Carbonic Anhydrase Inhibitors: Water-Soluble 4-Sulfamoylphenylthioureas as Topical Intraocular Pressure-Lowering Agents with Long-Lasting Effects

Angela Casini,[†] Andrea Scozzafava,[†] Francesco Mincione,[‡] Luca Menabuoni,[§] Marc A. Ilies,[||] and Claudiu T. Supuran*,[†]

Laboratorio di Chimica Inorganica e Bioinorganica, Università degli Studi di Firenze, Via Gino Capponi 7, I-50121 Florence, Italy, Istituto Oculistico, Università degli Studi di Firenze, Viale Morgagni 85, I-50134 Florence, Italy, Ospedale San Giovanni di Dio, S.A. Oculistica, Via Torregalli 3, I-50123 Florence, Italy, and Department of Chemistry, Faculty of Biotechnologies, University of Agricultural Sciences and Veterinary Medicine, B-dul Marasti nr. 59, 71331 Bucharest, Romania

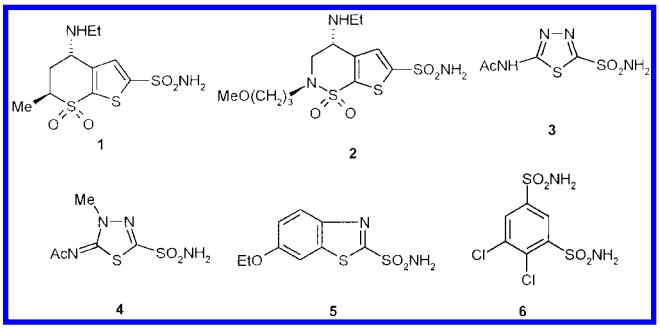
Received August 4, 2000

A series of sulfonamides has been obtained by reaction of 4-isothiocyanatobenzenesulfonamide with amines, amino acids, and oligopeptides. The new thiourea derivatives showed strong affinities toward isozymes I, II, and IV of carbonic anhydrase (CA, EC 4.2.1.1). In vitro inhibitory power was good (in the low-nanomolar range) for the derivatives of *β*-phenylserine and α-phenylglycine, for those incorporating hydroxy and mercapto amino acids (Ser, Thr, Cys, Met), hydrophobic amino acids (Val, Leu, Ile), aromatic amino acids (Phe, His, Trp, Tyr, DOPA), and dicarboxylic amino acids as well as di/tri/tetrapeptides among others. Such CA inhibitors displayed very good water solubility (in the range of 2−3%) mainly as sodium (carboxylate) salts, with pH values of the obtained solutions being 6.5−7.0. Some of these preparations (such as the derivatives of Ser, *β*-Ph-Ser, Leu, Asn, etc.) strongly lowered intraocular pressure (IOP) when applied topically, directly into the normotensive/glaucomatous rabbit eye, as 2% water solutions. It is interesting to note that not all the powerful CA inhibitors designed in the present study showed topical IOP-lowering effects (such as, for instance, the Cys and Lys derivatives, devoid of such properties) whereas the Pro, Arg, and oligopeptidyl thiourea derivatives showed reduced efficacy when administered topically. This may be due to the very hydrophilic nature of some of these compounds, whereas inhibitors with balanced hydro- and liposolubility also showed optimal in vivo effects. The interesting pharmacological properties of this new type of CA inhibitors, correlated with the neutral pH of their solutions used in ophthalmologic applications, make them attractive candidates for developing novel antiglaucoma drugs devoid of major ocular side effects.

Introduction

Sulfonamide inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) are extensively used in clinical medicine and as diagnostic tools, their main applications being in the treatment of glaucoma and macular edema.[1−3] Several such drugs are presently available, such as the recently introduced topical sulfonamides dorzolamide (**1**) and brinzolamide (**2**), in addition to the classical, systemically acting inhibitors acetazolamide (**3**), methazolamide (**4**), ethoxzolamide (**5**), and dichlorophenamide (**6**), which have been employed clinically for more than 45 years.[1−3]

Systemic inhibitors possess many undesired side effects due to inhibition of several CA isozymes (14 are presently known in higher vertebrates)[1] in tissues other than the target one, i.e., the eye (more precisely, the ciliary processes of the eye), and many agents such as **3**−**6** are presently used more in physiological studies or as diagnostic tools than as antiglaucoma drugs.[1] On the other hand, the two topically acting inhibitors **1** and



2 show a much more diminished number of side effects, together with an efficient reduction of intraocular pressure (IOP), when used alone or in combination with other drugs, such as prostaglandin analogues, *β*-blockers, etc., due to inhibition of the enzymes (mainly isozymes CA II and CA IV)[1−3] present in the ciliary processes, without appreciable inhibition of CAs from other tissues/organs.[1−4] These two sulfonamides **1** and **2** are both administered as hydrochloride salts, and this leads to frequent stinging sensations, burning or reddening of the eye, blurred vision, pruritus, and other local side effects. To reduce such inconveniences, many tentatives were ultimately reported to design topical antiglaucoma sulfonamides devoid of the above-mentioned side effects.[5−8] Among them, one approach consisted in attaching water-solubilizing "tails" to the

* To whom correspondence should be addressed. Fax: +39-055-2757555. Tel: +39-055-2757551. E-mail: cts@bio.chim.unifi.it.
[†] Laboratorio di Chimica Inorganica e Bioinorganica, Università degli Studi di Firenze.
[‡] Istituto Oculistico, Università degli Studi di Firenze.
[§] S.A. Oculistica.
[||] University of Agricultural Sciences and Veterinary Medicine.

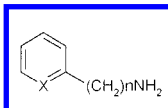
10.1021/jm001051+ CCC: $19.00
Published on Web 11/10/2000

molecules of aromatic/heterocyclic sulfonamides possessing amino, imino, or hydroxy moieties in their molecules.[6,7] Such tails included pyridinecarboximido, carboxypyridinecarboxamido, quinolinesulfonamido, picolinoyl, and isonicotinoyl, as well as amino acyl groups among others, whereas ring systems which have been derivatized by this procedure included 2-, 3-, or 4-aminobenzenesulfonamides, 4-(ω-aminoalkyl)benzenesulfonamides, 3-halogenosubstituted-sulfanilamides, 1,3-benzenedisulfonamides, 1,3,4-thiadiazole-2-sulfonamides, and benzothiazole-2-sulfonamides, as well as thienothiopyran-2-sulfonamides among others, and had as the main objective the possibility to formulate the eye drop solutions at pH values close to neutrality.[6−8]

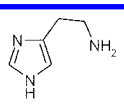
Continuing our previous research in this field,[6−8] we report here an alternative approach for obtaining water-soluble, strong CA inhibitors with putative applications as agents used in the treatment of ocular hypertension, with potentially no side effects due to the acidic pH of the eye drop solution. Thus, 4-isothiocyanatobenzenesulfonamide was reacted with amines, amino acids, or oligopeptides, and the thioureas obtained in this way showed excellent water solubilities either as sodium salts (for the amino acid/oligopeptide derivatives) or as hydrochlorides/triflates (in the case of the amine derivatives). The nucleophiles used in the above-mentioned syntheses were chosen in such a way as to possess pK_a values in the "physiological" range. More precisely, salts of these new inhibitors applied into the eye of the experimental animals generally possessed pH values in the range 6.5−7.0. Such salts were applied topically into the eye of normotensive or glaucomatous rabbits and produced a powerful, long-lasting reduction of IOP.

Results

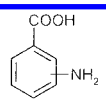
Synthesis. A large number of derivatives have been prepared by reaction of 4-isothiocyanatobenzenesulfonamide **A** with amines **7**−**13**, amino acids **14**−**38**, or oligopeptides **39**−**47**.



7: X=N; n = 1
8: X=N; n = 2
9: X=CH; n =2



10



11: 2-NH_2
12: 3-NH_2
13: 4-NH_2

14: Gly; **15**: Ala; **16**: beta-Ala; **17**: GABA; **18**: alpha-Ph-Gly; **19**: Ser; **20**: beta-Ph-Ser

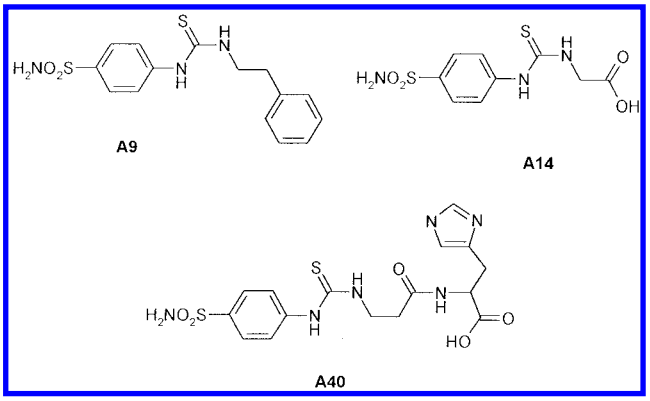
21: Thr; **22**: Cys; **23**: Met; **24**: Val; **25**: Leu; **26**: Ile; **27**: Asp; **28**: Asn; **29**: Glu; **30**: Gln

31: Pro; **32**: His; **33**: Phe; **34**: Tyr; **35**: DOPA; **36**: Trp; **37**: Lys; **38**: Arg; **39**: GlyGly

40: beta-AlaHis; **41**: HisGly; **42**: HisPhe; **43**: AlaPhe; **44**: LeuGly; **45**: AspAsp

46: ProGlyGly; **47**: $(Asp)_4$

The newly obtained derivatives will be numbered as **A7**−**A47**, denoting that the 4-H_2NO_2S-C_6H_4-NHCSNH- group is attached to the corresponding moiety of the starting nucleophile **7**−**47**. For instance, **A9** is the thiourea obtained by the reaction of phenethylamine **9** with 4-isothiocyanatobenzenesulfonamide **A**; **A14** is the thiourea obtained by reaction of glycine **14** with 4-isothiocyanatobenzenesulfonamide **A**; **A40** is the thiourea obtained by reaction of carnosine **40** with 4-isothiocyanatobenzenesulfonamide **A**; etc.



A9

A14

A40

CA Inhibitory Activity. The new sulfonamides **A7**−**A47** reported here were assayed for inhibition of three CA isozymes, two of them known to play a critical role in aqueous humor formation (CA II and CA IV), whereas the other one (CA I) is known to be important for the possible systemic side effects of such drugs (Table 1).[1]

Transcorneal Penetration of Drugs. Some physicochemical properties of several of the new sulfonamides reported here relevant for their pharmacological activity, such as buffer solubility or chloroform−buffer partition coefficient, are shown in Table 2. The in vitro transcorneal accession rates (k_{in}) of classical sulfonamides and topically active derivatives, such as dorzolamide and some of the new compounds reported in the present study, are also shown in Table 2.

IOP Measurements. In vivo IOP-lowering data with some of the most active CA inhibitors reported here in normotensive and glaucomatous rabbits, after topical administration of the drug, are shown in Tables 3 and 4, respectively.

The full time dependence of the IOP after dorzolamide and some of the new compounds reported here in normotensive albino rabbits is shown in Figure 1.

Distribution of Drugs in Ocular Fluids and Tissues. Ex vivo distribution data of some active compounds in ocular tissues and fluids after topical administration in normotensive rabbits are shown in Table 5.

Discussion

Chemistry. The synthesis of 4-isothiocyanatobenzenesulfonamide **A** has already been reported in 1946 by McKee and Bost,[9] in the search of more effective antibacterial sulfonamides. Still, this highly versatile compound has never been used for the preparation of sulfonamides possessing CA inhibitory properties. Compound **A** has been obtained by the reported literature procedure,[9] from sulfanilamide **B** and thiophosgene, and was subsequently reacted with a large number of amines, amino acids, and oligopeptides, of type **7**−**47**, leading to the new thioureas **A7**−**A47** (Scheme 1).[9,10]

The reaction generally proceeded in very good yields and without the formation of side products mainly for amines and simple amino acids. Still, in the case of oligopeptides (such as **39**−**47**) used as nucleophiles in these syntheses, a lot of tar was formed during the reaction, and pure compounds **A39**−**A47** could only be obtained after preparative HPLC.

The nucleophiles used for the preparation of the new compounds reported here (**7**−**47**) were chosen in such

Table 1. CA Inhibition Data with Standard Inhibitors **1**−**6**, 4-Isothiocyanatobenzenesulfonamide **A**, and New Sulfonamides **A7**−**A47** Reported in the Present Study

	K_i (nM)				K_i (nM)				K_i (nM)		
inhibitor	hCA I[a]	hCA II[a]	bCA IV[b]	inhibitor	hCA I[a]	hCA II[a]	bCA IV[b]	inhibitor	hCA I[a]	hCA II[a]	bCA IV[b]
dorzolamide, **1**	50000	9	43	**A17**	45	11	33	**A33**	30	6	13
acetazolamide, **3**	900	12	220	**A18**	40	6	15	**A34**	26	5	13
methazolamide, **4**	780	14	240	**A19**	28	3	15	**A35**	35	7	18
ethoxzolamide, **5**	25	8	13	**A20**	24	2	9	**A36**	47	11	24
dichlorophenamide, **6**	1200	38	380	**A21**	30	4	16	**A37**	97	12	55
A	5000	185	300	**A22**	25	5	10	**A38**	115	15	61
A7	135	45	76	**A23**	27	4	12	**A39**	54	15	32
A8	124	40	77	**A24**	23	5	13	**A40**	23	3	10
A9	125	42	75	**A25**	21	5	12	**A41**	13	1	6
A10	92	33	56	**A26**	22	4	10	**A42**	21	3	9
A11	55	13	29	**A27**	35	8	17	**A43**	23	4	13
A12	50	18	36	**A28**	27	6	11	**A44**	20	5	13
A13	59	16	40	**A29**	38	10	19	**A45**	50	15	32
A14	62	20	39	**A30**	40	9	23	**A46**	20	3	11
A15	54	12	36	**A31**	110	25	68	**A47**	59	10	27
A16	47	11	35	**A32**	32	5	15				

[a] Human (cloned) isozymes. [b] From bovine lung microsomes, by the esterase method.

Table 2. Solubility, Chloroform−Buffer Partition Coefficients, and in Vitro Corneal Permeability of Some Classical and New Sulfonamide CA Inhibitors

			$k_{in} \times 10^3$ (h^{-1})[c]	
compd	solubility[a] (mM)	log P[b]	cornea intact	no epithelium
dorzolamide[d]	60	2.0[e]	3.0	5.2
acetazolamide	3.2	0.001	0.37	7.0
methazolamide	12	0.06	1.90	13
ethoxzolamide	0.04	25	27	40
benzolamide	1.2	0.00001	0.1	0.4
A18[e]	54	3.652	5.9	14.3
A19[e]	73	1.459	3.4	8.0
A20[e]	48	2.314	4.9	12.3
A22[e]	65	0.09	0.3	0.7
A27[e]	60	0.05	0.1	0.8
A28[e]	51	1.759	3.1	7.8
A40[e]	52	0.725	2.2	5.0

[a] Solubility in pH 7.40 buffer, at 25 °C. [b] Chloroform−buffer partition coefficient. [c] Determined as described in refs 28, 29. [d] As hydrochloride, at pH 5.8. [e] As sodium salts, at pH 7.0.

a manner as to contain water-solubilizing moieties in the presence of acids/bases, such as the pyridine or imidazole moieties in the case of **7, 8,** and **10** (hydrochlorides[6,7] or triflates[6] of **A7**, **A8**, and **A10** would presumably lead to water-soluble CA inhibitors, but the compound unable to form such a salt − **A9** − has also been prepared for comparison and confirmed the hypothesis mentioned above). Thioureas obtained from other nucleophiles (**A11**−**A47**), on the other hand, easily form water-soluble sodium salts due to the presence of at least one carboxyl group in their molecules. It was previously noted in this laboratory[6a,11] that sulfonamides incorporating carboxylate moieties in their molecules might interact with a histidine cluster at the entrance of the hCA II active site (this is the isozyme that plays the most important function in aqueous humor secretion).[1−3] This cluster comprises the residues His 64 (at the center of the active site cavity) and His 4 and His 3 (at the rim of it) as well as several residues on the external surface of the enzyme, near the entrance to the active site cavity (His 10, His 15, His 17; hCA I numbering).[11] All imidazolic side chains of these residues may interact (better when in charged state, as imidazolium ions) with negatively charged inhibitors, containing, for instance, carboxylate anions, leading in this way to a supplementary stabilization of the enzyme−inhibitor adduct.[6] This is the reason so many compounds of this type have been designed and prepared in this study. The greatest majority of these new compounds contain one COO^- moiety (obviously, when in solution,

Table 3. Fall of IOP of Normotensive Rabbits (19.5 ± 2.1 mmHg)[a]

		ΔIOP ± SE[b] (mmHg)		
inhibitor	pH	30 min	60 min	90 min
dorzolamide	5.5	2.2 ± 0.15	4.1 ± 0.15	2.7 ± 0.10
A7	6.5	0.8 ± 0.10	4.0 ± 0.15	0.6 ± 0.20
A10	6.5	3.0 ±0.15	0.3 ± 0.25	0.0
A11	7.0	4.3 ±0.15	8.0 ± 0.25	0.9 ± 0.10
A14	7.0	3.0 ± 0.20	5.9 ± 0.20	0.5 ± 0.15
A15	7.0	4.0 ± 0.10	5.0 ± 0.30	5.2 ± 0.40
A17	7.0	5.0 ± 0.25	3.1 ± 0.15	2.9 ± 0.20
A18	7.5	7.0 ±0.15	2.3 ± 0.30	2.1 ± 0.10
A19	7.0	8.0 ±0.15	9.5 ± 0.20	9.1 ± 0.25
A20	7.0	9.0 ± 0.20	10.0 ± 0.30	9.6 ± 0.20
A22	7.0	0.0	0.0	0.0
A25	7.0	5.2 ±0.25	6.3 ± 0.30	6.0 ± 0.20
A26	7.0	2.0 ± 0.10	4.0 ± 0.20	0.3 ± 0.15
A27[c]	7.0	2.0 ± 0.15	2.6 ± 0.25	2.0 ± 0.10
A28	7.0	7.0 ± 0.35	10.1 ± 0.25	6.0 ± 0.30
A31	7.0	0.0	1.0 ± 0.25	1.0 ± 0.10
A32	7.0	4.0 ± 0.15	4.6 ± 0.30	4.0 ± 0.20
A33	7.0	4.0 ± 0.10	4.1 ± 0.20	3.0 ± 0.15
A37	7.0	0.0	0.0	0.0
A38	7.0	1.0 ± 0.15	1.2 ± 0.30	4.0 ± 0.20
A40	7.0	1.0 ± 0.15	1.2 ± 0.30	4.0 ± 0.20
A43	7.0	0.2 ± 0.15	0.2 ± 0.10	3.0 ± 0.20

[a] After treatment with 1 drop (50 μL) of a 2% CA inhibitor solution (as hydrochloride salt in the case of dorzolamide, **A7**, and **A10** and as sodium salts for the other derivatives, with the pH value shown) directly into the eye at 30, 60, and 90 min after administration. [b] $\Delta IOP = IOP_{control\ eye} - IOP_{treated\ eye}$ ($n = 3$). [c] Eye irritation observed.

Table 4. Fall of IOP of Glaucomatous Rabbits (33.5 ± 3.0 mmHg)[a]

		ΔIOP ± SE[b] (mmHg)		
inhibitor	pH	30 min	60 min	90 min
1[c]	5.5	3.6 ± 0.20	6.7 ± 0.30	4.2 ± 0.15
A19[d]	7.5	10.4 ± 0.35	15.0 ± 0.30	17.5 ± 0.35
A20[d]	6.5	10.5 ± 0.25	14.1 ± 0.35	16.4 ± 0.20
A28[d]	7.0	10.0 ± 0.40	13.0 ± 0.25	15.5 ± 0.40

[a] After treatment with 1 drop (50 μL) of a 2% CA inhibitor solution (with the pH value shown) directly into the eye at 30, 60, and 90 min after administration. [b] $\Delta IOP = IOP_{control\ eye} - IOP_{treated\ eye}$ ($n = 3$). [c] As hydrochloride salt. [d] As sodium salt.

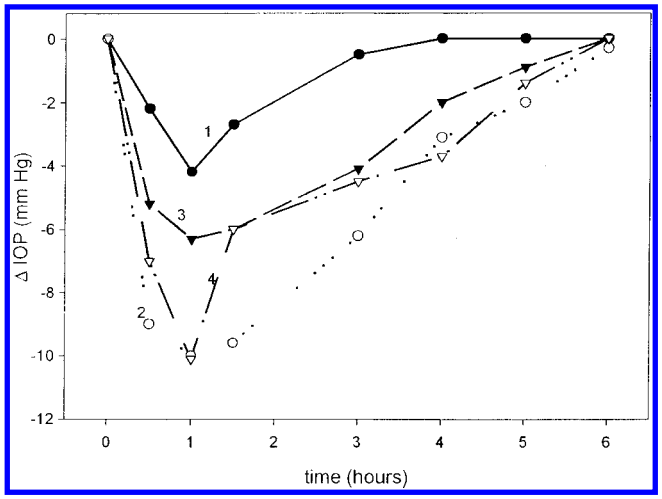


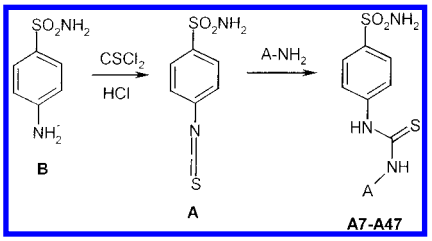
Figure 1. Effect of topically administered sulfonamide inhibitors (2% water solutions) on the IOP of normotensive albino rabbits: curve 1, dorzolamide (**1**) (hydrochloride salt, pH 5.5); curve 2, compound **A20** (sodium salt, pH 7.0); curve 3, compound **A25** (sodium salt, pH 7.0); curve 4, compound **A28** (sodium salt, pH 7.0).

Table 5. Ocular Tissue Concentrations after Corneal Application of 1 drop (50 μL) of a 2% Solution of Inhibitors **A20** and **A28** in Albino Rabbits[a]

	drug concentration (μM)[b]		
time (h)	cornea	aqueous humor	ciliary process
	1 (HCl)		
1	105 ± 5	32 ± 3	15 ± 3
2	39 ± 4	21 ± 2	6 ± 1
	A20		
1	163 ± 18	306 ± 21	53 ± 7
2	74 ± 5	65 ± 5	19 ± 2
	A28		
1	141 ± 15	285 ± 14	47 ± 5
2	54 ± 9	43 ± 3	12 ± 2

[a] With dorzolamide hydrochloride **1** as standard. [b] Mean ± standard deviation (n = 3).

Scheme 1. Synthesis of 4-Isothiocyanatobenzenesulfonamide **A** and the Thioureas **A7**−**A47**



as sodium salts, at the pH at which the experiments have been performed, i.e., 7.4 for the in vitro inhibition measurements and 6.5−7.0 for the in vivo experiments), but derivatives with two (**A45**) or four (**A47**) carboxylate moieties were also obtained. As will be discussed shortly, indeed, many such derivatives showed very good CA inhibitory properties.

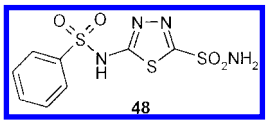
In Vitro CA Inhibition. CA inhibition data against three isozymes: hCA I, hCA II, and bCA IV (h = human; b = bovine isozyme) for the prepared compounds and standard inhibitors are shown in Table 1. All these compounds showed better CA inhibitory properties as compared to the parent sulfonamide **A** from which they were obtained, which is a moderately weak inhibitor, similarly to many benzene sulfonamides reported in the literature.[1,2,12,13] Thus, the four derivatives of aromatic/heterocyclic amines **A7**−**A10** (which are the most ineffective CA inhibitors among the new compounds reported here) showed affinities of 33−45 nM against hCA II, 56−76 nM against bCA IV, and 92−135 nM against the slow isozyme hCA I. Increased inhibitory power was observed for the derivatives obtained from **A** and aminobenzoic acids **11**−**13** or amino acids **14**−**38**. Thus, the most active such compounds were those incorporating β-phenylserine (**A20**), α-phenylglycine (**A18**), hydroxy (Ser, Thr; **A19**−**A21**) and mercapto (Cys, Met; **A22, A23**) amino acids, hydrophobic amino acids (Val, Leu, Ile; **A24**−**A26**), aromatic amino acids (His, Phe, Tyr, DOPA; **A32**−**A35**), and dicarboxylic (Asp, Glu, and their corresponding amides Asn, Gln; **A27**−**A30**) amino acid moieties. Such compounds possessed inhibition constants in the range of 2−10 nM against hCA II, 9−23 nM against bCA IV, and 21−40 nM against hCA I (being more active than the classical inhibitors of type **1**−**6** mentioned above, which have been used as standards in these measurements, Table 1). Slightly less active were, on the other hand, the derivatives of aminobenzoic acids (**A11**−**A13**) and those of Gly, Ala, β-Ala, and GABA (**A14**−**A17**), as well as those of Trp, Lys, and Arg (**A36**−**A38**), with inhibition constants in the range of 11−20 nM against hCA II, 24−61 nM against bCA IV, and 45−115 nM against hCA I (but one must mention that these compounds have the same potency as acetazolamide or methazolamide, clinically used CA inhibitors). Among all the thioureas reported here, the most ineffective CA inhibitor was the proline derivative **A31**, probably due to its relatively bulky nature (this compound is anyhow a strong inhibitor, comparable with the clinically used dichlorophenamide **6**). Strong inhibitory properties were, on the other hand, observed for the oligopeptidyl derivatives **A39**−**A47**. Thus, except for the Gly-Gly (**A39**) and Asp-Asp (**A45**) derivatives (which possess the same activity as methazolamide **4** against hCA II), all the other oligopeptidyl thioureas prepared in the present study were very potent inhibitors, with inhibition constants in the range of 1−10 nM against hCA II, 6−27 nM against bCA IV, and 13−59 nM against hCA I. Very good inhibitory properties were correlated with the presence of His moieties in such oligopeptidyl thioureas (such as **A40**−**A42**). It is also interesting to note the influence of the number of aspartyl residues, in some of the thioureas prepared in this study, on the CA inhibitory properties of the obtained compounds. Thus, among the Asp, Asp-Asp, and $(Asp)_4$ derivatives, best activity was observed for the simple mono derivative **A27**, whereas the most ineffective inhibitor was the dipeptidyl derivative **A45** (with the K_i against hCA II doubled as compared to that for **A27**). On the other hand, the tetrapeptide derivative **A47** regained much of the hCA II inhibitory activity but showed a much lower affinity for bCA IV and hCA I. It is difficult to explain at the present moment this intricate behavior. From all these data, the main SAR conclusion is that best inhibitors are obtained when the molecule of the thiourea is prolonged in the direction of the axis passing through the Zn(II) ion of the enzyme, the sulfonamide sulfur atom, and the thiourea nitrogen atom of the inhibitor, as was in fact explained theoretically by QSAR studies form our group for other series

of sulfonamide CA inhibitors[12,13] (the exceptions from this rule are just the aspartyl derivatives mentioned above: **A27**, **A45**, and **A47**). Among the three CA isozymes investigated here, the most susceptible to inhibition was hCA II followed by bCA IV and then hCA I. This is of great importance, since the sensitive isozymes are only those involved in aqueous humor secretion within the eye, i.e., CA II and CA IV.

IOP Lowering in Normotensive and Glaucomatous Rabbits. In vivo IOP-lowering experiments were done in normotensive and glaucomatous rabbits with many of the new compounds reported here, due to their strong CA II and CA IV inhibitory properties: **A7, A10, A11, A14, A15, A17**−**A22, A25**−**A28, A31**−**A33, A37, A38, A40,** and **A43**. The following may be noted regarding the data of Table 3: (i) Some of the new derivatives, such as **A19**, **A20**, and **A28**, provoked a very strong and rapid decrease of IOP when applied topically, with IOP lowering of 7.0−9.0 mmHg at 30 min after administration (compared with 2.2 mmHg IOP lowering after dorzolamide), which at 1 h amounted to 9.5−10.0 mmHg (versus 4.1 mmHg of dorzolamide), and this potent effect was maintained at 1.5 h (6.0−9.6 mmHg); furthermore, IOP returned to baseline values 6 h after administration. Thus, such new inhibitors are much more potent hypotensor agents, and their effect is much longer than that of the standard drug dorzolamide (Figure 1). It is interesting to note that two of these very effective IOP-lowering agents mentioned above are the Ser and β-Ph-Ser derivatives, whereas the third one is the Asn derivative. More surprisingly, the very similar Asp derivative (**A27**) is a modest IOP hypotensor agent, also producing eye irritation when administered topically (this was the only compound in this series that showed this unpleasant side effect). This fact also demonstrates that very small structural modifications (replacement of the $CONH_2$ moiety by the COOH one) lead to a completely different pharmacological profile for the two derivatives containing them, although their CA inhibitory properties are rather similar. (ii) Compounds such as **A11, A14, A15, A17, A18, A32,** and **A33** showed an IOP lowering of 3−7 mmHg at 30 min after administration (being more effective than dorzolamide), whereas at 1 h their effect was in the range of 2.3−8 mmHg and at 90 min of 0.5−5.2 mmHg. Thus, although more effective than the standard drug at 30 min after administration, many of these agents show a diminished effect at later times, probably due to their washing away from the ciliary processes. This may be due to an improper balance between lipo- and hydrophilicity for some of these compounds, as will be discussed shortly. (iii) Derivatives such as **A7, A10, A26, A38, A40,** and **A43** showed a modest effect in the decrease of IOP after administration, with IOP lowering of 0.2−3.0 mmHg after 30 min, 0.2−4.0 mmHg after 60 min, and 0.0−4.0 mmHg after 90 min. (iv) Compounds such as **A22**, **A31**, and **A37** did not reduce IOP after topical administration (or had a very modest such effect).

The same powerful IOP lowering after administration of some derivatives prepared in the present study, such as **A19**, **A20**, and **A28**, has been observed in glaucomatous rabbits (Table 4). In this case, at 30 min after topical administration pressure was lowered with 10.0−10.4 mmHg by the new sulfonamides (with dorzolamide the IOP lowering was 3.6 mmHg); at 60 min the IOP lowering with the first derivatives mentioned above was in the range of 13.0−15.0 mmHg (6.7 with **1**), whereas at 90 min it was of 15.5−17.5 mmHg (versus 4.2 mmHg with **1**). We stress again that the pH of all solutions of the new inhibitors described here, used in the in vivo experiments, were in the range of 6.5−7.0 pH units and that no eye irritation was observed after topical administration.

To this point it is important to return to the correlation between the physicochemical properties of the new CA inhibitors reported here (Table 2) and their in vivo activity as topical hypotensor agents. One of the most interesting observations of this work is that some of the newly obtained compounds, although acting as very potent hCA II (and bCA IV) inhibitors, showed an unexpectedly small IOP-lowering effect via the topical route, whereas structurally very similar derivatives acted as strong hypotensors (compare **A19** and **A22**, which differ minimally by the presence of an OH moiety in the first and an SH one in the second compound, or the example mentioned above, the Asp and Asn derivatives **A27** and **A28**). Already Maren[2] noted in his classical reviews that the most restrictive conditions needed for a sulfonamide to act as an effective IOP-lowering agent are to possess modest (but not insignificant) lipid solubility (attributable to its un-ionized form) accompanied by good water solubility (eventually conferred by the presence of ionizable groups of appropriate pK_a). As seen from data of Table 2, the very active compounds reported here that act as effective topical hypotensors, such as **A19**, **A20**, and **A28**, possessed just this type of balanced physicochemical properties, which led to good accession rates across the cornea followed by sustained inhibition of the ciliary processes enzyme. In contrast, the Cys (**A22**) or Asp (**A27**) derivatives mentioned above, although effective CA inhibitors and possessing very good water solubility, possess too low hydrophobicity (due to the presence of the second ionizable moiety, SH in the first case and COOH in the second one) and as a consequence an impaired penetration through the cornea (observe the low k_{in} values of Table 2, similar to those of benzolamide **48**). The same



48

seems to be true for other derivatives investigated here, such as the Lys, Arg, or oligopeptidyl derivatives (**A37**, **A38**, **A40,** and **A43**), which again showed relatively modest IOP lowering, although they were extremely efficient in vitro CA inhibitors. One must also mention that a very hydrophobic inhibitor (such as ethoxzolamide **6**) is also ineffective topically, although it possesses very high accession rates through the cornea. Still, this property also allows its rapid diffusibility into red blood cells, and in consequence its washing away from the ciliary processes due to the blood circulation. This might explain why some of the compounds reported here (such as the α-Ph-Gly derivative **A18** or the GABA derivative **A17**) initially showed a potent IOP lowering (at 30 min after administration), whereas this effect rapidly diminished at later periods after administration. One may

observe that **A18** was the most hydrophobic among the inhibitors investigated in some detail in this study (Table 2), and this probably leads not only to very good accession rates across the cornea but also to its rapid clearing from the ciliary processes, similarly to ethoxzolamide (**6**) mentioned above. Thus, this series of CA inhibitors proves in a clear-cut manner that in order to obtain hypotensors with prolonged duration of action via the topical route, the physicochemical and enzyme inhibitory properties must be fine-tuned in a very precise manner, and such compounds should possess a proper balance between lipo- and hydrophobicity, in addition to acting as potent hCA II inhibitors and possessing acceptable water solubility.

Drug Distribution in Ocular Fluids and Tissues. In Table 5 the drug distribution in ocular fluids and tissues is shown, after the topical administration of compounds **A20** and **A28**. It is seen that 1 and 2 h after topical administration of the drug, high levels of inhibitors were found in the cornea, aqueous humor, and ciliary processes. On the basis of the inhibition constant of these compounds, the fractional inhibition estimated in these tissues/fluids is of 99.5−99.9%,[2] proving the fact that the IOP decrease is indeed due to CA inhibition. Furthermore, as seen from the data of Table 5, the new compounds reported here tend to concentrate in the aqueous humor (concentrations of around 285−305 μM were detected 1 h after administration), whereas dorzolamide reaches much lower such concentrations (32 μM after 1 h). High concentrations of the inhibitor were maintained 2 h after administration too. Thus, the strong and long-lasting IOP-lowering properties of the new compounds reported here are probably due to this concentrating effect reached mainly in the aqueous humor but which is also present in the cornea and ciliary processes.

Conclusions

We report here a novel class of sulfonamides, obtained by reaction of 4-isothiocyanatobenzenesulfonamide with amines, amino acids, or oligopeptides. Many of the newly reported derivatives showed very good water solubility, at nearly neutral pH values, whereas their lipid solubility, hydrophobicity (log *P*), and accession rates across the cornea were those appropriate for acting as efficient topical IOP-lowering agents. Some of these new CA inhibitors possessed affinities in the nanomolar range for isozymes hCA II and bCA IV, acting as effective enzyme inhibitors in vitro. Several of the most active inhibitors strongly lowered IOP pressure in normotensive and glaucomatous rabbits, showing a prolonged duration of action as compared to dorzolamide. The new compounds reported here might lead to the development of more efficient antiglaucoma drugs from the class of the sulfonamide CA inhibitors.

Experimental Section

Chemistry. Melting points were recorded with a heating plate microscope and are not corrected. IR spectra were recorded in KBr pellets with a Carl Zeiss IR-80 instrument. ^{1}H NMR spectra were recorded in DMSO-d_6 as solvent, with a Bruker CPX200 or Varian 300 instrument. Chemical shifts are reported as δ values, relative to Me_4Si as internal standard. Elemental analyses were done by combustion for C, H, N with an automated Carlo Erba analyzer and were ±0.4% of the theoretical values. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Analytical and preparative HPLC was performed on a reversed-phase C_{18} Bondapack column, with a Beckman EM-1760 instrument. Sulfanilamide, thiophosgene, and nucleophiles (amines, amino acids, and oligopeptides) used in the synthesis were of highest purity, commercially available compounds (from Sigma-Aldrich, Fluka, E. Merck or Acros). Acetonitrile, acetone (E. Merck) and other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions.

General Procedure for the Preparation of Compounds A7−A47. An amount of 0.53 g (2.5 mmol) of 4-isothiocyanatobenzenesulfonamide **A**[9] and the stoichiometric amount of nucleophile **7−47** 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 either recrystallized from ethanol or ethanol−water (for the largest majority of thioureas described here) or purified by preparative HPLC in the case when the reaction mixture contained appreciable amounts of impurities (as evidenced by TLC). This was particularly the case for the oligopeptidyl thioureas **A39−A47**. Conditions used for the purification were: C_{18} reversed-phase μ-Bondapack or Dynamax-60A (25 × 250 mm) preparative columns; 90% acetonitrile/8% methanol/2% water, 30 mL/min.

1-(4-Sulfamoylphenyl)-3-[(1*H*-imidazol-4-yl)ethyl]thiourea (histamine derivative, A10): white crystals, mp 148−9 °C (ethanol−water 1:1, v/v). IR (KBr), cm^{-1}: 1150 (SO_2sym), 1245 (thioamide III), 1375 (SO_2as), 1550 (thioamide II), 1680 (thioamide I), 3060 (NH). ^{1}H NMR (DMSO-d_6), δ, ppm: 2.49 (t, 2H, J = 7.0, Hst CH_2); 2.99 (t, 2H, J = 7.0, Hst CSNHCH_2); 7.34 (m, 1H, imidazole CH); 7.52 (s, 2H, SO_2NH_2), 7.65 (d, $^3J_{HH}$ = 8.1, 2H, H_{ortho} of $H_2NO_2SC_6H_4$), 7.94 (d, $^3J_{HH}$ = 8.1, 2H, H_{meta} of $H_2NO_2SC_6H_4$); 8.21 (br s, 2H, NHCSNH); 8.35 (s, 1H, imidazole CH); 8.80 (s, 1H, imidazole NH). ^{13}C NMR (DMSO-d_6), δ, ppm: 33.3 (s, *C*H$_2$ of Hst); 37.6 (s, *C*H$_2$ of Hst); 123.7 (s, C-4 of Hst); 130.9 (s, C_{meta} of $H_2NO_2SC_6H_4$), 134.2 (s, C-5 of Hst); 135.0 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 137.3 (s, C-2 of Hst); 139.4 (s, NH*C*SNH), 144.6 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 148.0 (s, C_{para} of $H_2NO_2SC_6H_4$). Anal. Found: C, 44.13; H, 4.30; N, 21.39. $C_{15}H_{15}N_5O_2S_2$ requires C, 44.29; H, 4.65; N, 21.52.

1-(4-Sulfamoylphenyl)-3-(carboxymethyl)thiourea (glycine derivative, A14): white crystals, mp 214−6 °C (ethanol−water 1:2, v/v). IR (KBr), cm^{-1}: 1153 (SO_2sym), 1240 (thioamide III), 1375 (SO_2as), 1550 (thioamide II), 1682 (thioamide I), 1754 (COOH), 3065 (NH). ^{1}H NMR (DMSO-d_6), δ, ppm: 3.67 (s, 2H, CH_2 of Gly); 7.54 (s, 2H, SO_2NH_2), 7.63 (d, $^3J_{HH}$ = 8.1, 2H, H_{ortho} of $H_2NO_2SC_6H_4$), 7.94 (d, $^3J_{HH}$ = 8.1, 2H, H_{meta} of $H_2NO_2SC_6H_4$); 8.21 (br s, 2H, NHCSNH); 10.63 (br s, 1H, COOH). ^{13}C NMR (DMSO-d_6), δ, ppm: 40.8 (s, *C*H$_2$ of Gly); 131.5 (s, C_{meta} of $H_2NO_2SC_6H_4$), 135.4 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 139.7 (s, NH*C*SNH), 144.1 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 147.3 (s, C_{para} of $H_2NO_2SC_6H_4$); 179.6 (COOH). Anal. Found: C, 37.48; H, 3.75; N, 14.26. $C_9H_{11}N_3O_4S_2$ requires C, 37.36; H, 3.83; N, 14.52.

1-(4-Sulfamoylphenyl)-3-(1-carboxy-2-methylbutyl)-thiourea (isoleucine derivative, A26): white crystals, mp 179−81 °C (ethanol−water 1:1, v/v). IR (KBr), cm^{-1}: 1151 (SO_2sym), 1244 (thioamide III), 1376 (SO_2as), 1557 (thioamide II), 1676 (thioamide I), 1750 (COOH), 3065 (NH). ^{1}H NMR (DMSO-d_6), δ, ppm: 1.15 (d, $^3J_{HH}$ = 6.5, 3H, CH_3 of Ile), 1.21 (t, 3H, $^3J_{HH}$ = 6.7, CH_3 of Et moiety of Ile); 1.54 (m, 2H, CH_2 of Ile); 3.22 (m, 1H, EtC*H*(Me)- of Ile); 3.75 (m, 1H, NHC*H*CO of Ile), 7.51 (s, 2H, SO_2NH_2), 7.66 (d, $^3J_{HH}$ = 8.0, 2H, H_{ortho} of $H_2NO_2SC_6H_4$), 7.92 (d, $^3J_{HH}$ = 8.0, 2H, H_{meta} of $H_2NO_2SC_6H_4$); 8.20 (br s, 2H, NHCSNH); 10.42 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6), δ, ppm: 21.7 (s, CH*C*H$_3$ of Ile), 22.6 (s, *C*H$_3$CH$_2$ of Ile). 31.4 (s, *C*H$_2$ of Ile); 34.5 (s, *C*H(CH$_3$)$_2$ of Leu), 46.4 (s, Et*C*H(Me)- of Ile), 55.0 (s, NH*C*HCH$_2$ of Ile), 131.3 (s, C_{meta} of $H_2NO_2SC_6H_4$), 135.5 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 139.9 (s, NH*C*SNH), 144.3 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 147.8 (s, C_{para} of $H_2NO_2SC_6H_4$); 179.1 (COOH). Anal. Found: C, 45.33; H, 5.30; N, 11.97. $C_{13}H_{19}N_3O_4S_2$ requires C, 45.20; H, 5.54; N, 12.16.

1-(4-Sulfamoylphenyl)-3-(1-carboxy-2-phenylethyl)-thiourea (phenylalanine derivative, A33): white crystals, mp 223−4 °C (ethanol−water 1:1, v/v). IR (KBr), cm^{-1}: 1148 (SO_2sym), 1243 (thioamide III), 1376 (SO_2as), 1550 (thioamide II), 1684 (thioamide I), 1751 (COOH), 3065 (NH). ^{1}H NMR (DMSO-d_6), δ, ppm: 3.10−3.55 (m, 2H, CH_2CH of Phe), 4.08 (dd, $^3J_{HH}$ = 5.0, $^3J_{HH}$ = 7.8, 1H, CH_2CH of Phe), 7.29−7.51 (m, 5H, H_{arom} of Phe);7.57 (s, 2H, SO_2NH_2), 7.63 (d, $^3J_{HH}$ = 8.1, 2H, H_{ortho} of $H_2NO_2SC_6H_4$), 7.96 (d, $^3J_{HH}$ = 8.1, 2H, H_{meta} of $H_2NO_2SC_6H_4$); 8.20 (br s, 2H, NHCSNH); 10.71 (br s, 1H, COOH). ^{13}C NMR (DMSO-d_6), δ, ppm: 41.5 (s, CH$_2$CH of Phe), 59.3 (s, CH$_2$$C$H of Phe), 131.5 (s, C_{meta} of $H_2NO_2SC_6H_4$), 133.8 (s, C_{meta} of Phe), 134.4 (s, C_{ortho} of Phe), 135.4 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 139.7 (s, NHCSNH), 141.5 (s, C_{ipso} of Phe), 144.1 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 145.6 (s, C_{para} of Phe), 147.3 (s, C_{para} of $H_2NO_2SC_6H_4$); 179.3 (COOH). Anal. Found: C, 50.49; H, 4.60; N, 10.86. $C_{16}H_{17}N_3O_4S_2$ requires C, 50.65; H, 4.52; N, 11.07.

4-Sulfamoylphenylthioureido-β-alanylhistidine (carnosine derivative, A40): white crystals, mp 127−8 °C. IR (KBr), cm^{-1}: 1156 (SO_2sym), 1240 (thioamide III),1287 (amide I), 1375 (SO_2as), 1540 (amide I), 1550 (thioamide II), 1683 (thioamide I), 1720 (amide I), 1750 (COOH), 3065 (NH). ^{1}H NMR (DMSO-d_6), δ, ppm: 2.79−2.88 (m, 2H, CH_2 of β-Ala), 3.11−3.26 (m, 2H, CH_2 of β-Ala), 3.34−3.45 (m, 2H, CHCH_2 of His), 4.57−4.63 (m, 1H, CHCH$_2$ of His), 7.33 (s, 1H, CH-5 of His), 7.54 (s, 2H, SO_2NH_2), 7.63 (d, $^3J_{HH}$ = 8.1, 2H, H_{ortho} of $H_2NO_2SC_6H_4$), 7.94 (d, $^3J_{HH}$ = 8.1, 2H, H_{meta} of $H_2NO_2SC_6H_4$); 8.21 (br s, 2H, NHCSNH); 8.29 (br s, 1H, CONH); 8.35 (s, 1H, CH-2 of His); 8.80 (s, 1H, imidazole NH from His)10.63 (br s, 1H, COOH). ^{13}C NMR (DMSO-d_6), δ, ppm: 33.3 (s, CH$_2$ of His), 37.4 (s, NHCH$_2$CH$_2$ of β-Ala), 40.8 (s, CH$_2$$CH_2$CO of β-Ala), 59.6 (s, CHCH$_2$ of His), 122.2 (s, C-4 of His), 131.5 (s, C_{meta} of $H_2NO_2SC_6H_4$), 134.2 (s, C-5 of His), 135.3 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 137.5 (s, C-2 of His), 139.4 (s, NHCSNH), 144.1 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 147.1 (s, C_{para} of $H_2NO_2SC_6H_4$); 175.6 (s, CH$_2$$C$O of β-Ala), 180.1 (COOH). Anal. Found: C, 43.54; H, 4.33; N, 19.00. $C_{16}H_{20}N_6O_5S_2$ requires C, 43.63; H, 4.58; N, 19.08.

CA Inhibition. hCA I and hCA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog's group.[14] Cell growth conditions were those described in ref 15 and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[16] 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 29.3 kDa for CA II, respectively.[17,18] bCA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide.[19]

Initial rates of 4-nitrophenylacetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM-compatible PC.[20] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25 °C. A molar absorption coefficient ϵ of 18 400 M^{-1} cm^{-1} was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature.[20] Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. 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 10 min at room temperature prior to assay, to allow for the formation of the E·I complex. The inhibition constant K_i was determined as described by Pocker and Stone.[20] Enzyme concentrations were 3.6 nM for CA II, 9.1 nM for CA I, and 30 nM for CA IV (this isozyme has a decreased esterase activity[21] and higher concentrations had to be used for the measurements). Adult male New Zealand albino rabbits weighing 3−3.5 kg were used in the experiments (3 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 or sodium carboxylates) were obtained in distilled−deionized water. The pH of these solutions was in the range of 5.5−8.4.

Measurement of Tonometric IOP. IOP was measured using a Digilab 30R pneumatonometer (BioRad, Cambridge, MA) as described by Maren's group.[22,23] 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 are 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 eye and control eye, in this way minimizing the diurnal, seasonal, and interindividual variations commonly observed in the rabbit.[22,23] All data are expressed as mean ± SE, using a one-tailed t-test.

Ocular hypertension was elicited in the right eye of albino rabbits by the injection of α-chymotrypsin (from Sigma) as described by Melena et al.[24] The IOP of operated animals was checked after approximately 4 weeks, and animals with an elevated pressure of 30−35 mmHg were used at least 1 month after injection of α-chymotrypsin.

Drug Distribution in Ocular Fluids and Tissues. The general procedure of Maren's group has been followed.[22,23] The animals were killed with an intracardiac injection. Aqueous humor (both posterior and anterior chamber fluids) was withdrawn. Then, the cornea and anterior uvea (iris plus attached ciliary body) were dissected, rinsed well with water, blotted, weighed and put into 1−2 mL of water. For isolation of the ciliary processes, intact anterior uvea rings were placed on a Parafilm-covered piece of polystyrene foam in a Petri dish. The tissue has been wetted with normal saline and dissected under a microscope, when ciliary processes were liberated from their attachment to the iris, cut, weighed and put in 0.5 mL of distilled water. The tissues from 4 eyes (average weight of 8 mg/eye) were pooled for drug analysis. Samples were boiled for 5 min (in order to denature CA and free drug from the E·I complex), diluted and then incubated with a known amount of enzyme. The activity of the free enzyme and in the presence of the inhibitor were determined as described above. A calibration curve was used in order to determine the fractional inhibition in the different tissues, as described in refs 22, 23.

Determination of Water (Buffer) Solubility. A standard solution was prepared by dissolving a precisely weighted amount (generally 1 mg) of inhibitor in 10 mL of methanol. The UV absorption maximum of each compound was determined (with a Cary 3 spectrophotometer) eventually diluting the solution (with MeOH) as necessary. A saturated solution of each compound was then prepared by stirring magnetically a small volume of 0.039 M phosphate buffer (pH 7.4) in the presence of excess inhibitor for 3 h. The obtained saturated solution was filtered in order to remove solid compound through a Millipore 0.45-μm filter and scanned by UV at the wavelength of the absorption maximum previously determined. Total solubility was determined by the relationship: $C' = A'C/A$, where C = concentration of standard solution (mg/

mL), A = absorbance of standard solution, A' = absorbance of saturated solution, C' = concentration of saturated solution (mg/mL).[25]

Partition Coefficient Determinations. Chloroform−buffer partition coefficients were obtained by equilibrating the test compound between chloroform and 0.1 ionic strength pH 7.4 phosphate buffer. The concentration in each phase was determined by UV spectrophotometry or HPLC.[26]

Transcorneal Penetration of Drugs. The method of Maren et al.[27] with the modifications of Pierce's group[28,29] (for the HPLC assay of sulfonamides) was used. Excised rabbit corneas with either intact or denuded epithelium were used in these experiments. The pH was 7.4 and exposed area was of 1.2 cm^2. Concentrations of drug of 40−2000 μM were placed in the epithelial chamber and samples of fluid were collected from the endothelial chamber at different intervals, up to 4 h. Both chambers contained 6 mL. Drugs present in these fluids were assayed both by the HPLC method of Pierce et al.[28,29] or enzymatically.[27] The results of the drug analyses were used to calculate the rate constant of transfer across the cornea (k_{in}). As described by Pierce,[28,29] this value was determined by using the formula: k_{in} ($\times 10^3$ h^{-1}) = $[drug]_{endo}/[drug]_{epi} \times 60/t \times 1000$, where $[drug]_{endo}$ = concentration of drug on endothelial side, $[drug]_{epi}$ = concentration of drug on epithelial side, t = time (in min).

Acknowledgment. This research was financed by EU Grant ERB CIPDCT 940051 and by a grant from the Italian CNR − Target Project Biotechnology.

References

(1) (a) Supuran, C. T.; Scozzafava, A. Carbonic anhydrase inhibitors and their therapeutic potential. *Exp. Opin. Ther. Patents* **2000**, *10*, 575−600. (b) Supuran, C. T. Carbonic anhydrase inhibitors. In *Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism*; Puscas, I., Ed.; Helicon: Timisoara, Romania, 1994; pp 29−111.

(2) (a) Maren, T. H. The links among biochemistry, physiology and pharmacology in carbonic anhydrase mediated systems. In *Carbonic Anhydrase − From Biochemistry and Genetics to Physiology and Clinical Medicine*; Botré, F., Gros, G., Storey, B. T., Eds.; VCH: Weinheim, Germany, 1991; pp 186−207. (b) Maren, T. H. The development of topical carbonic anhydrase inhibitors. *J. Glaucoma* **1995**, *4*, 49−62.

(3) Sugrue, M. F. Pharmacological and ocular hypotensive properties of topical carbonic anhydrase inhibitors. *Prog. Ret. Eye Res.* **2000**, *19*, 87−112.

(4) Silver, L. H. Dose−response evaluation of the ocular hypotensive effect of brinzolamide ophthalmic suspension (Azopt). Brinzolamide dose−response study group. *Surv. Ophthalmol.* **2000**, *44* (Suppl. 2), 147−153.

(5) Chen, H. H.; Gross, S.; Liao, J.; McLaughlin, M.; Dean, T.; Sly, W. S.; May, J. A. 2*H*-Thieno [3,2-*e*]- and [2,3-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxides as ocular hypotensive agents: synthesis, carbonic anhydrase inhibition and evaluation in the rabbit. *Bioorg. Med. Chem.* **2000**, *8*, 957−975.

(6) (a) Scozzafava, A.; Menabuoni, L.; Mincione, F.; Briganti, F.; Mincione, G.; Supuran, C. T. Carbonic anhydrase inhibitors: Synthesis of water-soluble, topically effective intraocular pressure lowering aromatic/heterocyclic sulfonamides containing cationic or anionic moieties: is the tail more important than the ring? *J. Med. Chem.* **1999**, *42*, 2641−2650. (b) Scozzafava, A.; Briganti, F.; Mincione, G.; Menabuoni, L.; Mincione, F.; Supuran, C. T. Carbonic anhydrase inhibitors: Synthesis of water-soluble, amino acyl/dipeptidyl sulfonamides possessing long-lasting intraocular pressure-lowering properties via the topical route. *J. Med. Chem.* **1999**, *42*, 3690−3700.

(7) (a) Borras, J.; Scozzafava, A.; Menabuoni, L.; Mincione, F.; Briganti, F.; Mincione, G.; Supuran, C. T. Carbonic anhydrase inhibitors: Synthesis of water-soluble, topically effective intraocular pressure lowering aromatic/heterocyclic sulfonamides containing 8-quinoline-sulfonyl moieties: is the tail more important than the ring? *Bioorg. Med. Chem.* **1999**, *7*, 2397−2406. (b) Renzi, G.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors: Topical sulfonamide antiglaucoma agents incorporating secondary amine moieties. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 673−676. (c) Ilies, M.; Supuran, C. T.; Scozzafava, A.; Casini, A.; Mincione, F.; Menabuoni, L.; Caproiu, M. T.; Maganu, M.; Banciu, M. D. Carbonic anhydrase inhibitors. Sulfonamides incorporating furan-, thiophene- and pyrrole-carboxamido groups possess strong topical intraocular pressure lowering properties as aqueous suspensions. *Bioorg. Med. Chem.* **2000**, *8*, 2145−2155.

(8) (a) Supuran, C. T.; Briganti, F.; Menabuoni, L.; Mincione, G.; Mincione, F.; Scozzafava, A. Carbonic anhydrase inhibitors. Part 78. Synthesis of water-soluble sulfonamides incorporating β-alanyl moieties, possessing long lasting-intraocular pressure lowering properties via the topical route. *Eur. J. Med. Chem.* **2000**, *35*, 309−321. (b) Mincione, G.; Menabuoni, L.; Briganti, F.; Mincione, F.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. Part 79. Synthesis and ocular pharmacology of topically acting sulfonamides incorporating GABA moieties in their molecule, with long-lasting intraocular pressure lowering properties. *Eur. J. Pharm. Sci.* **1999**, *9*, 185−199.

(9) McKee, R. L.; Bost, R. W. *p*-Substituted phenyl isothiocyanates and some related thioureas. *J. Am. Chem. Soc.* **1946**, *68*, 2506−2507.

(10) (a) Browne, D. W.; Dyson, G. M. The inhibitory effect of substituents in chemical reactions. Part II. The reactivity of the isothiocyano group in substituted arylthiocarbimides. *J. Chem. Soc.* **1931**, 3285−33909. (b) Browne, D. W.; Dyson, G. M. The inhibitory effect of substituents in chemical reactions. Part III. The reactivity of the isothiocyanato group in substituted arylthiocarbimides. *J. Chem. Soc.* **1934**, 178−179.

(11) Briganti, F.; Mangani, S.; Orioli, P.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T. Carbonic anhydrase activators: X-ray crystallographic and spectroscopic investigations for the interaction of isozymes I and II with histamine. *Biochemistry* **1997**, *36*, 10384−10392.

(12) (a) Supuran, C. T.; Clare, B. W. Carbonic anhydrase inhibitors Part 24. A quantitative structure−activity relationship study of positively charged sulfonamide inhibitors. *Eur. J. Med. Chem.* **1995**, *30*, 687−696. (b) Maren, T. H.; Clare, B. W.; Supuran, C. T. Structure−activity studies of sulfonamide carbonic anhydrase inhibitors. *Roum. Chem. Quart. Rev.* **1994**, *2*, 259−282. (c) Clare, B. W.; Supuran, C. T. Carbonic anhydrase inhibitors Part 41. Quantitative structure−activity correlations involving kinetic rate constants of 20 sulfonamides from a noncongeneric series. *Eur. J. Med. Chem.* **1997**, *32*, 311−319. (d) Supuran, C. T.; Clare, B. W. Carbonic anhydrase inhibitors Part 47. Quantum chemical quantitative structure−activity relationships for a group of sulfanilamide Schiff base inhibitors of carbonic anhydrase. *Eur. J. Med. Chem.* **1998**, *33*, 489−500.

(13) (a) Supuran, C. T.; Clare, B. W. Carbonic anhydrase inhibitors. Part 57. Quantum chemical QSAR of a group of 1,3,4-thiadiazole and 1,3,4-thiadiazoline disulfonamides with carbonic anhydrase inhibitory properties. *Eur. J. Med. Chem.* **1999**, *34*, 41−50. (b) Clare, B. W.; Supuran, C. T. Carbonic anhydrase inhibitors. Part 61. Quantum Chemical QSAR of a group of benzenedisulfonamides. *Eur. J. Med. Chem.* **1999**, *34*, 463−474. (c) Clare, B. W.; Supuran, C. T. Carbonic anhydrase inhibitors. Part 86. A QSAR Study on some Sulfonamide Drugs which Lower Intra-Ocular Pressure. *Eur. J. Med. Chem.* **2000**, *35*, 859−865.

(14) Lindskog, S.; Behravan, G.; Engstrand, C.; Forsman, C.; Jonsson, B. H.; Liang, Z.; Ren, X.; Xue, Y. Structure−function relations in human carbonic anhydrase II as studied by site-directed mutagenesis. In *Carbonic anhydrase − From biochemistry and genetics to physiology and clinical medicine*; Botrè, F., Gros, G., Storey, B. T. Eds.; VCH: Weinheim, Germany, 1991; pp 1−13.

(15) Behravan, G.; Jonsson, B. H.; Lindskog, S. Fine-tuning of the catalytic properties of carbonic anhydrase. Studies of a Thr200-His variant of human isoenzyme II. *Eur. J. Biochem.* **1990**, *190*, 351−357.

(16) Khalifah, R. G.; Strader, D. J.; Bryant, S. H.; Gibson, S. M. Carbon-13 nuclear magnetic resonance probe of active site ionization of human carbonic anhydrase B. *Biochemistry* **1977**, *16*, 2241−2247.

(17) Lindskog, S.; Coleman, J. E. The catalytic mechanism of carbonic anhydrase. *Proc. Natl. Acad Sci. U.S.A.* **1964**, *70*, 2505−2508.

(18) Steiner, H.; Jonsson, B. H.; Lindskog, S. The catalytic mechanism of carbonic anhydrase. Hydrogen-isotope effects on the kinetic parameters of the human C isoenzyme. *Eur. J. Biochem.* **1975**, *59*, 253−259.

(19) Maren, T. H.; Wynns, G. C.; Wistrand, P. J. Chemical properties of carbonic anhydrase IV, the membrane-bound enzyme. *Mol. Pharmacol.* **1993**, *44*, 901−906.

(20) Pocker, Y.; Stone, J. T. The catalytic versatility of erythrocyte carbonic anhydrase. III. Kinetic studies of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate. *Biochemistry* **1967**, *6*, 668−678.

(21) Baird, T. T.; Waheed, A.; Okuyama, T.; Sly, W. S.; Fierke, C. A. Catalysis and inhibition of human carbonic anhydrase IV. *Biochemistry* **1997**, *36*, 2669−2678.

(22) Maren, T. H.; Brechue, W. F.; Bar-Ilan, A. Relations among IOP reduction, ocular disposition and pharmacology of the carbonic anhydrase inhibitor ethoxzolamide. *Exp. Eye Res.* **1992**, *55*, 73−79.

(23) Brechue, W. F.; Maren, T. H. pH and drug ionization affects ocular pressure lowering of topical carbonic anhydrase inhibitors. *Invest. Ophthalmol. Vis. Sci.* **1993**, *34*, 2581−2587.
(24) Melena, J.; Santafe, J.; Segarra-Domenech, J.; Puras, G. Aqueous humor dynamics in alpha-chymotrypsin-induced ocular hypertensive rabbits. *J. Ocul. Pharmacol. Ther.* **1999**, *15*, 19−27.
(25) Supuran, C. T.; Ilies, M. A.; Scozzafava, A. Carbonic anhydrase inhibitors. Part 29. Interaction of isozymes I, II and IV with benzolamide-like derivatives. *Eur. J. Med. Chem.* **1998**, *33*, 739−751.
(26) Scozzafava, A.; Briganti, F.; Ilies, M. A.; Supuran, C. T. Carbonic anhydrase inhibitors: Synthesis of membrane-impermeant low molecular weight sulfonamides possessing in vivo selectivity for the membrane-bound versus the cytosolic isozymes. *J. Med. Chem.* **2000**, *43*, 292−300.
(27) Maren, T. H.; Jankowska, L.; Sanyal, G.; Edelhauser, G. F.; The transcorneal permeability of sulfonamide carbonic anhydrase inhibitors and their effect on aqueous humor secretion. *Exp. Eye Res.* **1983**, *36*, 457−480.
(28) Pierce, W. M. Jr.; Sharir, M.; Waite, K. J.; Chen, D.; Kaysinger, K. K. Topically active ocular carbonic anhydrase inhibitors: Novel biscarbonylamidothiadiazole sulfonamides as ocular hypotensive agents. *Proc. Soc. Exp. Biol. Med.* **1993**, *203*, 360−365.
(29) Sharir, M.; Pierce, W. M. Jr.; Chen, D.; Zimmerman, T. J. Pharmacokinetics, acid−base balance and intraocular pressure effects of ethyloxaloylazolamide − A novel topically active carbonic anhydrase inhibitor. *Exp. Eye Res.* **1994**, *58*, 107−116.

JM001051+