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Carbonic anhydrase inhibitors: Design of thioureido sulfonamides with potent isozyme II and XII inhibitory properties and intraocular pressure lowering activity in a rabbit model of glaucoma

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Abstract—A new series of thioureido-substituted sulfonamides were prepared by reacting 4-isothiocyanato- or 4-isothiocyanato ethyl-benzenesulfonamide with amines, hydrazines, or amino acids bearing moieties that can lead to an enhanced hydrosolubility, such as 2-dimethylamino-ethylamine, fluorine-containing aromatic amines/hydrazines, an aminodiol, heterocyclic polyamines (derivatives of morpholine and piperazine), 4-aminobenzoic acid, or natural amino acids (Gly, Cys, Asn, Arg, and Phe). The new compounds showed good inhibitory properties against three physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isozymes, with K_Is in the range of 24–324 nM against the cytosolic isoform CA I, of 6–185 nM against the other cytosolic isozyme CA II, and of 1.5–144 nM against the transmembrane isozyme CA XII. Some of the new derivatives were also very effective in reducing elevated intraocular pressure in hypertensive rabbits as a glaucoma animal model. Considering that this is the first study in which potent CA II/CA XII inhibitors are designed and investigated in vivo, it may be assumed that the target isozymes of the antiglaucoma sulfonamides are indeed the cytosolic CA II and the transmembrane CA XII.

The treatment of glaucoma with inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1)[1–3] is very effective in reducing elevated intraocular pressure (IOP) characteristic of this disease,[4] with two topically acting inhibitors presently available for clinical use (dorzolamide **DZA** and brinzolamide **BRZ**),[5] in addition to the classical inhibitors used via the systemic route of administration (acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, and dichlorophenamide **DCP**).[6] However, systemic inhibitors provoke a wide range of deleterious side effects due to inhibition of the enzyme present in other tissues (kidneys, lungs, red cells, stomach, etc.) than in the eye.[1] The possibility of the topical administration of the classical drugs from this class, mentioned above, was extensively investigated by several researchers, but negative results have been constantly obtained, and for more than 40 years, it was considered that CA inhibitors (CAIs) could only be given systemically.[6] Important advances in this field have then been achieved by the Merck group, with the discovery of the first clinically used, topically effective antiglaucoma sulfonamide, dorzolamide **DZA**.[5] The approach for arriving to this compound (and in fact also to brinzolamide **BRZ**) is known as the 'ring approach,' as it involved the exploration of a wide range of ring systems to which sulfamoyl moieties were incorporated.[3] We have explored an alternative approach for the design of topically acting antiglaucoma sulfonamides, which

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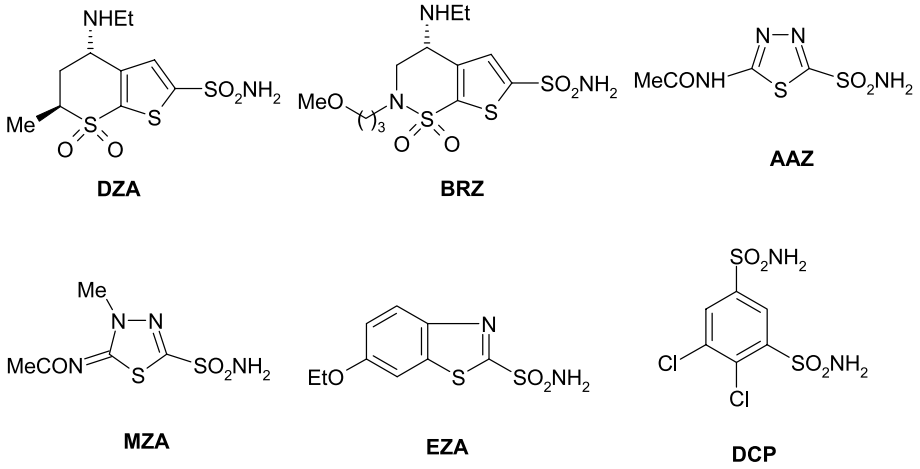
consisted in attaching tails that will induce the desired physicochemical properties (such as water solubility, enhanced penetrability through the cornea, etc.) to scaffolds of aromatic/heterocyclic sulfonamides also incorporating derivatizable moieties of the amino/hydroxy type, which has been denominated the 'tail approach.' [7–16]

The new sulfonamides reported here were prepared by reacting 4-isothiocyanato- or 4-isothiocyanatoethyl-benzenesulfonamide **A** ($n = 0$) and **B** ($n = 2$) with amines, hydrazines, or amino acids of types **1**–**15**, leading to thioureas **A1**–**A8** and **B1**–**B15**, respectively, by the procedure previously reported by this group (Scheme 1).[17,18,26]

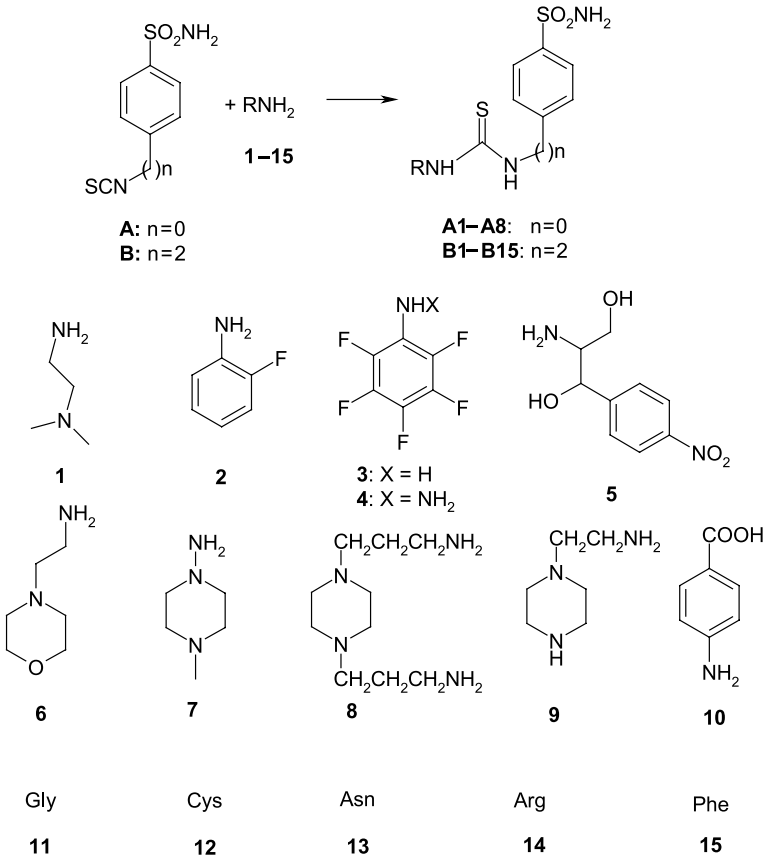


Some alkyl/aryl-thioureido moieties among the investigated tails were shown to induce favorable CA inhibitory properties as well as good physicochemical properties to a potential antiglaucoma agent incorporating them.[17–20] Such derivatives were prepared from the corresponding sulfonamide isothiocyanates and amino acids or amines, and were shown to behave as low nanomolar inhibitors of several physiologically relevant isozymes (of the 15 presently isolated in humans),[1] such as CA I, II, and IV.[17–20]

Until recently, it has been considered that the target isozymes of the antiglaucoma sulfonamides were CA II (a widely distributed cytosolic isoform present in high amounts in the eye)[21] and CA IV (a membrane-bound isoform, also present in eye tissues).[22] However, the original work of Maren et al.[22] has been carried out with the bovine isozyme, that is quite susceptible to inhibition with sulfonamides. Only recently, it was shown that the corresponding human isozyme, hCA IV, has a much lower affinity (in the micromolar range) for most of the clinically used CAIs.[23] Correlated with the fact that Lerman's group[24] recently showed that another membrane-associated isoform, CA XII, is overexpressed in glaucomatous (but not normal) eyes and that this isozyme is very much inhibited by most of the clinically used (or other types of simple sulfonamide) CAIs,[25] the original Maren's hypothesis[22] that CA IV is involved in the antiglaucoma effects of sulfonamides may be challenged. Here, we prove that a new series of thioureido sulfonamides acting as very efficient CA II and XII inhibitors, also show excellent IOP-lowering properties in an animal model of glaucoma. Thus, probably, the target isozymes for the antiglaucoma effects of sulfonamide CA inhibitors are the cytosolic CA II and the transmembrane CA XII, whereas the real role of CA IV should be reinvestigated in more detail.

Thioureido derivatives of sulfonamides were prepared earlier, being shown that such compounds may possess interesting biological activity as inhibitors of various CAs.[17,18,20,26] Recently, we have also reported the X-ray crystal structure of one such inhibitor, that is compound **A4** with the major cytosolic isozyme, hCA II.[19]

The high-resolution X-ray crystal structure of the **A4** adduct with hCA II showed the inhibitor to bind within the hydrophobic half of the enzyme active site, making extensive and strong van der Waals contacts with amino acid residues Gln92, Val121, Phe131, Leu198, Thr200, and Pro202, in addition to the coordination of the sulfonamide nitrogen to the Zn(II) ion of the active site, and the participation of the SO_2NH_2 group to a network of hydrogen bonds involving residues Thr199 and Glu106 (Fig. 1). Thus, this structure constitutes an important starting point for the design of other thioureido-containing sulfonamides with CA inhibitory properties. Since in previous reports,[17,18,20,26] we mainly investigated the preparation of such derivatives incorporating amino acid moieties, for the drug design of compounds reported here we concentrated on these as well as slightly different moieties. Thus, the reaction of isothiocyanates **A** and **B** (obtained from the corresponding amino-substituted benzenesulfonamide derivatives and thiophosgene, by the previously reported procedure)[17,26] was performed with amines bearing moieties that can lead to an enhanced hydrosolubility, such as 2-dimethylamino-ethylamine **1**, the fluorine-containing amines/hydrazines **2**–**4**, the aminodiol **5**, the heterocyclic polyamines **6**–**9**, 4-aminobenzoic acid **10**, as well as the natural amino acids Gly (**11**), Cys (**12**), Asn (**13**), Arg (**14**), and Phe (**15**), which were previously shown to lead to potent CAIs by reaction with isothiocyanate **A**.[17] In the case of amine **8**, the reaction involved two equivalents of isothiocyanates **A**/**B**, leading to bis-sulfonamides. These moieties present in amines/hydrazines/amino acids **1**–**15** were chosen on



Scheme 1.

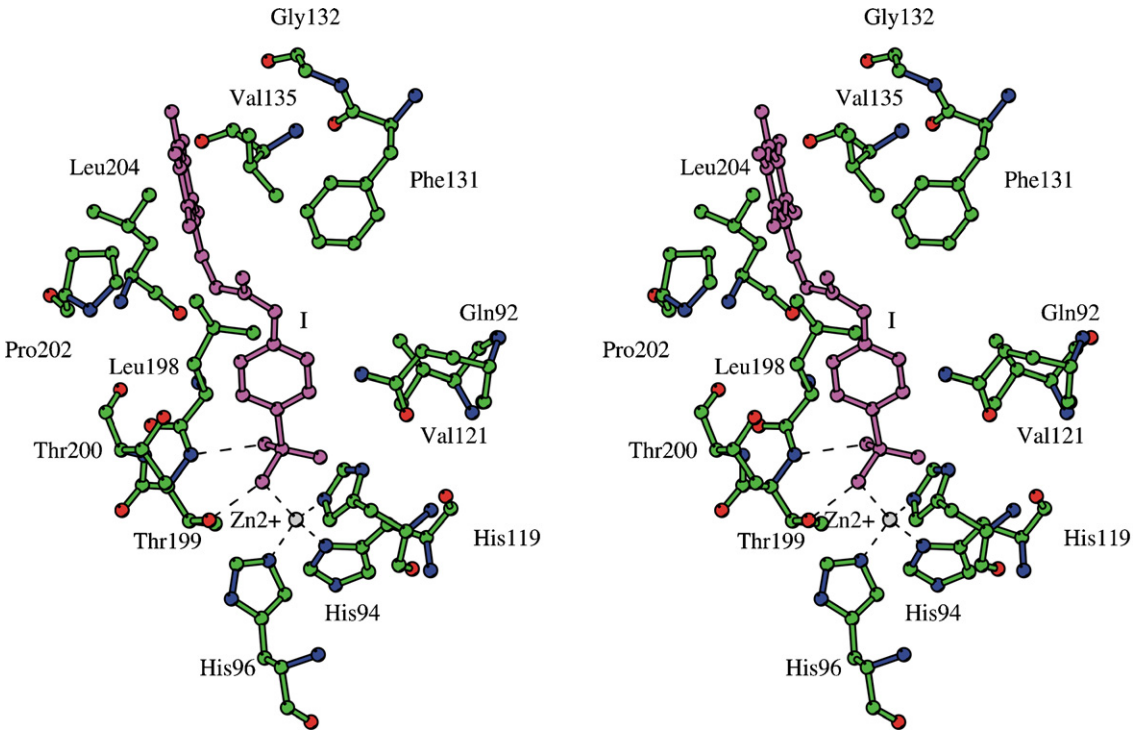


Figure 1. Stereo view of the active site region in the hCA II–**A4** complex showing the residues participating in recognition of the inhibitor molecule (in magenta). Hydrogen bonds and the active site Zn(II) ion coordination are also shown (dotted lines).[19]

one hand because presumably they can adopt a conformation within the CA active site similar to that of the perfluorophenylhydrazine moiety of **A4**, which, as discussed above, makes a lot of favorable interactions with critical amino acid residues involved in the binding of inhibitors. On the other hand, these moieties may lead to an enhanced hydrosolubility of such sulfonamides, which is another important factor for the in vivo studies of a potential antiglaucoma agent. Indeed, the moieties present in the 4-aminobenzoic acid derivatives **A10** and **B10**, or in the amino acid derivatives **A**,**B(11–15)** can make sodium salts with an enhanced solubility as compared to the free acid.[17] The aminodiol derivatives **A5** and **B5** may also show enhanced hydrosolubility due to the presence of many polar moieties in their molecules. On the other hand, the nitrogen-containing heterocycles present in **A**,**B(6–9)** or in the aliphatic derivatives **A1** and **A2** can be protonated, leading to hydrochlorides or similar salts which would also possess enhanced water solubility.[27] We already proved[28] that many of the fluorine-containing inhibitors show such a good parameter.[29]

Inhibition data of three physiologically relevant CA isozymes, the cytosolic, red blood cells human isozymes hCA I and II, and the transmembrane isozyme hCA XII with the new compounds reported here as well as the standard CA inhibitors are shown in Table 1.[30]

The following SAR can be noticed from the data of Table 1. First, against the slow red blood cell isozyme hCA I, the derivatives **A1**–**A8** and **B1**–**B15** reported here showed inhibition constants in the range of 24–324 nM. Thus, a first group of derivatives, such as **A1**, **A5**, **A8**, **B5**, **B8**, and **B10**, behave as medium potency hCA I inhibitors, with K_Is in the range of 100–324 nM. It may be observed that these compounds incorporate the rather bulky amino-diol moiety of **5**, the quite bulky bis-amine of **8** leading to bis-sulfonamides, as well as the 4-aminobenzoic acid moiety. All other derivatives investigated here showed much more effective hCA I inhibitory properties, with K_Is in the range of 24–95 nM, similarly to the clinically used drug **EZA**, and being thus more effective inhibitors than other clinically used CAIs (**AZA**, **MZA**, **DCP**, etc.). It should be observed the tremendous enhance of hCA I inhibitory properties of these new compounds over sulfanilamide, the lead molecule from which they can be considered to be derived. Except the pairs **A/B2**, **A/B3**, and **A/B8**, generally the sulfanilamide derivatives of type **A** are less efficient inhibitors as compared to the corresponding derivatives of 4-aminoethylbenzenesulfonamide of type **B**. Second, hCA II is generally considered the main therapeutic target of sulfonamide CAIs. Thus, affinity of newly designed inhibitors for this isozyme is of critical relevance. The new derivatives **A1**–**A8** and **B1**–**B15** reported here showed inhibition constants in the range of 6–185 nM, being much more inhibitory than the lead sulfanilamide (K_I of 300 nM). Medium potency inhibitors were **A7**, **A8**, **B8**, and **B10**, with K_Is in the range of 75–185 nM, and again they incorporate the bulky moiety of **8** and of 4-aminobenzoic acid **10**, in addition to the methylpiperazine **12** (which is less bulky). All other newly prepared compounds show very effective hCA II inhibitory properties, with K_Is in the range of 6–48 nM. Thus, most of the moieties investigated here for the design of CAIs lead to effective hCA II inhibitors. Again, the 4-aminoethylbenzensulfonamides of type **B** were generally more effective inhibitors as compared to the corresponding sulfanilamide derivatives **A**. Finally, the transmembrane isoform hCA XII was also very susceptible to inhibition by the thioureido-substituted sulfonamides reported in this article, with inhibition constants in the range of 1.5–144 nM. Medium potency hCA XII inhibitors were only the two bulkier derivatives **B8** and **B10** (K_Is in the range of 64–144 nM), whereas all the other compounds were much more effective. Particularly, strong hCA XII inhibitors were the amino acid derivatives **B11**–**B15** (K_Is in the range of 1.5–4.8 nM) and the aliphatic/aromatic/heterocyclic derivatives **B1**–**B7** (K_Is in the range of 1.7–4.9 nM). Generally, all the new compounds were more effective hCA XII inhibitors than the lead compound sulfanilamide (K_I of 37 nM). Clearly, this isoform is very prone to inhibition by this class of thioureido-substituted sulfonamides, followed

Table 1. Inhibition data of isozyme hCA I, II, and XII with compounds **A1**–**A7** and **B1**–**B15** as well as standard, clinically used CA inhibitors

Inhibitor	K_I[a] (nM)		
	hCA I[b]	hCA II[b]	hCA XII[c]
Dorzolamide	50,000	9	3.5
Brinzolamide	–	3	3
Acetazolamide	250	12	5.7
Methazolamide	780	14	3.4
Ethoxzolamide	25	8	22
Dichlorophenamide	1200	38	50
Sulfanilamide	28,000	300	37
A1	100	33	12
A2	24	7	13
A3	84	25	18
A4	79	19	12
A5	135	27	24
A6	73	13	10
A7	80	75	33
A8	250	150	50
B1	63	13	2.1
B2	77	6	1.9
B3	95	18	3.0
B4	61	10	2.3
B5	113	14	4.9
B6	54	9	1.7
B7	72	48	12
B8	313	185	64
B9	50	7	1.8
B10	324	159	144
B11	40	8	2.0
B12	24	6	1.5
B13	33	11	3.3
B14	74	16	4.8
B15	54	12	3.0

[a] Errors in the range of ±10% of the reported value, from three different determinations.
[b] Human cloned isozyme.
[c] Catalytic domain of the human, cloned isozymes, by the CO_2 hydrase, stopped flow assay.[30]

Table 2. Fall of IOP of glaucomatous rabbits (30.5 ± 3.0 mmHg), after treatment with one drop (50 μL) 2% solution of CA inhibitor (with the pH value shown below) directly into the eye, at 30, 60, and 90 min after administration

Inhibitor	pH	ΔIOP (mmHg)[a]			
		$t = 0$	$t = 30$ min	$t = 60$ min	$t = 90$ min
DZA[b]	5.5	0.0	3.6 ± 0.20	6.7 ± 0.30	4.2 ± 0.15
B6[b]	6.5	0.0	6.5 ± 0.20	6.9 ± 0.20	5.9 ± 0.35
B11[c]	7.0	0.0	3.0 ± 0.20	5.9 ± 0.20	5.5 ± 0.35
B13[c]	7.0	0.0	10.0 ± 0.40	13.0 ± 0.25	15.5 ± 0.40
B15[c]	7.0	0.0	4.0 ± 0.25	6.1 ± 0.35	6.4 ± 0.20

[a] $\Delta IOP = IOP_{control\ eye} - IOP_{treated\ eye}$; mean ± SEM ($n = 3$).
[b] As hydrochloride salt.
[c] As sodium salt.

by the rapid cytosolic isozyme II, whereas hCA I is less avid to be inhibited by these compounds.

Intraocular pressure lowering in hypertensive rabbits (a widely used animal model of glaucoma)[31,32] after treatment with 2% water solutions of some of the newly designed inhibitors is shown in Table 2, in comparison with dorzolamide (2%) treated animals.

The morpholine-based inhibitor **B6** (formulated as hydrochloride with a pH of 6.5 which was much less irritating to the eye than that of **DZA**, pH 5.5), as well as the amino acid thioureido derivatives **B11, B13**, and **B15** (formulated as sodium salts with a pH of 7, which is obviously nonirritating to the eye), all of them were potent CA II and XII inhibitors (Table 1), showed interesting IOP lowering effects in rabbits with hydrocortisol-induced eye hypertension. Thus, at 30-min post-administration, the new derivatives produced an IOP lowering of 3.0–10.0 mmHg (compared to 3.6 observed with dorzolamide); at 1-h post-administration, the fall of IOP was of 5.9–13 mmHg with the new inhibitors and 6.7 mmHg with **DZA**, whereas at 90 min, the data were of 5.9–15.5 mmHg with the new CAIs and 4.2 mmHg with **DZA**. It may be observed that the most effective IOP lowering agent was the asparagine derivative **B13** that produced potent and long lasting IOP lowering at all times, which were much better than those observed with the clinically used drug **DZA**. In addition, the pH of the **B13** solution is 7.0 (as compared to 5.5 of **DZA** hydrochloride), which is totally nonirritant to the eye.

In summary, a new series of thioureido-substituted sulfonamides were prepared by reacting 4-isothiocyanato- or 4-isothiocyanatoethyl-benzenesulfonamide with amines, hydrazines, or amino acids bearing moieties that can lead to an enhanced hydrosolubility, such as 2-dimethylamino-ethylamine, the fluorine-containing aromatic amines/hydrazines, an aminodiol, heterocyclic polyamines (derivatives of morpholine and piperazine), 4-aminobenzoic acid, or natural amino acids (Gly, Cys, Asn, Arg, and Phe). The new compounds showed good inhibitory properties against three physiologically relevant CA isozymes, with K_Is in the range of 24–324 nM against hCA I, of 6–185 nM against hCA II, and of 1.5–144 nM against hCA XII. Some of the new derivatives were also very effective in reducing elevated IOP in hypertensive rabbits as a glaucoma animal model. Considering that this is the first study in which potent hCA II/hCA XII inhibitors are designed and investigated in vivo, it may be assumed that the target isozymes of the antiglaucoma sulfonamides are indeed the cytosolic CA II and the transmembrane CA XII, whereas the role played by CA IV should be reinvestigated.

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29. *General procedure for the preparation of the compounds **A1–A7** and **B1–B15***. An amount of 2.5 mM of 4-isothiocyanato-benzenesulfonamides **A/B** and the stoichiometric amount of nucleophile **1–15** were suspended in 50–100 mL of dry acetone or acetonitrile and heated at reflux for 2–8 h (TLC control). The solvent was evaporated, and the crude product recrystallized from ethanol or ethanol–water when the title derivatives were obtained in excellent yields (85–95%). For **A8** and **B8**, the molar ratio of **A/B**:**8** used in the syntheses was of 2:1. 4-[3-(2-Dimethylaminoethyl)thioureido]-benzenesulfonamide A1, white crystals, mp 250–251 °C (dec); ^{1}H NMR (DMSO-d_6), δ, ppm (*J*, Hz): 2.38 (s, 6H, 2Me); 2.80 (t, 2H, CH_2 from aminoethyl); 2.94 (q, 2H, CH_2 from aminoethyl); 7.15 (s, 2H, SO_2NH_2), 7.70 (d, 2H, AA′BB′, 8.9), 7.88 (d, 2H, AA′BB′, 8.9), 8.49 (t, 1H, NHCS); 8.57 (s, 1H, NHCS). Anal. found: C, 43.35; H, 5.97; N, 18.25%; $C_{11}H_{18}N_4O_2S_2$ requires C, 43.69; H, 6.00; N, 18.53%. 4-[3-[2-Hydroxy-1-hydroxymethyl-2-(4-nitrophenyl)-ethyl]thioureido]- benzenesulfonamide B5, tan crystals, mp 175–177 °C (dec), ^{1}H NMR (DMSO-d_6), δ, ppm (*J*, Hz): 2.72(q, 1H, C*H*-NH, 5.9); 3.19 (dd, 1H, C*H* from CH_2OH, 6.2, 10.5); 3.36 (dd, 1H, C*H* from CH_2OH, 5.8, 10.5); 4.69 (d, 1H, C*H* from CHOH, 4.5); 7.23 (s, 2H, SO_2NH_2), 7.59 (d, 2H, ArH from $O_2NC_6H_4$, 8.8); 7.78 (d, 2H, AA′BB′, 8.9), 7.91 (d, 2H, AA′BB′, 8.9), 8.18 (d, 2H, ArH from $O_2NC_6H_4$, 8.8); 8.48 (m, 1H, NHCS); 8.60 (s, 1H, NHCS). Anal. found: C, 44.83; H, 4.13; N, 13.07%; $C_{16}H_{18}N_4O_6S_2$ requires C, 45.06; H, 4.25; N, 13.14%. 4-[3-[2-(4-Sulfamoylphenyl)-ethyl]-thioureido]-benzoic acid B10, white crystals, mp 212–213 °C (dec), ^{1}H NMR (DMSO-d_6), δ, ppm (*J*, Hz): 2.91 (t, 2H from ethylthioureido, 7.1), 3.49 (q, 2H from ethylthioureido, 6.7), 7.32 (s, 2H, SO_2NH_2), 7.43 (d, 2H, AA′BB′, 8.2), 7.46 (d, 2H, AA′BB′, 8.1), 7.76 (d, 2H, AA′BB′, 8.2), 7.79 (d, 2H, AA′BB′, 8.1), 8.49 (t, 1H, NHCS, 5.6); 8.63 (s, 1H, NHCS). Anal. found: C, 50.49; H, 4.60; N, 11.00%; $C_{16}H_{17}N_3O_4S_2$ requires C, 50.65; H, 4.52; N, 11.07%.
30. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An SX.18*M*V-R Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) 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, to allow for the formation of the E–I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier,[16,26] and represent the mean from at least three different determinations. The recombinant enzymes (hCA I, hCA II, and hCA XII) were obtained as reported earlier by this group.[16,25]
31. Adult male New Zealand albino rabbits weighing 3–3.5 kg were used in the experiments (three animals were used for each inhibitor studied). The experimental procedures conform to the Association for Research in Vision and Ophthalmology Resolution on the use of animals. The rabbits were kept in individual cages with food and water provided ad libitum. The animals were maintained on a 12 h:12 h light/dark cycle in a temperature controlled room, at 22–26 °C. Solutions of inhibitors (2%, by weight, as hydrochlorides, triflates, or sodium carboxylates) were obtained in distilled-deionized water. The pH of these solutions was in the range of 5.5–8.4. IOP was measured using a Tono-Pen XL tonometer (Medtronic Solan, USA) as described earlier. The pressure readings were matched with two-point standard pressure measurements at least twice each day using a Digilab Calibration verifier. All IOP measurements were done by the same investigator with the same tonometer. One drop of 0.2% oxybuprocaine hydrochloride (novesine, Sandoz) diluted 1:1 with saline was instilled in each eye immediately before each set of pressure measurements. IOP was measured three times at each time interval, and the means reported. IOP was measured first immediately before drug administration, then at 30 min after the instillation of the pharmacological agent, and then each 30 min for a period of 4–6 h. For all IOP experiments, drug was administered to only one eye, leaving the contralateral eye as an untreated control. The ocular hypotensive activity is expressed as the average difference in IOP between the treated and the control eye, in this way minimizing the diurnal, seasonal, and interindividual variations commonly observed in the rabbit. Ocular hypertension was elicited in the right eye of albino rabbits by the

instillation of hydrocortisol (Sigma) as described by Melena et al.[32] The IOP of treated animals was checked daily, and after approximately 3–4 weeks, an elevated pressure of 33–35 mmHg has been achieved. Such animals were used in the IOP measurement experiments.

32. Melena, J.; Santafe, J.; Segarra-Domenech, J.; Puras, G. *J. Ocul. Pharmacol. Ther.* **1999**, *15*, 19.