Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Xanthates and Trithiocarbonates Strongly Inhibit Carbonic Anhydrases and Show Antiglaucoma Effects in Vivo

Fabrizio Carta,[†] Atilla Akdemir,[‡] Andrea Scozzafava,[†] Emanuela Masini,[§] and Claudiu T. Supuran*[,†,∥]

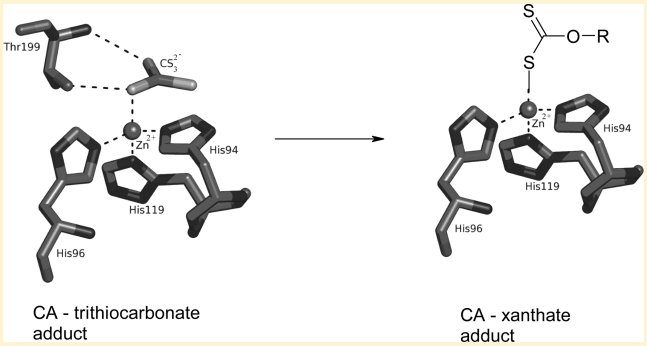
[†]Laboratorio di Chimica Bioinorganica, Polo Scientifico, Università degli Studi di Firenze, Rm. 188 Via della Lastruccia 3, 50019 Sesto Fiorentino (Florence), Italy

[‡]Department of Pharmacology, Faculty of Pharmacy, Bezmialem Vakif University, Vatan Caddesi, 34093 Fatih, Istanbul, Turkey

[§]Preclinical and Clinical Pharmacology Section, NEUROFARBA Department, Università degli Studi di Firenze, Viale Pierracini 6, 50139 Florence, Italy

[∥]Sezione di Scienze Farmaceutiche e Nutraceutiche, NEUROFARBA Department, Università degli Studi di Firenze, 50019 Sesto Fiorentino (Florence), Italy

ABSTRACT: Dithiocarbamates (DTCs) were recently discovered as carbonic anhydrase (CA, EC 4.2.1.1) inhibitors. A series of xanthates and a trithiocarbonate, structurally related to the DTCs, were prepared by reaction of alcohols/thiols with carbon disulfide in the presence of bases. These compounds were tested for the inhibition of four human (h) isoforms, hCA I, II, IX, and XII, involved in pathologies such as glaucoma (CA II and XII) or cancer (CA IX). Several low nanomolar xanthate/trithiocarbonate inhibitors targeting these CAs were detected. A docking study of some xanthates within the CA II active site showed that these compounds bind in a similar manner with the dithiocarbamates, coordinating monodentately to the Zn(II) ion from the enzyme active site. Several xanthates showed potent intraocular pressure lowering activity in two animal models of glaucoma via the topical administration. Xanthates and thioxanthates represent two novel, promising classes of CA inhibitors.



INTRODUCTION

The classical carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) are the sulfonamides and their isosteres (sulfamates, sulfamides, etc).[1−4] However, most of these compounds indiscriminately inhibit many of the 16 CA isoforms known to date in mammals.[1−3] Thus, efforts have been made to find different CAIs, from the sulfonamide, sulfamate, and sulfamide ones. Indeed, recently, the coumarins were discovered as mechanism-based inhibitors which act as prodrugs and bind in a very different mode compared to sulfonamides and their isosteres,[4] whereas some polyamines (such as spermine),[5] as well as a range of phenols[6] were also investigated and showed such interesting properties and novel mechanisms of inhibition.[4−6] Among the CAIs investigated to date, there were also the inorganic anions which coordinate to the zinc ion from the enzyme active site.[1,7,8] Indeed, trithiocarbonate (CS_3^{2-}), an anion similar to carbonate, has recently been investigated and shown to constitute a "lead" for novel CAIs.[8] The X-ray crystal structure for the adduct of trithiocarbonate (CS_3^{2-}) bound to hCA II has been reported, being shown that the inhibitor binds to the Zn^{2+} in the hCA II active site in a slightly distorted tetrahedral geometry of the metal ion,[7a] occupying a position similar to that observed in the case of hCA II−bicarbonate complex (Figure 1A).[7b] Trithiocarbonate was found monocoordinated to the Zn(II) ion from the enzyme active site by means of one of its sulfur atoms. The same sulfur made a hydrogen bond to the OH of Thr199, whereas a second sulfur atom participated to another hydrogen bond with the NH group of the same amino acid residues, Thr199. This binding mode explained the rather good affinity of this inhibitor to many of the CA isoforms investigated to date.[7a] On the basis of this binding mode of a millimolar inhibitor, trithiocarbonate (CS_3^{2-}), we discovered that other compounds incorporating this new zinc-binding group (ZBG), i.e., the CS_2^- one, such as the dithiocarbamates (DTCs), act as even stronger CAIs. These inhibitors bind to the Zn(II) ion from the enzyme active site similar to the inorganic anion trithiocarbonate (Figure 1B).[9−11] Indeed, we have recently demonstrated that many primary/secondary dithiocarbamates, possessing the general formula $R^1R^2N\text{-}CS_2^-M^+$ act as highly efficient CAIs against many α and β class CAs (of mammalian, fungal, or bacterial origin).[9−11]

X-ray crystal structures were also reported[9,11] for three DTCs complexed to hCA II, compounds **A**−**C** (Figure 1B). These DTCs inhibited isoform hCA II with K_Is of 25, 41, and 0.95 nM, respectively, and the transmembrane, tumor-associated hCA IX with K_Is of 53, 757, and 6.2 nM, respectively.[9,11] As seen from Figure 1B, the binding mode of the

Received: March 21, 2013
Published: May 6, 2013

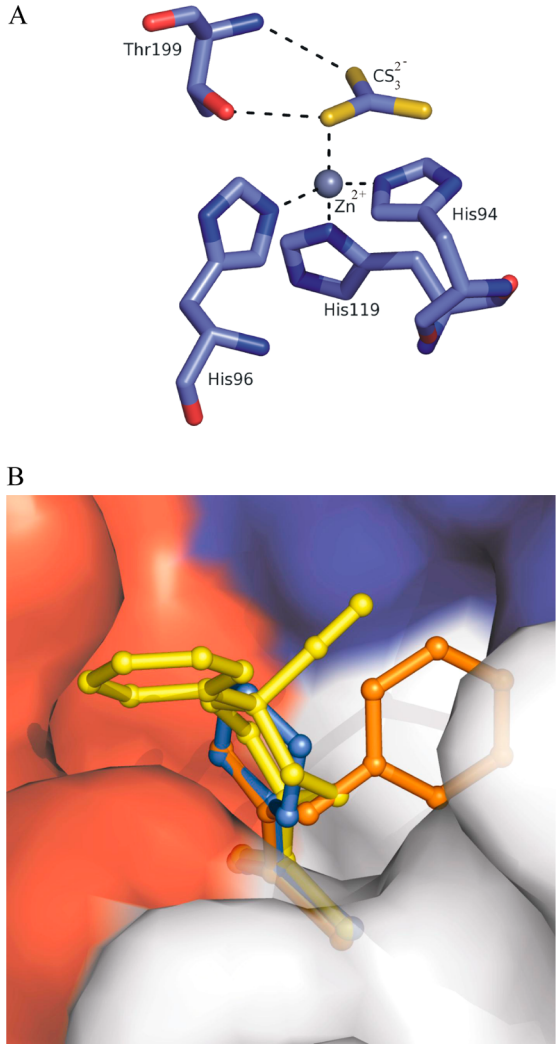
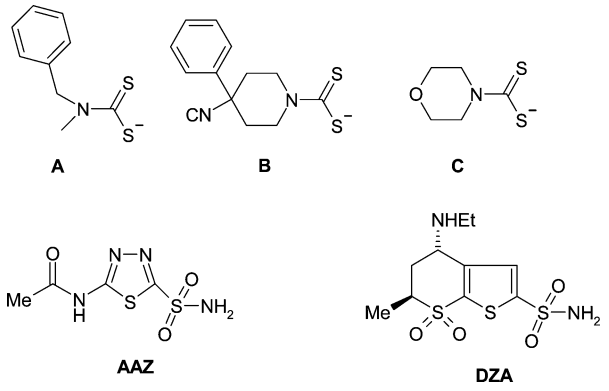


Figure 1. (A) Trithiocarbonate (CS_3^{2-}), a recently investigated CAI,[8] binds to the Zn^{2+} in the hCA II active site in a slightly distorted tetrahedral geometry of the metal ion, occupying a position similar to that observed in the case of hCA II–bicarbonate complex. The protein zinc ligands (His94, 96, and 119) and Thr199 are shown (PDB code 3K7K). (B) Structural superposition of dithiocarbamates CAIs bound to hCA II (as determined by X-ray crystallography).[9,11] **A** is shown in orange (PDB code 3P58), **B** in yellow (PDB code 3P5L), and **C** is reported in blue (PDB code 3P5A). The hCA II active site is represented as a surface model (hydrophobic side in red, hydrophilic side in blue).



ZBG present in **A**–**C** is identical to that of trithiocarbonate (Figure 1B), with one sulfur atom coordinated to the zinc ion, but the organic scaffold present in the DTCs was observed to make extensive contacts with several amino acid residues from the active site, which explains the wide range of inhibitory power of these derivatives (from the subnanomolar to the micromolar range, for the entire series of around 30 DTCs reported so far).[9–11] For example, the benzyl (hydrophobic) moiety of **A** was observed orientated toward the hydrophilic half of the hCA II active site. For derivative **B**, the cyano fragment pointed toward the hydrophilic part whereas the phenyl moiety was orientated toward the hydrophobic half of the hCA II cavity (Figure 1B). Interestingly, the small and compact morpholine dithiocarbamate **C** was found in the middle of the active site cavity, and its six-membered ring was not superposable to the one present in the compound **B**. Such a variability of orientation of the scaffold is of great interest for designing CAIs, as in the case of the sulfonamides this variability was not observed even considering the huge number of sulfonamides for which the structure is available in complex with various CA isoforms.[1–3] The highly water-soluble DTC **C** was also effective in vivo as an antiglaucoma agent when administered topically directly to the eye of hypertensive rabbits,[11] a widely used animal model of glaucoma.[12]

Xanthates and thioxanthates (trithiocarbonates) contain the same ZBG found in CS_3^{2-} and in DTCs, and such compounds have never been investigated as CAIs. Here we report that the xanthates ($RO\text{-}CS_2^-M^+$) and thioxanthates (organic trithiocarbonates, $RS\text{-}CS_2^-M^+$) represent two new classes of potent CAIs, with a mechanism of action different from that of the sulfonamides but similar to that of the DTCs. Furthermore, we prove that some of these highly water-soluble compounds possess excellent intraocular pressure (IOP) lowering properties in an animal model of glaucoma,[12] making them interesting candidates for the development of antiglaucoma drugs based on inhibitors of CAs.

Chemistry. The rationale of this work was a very simple one: considering CS_3^{2-} and DTCs ($R^1R^2N\text{-}CS_2^-M^+$) as lead molecules, we replaced the nitrogen atom from the DTCs by the oxygen or sulfur atoms, leading thus to xanthates ($RO\text{-}CS_2^-M^+$) and trithiocarbonates ($RS\text{-}CS_2^-M^+$), respectively.

Xanthates **1**–**18**, and the thioxanthate (**19**) (as well as one DTC, **20**) were prepared as illustrated in Scheme 1, by reaction

Scheme 1. Preparation of Xanthates 1, 2, 4–17, Trithiocarbonate (Thioxanthate) 19, and DTC 20 from the Corresponding Alcohol, Thiol or Amine and Carbon Disulfide in the Presence of Base[a]

[a]Compounds **3** and **18** were purchased.

of the corresponding nucleophile (alcohol, thiol, or amine) and CS_2 in the presence of a strong base, KOH, by the literature procedures.[9–13]

The moieties R present in compounds **1**–**19** reported here, include simple aliphatic (C_1–C_8), cycloaliphatic (cyclopentyl, cyclohexyl, saturated rings), arylalkyl-, heterocyclyl, as well as polycyclic rings, possibly substituted with aminoalkyl or Boc-protected-amino moieties. These scaffolds were incorporated in these compounds in order to generate chemical diversity, as it has been observed for the DTCs investigated earlier[9–11] that the nature of the organic scaffold is the main factor influencing inhibitory activity against various CA isoforms (and also that its

dx.doi.org/10.1021/jm400414j | J. Med. Chem. 2013, 56, 4691–4700

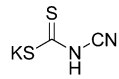
binding to the enzyme active site is quite variable, as mentioned above; see also Figure 1B). A special mention regards the only DTC investigated here, compound **20**, which was obtained by reaction of cyanamide (H_2NCN) with carbon disulfide and potassium hydroxide (Scheme 1). Compound **20** does not possess an organic scaffold as compounds **1**−**19** or the DTCs investigated earlier,[9−11] being thus a special case of quite a simple, almost "inorganic", derivative.

CA Inhibition. Compounds **1**−**20** were assayed[14] for the inhibition of four physiologically relevant CA isoforms, hCA I, II, IX, and XII. All of them are drug targets: hCA I, II, and XII are for ophthalmologic diseases, mainly glaucoma,[1] whereas CA IX and XII are for antitumor drugs/tumor imaging agents.[1,2] Inhibition data with the sulfonamide, clinically used agent acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also reported in Table 1 for comparison.

Table 1. Human (h) CA I, II, IX and XII Inhibition Data[a] with Xanthates 1−18, Thioxanthate (Trithiocarbonate) 19 and Dithiocarbamate 20[14]

R-O-CSS$^-$K$^+$ **1-18**

R-S-CSS$^-$K$^+$ **19**



20

No	R	K_I (nM)*			
		hCA I	hCA II	hCA IX	hCA XII
1	Me	687±32	346±23	631±54	808±46
2	Et	604±18	305±27	566±50	814±65
3	i-Pr	401±31	307±14	531±28	685±43
4	i-Amyl	638±50	298±21	322±16	561±50
5	n-C_5H_{11}	384±17	361±28	403±38	766±39
6	n-C_8H_{17}	351±21	337±30	72.1±6	810±76
7	cyclopentyl	532±42	366±22	47.2±3	74.7±6
8	cyclohexyl	795±61	293±14	78.8±7	75.6±4
9	Me_2N-CH_2CH_2	327±15	60.4±5	760±45	745±29
10	Me_2N-$CH_2CH_2OCH_2CH_2$	45.1±2	8.1±0.4	831±59	622±52
11	Boc-NH-$(CH_2)_4$	5.6±03	9.5±0.8	622±30	705±26
12	Boc-NH-$(CH_2)_6$	6.3±0.4	59.0±3	765±41	744±53
13	$PhCH_2$	76.1±6	45.1±2	8.1±0.6	6.0±0.3
14	Ph_2CHCH_2	64.1±5	5.4±0.4	58.0±4	63.3±4
15	2-Pyridyl-CH_2CH_2	74.2±7	13.1±1.2	4.3±0.2	3.6±0.3
16	1-Adamantyl-CH_2	81.3±6	21.5±1.8	776±54	750±52
17	1-Adamantyl-CH_2CH_2	59.1±4	6.4±0.5	779±69	737±68
18		63.4±3	6.6±0.4	643±37	835±59
19	$PhCH_2$	73.5±5	75.7±6	7.8±0.5	6.7±0.6
20	-	531±26	73.6±7	678±51	709±62
AAZ	-	250±13	12±0.9	25±1.7	5.7±0.3

[a]*From three different CO_2 hydrase stopped-flow method assay, mean ± standard error (from three different determinations).[14]

dx.doi.org/10.1021/jm400414j | J. Med. Chem. 2013, 56, 4691−4700

The following structure−activity relationship (SAR) can be observed for the CA inhibition data with **1**−**20** investigated here:

i. . The cytosolic isoform hCA I was modestly inhibited by compounds **1**−**9** (xanthates) and DTC **20**, with K_Is in the range of 327−795 nM. Thus, the aliphatic, *n*-alkyl, and cycloalkyl xanthates were ineffective CAIs, together with the cyanamide DTC **20**. Medium inhibitory activity was seen for some of the xanthates investigated here, i.e., compounds **10**, **13**−**18**, and the trithiocarbonate **19**, which showed inhibition constants in the range of 45.1−81.3 nM, being thus much more effective CAIs against this isoform compared to the sulfonamide acetazolamide (AAZ) (K_I of 250 nM). These derivatives incorporate a longer aminoalkyloxy moiety (derivative **10**) as well as arylalkyl or polycyclic, bulkier scaffolds (compounds **13**−**18**). Xanthates **11** and **12**, incorporating Boc-substituted aliphatic alcohols (C_4 and C_6) were on the other hand highly effective hCA I inhibitors, with K_Is in the range of 5.6−6.3 nM. Thus, the structure−activity relationship (SAR) for the inhibition of hCA I is rather straightforward: alkyl- and cycloalkyl xanthates with C_1−C_8 carbon atoms are weak inhibitors, whereas activity increases with the incorporation of heteroatoms in the aliphatic chain (as in **10**−**12**) or the presence of arylalkyl and polycyclic moieties (as in **13**−**18**)..

ii. . Against the physiologically dominant isoform hCA II, the C_1−C_8 aliphatic xanthates **1**−**8** showed again weak inhibitory power, with K_Is in the range of 293−366 nM (with a rather irrelevant variatiation in the inhibitory power with the increase of the chain from C_1 to C_8, or its ramification/cyclization). All these compounds incorporate similar alkyl or cycloalkyl moieties and showed a compact behavior of weak CAIs. A number of other derivatives, among which the xanthates **12**, **13**, **16**, the thioxanthate **19**, and the DTC **20** were medium potency hCA II inhibitors, with K_Is in the range of 21.5−75.7 nM (Table 1). Some of the xanthates investigated here, i.e., **10**, **11**, **14**, **15**, **17**, and **18**, were highly effective, low nanomolar hCA II inhibitors, with K_Is in the range of 5.4−13.1 nM (being more effective or similar as the clinically used sulfonamide AAZ). Interestingly, they incorporate various scaffolds, such as the bulky 1,1-diphenylethyl one in **14**, the adamantylethyl one in **17**, the cyclohexyl-norbornyl one in **18**, as well as the *N*,*N*-dimethylaminoethoxy-ethyl- or Boc-aminobutyl (in **10** and **11**, respectively). To be noted that increasing the length of the scaffold by an ethylenoxy moiety (from **9** to **10**) leads to a 7.45-fold increase of hCA II inhibitory potency (Table 1). However, for the pair of Boc-aminoalkyl derivatives **11** and **12**, the shorter derivative 11 was 6.2-fold a better hCA II inhibitor compared to the longer analogue **12**..

iii. . The tumor-associated hCA IX was also modestly inhibited by xanthates **1**−**5**, **9**−**12**, **16**−**18**, and **20**, with K_Is in the range of 322−831 nM. SAR was here more complicated compared to hCA I and II discussed above, as the ineffective inhibitors incorporate many types of scaffolds (alkyl, amino-alkyl, Boc-amino-alkyl, adamantyl, and other polycyclic rings). The simple DTC **20** was also an ineffective hCA IX inhibitor (K_I of 678 nM). Four of the investigated xanthates, i.e., **6**−**8** and **14**, were medium potency hCA IX inhibitors, with K_Is in the range of 47.2−78.8 nM. They incorporate the long *n*-alkyl chain with 8 carbon atoms (compound **8**), the cycloalkyl C_5 and C_6 rings as well as the bulky diphenylethyl moieties. Two xanthates, **13** and **15**, and the thioxanthate **19**, were highly effective hCA IX inhibitors, with K_Is in the range of 4.3−8.1 nM. They incorporate the benzyl or 2-pyridylethyl moieties. Thus, also here SAR is straightforward, with few scaffolds (e.g., arylalkyl and hetarylalkyl), leading to highly effective xanthate hCA IX inhibitors..

iv. . A rather similar inhibition profile (with hCA IX) was seen for the inhibition of hCA XII with the investigated compounds. Indeed, **1**−**6**, **9**−**12**, **16**−**18**, and **20** were weak hCA XII inhibitors, with K_Is in the range of 561−835 nM. Three of the investigated xanthates, i.e., **7**, **8**, and **14**, were medium potency hCA XII inhibitors, with K_Is in the range of 63.3−75.6 nM. The best hCA XII inhibitors were again, as for hCA IX, xanthates, **13** and **15**, and the thioxanthate **19**, with K_Is in the range of 3.6−6.7 nM..

v. . There is a net distinction in the inhibition profile of the two cytosolic (hCA I and II) over the two transmembrane CA isoforms (hCA IX and XII) with these new classes of inhibitors. Indeed, for the cytosolic enzymes, the best, low nanomolar inhibitors incorporated Boc-aminoalkyl (hCA I) or diphenylethyl, adamanthylethyl, and other such bulkier polycyclic ring systems. For the transmembrane isoforms, the best inhibitors incorporated benzyl and 2-pyridylethyl moieties. For example, **18** was a highly selective hCA II inhibitor, **11** and **12** were selective hCA I-inhibitors, whereas xanthates **13** and **15** and the thioxanthate **19** were hCA IX/XII selective (over hCA I and II) inhibitors.

Inhibition Mechanism of Xanthates and Thioxanthates.

To understand the CA inhibition mechanism with xanthates and thioxanthates, we performed some theoretical studies.

Compounds **10** and **14** were built and converted to three-dimensional structures using the MOE software package.[15] The zinc-binding function was assigned a negative charge ($-CS_2^-$) because it was expected to interact with the Zn^{2+} ion of the CA active site as was also observed for the crystal structure of hCA II in complex with 4-cyano-4-phenylpiperidine-1-carbodithioate **B** (PDB file: 3P5L). Subsequently, partial atomic charges were calculated and the molecules were energy-minimized according to a steepest-descent protocol using the MMFF94x force field in MOE. The ligands were saved as a multi-mol2 file.

The crystal structures of hCA II (PDB 3P5L; 1.50 Å) in complex with 4-cyano-4-phenylpiperidine-1-carbodithioate (**B**) was obtained from the Protein Data Bank. The water molecules, ligand, and DMSO were deleted. Hydrogen atoms and charges were added to the protein using the protonate 3D tool of the MOE software package,[15] and a steepest descent energy minimization was applied (MMFF94x forcefield). The protein was saved as a mol2-file. Compounds **10** and **14** were docked into the crystal structure of hCA II (PDB 3P5L; 1.50 Å) using the GOLD Suite docking package[16] and the ChemScore scoring function (25 docking per ligand) (Figure 2). The binding pocket was defined as all residues within 12 Å of the N22 nitrogen atom of compound **B**. No restrictions were applied for the ligand conformations and binding poses during the docking procedure.

As seen from Figure 2, xanthates **10** and **14** coordinate monodentately to the Zn(II) from the enzyme active site, almost in a superposable manner with the DTC **B** for which the X-ray crystal structure was reported earlier.[9] The zinc−sulfur distances, evidenced in Figure 2B, were of 2.46 Å for xanthate **10**, of 2.12 Å for xanthate **14**, and of 2.31 Å for DTC **B**, respectively. The sulfur atoms of these xanthates/DTC also form hydrogen bonds with the side chain hydroxyl of Thr199 (for compound **10**, of 3.0 Å, for compound **14**, of 3.5 Å, for DTC **B**, of 3.1 Å; Figure 2). The organic scaffolds of xanthates **10** and **14** adopt extended conformation within the enzyme

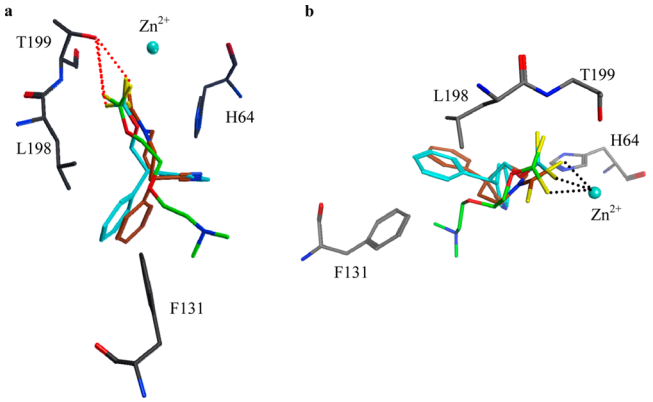


Figure 2. (a). The docked poses of compounds **10** (green) and **14** (turquoise) inside the active site of hCA II (PDB 3P5L).[9] Both xanthates have their Zn^{2+}-binding CS_2^- moieties at a similar location as the one from the DTC 4-cyano-4-phenylpiperidine-1-carbodithioic acid (ligand **B** of 3P5L, shown brown). The phenyl groups of ligands **14** and **B** interact hydrophobically with Phe131 (π-stacking). (b) Alternative view for the binding of compounds **10**, **14**, and **B** to the hCA II active site (the Zn–sulfur interaction shown as black dotted lines).

active site (Figure 2A), similar to the binding of the DTC **B**, determined by X-ray crystallography.[9] Indeed, one phenyl group of **14** and the phenyl moiety present in the DTC **B** interacted with Phe131 through an interesting π-stacking observed earlier for some sulfonamide derivatives incorporating aromatic moieties.[17] Other amino acid residues involved in the binding of xanthates **10** and **14**, as determined through these docking studies, were His64, Leu198, and, as mentioned above, Thr199 (Figure 2A,B). All these favorable interactions, in which the two compounds participate with the metal ion from the enzyme active site and amino acid residues, may explain the excellent in vitro inhibition measured against this isoform (of 8.1 nM for **10**, and of 5.4 nM for **14**, respectively, see Table 1).

IOP Lowering with Xanthate CAIs. Some of the best in vitro hCA II inhibitors (hCA II is the predominant isoform from the ciliary bodies, being responsible of aqueous humor secretion),[18] such as derivatives **10**, **11**, **14**, **15**, and **17**, were initially screened in a normotensive rabbit model[19] for their possible effects as IOP lowering agents (Figure 3). Data of Figure 3 show promising activities for most of these compounds, with a decrease of the IOP of 1.0–3.5 mmHg after administration of 50 μL of solution of 2% xanthate directly into the eye. Mention should be made that the standard, clinically used drug dorzolamide (DZA), a sulfonamide CAI, is ineffective as an IOP lowering agent in this model.

Furthermore, to establish whether this new class of CAI may be an alternative to the sulfonamides with antiglaucoma action,[18] we have tested their efficacy in a hypertensive rabbit model described earlier by us.[12] In the carbomer model,[12] a high IOP of 35–40 mmHg can be achieved, which simulates better the human diseases characterized by IOP levels in this range. As seen from data of Figure 4, administration of xanthates **10**, **11**, **14**, **15**, and **17** (50 μL solution of 2% xanthate directly into the eye) as well as DZA (as standard drug) led to a strong IOP lowering of 4.0–12.0 mmHg postadministration. The maximal effect of DZA was of 4 mmHg lowering, at around 1 h postadministration, whereas many of the xanthate CAIs were 2–3 times more effective compared to DZA. The peak of IOP lowering with the xanthates was at around 50–100 min postadministration (depending on the xanthate), and the effect was also longer lasting compared to DZA (Figure 4). In fact, an appreciable IOP lowering was still seen up to 200–250 min postadministration (in the case of DZA, the effect vanished or was very minor after 150 min). The structure (and CA inhibitory power of the xanthate) clearly influenced the IOP lowering effects. The best compound was **14** (K_I of 5.4 nM against hCA II), which led to an IOP lowering of 12 mmHg (3 times as high as the equivalent concentration of DZA), t = 100 min postadministration. This effect was rather prolonged, for more than 250 min (Figure 4). The next best

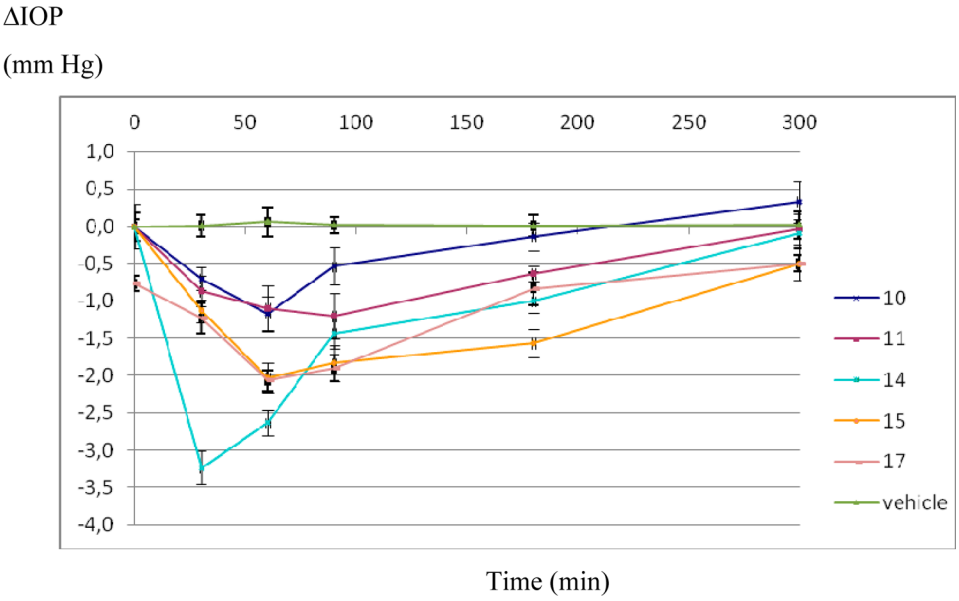


Figure 3. ΔIOP (mm Hg) versus time (min), in normotensive rabbits, treated with 50 μL of 2% solution of xanthates **10**, **11**, **14**, **15**, and **17**. Errors were in the range of 10–15% of the reported IOP values (from three different measurements for each of the four animals in the study group) and were statistically significant (p = 0.045, by Student's t test). Dorzolamide DZA was ineffective as IOP lowering agent in this model, and its IOP lowering curve was superimposable to that of the vehicle (within the limits of the experimental error, data not shown).

dx.doi.org/10.1021/jm400414j | J. Med. Chem. 2013, 56, 4691–4700

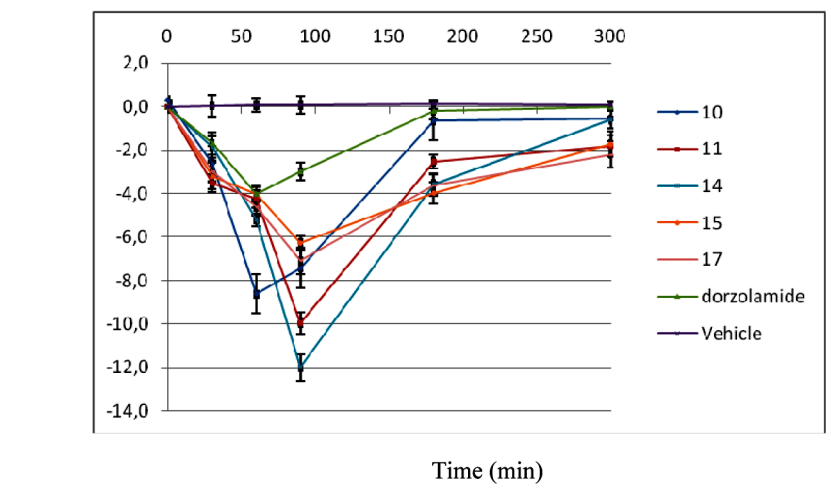


Figure 4. ΔIOP (mm Hg) versus time (min), in hypertensive rabbit eyes treated with 50 μL of 2% solution of xanthates **10**, **11**, **14**, **15**, and **17**, and dorzolamide DZA as standard drug. Errors were in the range of 10−15% of the reported IOP values (from three different measurements for each of the four animals in the study group) and were statistically significant (p = 0.045, by Student's t test).

compounds as IOP lowering agents were **11** and **10** (K_Is of 9.5 and 8.2 nM, respectively). It is interesting to note that these derivatives possess a balanced hydro-/lipophilicity, as they contain both a highly hydrophilic moiety (the xanthate group) as well as hydrophobic organic moieties.

CONCLUSIONS

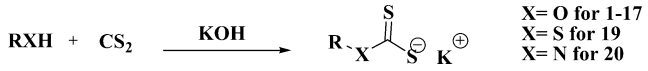
The xanthates and thioxanthates represent a new class of CAIs. Compounds with all type of activity were reported from micromolar to low nanomolar inhibitors against four isoforms: the cytosolic hCA I and II and the transmembrane hCA IX and XII. Furthermore, isoform-selective compounds were identified against all these CAs: **18** was a selective hCA II-inhibitor, **11** and **12** were selective hCA I-inhibitors, whereas xanthates **13** and **15** and the thioxanthate **19** were hCA IX/XII selective (over hCA I and II) inhibitors. The binding mode of these compounds to hCA II closely resembles the binding of the DTCs, which has been investigated by means of X-ray crystallography. One sulfur atom of the CS_2^- ZBG was found coordinated to the metal ion at a distance of 2.1−2.5 Å; furthermore, the same sulfur formed a hydrogen bond with the side chain hydroxyl of Thr199 (of 3.0−3.5 Å), whereas the rest of the scaffold interacted hydrophobically with amino acid residues involved in the binding of other classes of CAIs (such as the sulfonamides and the DTCs), among which are His64, Phe131, and Leu198. In vivo, in animal models of glaucoma in normotensive/hypertensive rabbits, some of the effective hCA II inhibitor xanthates showed effective IOP lowering after direct administration within the eye (as 2% solutions).

EXPERIMENTAL SECTION

Chemistry. Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Alfa Aesar, and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR, DEPT-135, DEPT-90, HSQC, HMBC) spectra were recorded using a Bruker Advance III 400 MHz spectrometer in DMSO-d_6. Chemical shifts are reported in parts per million (ppm), and the coupling constants (J) are expressed in hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; sept, septet; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; dd, double of doubles; appt, apparent triplet; appq, apparent quartet. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck Silica Gel 60 (230−400 mesh ASTM) as the stationary phase, and ethyl acetate/*n*-hexane were used as eluents. Melting points (mp) were carried out in open capillary tubes and are uncorrected. Purity was determined by HPLC and was >95% for all the compounds reported in the paper.

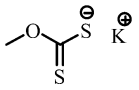
O-Isopropylxanthic acid potassium salt **3** and *O*-tricyclo[5.2.1.02,6]-dec-9-yl dithiocarbonate potassium salt **18** were purchased from Aldrich as used

General Procedure for the Synthesis of Compounds 1−20. Primary alcohols (1.0 equiv) were treated with KOH (1.0 equiv for



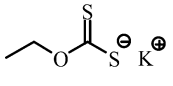
1−**17**, 2.0 equiv for **20**) and carbon disulfide (1.2 equiv) in diethyl ether. The precipitates formed were collected by filtration, washed with diethyl ether, and dried in vacuo to afford the title products.

Synthesis of Potassium O-Methyl Carbonodithioate Salt ***1***. Methanol (3.0 mL) was treated with crushed potassium hydroxide



(1.0 equiv) followed by addition of carbon disulfide (1.2 equiv). The solution was stirred at rt and then concentrated in vacuo to give a residue that was triturated from diethyl ether to afford the title product as a pale-yellow solid. Potassium *O*-methyl carbonodithioate salt (**1**): 89% yield; δ_H (400 MHz, DMSO-d_6) 3.40 (s); δ_C (100 MHz, DMSO-d_6) 58.5, 231.4 (*C*═S); m/z (ESI Negative), 106.96 [M − K]$^-$.

Synthesis of Potassium (Carbodithioethoxy)ethane ***2***. Ethanol (3.0 mL) was treated with crushed potassium hydroxide (1.0 equiv),

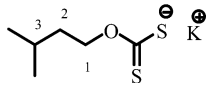


and the solution was treated with carbon disulfide (1.2 equiv). The solution was stirred at rt and then concentrated in vacuo to give a residue that was triturated from diethyl ether to afford the title product

dx.doi.org/10.1021/jm400414j | J. Med. Chem. 2013, 56, 4691−4700

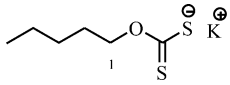
as a pale-yellow solid. Potassium (carbodithioatooxy)ethane (**2**): 90% yield; δ_H (400 MHz, DMSO-d_6) 2.21 (3H, t, J 6.2, CH_3), 4.26 (2H, q, J 6.2, CH_2); δ_C (100 MHz, DMSO-d_6) 15.4, 67.0, 230.8 (C═S); m/z (ESI Negative), 120.98 $[M - K]^-$.

*Synthesis of Potassium O-(3-Methyl-butyl) Carbonodithioate Salt **4**.* 3-Methyl-butan-1-ol (0.2g, 1.0 equiv) was treated with freshly



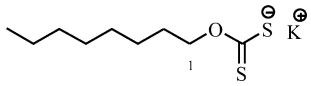
crushed potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a light-brown solid. Potassium *O*-(3-methyl-butyl) carbonodithioate salt **4**: 78% yield; δ_H (400 MHz, DMSO-d_6) 0.91 (6H, d, J 6.8, 2 × CH_3), 1.50 (2H, q, J 6.8, 2-H_2), 1.69 (1H, m, 3-H), 4.26 (2H, t, J 6.8, 1-H_2); δ_C (100 MHz, DMSO-d_6) 23.5, 25.7, 38.4, 69.9, 230.9 (C═S); m/z (ESI Negative), 163.03 $[M - K]^-$.

*Synthesis of Potassium O-Pentyl Carbonodithioate Salt **5**.* Pentan-1-ol (0.2g, 1.0 equiv) was treated with freshly crushed



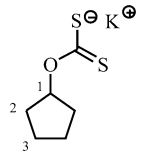
potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-pentyl carbonodithioate salt **5**: 95% yield; δ_H (400 MHz, DMSO-d_6) 0.92 (3H, t, J 6.4, CH_3), 1.34 (4H, m), 1.62 (2H, m), 4.21 (2H, t, J 6.8, 1-H_2); δ_C (100 MHz, DMSO-d_6) 14.9, 22.9, 28.9, 29.2, 71.4, 231.0 (C═S); m/z (ESI Negative), 163.03 $[M - K]^-$.

*Synthesis of Potassium O-Octyl Carbonodithioate Salt **6**.* Octan-1-ol (0.2g, 1.0 equiv) was treated with freshly crushed potassium hydroxide



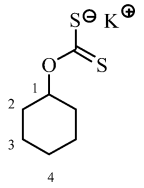
(1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-octyl carbonodithioate salt **6**: 97% yield; δ_H (400 MHz, DMSO-d_6) 0.90 (3H, t, J 6.4, CH_3), 1.31 (10H, m), 1.61 (2H, m), 4.20 (2H, t, J 6.8, 1-H_2); δ_C (100 MHz, DMSO-d_6) 14.3, 23.1, 28.7, 28.9, 29.0, 29.1, 29.2, 72.0, 230.9 (C═S); m/z (ESI Negative), 205.07 $[M - K]^-$.

*Synthesis of Potassium O-Cyclopentyl Carbonodithioate Salt **7**.* Cyclopentan-1-ol (0.2g, 1.0 equiv) was treated with freshly crushed



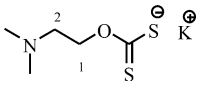
potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-cyclopentyl carbonodithioate salt **7**: 67% yield; δ_H (400 MHz, DMSO-d_6) 1.50−1.86 (8H, m, 2 × 2-H_2, 2 × 3-H_2), 5.61 (1H, m, 1-H); δ_C (100 MHz, DMSO-d_6) 24.6, 33.3, 82.6, 230.4 (C═S); m/z (ESI Negative), 161.01 $[M - K]^-$.

*Synthesis of Potassium O-Cyclohexyl Carbonodithioate Salt **8**.* Cyclohexan-1-ol (0.2g, 1.0 equiv) was treated with freshly crushed



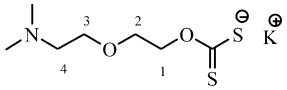
potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-cyclohexyl carbonodithioate salt **8**: 67% yield; δ_H (400 MHz, DMSO-d_6) 1.20−2.00 (10H, m), 5.22 (1H, m, 1-H); δ_C (100 MHz, DMSO-d_6) 24.9, 26.2, 32.5, 78.4, 230.2 (C═S); m/z (ESI Negative), 175.03 $[M - K]^-$.

*Synthesis of Potassium O-(2-Dimethylamino-ethyl) Carbonodithioate Salt **9**.* 2-Dimethylaminoethan-1-ol (0.2g, 1.0 equiv) was



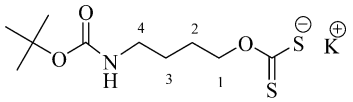
treated with freshly crushed potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-(2-dimethylamino-ethyl) carbonodithioate salt **9**: 85% yield; δ_H (400 MHz, DMSO-d_6) 2.19 (6H, s, 2 × CH_3), 2.51 (2H, t, J 6.6, 2-H_2), 4.31 (2H, t, J 6.6, 1-H_2); δ_C (100 MHz, DMSO-d_6) 46.5, 58.6, 69.4, 230.6 (C═S); m/z (ESI Negative), 164.02 $[M - K]^-$.

*Synthesis of Potassium O-[2-(4-Dimethylamino-ethoxy)-ethyl] Carbonodithioate Salt **10**.* 2-(4-Dimethylamino-ethoxy)-ethanol



(0.2g, 1.0 equiv) was treated with freshly crushed potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-[2-(4-dimethylamino-ethoxy)-ethyl] carbonodithioate salt **10**: 80% yield; δ_H (400 MHz, DMSO-d_6) 2.18 (6H, s, 2 × CH_3), 2.42 (2H, t, J 6.4, 3-H_2), 3.62 (2H, t, J 6.4, 2-H_2), 4.33 (2H, t, J 6.4, 1-H_2); δ_C (100 MHz, DMSO-d_6) 46.5, 59.3, 69.5, 69.6, 70.7, 230.5 (C═S); m/z (ESI Negative), 208.05 $[M - K]^-$.

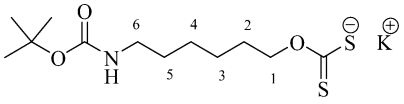
*Synthesis of Potassium O-4-(tert-Butoxycarbonylamino)butyl Carbonodithioate **11**.* *tert*-Butyl 4-hydroxybutylcarbamate (0.1g,



1.0 equiv) was treated with freshly crushed potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-4-(*tert*-butoxycarbonylamino)butyl carbonodithioate **11**: 68% yield; δ_H (400 MHz, DMSO-d_6) 1.41 (12H, 2-H_2/3-H_2, 3 × CH_3), 1.59 (2H, m, 2-H_2/3-H_2), 2.96 (2H, q, J 6.2, 4-H_2), 4.19 (2H, t, J 6.2, 1-H_2), 6.84 (1H, brt, J 6.2, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 25.4, 26.2, 28.2, 28.4, 42.0, 70.0, 154.6, 230.2 (C═S); m/z (ESI Negative), 264.07 $[M - K]^-$.

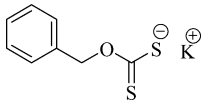
dx.doi.org/10.1021/jm400414j | *J. Med. Chem.* 2013, 56, 4691−4700

Synthesis of Potassium O-6-(tert-Butoxycarbonylamino)hexyl Carbonodithioate ***12***. *tert*-Butyl 6-hydroxyhexylcarbamate (0.1g,



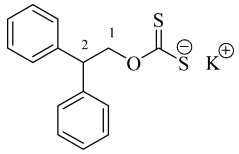
1.0 equiv) was treated with freshly crushed potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-6-(*tert*-butoxycarbonylamino)hexyl carbonodithioate **12**: 70% yield; δ_H (400 MHz, DMSO-d_6) 1.30 (4H, m, 2 × CH_2), 1.41 (12H, s, CH_2, 3 × CH_3), 1.60 (2H, m, CH_2), 2.93 (2H, q, *J* 6.2, 6-H_2), 4.20 (2H, t, *J* 6.2, 1-H_2), 6.80 (1H, brt, *J* 6.2, exchange with D_2O, N*H*); δ_C (100 MHz, DMSO-d_6) 26.4, 27.1, 29.2, 29.5, 30.4, 40.7, 71.4, 78.2, 156.5, 230.9 (*C*═S); *m*/*z* (ESI Negative), 292.10 $[M - K]^-$.

Synthesis of Potassium O-Benzyl Carbonodithioate ***13***. Benzyl alcohol (0.2g, 1.0 equiv) was treated with freshly crushed potassium



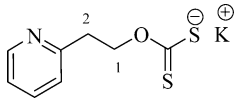
hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-benzyl carbonodithioate **13**: 87% yield; δ_H (400 MHz, DMSO-d_6) 5.38 (2H, s, CH_2), 7.32 (5H, m, Ar−H); δ_C (100 MHz, DMSO-d_6) 72.9, 128.1, 128.8, 129.0, 139.2, 230.2 (*C*═S); *m*/*z* (ESI Negative), 182.99 $[M - K]^-$.

Synthesis of Potassium O-(2,2-Diphenyl-ethyl) Carbonodithioate Salt ***14***. 2,2-Diphenyl-ethanol **1** (0.1g, 1.0 equiv) was treated with



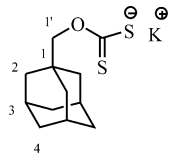
freshly crushed potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a light-brown solid. Potassium *O*-(2,2-diphenyl-ethyl) carbonodithioate salt **14**: 71% yield; δ_H (400 MHz, DMSO-d_6) 4.48 (1H, t, *J* 7.6, 2-H), 4.79 (2H, d, *J* 7.6, 1-H_2), 7.22 (2H, m, Ar−H), 7.32 (8H, m, Ar−H); δ_C (100 MHz, DMSO-d_6) 50.5, 73.8, 127.2, 123.0, 129.3, 143.5, 230.4 (*C*═S); *m*/*z* (ESI Negative), 295.37 $[M - K]^-$.

Synthesis of Potassium O-2-(Pyridin-2-yl)ethyl Carbonodithioate ***15***. 2-(Pyridin-2-yl)ethan-1-ol (0.2g, 1.0 equiv) was treated with



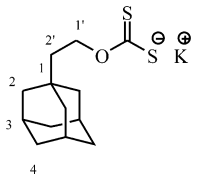
freshly crushed potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a pale-yellow solid. Potassium *O*-2-(pyridin-2-yl)ethyl carbonodithioate **15**: 90% yield; δ_H (400 MHz, DMSO-d_6) 3.10 (2H, t, *J* 6.8, 2-H_2), 4.56 (2H, d, *J* 6.8, 1-H_2), 7.25 (1H, m, Ar−H), 7.34 (1H, d, *J* 8.0, Ar−H), 7.47 (1H, t, *J* 8.0, Ar−H), 8.52 (1H, d, *J* 8.0, Ar−H); δ_C (100 MHz, DMSO-d_6) 38.1, 70.5, 122.4, 124.2, 137.3, 149.9, 159.8, 230.6 (*C*═S); *m*/*z* (ESI Negative), 198.01 $[M - K]^-$.

Synthesis of Potassium O-Methyl-1-adamantyl Carbonodithioate ***16***. Adamantane-methyl-1-ol (0.1g, 1.0 equiv) was treated with freshly



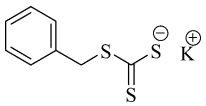
crushed potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a white solid. Potassium *O*-methyl-1-adamantyl carbonodithioate **16**: 60% yield; δ_H (400 MHz, DMSO-d_6) 1.56 (6H, s, 3 × 2-H_2), 1.67 (6H, m, 3 × 4-H_2), 1.98 (3H, brs, 3 × 3-H), 3.84 (2H, s, 1′-H_2); δ_C (100 MHz, DMSO-d_6) 26.8, 28.4, 37.8, 40.0, 72.0, 230.0 (C═S); *m*/*z* (ESI Negative), 241.07 $[M - K]^-$.

Synthesis of Potassium O-Ethyl-1-adamantyl Carbonodithioate ***17***. Adamantane-ethyl-1-ol (0.1g, 1.0 equiv) was treated with freshly



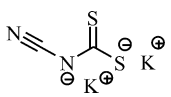
crushed potassium hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a white solid. Potassium *O*-2-(pyridin-2-yl)ethyl carbonodithioate **17**: 65% yield; δ_H (400 MHz, DMSO-d_6) 1.43 (2H, t, *J* 6.8, 2′-H_2), 1.54 (6H, s, 3 × 2-H_2), 1.69 (6H, m, 3 × 4-H_2), 1.95 (3H, brs, 3 × 3-H), 4.28 (2H, t, *J* 6.8, 1′-H_2); δ_C (100 MHz, DMSO-d_6) 26.4, 28.2, 37.9, 40.0, 42.2, 68.0, 230.2 (C═S); *m*/*z* (ESI Negative), 255.09 $[M - K]^-$.

Synthesis of Potassium Benzyl Carbonotrithioate ***19***. Benzylmercaptan (0.2g, 1.0 equiv) was treated with freshly crushed potassium



hydroxide (1.0 equiv) and carbon disulfide (1.1 equiv) in diethyl ether (15 mL). The suspension was stirred at rt for 3 h, and the white precipitate formed was collected by filtration, washed with diethyl ether (2 × 15 mL), and dried in vacuo to afford the desired product as a bright-yellow solid. Potassium benzyl carbonotrithioate **19**: 66% yield; δ_H (400 MHz, DMSO-d_6) 4.40 (2H, s, CH_2), 7.28 (5H, m, Ar−H); δ_C (100 MHz, DMSO-d_6) 46.1, 127.1, 129.0, 129.1, 129.6, 140.2, 239.6 (*C*═S); *m*/*z* (ESI Negative), 198.97 $[M - K]^-$.

Synthesis of Dipotassium Cyanodithioimidocarbonate ***20***.[12] Cyanamide (0.2g, 1.0 equiv) was dissolved in absolute ethanol (2.0 mL)



and treated with carbon disulfide (1.1 equiv), followed by addition of a solution of potassium hydroxide (2.0 equiv) in the same solvent (5.0 mL). The solution was stirred at rt for 4 h, and the precipitate formed was collected by filtration, washed with diethyl ether (3 × 15 mL), and dried under vacuo to afford the title compounds as a white solid. Dipotassium cyanodithioimidocarbonate **20**: 76% yield; δ_C (100 MHz, DMSO-d_6) 122.4, 228.2 (C═S); *m*/*z* (ESI Negative), 115.95 $[M - 2K]^-$.

dx.doi.org/10.1021/jm400414j | *J. Med. Chem.* 2013, 56, 4691−4700

CA Inhibition. An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer and 20 mM Na_2SO_4 (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.[13] 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 were 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 (0.1 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 nonlinear least-squares methods using PRISM 3, as reported earlier,[11] and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier.[9−11]

Normotensive Rabbit IOP Lowering Studies. Male white New Zealand rabbits weighing 1500−2000 g were used in these studies. Animals were anaesthetized using Zoletil (Tiletamine chloride plus Zolazepam chloride, 3 mg/kg body wt, im) and injected with 0.1 mL hypertonic saline solution (5% in distilled water) into the vitreous of both eyes. IOP was determined using a tonometer (Tono-pen Avia Tonometer, Reichhert Inc. Depew, NY 14043, USA) prior to hypertonic saline injection (basal) at 0.5, 1.0, 1.5, 3, and 6 h thereafter. Vehicle (phosphate buffer 7.00 plus DMSO 2%) or drugs were instilled immediately after the injection of hypertonic saline. Eyes were randomly assigned to different groups. Vehicle or drug (0.50 mL) were directly instilled into the conjunctival pocket at the desired doses (usually 2%).[19]

Hypertensive Rabbit IOP Lowering Studies. Adult male New Zealand Albino rabbits weighing 2−2.5 kg were employed in this study. The animals were utilized in groups of eight for each of the chosen specific treatments. The experimental procedures conformed to those of the Declaration of Helsinki and with the Guidelines for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and were conducted upon authorization of the Italian Regulations on Protection of Animals used for experimental and other scientific purpose (DM 116/1992) as well as with the European Union Regulations (OJ of ECL 358/1, 12/12/1986), and the experimental protocol was approved by the local animal care committee of the University of Florence (Florence, Italy). The rabbits were kept in individual cages; food and water were provided ad libitum. The animals were identified with a tattoo on the ear, numbered consecutively, and maintained on a 12 h−12 h light/dark cycle in a temperature controlled room (22−23 °C). All selected animals were examined before the beginning of the study and were determined to be normal on ophthalmic and general examinations. Glaucoma was induced by injection of 0.1 mL of 0.25% carbomer (Siccafluid, FarMila−THEA Pharmaceuticals) into the anterior eye chamber bilaterally in New Zealand albino rabbits anesthetized with tiletamine and zolazepam (Zoletil 100, 0.05 mg/kg body wt) plus xilazine (Xilor 2%, 0.05 mL/kg body wt) im, by the procedure previously reported.[12] IOP was measured before carbomer injection and after 1, 2, and 4 h the first day and three times a day until stabilization and then every 24 h. All rabbits treated with carbomer presented a net increase in IOP. One drop of 0.2% oxybuprocaine hydrochloride (Novesine, Sandoz) diluted 1:1 with sterile saline was instilled in each eye immediately before each set of pressure measurements. IOP was measured using a Tono-Pen XL tonometer (Medtronic Solan, USA) as reported by earlier.[12] The pressure readings were matched with two-point standard pressure measurements at 1, 2, 4, and 8 h after the instillation of the drug and once a day for the following days using a Digilab calibration verifier. All IOP measurements were done by the same investigators using the same tonometer. As soon as a stable IOP increase was obtained, the animals were treated with the drugs in study. The efficacy of the different drugs in lowering IOP was evaluated after drug administration over 4 h, with the following schedule: before and after 30, 60, 90, 120, 240, and 300 min after drug administration. The treatment was performed in three animals per drug in one eye and compared to the contralateral eye treated with vehicle. A group of four nonglaucomatous albino rabbits was treated with the drugs of this study and used as control. At the end of the experiments, the animals were killed with a lethal dose of Pentothal (Abbott SpA, Campoverde di Aprilia, IT).

AUTHOR INFORMATION

Corresponding Author

*Phone: +39-055-457-3005. Fax: +0-39-055-457-3385. E-mail: claudiu.supuran@unifi.it.

Notes

The authors declare the following competing financial interest(s): The compounds described in the manuscript and their antiglaucoma action have been patented.

ACKNOWLEDGMENTS

This work was financed in part by two EU projects, Metoxia and Dynano.

ABBREVIATION USED

AAZ, acetazolamide; CA, carbonic anhydrase; CAI, CA inhibitor; hCA, human CA; DZA, dorzolamide; DTC, dithiocarbamate; IOP, intraocular pressure

REFERENCES

(1) (a) Smith, K. S.; Jakubzick, C.; Whittam, T. S.; Ferry, J. G. Carbonic anhydrase is an ancient enzyme widespread in prokaryotes. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 15184−15189. (b) Aggarwal, M.; Boone, C. D.; Kondeti, B.; McKenna, R. Structural annotation of human carbonic anhydrases. *J. Enzyme Inhib. Med. Chem.* **2013**, *28*, 267−277. (c) Alterio, V.; Di Fiore, A.; D'Ambrosio, K.; Supuran, C. T.; De Simone, G. Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? *Chem. Rev.* **2012**, *112*, 4421−4468. (d) Supuran, C. T. Carbonic anhydrases. *Bioorg. Med. Chem.* **2013**, *21*, 1377−1378. (e) Supuran, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nature Rev. Drug Discovery* **2008**, *7*, 168−181.

(2) (a) Aggarwal, M.; McKenna, R. Update on carbonic anhydrase inhibitors: a patent review (2008−2011). *Expert. Opin. Ther. Pat.* **2012**, *22*, 903−915. (b) Supuran, C. T. Structure-based drug discovery of carbonic anhydrase inhibitors. *J. Enzyme Inhib. Med. Chem.* **2012**, *27*, 759−772. (c) Supuran, C. T.; Scozzafava, A.; Casini, A. Carbonic anhydrase inhibitors. *Med. Res. Rev.* **2003**, *23*, 146. (d) Pastorekova, Parkkila, S.; Pastorek, J.; Supuran, C. T. Carbonic anhydrases: current state of the art, therapeutic applications and future prospects. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199−229. (e) Supuran, C. T.; Scozzafava, A.; Casini, A. Development of sulfonamide carbonic anhydrase inhibitors (CAIs). In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C.T., Scozzafava, A., Conway, J. Eds.; CRC Press: Boca Raton, FL, 2004; pp 67−147; (f) Neri, D.; Supuran, C. T. Interfering with pH regulation in tumours as a therapeutic strategy. *Nature Rev. Drug Discovery* **2011**, *10*, 767−777.

(3) (a) Supuran, C. T. Carbonic anhydrases: from biomedical applications of the inhibitors and activators to biotechnologic use for CO_2 capture. *J. Enzyme Inhib. Med. Chem.* **2013**, *28*, 229−230. (b) Supuran, C. T. Carbonic anhydrase inhibitors. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 3467−3474. (c) Supuran, C. T. Carbonic anhydrase inhibitors and activators for novel therapeutic applications. *Future Med. Chem.* **2011**, *3*, 1165−1180. (d) Nishimori, I.; Minakuchi, T.; Maresca, A.; Carta, F.; Scozzafava, A.; Supuran, C. T. The β-carbonic anhydrases from *Mycobacterium tuberculosis* as drug targets. *Curr. Pharm. Des.* **2010**, *16*, 3300−3309.

(4) (a) Maresca, A.; Temperini, C.; Vu, H.; Pham, N. B.; Poulsen, S. A.; Scozzafava, A.; Quinn, R. J.; Supuran, C. T. Non-zinc mediated inhibition of carbonic anhydrases: coumarins are a new class of suicide inhibitors. *J. Am. Chem. Soc.* **2009**, *131*, 3057−3062. (b) Maresca, A.; Temperini, C.; Pochet, L.; Masereel, B.; Scozzafava, A.; Supuran, C. T. Deciphering the mechanism of carbonic anhydrase inhibition with coumarins and thiocoumarins. *J. Med. Chem.* **2010**, *53*, 335−344.

(5) Carta, F.; Temperini, C.; Innocenti, A.; Scozzafava, A.; Kaila, K.; Supuran, C. T. Polyamines inhibit carbonic anhydrases by anchoring to the zinc-coordinated water molecule. *J. Med. Chem.* **2010**, *53*, 5511−5522.

(6) (a) Innocenti, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. Interactions of phenols with the 12 catalytically active mammalian isoforms (CA I−XIV). *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1583−1587. (b) Innocenti, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. Inhibition of mammalian isoforms I−XIV with a series of substituted phenols including paracetamol and salicylic acid. *Bioorg. Med. Chem.* **2008**, *16*, 7424−7428. (c) Davis, R. A.; Innocenti, A.; Poulsen, S. A.; Supuran, C. T. Carbonic anhydrase inhibitors. Identification of selective inhibitors of the human mitochondrial isozymes VA and VB over the cytosolic isozymes I and II from a natural product-based phenolic library. *Bioorg. Med. Chem.* **2010**, *18*, 14−18. (d) Davis, R. A.; Hofmann, A.; Osman, A.; Hall, R. A.; Mühlschlegel, F. A.; Vullo, D.; Innocenti, A.; Supuran, C. T.; Poulsen, S. A. Natural Product-Based Phenols as Novel Probes for Mycobacterial and Fungal Carbonic Anhydrases. *J. Med. Chem.* **2011**, *54*, 1682−1692.

(7) (a) Innocenti, A.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. Inhibition of cytosolic isoforms I, II, III, VII and XIII with less investigated inorganic anions. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1855−1857. (b) Innocenti, A.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. Inhibition of transmembrane isoforms IX, XII and XIV with less investigated inorganic anions. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 1548−1550.

(8) Temperini, C.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. X-Ray crystal studies of the carbonic anhydrase II−trithiocarbonate adduct—an inhibitor mimicking the sulfonamide and urea binding to the enzyme. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 474−478.

(9) Carta, F.; Aggarwal, M.; Maresca, A.; Scozzafava, A.; McKenna, R.; Supuran, C. T. Dithiocarbamates: a new class of carbonic anhydrase inhibitors. Crystallographic and kinetic investigations. *Chem. Commun.* **2012**, *48*, 1868−1870.

(10) (a) Maresca, A.; Carta, F.; Vullo, D.; Supuran, C. T. Dithiocarbamates strongly inhibit the beta-class carbonic anhydrases from *Mycobacterium tuberculosis*. *J. Enzyme Inhib. Med. Chem.* **2013**, *28*, 407−411. (b) Monti, S. M.; Maresca, A.; Carta, F.; De Simone, G.; Mühlschlegel, F. A.; Scozzafava, A.; Supuran, C. T. Dithiocarbamates strongly inhibit the beta-class fungal carbonic anhydrases from *Cryptococcus neoformans*, *Candida albicans* and *Candida glabrata*. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 859−862.

(11) Carta, F.; Aggarwal, M.; Maresca, A.; Scozzafava, A.; McKenna, R.; Masini, E.; Supuran, C. T. Dithiocarbamates strongly inhibit carbonic anhydrases and show antiglaucoma action in vivo. *J. Med. Chem.* **2012**, *55*, 1721−1730.

(12) Fabrizi, F.; Mincione, F.; Somma, T.; Scozzafava, G.; Galassi, F.; Masini, E.; Impagnatiello, F.; Supuran, C. T. A new approach to antiglaucoma drugs: carbonic anhydrase inhibitors with or without NO donating moieties. Mechanism of action and preliminary pharmacology. *J. Enzyme Inhib. Med. Chem.* **2012**, *27*, 138−147.

(13) Seltzer, R. The reactions of organotin chlorides with the cyanodithioimidocarbonate anion. *J. Org. Chem.* **1968**, *33*, 3896−900.

(14) Khalifah, R. G. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **1971**, *246*, 2561−2573.

(15) *MOE*, version 2012.10; Chemical Computing Group: Montreal, Canada, 2012.

(16) *GOLD*, version 5.1; CCDC: Cambridge, UK.

(17) (a) Menchise, V.; De Simone, G.; Alterio, V.; Di Fiore, A.; Pedone, C.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors: stacking with Phe131 determines active site binding region of inhibitors as exemplified by the X-ray crystal structure of a membrane-impermeant antitumor sulfonamide complexed with isozyme II. *J. Med. Chem.* **2005**, *48*, 5721−5727. (b) Wagner, J.; Avvaru, B. S.; Robbins, A. H.; Scozzafava, A.; Supuran, C. T.; McKenna, R. Coumarinyl-substituted sulfonamides strongly inhibit several human carbonic anhydrase isoforms: solution and crystallographic investigations. *Bioorg. Med. Chem.* **2010**, *18*, 4873−4878. (c) Biswas, S.; Aggarwal, M.; Guzel, O.; Scozzafava, A.; McKenna, R.; Supuran, C. T. Conformational variability of different sulfonamide inhibitors with thienyl-acetamido moieties attributes to differential binding in the active site of cytosolic human carbonic anhydrase isoforms. *Bioorg. Med. Chem.* **2011**, *19*, 3732−3738. (d) Hen, N.; Bialer, M.; Yagen, B.; Maresca, A.; Aggarwal, M.; Robbins, A. H.; McKenna, R.; Scozzafava, A.; Supuran, C. T. Anticonvulsant 4-aminobenzenesulfonamide derivatives with branched-alkylamide moieties: X-ray crystallography and inhibition studies of human carbonic anhydrase isoforms I, II, VII and XIV. *J. Med. Chem.* **2011**, *54*, 3977−3981. (e) Gitto, R.; Agnello, S.; Ferro, S.; Vullo, D.; Brynda, J.; Mader, P.; Supuran, C. T.; Chimirri, A. Identification of 3,4-dihydroisoquinoline-2(1*H*)-sulfonamides as Potent Carbonic Anhydrase Inhibitors: Synthesis, Biological Evaluation and Enzyme-ligand X-ray studies. *J. Med. Chem.* **2010**, *53*, 2401−2408.

(18) Carta, F.; Supuran, C. T.; Scozzafava, A. Novel therapies for glaucoma: a patent review 2007−2011. *Expert Opin. Ther. Pat.* **2012**, *22*, 79−88.

(19) Krauss, A. H. P.; Impagnatiello, F.; Toris, C. B.; Gale, D. C.; Prasanna, G.; Borghi, V.; Chiroli, V.; Chong, W. K. M.; Carreiro, S. T.; Ongini, E. Ocular hypotensive activity of BOL-303259-X, a nitric oxide donating prostaglandin F2α agonist, in preclinical models. *Exp. Eye Res.* **2011**, *93*, 250−255.