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?[1]

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Reaction of several aromatic/heterocyclic sulfonamides containing a free amino, imino, hydrazino, or hydroxyl group, with 2,3-pyridinedicarboxylic anhydride or 2,6-pyridinedicarboxylic acid in the presence of carbodiimide derivatives, afforded two series of water-soluble (as hydrochloride, triflate, or carboxylate salts) compounds. The new derivatives were assayed as inhibitors of the zinc enzyme carbonic anhydrase (CA) and more precisely of three of its isozymes, CA I, II (cytosolic forms), and IV (membrane-bound form), involved in important physiological processes. Efficient inhibition was observed against all three isozymes, but especially against CA II and IV (in nanomolar range), the two isozymes known to play a critical role in aqueous humor secretion within the ciliary processes of the eye. Some of the best inhibitors synthesized were applied as 2% water solutions directly into the eye of normotensive and glaucomatous albino rabbits. Very strong and long-lasting intraocular pressure (IOP) lowering was observed with many of them. This result prompted us to reanalyze the synthetic work done by other groups for the design of water-soluble, topically effective antiglaucoma sulfonamides. According to these researchers, the IOP-lowering effect is due to the intrinsic nature of the specific heterocyclic sulfonamide considered, among which the thienothiopyran-2-sulfonamide derivatives represent the best-studied case. Indeed, the first agents developed for topical application, such as dorzolamide, are derivatives of this ring system. To prove that the tail (in this case the pyridinecarboxylic moieties) conferring water solubility to a sulfonamide CA inhibitor is more important than the ring to which the sulfonamido group is grafted, we also prepared dorzolamide derivatives incorporating such moieties. These new compounds possess good water solubility as hydrochloride or carboxylate salts, balanced by a relatively modest lipid solubility. They are strong CA II inhibitors and are able to lower IOP in experimental animals more than the parent derivatives. Our conclusion is that the tail conferring water solubility to such an enzyme inhibitor is more important for topical activity as an antiglaucoma drug, than the heterocyclic/aromatic ring to which the sulfonamido moiety is grafted.

Introduction

The sulfonamides represent an important class of biologically active compounds, with at least five different classes of pharmacological agents obtained from the sulfanilamide structure as lead, the derivative initially studied by Domagk[2] as the first modern chemotherapeutic drug. Indeed, the antibacterial sulfonamides[3] continue to play an important role in chemotherapy, alone or in combination with other drugs,[4] and the sulfonamides that inhibit the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) possess many applications as diuretic, antiglaucoma, or antiepileptic drugs among others.[5−7] The hypoglycemic sulfonamides are extensively used in the treatment of some forms of diabetes,[8] whereas the thiazides and high-ceiling diuretics might be considered as a fortunate development of the CA inhibitors,[9] but these compounds possess a different pharmacological profile, independent of CA inhibition.[10,11] Finally, some antithyroid drugs have also been developed starting from the sulfonamide structure as lead molecule.[12]

The second class of the above-mentioned pharmacological agents, i.e., the sulfonamides with CA inhibitory action, have been thoroughly investigated in the last 10 years, searching for a topically effective antiglaucoma drug.[13−19] The possibility of administering a sulfonamide via the topical route directly into the eye, although investigated since the 1950s,[20,21] has been totally unsuccessful, whereas the systemic administration, quite useful in lowering intraocular pressure (IOP), was generally accompanied by undesired side effects, due to CA inhibition in tissues other than the eye.[21] In 1983, Maren's group[13] postulated that a water-soluble sulfonamide, possessing a relatively balanced lipid solubility as well as strong enough CA inhibitory properties, would be an effective IOP-lowering drug via the topical

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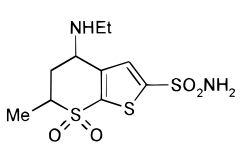
route, but at that moment no inhibitors possessing such physicochemical properties existed, as the organic chemistry of this class of compounds remained relatively little studied. Water-soluble sulfonamide CA inhibitors started to be developed in several laboratories soon thereafter,[13−19] and in 1995 the first of such pharmacological agents, dorzolamide (**1**), entered clinical use in the United States and Europe.[22] A second compound, brinzolamide (**2**), structurally quite similar to dorzolamide, has also recently been approved for the topical treatment of glaucoma in the United States.[23]



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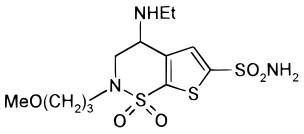

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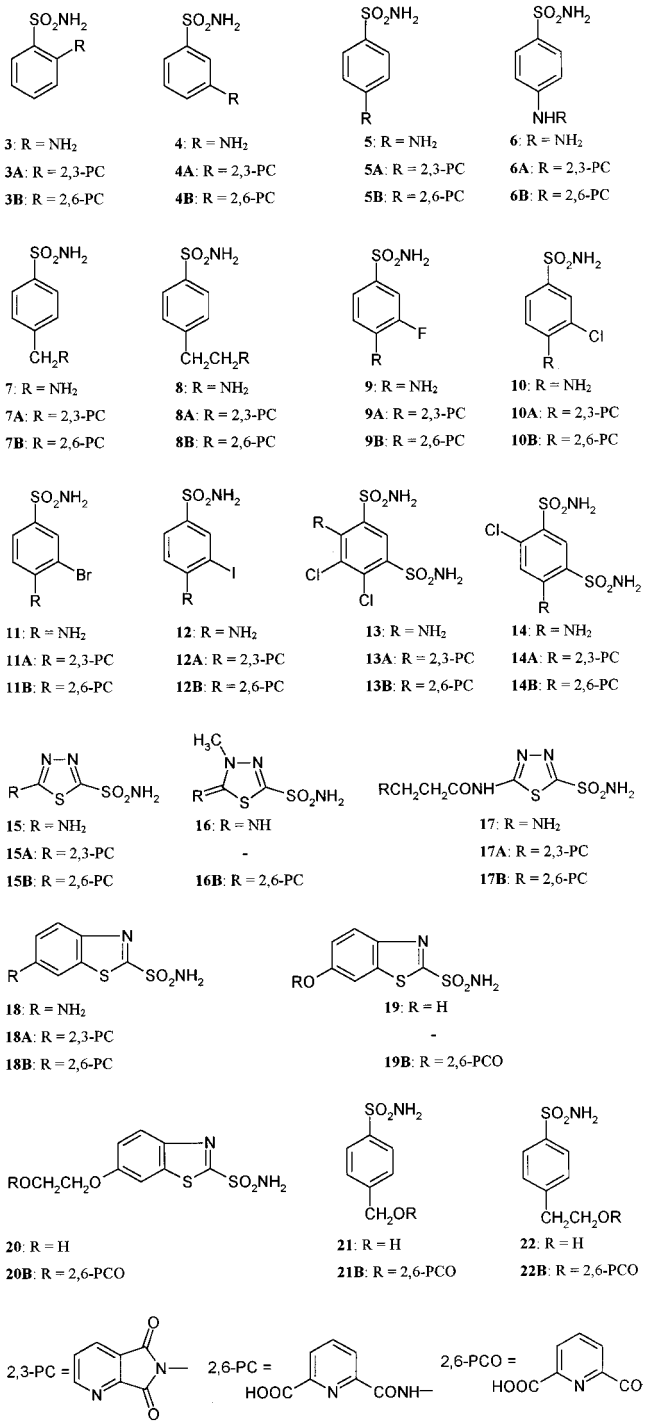
Thus, in a series of interesting papers,[15,24−28] the Merck, Sharp and Dohme group has developed the synthesis of a large series of bicyclic heterocyclic sulfonamides (derivatives of benzo[*b*]thiophene,[24] benzothiazole,[24] benzofuran,[25] indole,[25] thieno[2,3-*b*]pyrrole,[26] thienothiophene,[27] thienofuran,[28] and thienothiopyran[15,28]), which were then tested as IOP-lowering agents and led to the selection of the above-mentioned drug (dorzolamide). Still, the greatest majority of the synthesized compounds proved to be potent allergens in vivo, since their sulfonamido group was nucleophilically displaced by reduced glutathione or other nucleophiles present in the reaction medium. More than that, the only compounds with acceptable water solubility proved to be hydrochlorides of amino derivatives of the thienothiopyran sulfonamides of the dorzolamide type.[15,28] Obviously, the approach followed by this group consisted in the exploration of as many as possible heterocyclic ring systems on which the sulfonamido moiety should be grafted, and this approach was extremely beneficial for the chemistry of this class of compounds. Still, this approach seem to us to not be the only way to design topically active IOP-lowering agents, and we decided to explore the opposite one, i.e., to graft moieties that would ensure water solubility (as salts of a strong acid/base for instance) on the classical ring systems of the aromatic/heterocyclic sulfonamides possessing CA inhibitory properties.

In this paper we report the reaction of 20 aromatic/heterocyclic sulfonamides containing a free amino, imino, hydrazino, or hydroxyl group, with 2,3-pyridinedicarboxylic anhydride or 2,6-pyridinedicarboxylic acid (in the presence of carbodiimide derivatives), which afforded two series of water-soluble (as hydrochloride, triflate, or carboxylate salts) sulfonamides with strong CA inhibitory properties. Furthermore, dorzolamide has been similarly derivatized, at its secondary amino group, and the obtained compounds also possessed good water solubility as hydrochloride or sodium salts. The new compounds reported here were tested for the inhibition of three CA isozymes: hCA I, hCA II, and bCA IV (h = human, b = bovine isozymes). Affinities in the nanomolar range were detected for some compounds for isozymes II and IV. The most active derivatives were assayed in vivo in normotensive and glaucomatous rabbits, for their IOP-lowering properties. Very strong and long-lasting IOP lowering was observed for many of them. Transcorneal accession rates for some of the new compounds were also determined, indicating a facilitated penetration of our compounds as compared to dorzolamide and other heterocyclic sulfonamides.

Chart 1

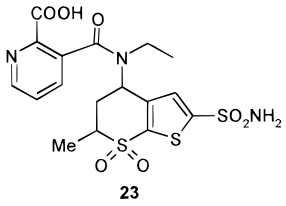


Results

Synthesis. Compounds prepared by reaction of 2,3-pyridinedicarboxylic anhydride or 2,6-pyridinedicarboxylic acid with aromatic/heterocyclic sulfonamides **3**−**22**, of type **3A**−**18A** and **3B**−**22B**, are shown in Chart 1.

Nonexceptional routine synthetic procedures were employed for the reaction of amines/alcohols/phenols

with anhydrides or carboxylic acids, as reported previously by Whitesides[16] or this group.[18,19] Reaction of dorzolamide (**1**) with the above-mentioned pyridinedicarboxylic acid derivatives afforded a mixture of two compounds, **23** and **24**, for the first reaction involving the 2,3-pyridinedicarboxylic anhydride and one compound, an isomer of the first two, **25**, in the case of the 2,6-pyridinedicarboxylic acid. Mention should be made that the first two isomers, **23** and **24**, could not be separated successfully by means of preparative HPLC, due to their very similar retention times, and some in vitro and in vivo experiments were done with the mixture of the two compounds. Due to the symmetric nature of the 2,6-pyridinedicarboxylic acid, its reaction with the above-mentioned sulfonamides afforded only one compound and not a mixture.



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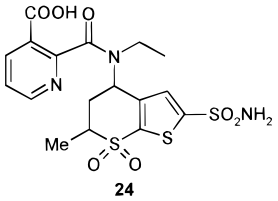

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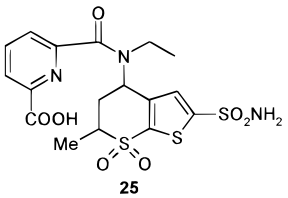

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Carbonic Anhydrase Inhibitory Activity. Inhibition data against the esterase activity of three CA isozymes, hCA I, hCA II, and bCA IV, with compounds **1**−**25** are shown in Table 1.

Some physicochemical properties of the new compounds, relevant for their pharmacological activity, such as buffer solubility, chloroform−buffer partition coefficient, rate constant of transfer across the cornea (k_{in}), are 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 administration of 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 the topical administration in normotensive rabbits are shown in Table 5.

Transcorneal Penetration of Drugs. The data of the in vitro transcorneal accession rates (k_{in}) for classical sulfonamides and topically active derivatives, such as dorzolamide and some of the new compounds reported in the present study, are shown in Table 2.

Discussion

Chemistry. Previous QSAR studies on large series of sulfonamide CA inhibitors[29] suggested that strong CA inhibitors might be designed by incorporating heterocyclic moieties in the molecule of aromatic/heterocyclic sulfonamides with known CA inhibitory properties. Key findings of these studies indicated that the enhancement of CA inhibitory activity is correlated with increased positive charges on a heterocyclic ring attached to such molecules, as well as with "long" inhibitor molecules per se (i.e., molecules extending on the direction passing through the Zn(II) ion of the enzyme, the sulfonamide

Table 1. CA Inhibition Data with Standard Inhibitors **1** and **2**, Parent Sulfonamides **3**−**22**, and New Derivatives Reported in the Present Study, Against Isozymes I, II, and IV

	K_i[a] (nM)		
inhibitor	hCA I[b]	hCA II[b]	bCA IV[c]
dorzolamide (**1**)	50000	9	45
brinzolamide[d] (**2**)		3.2	45.3
3	45400	295	1310
4	25000	240	2200
5	28000	300	3000
6	78500	320	3215
7	25000	170	2800
8	21000	160	2450
9	8300	60	180
10	9800	110	320
11	6500	40	66
12	6000	70	125
13	6100	28	175
14	8400	75	160
15	8600	60	540
16	9300	19	355
17	455	3	125
18	70	9	19
19	55	8	17
20	50	7	15
21	24000	125	560
22	18000	110	450
3A	21000	280	310
4A	20000	250	305
5A	15500	132	170
6A	21500	275	300
7A	1040	42	79
8A	845	9	54
9A	540	10	46
10A	620	44	79
11A	605	31	75
12A	610	33	70
13A	500	12	69
14A	600	9	60
15A	39	8	18
17A	18	3	10
18A	12	4	9
3B	20000	260	300
4B	18500	242	285
5B	15000	121	150
6B	20600	270	304
7B	860	36	70
8B	550	7	40
9B	510	8	44
10B	600	25	66
11B	575	30	69
12B	600	31	62
13B	450	10	55
14B	370	9	48
15B	36	6	21
16B	35	7	19
17B	19	2	9
18B	18	3	11
19B	16	2	13
20B	15	3	10
21B	2050	20	120
22B	2000	15	105
23+**24**	450	5	18
25	400	5	17

[a] Standard error for the determination of K_i's was 5−10% (from two different assays). [b] Human (cloned) isozyme. [c] Isolated from bovine lung microsomes. [d] Data from ref 46.

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

compound	solubility[a] (mM)	log P[b]	$k_{in} \times 10^3$ (h^{-1})[c] cornea intact	$k_{in} \times 10^3$ (h^{-1})[c] no epithelium
acetazolamide[d]	3.2	0.001	0.37	7
ethoxzolamide[d]	0.04	25	40	330
1	60[e]	2.0[e]	3.0	5.2
15A	72[f]	0.315	2.5	7.6
17A	81[f]	0.449	3.8	9.7
9B	66[f]	0.117	1.6	7.0
15B	83[g]	0.085	1.1	6.4
16B	79[g]	0.428	3.6	8.5
25	58,[f] 64[g]	1.736	2.8	5.9

[a] Solubility in pH 7.40 buffer, at 25 °C. [b] Chloroform−buffer partition coefficient. [c] Determined as described in refs 12, 48, and 49. [d] Data from ref 13. [e] As hydrochloride, at pH 5.8, from ref 44. [f] As hydrochloride salts. [g] As sodium carboxylate salts.

Table 3. Fall of IOP of Normotensive Rabbits (20.5 ± 2.6 mmHg) after Treatment with 1 drop (50 μL) of 2% Solution of CA Inhibitor (as hydrochloride or sodium carboxylate salt, with pH value shown) Directly into the Eye, at 30, 60, and 90 min after Administration

inhibitor	pH	ΔIOP (mmHg)[a] $t = 0$ min	$t = 30$ min	$t = 60$ min	$t = 90$ min
1·HCl	5.5	0	2.2 ± 0.10	4.1 ± 0.15	2.7 ± 0.08
8A·HCl	5.5	0	2.7 ± 0.12	4.8 ± 0.15	4.2 ± 0.13
15A·HCl	5.5	0	5.4 ± 0.10	9.0 ± 0.14	8.1 ± 0.13
17A·HCl	5.8	0	5.8 ± 0.10	9.2 ± 0.11	9.0 ± 0.12
18A·TfH[b]	5.9	0	2.0 ± 0.05	4.4 ± 0.12	4.0 ± 0.09
(23+24) HCl	5.5	0	2.4 ± 0.08	5.2 ± 0.11	3.5 ± 0.10
9B·HCl	5.5	0	2.5 ± 0.06	4.2 ± 0.10	3.9 ± 0.12
9B·Na salt	8.5	0	2.3 ± 0.10	4.8 ± 0.08	4.5 ± 0.13
15B·HCl	5.4	0	5.5 ± 0.05	9.2 ± 0.07	9.9 ± 0.10
15B Na salt	8.0	0	4.5 ± 0.06	9.3 ± 0.10	10.5 ± 0.12
16B·HCl	5.6	0	4.1 ± 0.12	8.0 ± 0.09	9.5 ± 0.11
20B·HCl	5.5	0	4.0 ± 0.15	7.2 ± 0.13	9.0 ± 0.11
25·HCl	5.5		3.5 ± 0.10	6.8 ± 0.09	9.1 ± 0.12
25·Na salt	7.4	0	3.5 ± 0.05	7.2 ± 0.08	9.8 ± 0.11
25·Na salt	8.4	0	3.6 ± 0.07	7.7 ± 0.12	9.9 ± 0.10

[a] $\Delta IOP = IOP_{control\ eye} - IOP_{treated\ eye}$; mean ± average spread ($n = 3$). [b] TfH = triflic acid, CF_3SO_3H.

Table 4. Fall of IOP of Glaucomatous Rabbits (34 ± 2.5 mmHg) after Treatment with 1 drop (50 μL) of 2% Solution of CA Inhibitor (as hydrochloride or sodium carboxylate salt, with pH value shown) Directly into the Eye, at 30, 60, and 90 min after Administration

inhibitor	pH	ΔIOP (mmHg)[a] $t = 0$ min	$t = 30$ min	$t = 60$ min	$t = 90$ min
17A·HCl	5.8	0	9.1 ± 0.10	13.2 ± 0.11	15.0 ± 0.12
15B·HCl	5.4	0	8.5 ± 0.15	12.2 ± 0.15	14.9 ± 0.12
15B·Na salt	8.0	0	8.5 ± 0.10	12.3 ± 0.10	15.0 ± 0.12
25·HCl	5.5	0	6.5 ± 0.10	9.9 ± 0.12	12.1 ± 0.10
25·Na salt	7.4	0	6.5 ± 0.11	10.1 ± 0.14	11.8 ± 0.13

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

nitrogen atom, and the long axis of the inhibitor), whereas weakening of activity could be attributed to increasing HOMO energies and anisotropic polarizabilities due to London forces.[29]

Examining different potential moieties that might be attached to aromatic/heterocyclic sulfonamide CA inhibitors, it appeared thus of great interest to try pyridinecarboxylate type substitutions, since they should satisfy all conditions for enhancement of CA inhibitory

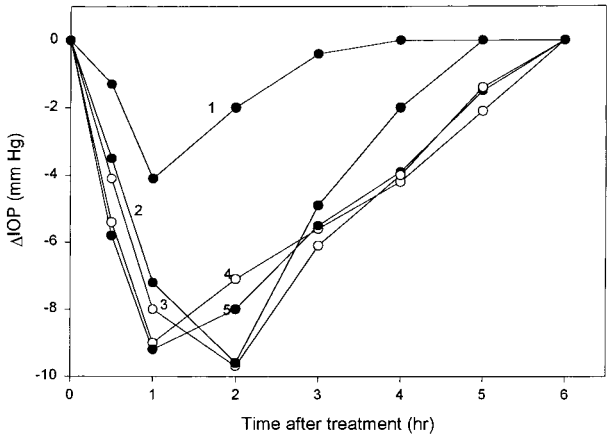


Figure 1. Effect of topically administered sulfonamide inhibitors (2% water solutions) on the IOP of normotensive albino rabbits: curve 1, dorzolamide (**1**) (hydrochloride salt, pH 5.5); curve 2, compound **25** (sodium salt, pH 7.20); curve 3, compound **16B** (as hydrochloride salt, pH 5.6); curve 4, compound **15A** (as hydrochloride salt, pH 5.5); curve 5, compound **17A** (as hydrochloride salt, pH 5.8).

Table 5. Ocular Tissue Concentrations (μM) after 1 and 2 h following Corneal Application of 1 drop (50 μL) of 2% Solution of Sulfonamides **1** (dorzolamide·HCl), **17A**·HCl, and **25** (as sodium salt) in Normotensive Albino Rabbits

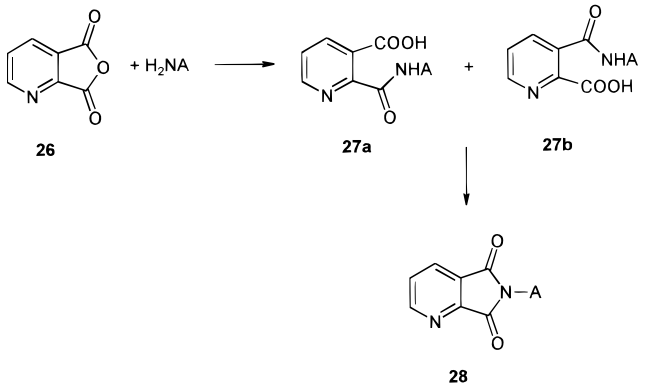
inhibitor	time (h)	drug concentration (μM)[a] cornea	aqueous humor	ciliary process
1	1	105 ± 5	32 ± 3	15 ± 3
·HCl	2	39 ± 4	21 ± 2	6 ± 1
17A	1	158 ± 5	280 ± 10	50 ± 3
·HCl	2	45 ± 6	42 ± 3	12 ± 1
25	1	113 ± 4	275 ± 8	47 ± 2
Na salt	2	44 ± 8	39 ± 2	10 ± 2

[a] Mean ± standard deviation ($n = 3$).

properties mentioned above. Two pyridinedicarboxylic acid derivatives have been selected for this study, 2,3-pyridinedicarboxylic anhydride (**26**), and 2,6-pyridinedicarboxylic acid, together with a large number of aromatic/heterocyclic sulfonamides possessing free amino, imino, hydrazino, or hydroxy moieties in their molecule. Aromatic sulfonamides such as sulfanilamide and metanilamide, which would lead to weaker CA inhibitors[17−19] were also included in the study to demonstrate that the proposed approach for the design of very efficient as well as weak enzyme inhibitors is a general one. Reactions of sulfonamides **1** or **3**−**18** with 2,3-pyridinedicarboxylic anhydride afforded the new compounds **3A**−**18A**, **23**, and **24**. The synthesis has generally been performed in acetonitrile as solvent, with azeotropic elimination of water from the system.[18]

The reaction of a carboxylic anhydride, such as **26**, with a nucleophile, such as an amine, is a relatively simple process: in the first step a carboxamide derivative **27** is formed, which in more energetic conditions can be cyclized to a carboximide of type **28**. In the particular case of **26**, due to its asymmetric molecule, two carboxamides, **27a**,**b**, are initially formed, which then both cyclize to the same carboximide **28** (Scheme 1). In the case of the reaction of **26** with an imine or an alcohol/phenol, obviously, the second step mentioned above cannot take place. Indeed, during the preparation of compounds **3A**−**18A**, intermediates of type **27a**,**b** were evidenced by both TLC as well as HPLC, and the reaction could be stopped at this stage (without cycliza-

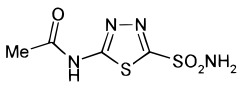
Scheme 1



tion). Still, the two isomers **27a**,**b** could not be separated by means of HPLC and other purification procedures, and some preliminary enzyme inhibition experiments were performed with the mixture of isomers. On the other hand, the CA inhibitory properties of such compounds (data not shown) were only slightly different from those of the corresponding 2,6-pyridinedicarboxylic acid derivatives **3B**−**18B** (with which they are isomers).

Reaction of sulfonamides **1** or **3**−**22** with 2,6-pyridinedicarboxylic acid **29**, in the presence of EDCI (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide), afforded the new derivatives **3B**−**22B** and **25** by the procedure previously reported by the Whitesides' group[16] for structurally related aromatic sulfonamides. Reaction of dorzolamide (**1**) with 2,3-pyridinedicarboxylic anhydride afforded two isomeric derivatives, **23** and **24**, which could not be separated successfully by means of preparative HPLC or other purification techniques. On the other hand, another isomer of the two compounds mentioned above, **25**, in pure state this time, has been prepared by reaction of **1** with 2,6-pyridinedicarboxylic acid, in the presence of EDCI.

Hydrochlorides of the new derivatives were then prepared by reacting the free bases **3A**−**18A**, **23**, **24**, or **3B**−**22B, 25** with a methanolic HCl solution. Similarly were obtained the triflate salts, by reaction of the previously mentioned free bases with triflic acid in water as solvent. Carboxylate sodium salts of derivatives **3B**−**22B** and **25** were obtained by reaction of the free acids with the stoichiometric amount of sodium bicarbonate or hydroxide, in water as solvent. These salts possess good water solubility, generally in the range of 2−5% (by weight), i.e., in the range 50−80 mM (Table 2). Triflates and hydrochlorides possess quite similar water solubilities (data not shown), so that the largest majority of studies with the cationic derivatives have been performed with hydrochlorides. The pH of such solutions is in the range of 5.0−8.5, making them appropriate for topical application directly into the eye. As observed in Table 2, some of the newly obtained compounds, for which detailed pharmacological data were obtained, possess a relatively moderate lipid solubility, similarly to that of dorzolamide (**1**). In fact, Maren[12] noted in his classical review that one of the conditions needed for a sulfonamide to act as an effective IOP-lowering agent is to possess a modest lipid solubility (attributed to its un-ionized form), accompanied by a good water solubility (conferred by the presence of ionizable groups of appropriate pK_a).[12] To illustrate the importance of water/lipid solubility and corneal accession rates for the topical activity of a sulfonamide CA inhibitor, data for two topically inactive compounds, acetazolamide (**30**) and ethoxzolamide (**31**), are also shown in Table 2. These two classical high-affinity CA inhibitors (K_i of 8 nM against hCA II for **30** and 1 nM for **31**) possess a too low lipid solubility (in neutral form) correlated with a high water solubility (as sodium salt—the case of acetazolamide) and are inactive topically due to poor penetration across the cornea. On the other hand, unlike acetazolamide, ethoxzolamide is very lipid-soluble, and although its rate constants are very high, the actual amount delivered into the ciliary processes is too small due to the very low water solubility of the drug. More than that, due to these properties the drug diffuses rapidly from the eye into the blood. As seen from data of Table 2, the compounds reported here possessed excellent water solubility, balanced by a modest lipid solubility. Their accession rates across the cornea were thus of the same order of magnitude as those of dorzolamide.



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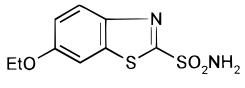

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The new compounds reported in the present work were characterized by standard chemical and physical methods (elemental analysis, within ±0.4% of the theoretical values; IR; ^1H and ^{13}C NMR spectroscopy) that confirmed their structure (see Experimental Section for details) and were assayed for the inhibition of isozymes hCA I, hCA II, and bCA IV (Table 1).

In Vitro CA Inhibition. Inhibition data against three CA isozymes, hCA I, hCA II, and bCA IV, with the new derivatives (Table 1) prove that the pyridinecarboxyl moiety containing derivatives reported here generally behave as strong inhibitors, with increased affinities as compared to the parent compounds from which they were prepared (the sulfonamides **3**−**22**). The affinities of the obtained inhibitor generally varied in the following way, based on the parent sulfonamide from which it was prepared: the derivative of *p*-hydrazinobenzenesulfonamide **6** < the orthanilamide **3** ≃ the metanilamide **4** < the sulfanilamide **5** < the homosulfanilamides **7** < the *p*-(aminoethyl)benzenesulfonamides **8** ≃ the halogeno-substituted sulfanilamides **9**−**12** ≃ the 1,3-benzenedisulfonamides **13** and **14** < the 1,3,4-thiadiazole-2-sulfonamides **15** and **17** ≃ 4-methyl-δ^2-1,3,4-thiadiazoline-2-sulfonamide **16** ≃ the dorzolamide derivative **1** ≃ the benzothiazole-2-sulfonamides **18**−**20**. Based on the carboxylic acid from which they were obtained, the 2,6-pyridinedicarboxylic acid derivatives were slightly more effective CA inhibitors as compared to the corresponding 2,3-pyridinedicarboxylic acid derivatives. All three CA isozymes investigated here were susceptible to inhibition with this type of sulfonamides, with hCA II and bCA IV the most sensitive, whereas hCA I was generally less susceptible to inhibition as compared to the first two isozymes.

IOP Lowering in Normotensive and Glaucomatous Rabbits. The promising in vitro CA inhibitory activity as well as other physicochemical properties mentioned above for some of the newly prepared com-

pounds prompted us to investigate their effect in vivo on the IOP after topical application directly into the eye, in normotensive and glaucomatous rabbits (frequently used as an animal model of glaucoma).[3−15,22,23]

The compounds selected for in vivo studies were among the most active in vitro inhibitors against hCA II and IV, in the prepared series, and possessed other favorable properties such as a moderate lipid solubility, good accession rates across the cornea, etc. Such compounds included among others: **8A**, **15A**, **17A**, **18A**, **23**+**24**, **9B**, **15B**, **16B**, **20B**, and **25**. The following facts should be noted regarding the data of Table 3. Some of the new compounds investigated in vivo, such as **8A**, **18A**, **23**+**24**, and **9B**, showed IOP-lowering effects generally of the same order of magnitude as those of dorzolamide (**1**). Thus, after 0.5 or 1 h, these were around 2.0−2.7 and 4.0−5.2 mmHg, respectively, for both dorzolamide as well as the new derivatives. An important difference between the two groups of drugs appears at longer periods after the administration, since unlike dorzolamide, which diminishes its power of action to an IOP lowering of 2.7 mmHg after 90 min, the new compounds mentioned above maintained a much more effective IOP lowering, in the range of 4.2−4.8 mmHg, comparable to that observed at 1 h after their administration. A second group of inhibitors, such as **15A**, **17A**, **15B**, **16B**, **20B**, and **25**, showed much more effective IOP lowering as compared to dorzolamide **1**, both after 30 min from the administration of the inhibitor within the rabbit eye as well as at longer times (1, 1.5, and 2−6 h, respectively). Thus, after 30 min, the IOP lowering was in the range of 3.6−5.8 mmHg with the new compounds mentioned above (the least active was just **25**, a dorzolamide derivative) and only 2.2 mmHg with dorzolamide. At 1 h after the administration the new compounds generally fared doubly as well as the clinically used drug **1** (7.0−9.3 mmHg for the new derivatives versus 4.1 mmHg for dorzolamide), and this strong effect was maintained after another 0.5 h (whereas it is halved in the case of **1**, where the pressure decrease amounts to 2.7 mmHg after 90 min). Both cationic compounds (hydrochlorides, such as **8A**, **15A**, **17A**, **18A**, **9B**, **15B**, **16B**, **20B**, and **25**) as well as anionic derivatives (as sodium carboxylates, such as **9B**, **15B**, **16B**, **20B**, and **25**) were equally active as IOP-lowering agents, with a slightly better action of the hydrochlorides at shorter periods after administration, whereas the sodium salts seemed to be more active at later times (obviously for the same amphoteric compound, which has been used both as a hydrochloride and as a sodium salt; for instance one should compare the data at different pH values of **25**—Tables 3 and 4).

But one of the most interesting findings is that IOP remains low for longer periods (3−6 h) after the topical administration of the new type of compounds reported in this paper, as compared to the standard drug dorzolamide (Figure 1). As seen from Figure 1, compounds such as **15A** and **17A** possessed maximal IOP-lowering effects at 1 h after administration, similarly to dorzolamide. The main difference between them was that the new compounds acted as much more potent IOP-lowering agents, and at 3 or 4 h after administration (when the effects of dorzolamide completely vanished) they still diminished eye pressure appreciably (4.8−6.2 mmHg). IOP generally returned at the baseline values after 5−6 h after administration of the drug. Other derivatives reported by us, such as **16B** and **25**, possessed a maximal IOP reduction after 2 h, their potent effect also being maintained for the next 3−4 h. Thus, all these derivatives are longer lasting IOP-lowering agents as compared to the clinically available drug dorzolamide.

The above findings also apply for the glaucomatous rabbits experiments (Table 4), but the IOP are much more important as compared to those of normotensive rabbits. Thus, IOP reductions of around 9 mmHg were generally observed after 30 min, whereas at longer periods, these amounted to 12−15 mmHg. No important differences between the cationic and anionic inhibitors were observed. The long-lasting effect mentioned above has also been evidenced for the glaucomatous rabbit experiments (data not shown).

Drug Distribution in Ocular Fluids and Tissues. Table 5 shows ex vivo data obtained in normotensive rabbits after the topical administration of two of the most active topical inhibitors in the prepared series, i.e., compounds **17A** and **25**, as well as the standard drug **1**. It can be observed that at 1 and 2 h after topical administration of the drug, high levels of inhibitors were found in the cornea, aqueous humor, and ciliary processes. Based on the inhibition constants of these compounds (3 nM for CA II for **17A** and 5 nM for CA II for **25**), the fractional inhibition estimated in these tissues/fluids is of 99.5−99.9%, indicating 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, such as **17A**, **25**, etc., tend to concentrate in the aqueous humor (concentrations of around 275−280 μM were detected after 1 h from administration), whereas dorzolamide reaches much lower concentrations (32 μM after 1 h). High concentrations of the inhibitor were maintained at 2 h from administration too. Concentrations of the new compounds **17A** and **25** in the cornea and ciliary processes are also enhanced as compared to those of dorzolamide (**1**), but the differences are not so dramatic as those from the aqueous humor. Thus, one may conclude that the strong and long-lasting IOP-lowering properties of the new compounds are due to this concentrating effect reached mainly in the aqueous humor, but which is also present in the cornea and ciliary processes. The mechanism by which such high concentrations of active compounds reach these tissues is unexplained for the moment.

Conclusions

We report here a general approach for the preparation of water-soluble, topically effective antiglaucoma sulfonamides by attaching water-solubilizing moieties (such as pyridinecarboximido, carboxypyridinecarboxamido, etc.) to well-known aromatic/heterocyclic sulfonamides. By applying simple chemical reactions between a heterocyclic anhydride (2,3-pyridinedicarboxylic anhydride) or a heterocyclic dicarboxylic acid (2,6-pyridinedicarboxylic acid) with aromatic/heterocyclic sulfonamides containing free amino, imino, hydrazino, or hydroxy groups, two series of new compounds were prepared. Ring systems which have been derivatized by the above-mentioned procedures included: 2-, 3-, and 4-aminoben-

zenesulfonamides, 4-(ω-aminoalkyl)benzenesulfonamides, 3-halogeno-substituted-sulfanilamides, 1,3-benzenedisulfonamides, 1,3,4-thiadiazole-2-sulfonamides, benzothiazole-2-sulfonamides, and thienothiopyran-2-sulfonamides among others, and were chosen in such a way as to demonstrate that the proposed approach is a general one. Derivatives in the first series of compounds formed water-soluble salts by reaction with strong acids, such as hydrochloric or trifluoromethanesulfonic acid, with protonation of the pyridine nitrogen atom. Derivatives of the second series equally formed salts of the same type by reaction with acids, as well as sodium carboxylate salts, by reaction with sodium hydroxide or bicarbonate. Both types of salts possessed good water solubility, in the range of 2−5%, whereas their lipid solubility, hydrophobicity (log *P*), and accession rates across the cornea were those appropriate for acting as efficient topical IOP-lowering agents. Many of the reported inhibitors possessed affinities in the nanomolar range for isozymes hCA II and bCA IV, acting as effective enzyme inhibitors in vitro. Some of the most active inhibitors strongly lowered IOP pressure in normotensive and glaucomatous rabbits, showing a highly prolonged duration of action as compared to dorzolamide. The new compounds reported here might lead to the development of more efficient and inexpensive antiglaucoma drugs (the presently available topical antiglaucoma sulfonamides dorzolamide and brinzolamide are quite expensive drugs, whereas the patients most affected are generally the elderly of more than 60 years).

Experimental Section

General. Melting points, heating plate microscope (not corrected); IR spectra, KBr pellets, 400−4000 cm^{-1} Perkin-Elmer 16PC FTIR spectrometer; ^1H NMR spectra, Varian 300CXP apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard); elemental analysis: Carlo Erba Instrument CHNS elemental analyzer, model 1106. 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. Sulfonamides **3**−**22** used in synthesis were either commercially available compounds (from Sigma, Acros, or Aldrich) or were prepared as described previously: 4-hydrazinobenzenesulfonamide **6** by diazotization of sulfanilamide followed by reduction of the diazonium salt with tin(II) chloride;[30] halogenosulfanilamides **9**−**12** by halogenation of sulfanilamide as reported in the literature;[31] compound **17** from 5-amino-1,3,4-thiadiazole-2-sulfonamide (obtained from acetazolamide)[32] by acylation with the phthalimido derivative of β-alanine, followed by hydrazinolysis;[33] imine **16** by deprotection of methazolamide with concentrated hydrochloric acid.[32] The benzothiazole-2-sulfonamide derivatives **18**−**20** were prepared as described in ref 24, whereas the alcohols **21** and **22** were prepared from the corresponding amines by diazotization followed by hydrolysis of the diazonium salts. Dorzolamide (**1**) was prepared as described in the literature[34] or was obtained from Merck, Sharp and Dohme. 2,3-Pyridinedicarboxylic anhydride was from Aldrich, whereas 2,6-pyridinedicarboxylic acid, EDCI, triflic acid, and triethylamine were from Sigma Chemical Co. Acetonitrile (E. Merck) or 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 3A−18A and 23+24. An amount of 1 mM sulfonamide **3**−**18** or **1** was dissolved/suspended in 25 mL of anhydrous acetonitrile and then treated with 150 mg (1 mM) of 2,3-pyridinedicarboxylic anhydride. The reaction mixture was heated at reflux for 15−20 h, with azeotropic elimination of water from the system. By means of TLC the conversion of all the sulfonamide to the corresponding pyridinedicarboximido derivatives was monitored. When the reaction was completed, the solvent was evaporated until a small volume of the reaction mixture was obtained. Generally the new compounds crystallized spontaneously by leaving the above mixture at 4 °C overnight. In some cases, the concentrated liquor obtained after the evaporation of the solvent was poured into 50 mL of cold water; then the reaction products precipitated and were filtered. The prepared compounds were recrystallized from ethanol or ethanol−water (1:1, v/v). Yields were in the range of 70−90%. Hydrochlorides of the new derivatives were obtained from the free bases and a methanolic HCl solution, in methanol as solvent. The hydrochlorides precipitated by leaving the above mixtures at 4 °C overnight. The hydrochlorides were analyzed for the presence of Cl^- by potentiometric titrations. The obtained data were ±0.5% of the theoretical data calculated for the proposed formulas (data not shown). Triflate salts were similarly obtained from the free bases and the stoichiometric amount of triflic acid, in water as solvent.

General Procedure for the Preparation of Compounds 3B−22B and 25. An amount of 1 mM sulfonamide **3**−**22** or **1** was dissolved/suspended in 25 mL of anhydrous acetonitrile or acetone and then treated with 170 mg (1 mM) of 2,6-pyridinedicarboxylic acid and 190 mg (1 mM) of EDCI·HCl. The reaction mixture was magnetically stirred at room temperature for 15 min, then 30 mL (2 mM) of triethylamine was added, and stirring was continued for 16 h at 4 °C. 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 was removed in vacuo. Preparative HPLC (C_{18} reversed-phase Bondapack or Dynamax-60A (25 × 250 mm) columns; 90% acetonitrile/8% methanol/2% water, 30 mL/min) afforded the pure compounds as colorless solids.

4-(2,3-Pyridinecarboximido)benzenesulfonamide (5A): white crystals, mp 261−3 °C dec; IR (KBr) cm^{-1} 1150 (SO_2^{sym}), 1290 (amide III), 1345 (SO_2^{as}), 1560 (amide II), 1690 (amide I), 3360 (NH_2); ^1H NMR (DMSO-d_6) δ_A 7.18, δ_B 7.75 (AA′BB′system, 4H, J_{AB} = 7.9 Hz, ArH from 4-sulfamoylphenyl), 7.56 (br s, 2H, SO_2NH_2), 7.84−8.27 (m, 3H, ArH from pyridine); ^{13}C NMR (DMSO-d_6) δ 118.5, 118.9, 124.6, 125.0, 128.7, 129.9, 130.4, 135.7, 138.5, 175.8, 176.2. Anal. ($C_{13}H_9N_3O_4S$) C, H, N.

5-(6-Carboxypyridine-2-carboxamido)-1,3,4-thiadiazole-2-sulfonamide (15B): white crystals; mp > 300 °C; IR (KBr) cm^{-1} 1180 (SO_2^{sym}), 1295 (amide III), 1360 (SO_2^{as}), 1560 (amide II), 1690 (amide I), 1770 (COOH), 3060 (NH), 3375 (NH_2); ^1H NMR (DMSO-d_6) 7.25 (br s, 2H, SO_2NH_2), 7.62 (t, 1H, H^4 of pyridine), 7.93 (d, 2H, H^3 and H^5 of pyridine), 8.10 (s, 1H, CONH), 12.10 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6) 121.5, 125.9, 137.2, 159.3 (C-2 of thiadiazole), 170.4 (C-5 of thiadiazole), 176.9 (CONH), 182.5 (COOH). Anal. ($C_{15}H_{13}N_3O_4S_2$) C, H, N.

5,6-Dihydro-4-[*N*-6-carboxypyridine-2-carboxamido-(ethylamido)]-6-methyl-4*H*-thieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-dioxide (25): white crystals; mp > 300 °C; IR (KBr) cm^{-1} 1133 (SO_2^{sym}), 1295 (amide III), 1345 (SO_2^{as}), 1565 (amide II), 1680 (amide I), 1775 (COOH), 3060 (NH), 3360 (NH_2); ^1H NMR (DMSO-d_6) 1.29 (d, 3H, Me), 1.39 (t, 3H, Me from ethyl), 2.55 (m, 1H, CH), 2.80 (m, 1H, CH), 3.05−3.20 (m, 2H, CH_2 from ethyl), 4.37 (m, 2H, CH_2), 7.63 (t, 1H, H^4 of pyridine), 7.90 (d, 2H, H^3 and H^5 of pyridine), 8.03 (s, 1H, CH, ArH from thienyl), 8.25 (br s, 2H, SO_2NH_2), 12.07 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 10.0, 11.1, 30.6, 40.8, 49.3, 51.5, 121.7, 125.8, 130.7, 137.3, 137.5, 141.9, 149.8, 176.9 (CONH), 182.5 (COOH). Anal. ($C_{15}H_{13}N_3O_4S_2$) C, H, N.

Enzyme Preparations. 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 as described by Lidskog et al.[35] (the two plasmids were a gift from Prof. Sven

Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group,[36] and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[37] 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.[38,39] CA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide.[40]

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM-compatible PC.[41] 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 e 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.[41] Nonenzymatic 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.[41] Enzyme concentrations were 3.5 nM for hCA II, 12 nM for hCA I, and 36 nM for bCA IV (this isozyme has a decreased esterase activity[42] and higher concentrations had to be used for the measurements).

Measurement of Tonometric IOP. 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 Digilab 30R pneumatonometer (BioRad, Cambridge, MA) as described by Maren's group.[43,44] 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 were reported. IOP was measured first immediately before drug administration, then at 30 min after the instillation of the pharmacological agent, and then every 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.[43,44] 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 Sugrue et al.[45] 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 the injection of α-chymotrypsin.

Drug Distribution in Ocular Fluids and Tissues. The general procedure of Maren's group has been followed.[43,44] 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 was wetted with normal saline and dissected under a microscope; then ciliary processes were liberated from their attachment to the iris, cut, weighed, and put in 0.5 mL of distilled water. The tissue from four eyes (average weight of 8 mg/eye) was 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 activity 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 43 and 44.

Determination of Water (Buffer) Solubility. A standard solution was prepared by dissolving a precisely weighed 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-mm 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 the saturated solution; C' = concentration of the saturated solution (mg/mL).[46]

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.[7,47]

Transcorneal Penetration of Drugs. The method of Maren et al.[13] with the modifications of Pierce's group[48,49] (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 1.2 cm^2. Concentrations of drug of 40−2000 mM 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 by the HPLC method of Pierce et al.[48,49] or enzymatically.[7,13] The results of the drug analyses were used to calculate the rate constant of transfer across the cornea (k_{in}). As described by Pierce,[48,49] 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).

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