, 174.1 (*C*=O), 209.5 (*C*=S);*m*/*z*(ESI negative) 217.0 [M-2 K]$^-$.

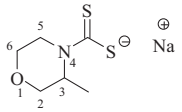
Potassium 3-(*tert*-butoxycarbonylamino) piperidine-1-carbodithioate **16b**



16b

Potassium 3-(tert-butoxycarbonyl)piperidine-1-carbodithioate ***16b***: 95% yield; mp 99–100 °C; δ_H (400 MHz, DMSO-d_6) 1.30 (2H, m), 1.42 (9H, s, 3× *CH*$_3$), 1.60 (1H, m), 1.80 (1H, m), 5.30 (2H, m), 6.70 (1H, br s, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 24.3, 29.2, 31.8, 48.1, 50.2, 54.4, 78.4, 155.6 (*C*=O), 215.2 (*C*=S), *m*/*z* (ESI negative) 275.09 [M−K]$^-$.

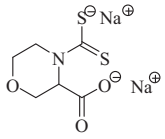
Sodium 3-methylmorpholine-4-carbodithioate **17b**



17b

Sodium 3-methylmorpholine-4-carbodithioate ***17b***: 96% yield; mp 108–109 °C (dec); δ_H (400 MHz, D_2O) 1.27 (3H, d, *J* 4.0), 3.36 (1H, m), 3.49 (1H, m), 3.63 (1H, dd, *J* 12.0 4.0), 3.76 (1H, dt, *J*11.0 1.2), 3.95 (1H,dd; *J* 11.0 4.0), 5.34 (1H, m), 5.70 (1H, m); δ_C (100 MHz, D_2O) 14.0, 46.4, 54.5, 66.8, 70.4, 209.7 (*C*=S); *m*/*z* (ESI negative)176.08 $[M-Na]^-$.

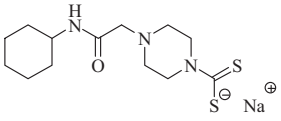
Disodium 4-dithiocarboxylatomorpholine-3-carboxylate **18b**



18b

Disodium 4-dithiocarboxylatomorpholine-3-carboxylate ***18b***: 97% yield; mp 111–112 °C (dec); δ_H (400 MHz, D_2O) 3.43 (2H, m), 3.60 (1H, dd, *J* 12.03.7), 3.85 (1H, *J* 12.0 2.8), 4.35 (1H, d, *J*12.0), 5.37 (1H, dd, *J*12.0 2.8), 6.15 (1H, m); δ_C (100 MHz, D_2O) 49.0, 64.0, 66.6, 68.6, 176.2 (*C*=O), 211.7 (*C*=S); *m*/*z* (ESI negative) 228.00 $[M-Na]^-$.

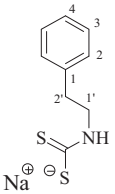
Sodium 4-[2-(cyclohexylamino)-2-oxoethyl]piperazine-1-carbodithioate **19b**



19b

Sodium 4-[2-(cyclohexylamino)-2-oxoethyl]piperazine-1-carbodithioate ***19b***: 92% yield; mp 99–100 °C; δ_H (400 MHz, DMSO-d_6) 1.24 (4H, m), 1.69 (6H, m), 2.37 (4H, t, *J* 5.2), 2.91 (2H, s); 3.63 (1H, m), 4.37 (4H, t, *J* 5.2), 7.54 (1H, s, N-*H*); δ_C (100 MHz, DMSO-d_6) 25.5, 26.1, 33.2, 48.0, 49.7, 53.8, 61.9, 168.9 (*C*=O), 215.1 (*C*=S), *m*/*z* (ESI negative) 300.30 $[M-Na]^-$.

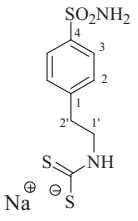
Sodium phenethylcarbamodithioate **20b**



20b

Sodium phenethylcarbamodithioate ***20b***: 70% yield; mp 87–88 °C; δ_H (400 MHz, DMSO-d_6) 2.81 (2H, t, *J* 8.0, 2′-H_2), 3.61 (2H, m, 1′-H_2), 7.29 (5H, m, Ar-H), 8.0 (1H, brt, *J* 6.0, exchange with D_2O, N-*H*); δ_C (100 MHz, DMSO-d_6) 35.6, 48.9, 126.8, 129.2, 129.5, 141.1, 215.4 (*C*=S), *m*/*z* (ESI negative) 196.03 $[M-Na]^-$. Experimental data in agreement with reported data.[23,29c]

Sodium 4-sulfamoylphenethylcarbamodithioate **21b**



21b

Sodium 4-sulfamoylphenethylcarbamodithioate ***21b***: 67% yield; mp 220–221 °C (dec); δ_H (400 MHz, DMSO-d_6) 2.91 (2H, t, *J* 8.0, 2′-H_2), 3.62 (2H, q, *J* 8.0, 1′-H_2), 7.30 (2H, br s, exchange with D_2O, SO_2NH_2), 7.44 (2H, d, *J* 8.0, Ar-H), 7.77 (2H, d, *J* 8.0, Ar-H), 8.06 (1H, brt, *J* 8.0, exchange with D_2O, N-*H*); δ_C (100 MHz, DMSO-d_6) 35.2, 48.4, 126.6, 123.0, 142.7, 145.5, 216.0 (*C*=S); *m*/*z* (ESI negative) 275.17 $[M-Na]^-$.

4.3. CA inhibition assay

A stopped-flow instrument (SX.18MV-R Applied Photophysics model) was used for assaying the CA-catalyzed CO_2 hydration activity.[24] 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.[30,31] 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 the enzyme activity is at half maximal. All enzymes used were recombinant, produced in *Escherichia coli* as reported earlier.[30,31] The concentrations of enzymes used in the assay were: hCA I, 12.0 nM; hCA II, 9.2 nM; hCA IX, 8.3 nM and hCA XII, 9.4 nM.

4.4. Normotensive rabbit IOP lowering studies

Male New Zealand albino rabbits weighing 1500−2000 g were used in these studies. Animals were anaesthetized using Zoletil (Tiletamine chloride + Zolazepam chloride, 3 mg/kg body weight, im injection) and injected with 0.1 mL hypertonic saline solution (5% in distilled water) into the vitreous of both eyes.[28,32] IOP was measured by using a digital tonometer (TonoPen Avia Tonometer, Reichhert Inc. Depew, NY 14043, USA) prior to hypertonic saline injection (basal) at 1, 2 and 3 h after administration of the drug. Vehicle (phosphate buffer, pH 7.0 plus DMSO 2%) or drugs were instilled immediately after the injection of hypertonic saline. Eyes were randomly assigned to different groups. Four different animals were used for each tested compound. Vehicle or drug (0.50 mL) were directly instilled into the conjunctive pocket at the desired doses (1–2%).[32] The IOP was followed for 3 h after drug administration. Experiments with animals were conducted in agreement with current ethical guidelines and norms approved by the ethical committee of our university.

4.5. Molecular modelling studies

Both stereoisomers of compound **16b** were built using the MOE software package (version 2013.0802, Chemical Computing Group Inc., Montreal, Canada). The carbodithioate moiety was assigned a negative charge (*R*-CS_2^-), partial atomic charges were calculated and a steepest-descent energy-minimization was performed using the MMFF94x forcefield. A high-resolution crystal structures of hCA II has been used (PDB files 3B4F). All non-protein atoms were deleted except the Zn^{2+} ions. Hydrogen atoms and partial charges were added with the 'Protonate 3D' utility of MOE and a steepest-descent energy minimization was applied (MMFF94x forcefield). Docking calculations of both stereoisomers of compound **16b** into the hCA II crystal structure was performed with the GOLD Docking programme (version 5.2, CCDC, Cambridge, UK) and the ChemScore scoring function (25 dockings). All residues within 12 Å of the Zn^{2+} ion were assigned as being part of the binding pocket.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.03.068.

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