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Carbonic Anhydrase Inhibitors: Topically Acting Antiglaucoma Sulfonamides Incorporating Esters and Amides of 3- and 4-Carboxybenzolamide

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Abstract—Reaction of 3- and 4-carboxybenzenesulfonyl chloride with 5-amino-1,3,4-thiadiazole-2-sulfonamide/5-imino-4-methyl-δ^2-1,3,4-thiadiazoline-2-sulfonamide afforded two series of benzolamide analogues to which the carboxyl moiety has been derivatized as esters or amides, in order to reduce their very polar character. The new derivatives showed low nanomolar affinity for three carbonic anhydrase (CA) isozymes, CA I, II and IV, and were effective as topical antiglaucoma agents in normotensive rabbits. Efficacy of several of the new sulfonamides reported was better than that of the standard drugs dorzolamide and brinzolamide, whereas their duration of action was prolonged as compared to that of the clinically used drugs.

Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc enzymes, present in *Archaea*, prokaryotes and eukariotes, being encoded by three distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, bacteria, algae and cytoplasm of green plants), the β-CAs (predominantly in bacteria, algae and chloroplasts of both mono- as well as dicotyledons) and the γ-CAs (mainly in *Archaea* and some eubacteria), respectively.[1,2] In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) were described, with very different subcellular localization and tissue distribution.[1,2] Basically, there are several cytosolic forms (CA I-III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII and CA XIV), one mitochondrial form (CA V) as well as a secreted CA isozyme, CA VI. [1,2] These enzymes catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as the gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes. [1,2] Many of these isozymes were important targets for the design of inhibitors with clinical applications, such as for obtaining different diuretic types, or drugs useful in the treatment and prevention of a variety of diseases such as glaucoma, epilepsy, congestive heart failure, mountain sickness, gastric and duodenal ulcers, epilepsy and other neurological disorders, or osteoporosis among others.[1-3] Several such derivatives (acetazolamide **AZA**, methazolamide **MZA**, ethoxzolamide **EZA** and dichlorophenamide **DCP**) were successfully used in clinical medicine for the last 45 years. More recently, dorzolamide **DZA** and brinzolamide **BRZ** have also found important therapeutic applications as ocular (mainly antiglaucoma) drugs.[1–3]

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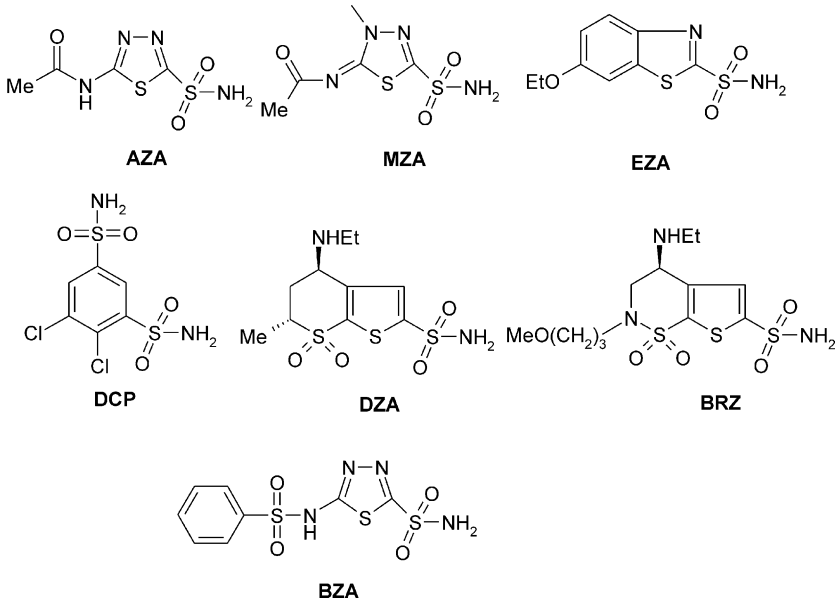


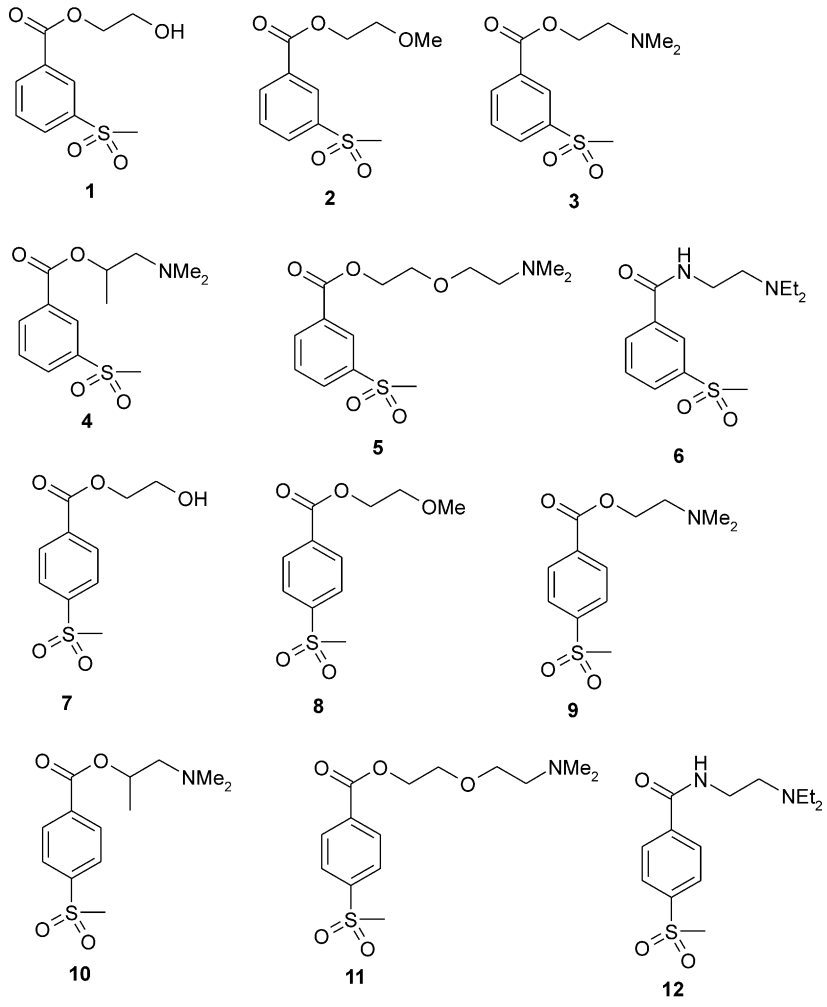
Two main classes of CA inhibitors (CAIs) are known: the metal complexing anions, and the unsubstituted sulfonamides, which bind to the Zn(II) ion of the enzyme either by substituting the non-protein zinc ligand, or add to the metal coordination sphere generating trigonal-bipyramidal species[1–3]. Sulfonamides, which are the most important CAIs bind in a tetrahedral geometry of the Zn(II) ion, in deprotonated state, with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II); anions may bind either in tetrahedral geometry of the metal ion, or as trigonal-bipyramidal adducts.[1–3] X-ray crystallographic structures are available for many adducts of sulfonamide inhibitors with isozymes CA I, II and IV.[2,4,5] In all these adducts, the deprotonated sulfonamide is coordinated to the Zn(II) ion of the enzyme, and its NH moiety donates a hydrogen bond to the Oγ of Thr 199, which in turn donates a hydrogen bond to the carboxylate group of Glu 106. One of the oxygen atoms of the SO_2NH moiety also participates in a hydrogen bond with the backbone NH moiety of Thr 199, and extensive hydrophobic and van der Waals interactions between the heterocyclic/aromatic part of the inhibitor molecule and active site amino acid residues, assure a strong affinity of sulfonamides to the CA active site (which may arrive to the nanomolar range in some cases).[1–5]

In our laboratory an original approach has recently been developed for obtaining high affinity CAIs with a multitude of pharmacological uses, such as topically acting antiglaucoma drugs, antitumor agents or diagnostic tools for PET among others.[6–10] This approach (the so-called 'tail approach') consists in using well-known aromatic/heterocyclic sulfonamide scaffolds to which tails that will induce water solubility or other desired physico-chemical properties are attached at the amino, hydroxy, imino or hydrazino moieties contained in the precursor sulfonamides.[6–10]

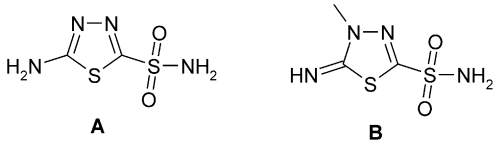
In this paper, we report novel types of CAIs prepared by the tail approach. These compounds incorporate carboxyphenylsulfonamide tails derivatized as esters and amides, and were designed in such a way as to assure a good penetration through the cornea, for optimizing their topical antiglaucoma properties.

Chemistry

Benzolamide **BZA**, is a very potent CAI, and although quite similar structurally with acetazolamide, **AZA**, the CAI par excellence, it possesses different physico-chemical properties, due to the presence of the second sulfonamide moiety in its molecule.[11,12] In consequence, **BZA** is much more polar than **AZA**, does not possess antiglaucoma properties when given systemically (in contrast to acetazolamide or methazolamide), and to a certain extent, crosses biological membranes with much more difficulty as compared to other CAIs in clinical use.[1,11] This was the rationale that lead to the proposal that it acts as a renal-specific CAI, but no drug house developed this agent for wide clinical use, and presently **BZA** is still an orphan drug.[11] Due to its good CA inhibitory properties, as well as interesting physico-chemical properties, **BZA** has been used as lead for the design of CAIs by our group.[12] Indeed, a large number of benzolamide analogues possessing diversely substituted phenyl moieties, as well as the corresponding 4-methyl-thiadiazoline-sulfonamides (incorporating the methazolamide scaffold) have been prepared and assayed as CAIs.[12] Some of these derivatives, such as for example 5-(2-carboxyphenylsulfonamido)-1,3,4-thiadiazole-2-sulfonamide (2-carboxy-**BZA**) as well as the corresponding 4-methyl-thiadiazoline derivative showed sub-nanomolar affinity for hCA II (the main enzyme involved in aqueous humor secretion within the eye)[1,2] and very good inhibition profiles against other isozymes (such as CA I and IV), but were devoid of topical antiglaucoma properties (unpublished results from this laboratory).[12] It appeared thus of interest on the one hand to prepare the 3-carboxy- and 4-carboxy-isomers of 2-carboxy-**BZA**, which were not reported in



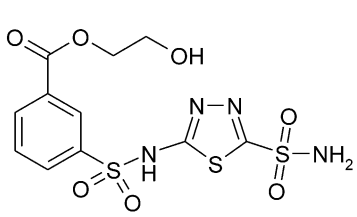
Tails **1-12**



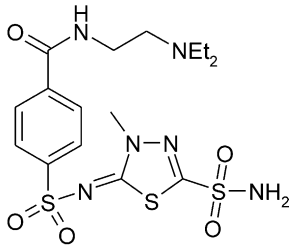
Heads **A** and **B**

the preceding study,[12] and on the other one, to reduce their very polar character by derivatizing the carboxyl moiety, in order to investigate whether this will lead to enhanced ocular penetration, and thus topical antiglaucoma activity. Thus, a series of esters and amides of 3-carboxy-**BZA**, and 4-carboxy-**BZA** were prepared, together with the corresponding thiadiazoline-sulfonamides. These compounds formally incorporate tails **1**–**12** which were attached to heads **A** and **B** of the parent sulfonamides from which acetazolamide and methazolamide were derived. As for other compounds prepared by the tail aproach,[1] sulfonamides reported here are denominated by a figure specifying the tail, followed by a letter specifying the head to which this tail has been attached. For example, **1A** is the ethylene glycol ester of 3-carboxybenzolamide, whereas **12B** is the 2-*N,N*-diethylaminoethylamide of 5-(4-carboxybenzenesulfonyl-imido)-4-methyl-δ^2-1,3,4-thiadiazoline-2-sulfonamide.

Compounds reported here were prepared as shown in Scheme 1. The key intermediates **13**–**16** were obtained by arylsulfonylation of 5-amino-1,3,4-thiadiazole-2-sulfonamide (**A**)/5-imino-4-methyl-δ^2-1,3,4-thiadiazoline-2-sulfonamide (**B**) with 3- or 4-carboxyphenylsulfonyl chloride, as reported previously for structurally related benzolamide derivatives.[7,12–15] Coupling of these 3- and 4-carboxybenzolamide derivatives **13**–**16** with different alcohols/amines in the presence of carbodiimides (such as EDCI) led to the new sulfonamides of types (**1**–**12**)**A**,**B**, by the same procedure reported previously for preparing topically actings antiglaucoma sulfonamides incorporating amino acyl tails.[6–10,16] In a variant of these syntheses, the key intermediates **13**–**16** were converted to the corresponding acyl chlorides by treatment with thionyl chloride, and the obtained acyl chlorides were then reacted with alcohols/amines in standard conditions[17] (Scheme 1). The presence of hydroxyethyl,



1A



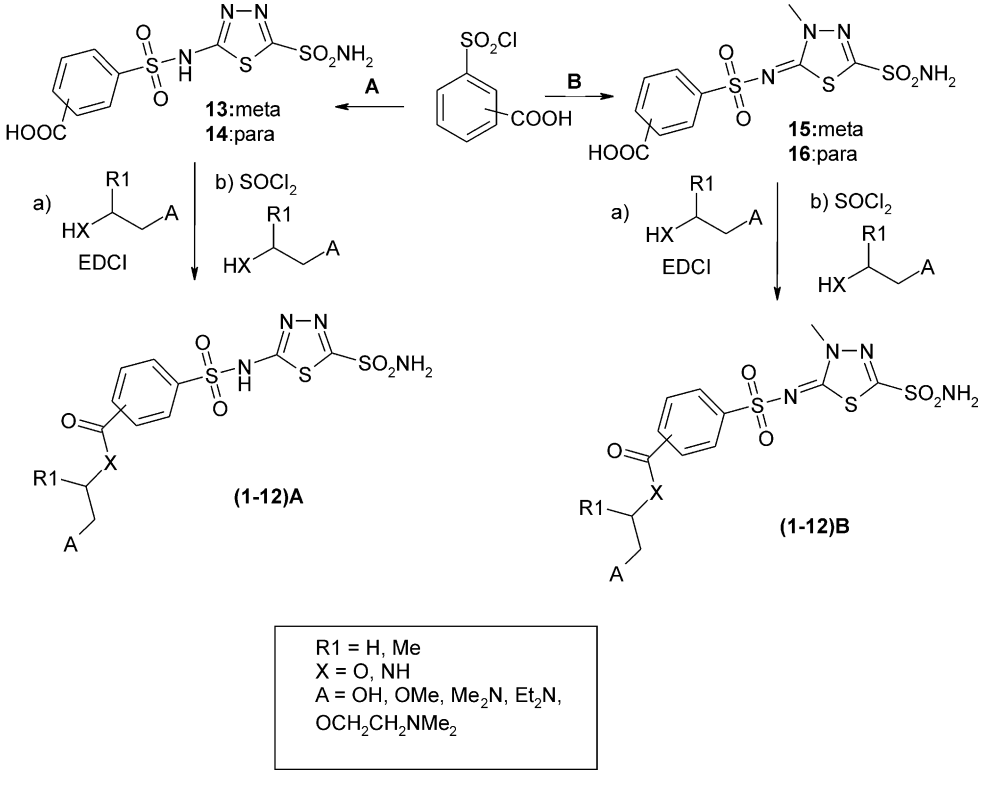
12B

methoxyethyl, dialkylaminoethyl or dialkylaminoethoxyethyl moieties in the tails **1**–**12** investigated here is considered beneficial for improving pharmacological properties of these new CAIs, such as for example water solubility, balanced water/lipid solubility; penetrability of the drug through the cornea, etc., and this was shown indeed to be the case (see later in the text).

CA inhibition

Inhibition data of Table 1 show that the new compounds reported here act as very strong inhibitors of the physiologically relevant isozymes I, II and IV, similarly with the clinically used derivatives acetazolamide, methazolamide, ethoxzolamide or benzolamide. Thus, the parent carboxy-derivatives are already potent inhibitors of all these isozymes, with inhibition constants in the range of 45–73 nM against hCA I, 4.6–5.9 nM against hCA II and 12–15 nM against bCA IV. Enlarging the carboxyphenylsulfonyl tail as in esters/amides (**1**–**12**)**A**,**B** generally leads to an increase of the affinity for these isozymes, with K_is in the 10–23 nM range against hCA I, 1.3–5.2 nM against hCA II and 6–14 nM against bCA IV (Table 1).[20]

The following SAR can be also evidenced for the new esters/amides (**1**–**12**)**A**,**B**: (i) the ethylene glycol esters **1A**, **7A**, **1B** and **7B** were generally less effective than the corresponding methoxy derivatives (**2A**, **8A**, **2B** and **8B**), which in turn were less effective than the corresponding N,N-dimethylamino-substituted compounds **3A**, **9A**, **3B** and **9B** (against hCA II), but differences of activity were rather small (ii) the presence of the methyl group in the ester tail, such as in **4A**,**B** and **10A**,**B** did not significantly influence the CA inhibitory activity of these compounds, whereas the elongation of the tail, such as in compounds **5A**,**B** and **11A**,**B** leads to some of the best inhibitors in this series; (iii) the amides **6A**,**B** and **12A**,**B** also led to some of the best inhibitors in this series; (iv) the *para*-substituted derivatives were slightly better inhibitors than the corresponding *meta*-substituted derivatives (with the exception of bCA IV, for which the *para*-type tails were slightly less effective



Scheme 1.

Table 1. CA inhibition data with standard inhibitors, the parent sulfonamides **13**–**16** and some of the new derivatives reported in the present study, against isozymes I, II and IV

Inhibitor	K_i (nM)[a]		
	hCA I[a]	hCA II[b]	bCA IV[c]
AZA	200	7	120
MZA	100	9	145
EZA	25	8	13
BZA	15	9	12
DZA	50,000	9	43
BRZ	—	3	45
13	68	5.1	13
14	45	4.6	12
15	73	5.9	15
16	50	5.4	14
1A	16	3.1	8
2A	15	2.9	8
3A	15	2.1	7
4A	17	2.0	8
5A	13	1.6	6
6A	14	1.7	7
7A	14	2.9	11
8A	12	2.8	10
9A	11	1.5	10
10A	13	1.4	10
11A	12	1.3	7
12A	10	1.4	8
1B	23	5.2	10
2B	17	3.3	9
3B	16	2.9	10
4B	18	2.2	12
5B	15	2.0	8
6B	16	1.9	7
7B	20	4.0	14
8B	16	3.1	12
9B	13	2.4	14
10B	15	1.8	13
11B	13	1.7	9
12A	12	1.7	8

[a]From triplicate experiments (errors in the range of 5-10% of the reported value).
[b]Human cloned isozyme.[18]
[c]Isolated from bovine lung.[19]

inhibitors); (v) compounds incorporating the 1,3,4-thiadiazole-2-sulfonamide moiety (head **A**) were more effective CAIs as compared to the corresponding derivatives incoporating the thiadiazoline-sulfonamide scaffold (head **B**).

Topical antiglaucoma activity

As seen from data of Table 2, in vivo, in normotensive rabbits, some of the new sulfonamides reported here (which possess very good water solubility—data not shown) exhibited effective intraocular pressure (IOP) lowering after topical administration, with pressure reductions of 2.5–10.5 mm Hg at half an hour (compared to 1.9 mm Hg for dorzolamide and 2.9 mm Hg for brinzolamide), 2.5–8.5 mm Hg at 1 h (4.0 for **DZA**, and 3.2 for **BRZ**, respectively), 2.0–10.1 mm Hg at 90 min (compared to 2.1 mm Hg for **DZA**, and 6.3 for **BRZ**).[21] An important feature of some representatives of the new class of CA inhibitors reported here (such as **6A**, **9A**, **10A** and **12A** among others) was that IOP remained low for longer periods (3–5 h) after their topical administration, as compared to the standard drug, for which the

Table 2. Fall of IOP of normotensive rabbits (20.5±2.1 mm Hg), after treatment with one drop (50 μL) 2% water solution of CA inhibitor directly into the eye, 60 and 90 min after administration

Inhibitor	pH	ΔIOP (mm Hg)[a]				
		$t=0$	30 min	60 min	90 min	240 min
Dorzolamide	5.5	0	1.9±0.2	4.0±0.3	2.1±0.2	0
Brinzolamide	5.5	0	2.9±0.1	3.2±0.3	6.3±0.4	1.3±0.2
1A	7	0	10.5±0.55	4.8±0.15	2.8±0.2	0.5±0.1
2A	7	0	6.0±0.3	2.5±0.2	2.0±0.3	0
3A	7	0	4.5±0.15	4.9±0.1	7.1±0.4	5.7±0.4
4A	7	0	2.5±0.2	6.7±0.15	8.5±0.2	7.9±0.5
5A	7	0	4.9±0.3	4.5±0.3	4.1±0.3	3.3±0.2
6A	7	0	6.5±0.4	6.9±0.2	9.1±0.5	8.0±0.3
9A	7	0	4.5±0.25	5.5±0.25	8.0±0.4	7.6±0.4
10A	7	0	4.8±0.4	5.9±0.3	8.2±0.3	8.0±0.2
11A	7	0	2.7±0.1	3.9±0.15	5.7±0.2	6.3±0.4
12A	7	0	3.6±0.2	8.5±0.3	10.1±0.4	8.7±0.4

[a]$\Delta IOP = IOP_{control\ eye} - IOP_{treated\ eye}$; mean±SE ($n=3$).

effect lasted generally no longer than 2–3 h (data not shown). Some of the new derivatives reported here, such as **1A** and to a lesser extent **2A** showed a very sharp decrease of IOP after 30 min post-administration, but this good effect vanished rather quickly (in the next 30 min), probably because the inhibitor has been washed away from the eye tissue due to the blood circulation. Clearly such inhibitors did not possess the desired physico-chemical properties for a prolonged duration of action. On the contrary, several other compounds, such as **6A**, **9A**, **10A** and **12A** among others, showed a good IOP lowering effect after 30 min (around 3.6–6.5 mm Hg), which continued to increase both at one h, as well as at 90 min post-administration, when the maximal peak of IOP lowering has been achieved (of 8.0–10.1 mm Hg). These inhibitors may indeed be considered as valuable candidates for achieving long-lasting IOP lowering after topical administration of sulfonamide CAIs. Furthermore, the pH of the eye drops of new CAIs reported here were neutral, as compared to the acidic solutions of dorzolamide and brinzolamide (pH 5.5) which produce eye irritation in many patients.[22]

Conclusions

Two new series of sulfonamides have been synthesized by using the tail approach and benzolamide as lead molecule. These are derivatives of 3-carboxy- and 4-carboxy-benzolamide (as well as the homologous 4-methyl-1,3,4-thiadiazoline-2-sulfonamides) to which the carboxyl moiety has been either esterified or amidated in order to reduce the very polar character of the lead, which is ineffective as antiglaucoma agent. The new sulfonamides reported showed low nanomolar activity against isozymes CA I, II and IV, and some of them were effective in normotensive rabbits in reducing IOP after topical administration as 2% solutions. Their efficacy was better than that of the standard drugs dorzolamide and brinzolamide, whereas the duration of action was also prolonged as compared to that of the clinically used drugs.

Acknowledgements

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15. An amount of 10 g (55 mM) 5-amino-1,3,4-thiadiazole-2-sulfonamide was dissolved in 30 mL NaOH 2.5 N, under stirring at room temperature. The obtained clear solution was cooled at 10 °C, then a 1.3 molar excess of the corresponding 3- or 4-carboxybenzenesulfonyl chloride (from Sigma-Aldrich, Milan, Italy), together with 50 mL NaOH 5 N were added simultaneously, in five portions, maintaining the temperature at 10 °C. The dark brown solution obtained was stirred overnight at room temperature, then the pH was adjusted to 1.5–2 with 6 N HCl. The crude precipitate was filtered and suspended in 100 mL HCl 1:1 in order to dissolve the unreacted amine. The obtained solid was suspended in 80 mL of water and treated dropwise with concentrated (25%) NH_3 solution until pH 9 was reached. The solution of the sulfonamide/carboxylate ammonium salt was brought dropwise to pH 1.5–2 with 6 N HCl. Recrystallization from 300 mL of water with active charcoal (0.5–0.7 g) afforded the pure products, **13** and **14**, respectively (overall yields were in the range of 30–40%). 4-Carboxybenzolamide **14**, colorless crystals, mp 235–237 °C (dec.); ^{1}H NMR (DMSO-d_6, ppm): 7.31 (s, NH_2); 7.39 (d, ArH, AA′BB′, 8.4); 7.72 (d, ArH, AA′BB′, 8.4); 8.83 (br s, SO_2NH); ^{13}C NMR (DMSO-d_6, ppm): 168.08 (COOH), 166.17, 158.32 ($C_{thiadiazole}$), 144.70 (C-1 of 4-HOOC-C_6H_4), 134.52 (C-4 of 4-HOOC-C_6H_4), 130.26 (C-2 of 4-HOOC-C_6H_4), 126.24 (C-3 of 4-HOOC-C_6H_4); elem. anal. data: found, C, 29.46; H, 2.13; N, 15.10%; $C_9H_8N_4O_6S_3$ requires: C, 29.67; H, 2.21; N, 15.38%.
16. An amount of 1 mM **13**–**16** was dissolved in 25 mL of anhydrous acetonitrile and then treated with 2 mM of alcohol/amine (ethylene glycol; 2-methoxyethanol; 2-*N,N*-dimethylaminoethanol; 1-methyl-2-*N,N*-dimethylamino-ethanol; 2-(2-*N,N*-dimethylaminoethoxy)-ethanol; 2-*N,N*-diethylaminoethylamine, all commercially available from Sigma-Aldrich, Milan, Italy). An amount of 190 mg (1 mM) of EDCI HCl was then added and the reaction mixture was magnetically stirred at room temperature for 15 min, then 30 μL (2mM) of triethylamine were added and stirring was continued for 8–16 h at 4 °C (TLC control). The solvent was evaporated in vacuo and the residue taken up in ethyl acetate (5 mL), poured into a 5% solution of sodium bicarbonate (5 mL) and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate and filtered, and the solvent removed in vacuo. The obtained oils were were recrystallized from ethanol–water or methanol, or purified by means of preparative HPLC (C_{18} reversed-phase μ-Bondapack or Dynamax-60A (25×250 mm) columns; 90% acetonitrile/8% methanol/2% water, 30 mL/min). Yields were in the range of 50–54%. **12A**, white crystals, mp 197–9 °C; ^{1}H NMR (DMSO-d_6, ppm): 1.86 (t, 6H, 2Me from Et, 6.9 Hz); 2.84 (q, 4H, CH_2 from 2 Et, 7.3 Hz); 2.90 (t, 2H, CH_2CH_2N, 7.2 Hz); 3.47 (q, 2H, CH_2NH, 6.5 Hz); 7.37 (d, ArH, AA′BB′, 8.4 Hz); 7.73 (d, ArH, AA′BB′, 8.4 Hz); 8.85 (br s, SO_2NH); ^{13}C NMR (DMSO-d_6, ppm): 171.12 (CONH), 166.237, 158.31 ($C_{thiadiazole}$), 144.82 (C-1 of 4-HOOC-C_6H_4), 134.65 (C-4 of 4-HOOC-C_6H_4), 130.43 (C-2 of 4-HOOC-C_6H_4), 124.98 (C-3 of 4-HOOC-C_6H_4); 42.35 (CH_2); 42.12 (CH_2); 40.81 (CH_2); 16.54 (Me); elem. anal. data: found, C, 39.13; H, 4.68; N, 18.05%; $C_{15}H_{22}N_6O_5S_3$ requires: C, 38.95; H, 4.79; N, 18.17%.
17. Carboxyl-sulfonamides **13**–**16** (1 mM) were suspended in 20 mL of anhydrous benzene and a 2-fold excess of $SOCl_2$ was added. The reaction mixture was refluxed for 3–5 h till the formation of the acyl chloride was complete (TLC control), the solvent and excess thionyl chloride were removed in vacuo, and the obtained acyl halides dissolved in 10 mL of anhydrous acetonitrile. To this solution was added the 2-fold excess amount of alcohols/amines mentioned above, and the acylation reaction was followed by TLC. The reaction mixture has been worked out as described above. Yields were in the range of 70–75%.
18. Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/

hCA I and pACA/hCA II described by: Lindskog, S.; Behravan, G.; Engstrand, C.; Forsman, C.; Jonsson, B. H.; Liang, Z.; Ren, X.; Xue, Y. In *Carbonic Anhydrase—From Biochemistry and Genetics to Physiology and Clinical Medicine*, Botrè, F., Gros, G., Storey, B. T. Eds.: VCH, Weinheim 1991; p 1. Cell growth conditions were those described by this group, and enzymes were purified by affinity chromatography. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM^{-1} cm^{-1} for CA I and 54 $mM^{-1}.cm^{-1}$ for CA II, respectively, based on $M_r = 28.85$ kDa for CA I, and 29.30 kDa for CA II, respectively. CA IV was isolated from bovine lung microsomes as described by Maren et al.[19] and its concentration has been determined by titration with ethoxzolamide.

19. Maren, T. H.; Wynns, G. C.; Wistrand, P. J. *Mol. Pharmacol.* **1993**, *44*, 901.

20. A stopped flow variant of the Pocker and Stone spectrophotometric method (Pocker, Y.; Stone, J. T. *Biochemistry* **1967**, *6*, 668) has been employed, using an SX.18MV-R Applied Photophysics stopped flow instrument, as described previously.[9,10]

21. 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) were obtained in distilled deionized water. The pH of these solutions was in the range of 6.5–7.0. IOP was measured using a Tono-Pen XL tonometer (Medtronic Solan, USA) as reported earlier.[9,10] 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. 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 control eye, in this way minimizing the diurnal, seasonal and interindividual variations commonly observed in the rabbit.[9]

22. (a) Sugrue, M. F. *Progr. Ret. Eye Res.* **2000**, *19*, 87. (b) Silver, L. H. *Surv. Ophthalmol.* **2000**, *44*, 147.