Carbonic Anhydrase Inhibitors: Synthesis of Water-Soluble, Aminoacyl/Dipeptidyl Sulfonamides Possessing Long-Lasting Intraocular Pressure-Lowering Properties via the Topical Route[1]

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Received April 19, 1999

Reaction of 26 aromatic/heterocyclic sulfonamides containing amino, imino, hydrazino, or hydroxyl groups with Boc-Gly, Boc-Sar, TrS-Crt, or Boc-Gly-Gly (Sar = sarcosine, *N*-Me-Gly; Crt = creatine, *N*-amidinosarcosine; TrS = tritylsulfenyl; Boc = *tert*-butoxycarbonyl) in the presence of carbodiimide derivatives afforded after removal of the protecting groups a series of water-soluble compounds (as salts of strong acids, such as hydrochloric, trifluoroacetic, or trifluoromethanesulfonic). 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 and especially against CA II and IV (in the 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 into the eye of normotensive or glaucomatous albino rabbits, when strong and long-lasting intraocular pressure (IOP) lowering was observed with many of them. Thus, the aminoacyl/dipeptidyl tail conferring water solubility to these sulfonamide CA inhibitors coupled with strong enzyme inhibitory properties and balanced lipid solubility seem to be the key factors for obtaining compounds with effective topical antiglaucoma activity.

Introduction

In previous contributions from this laboratory[1–3] it was shown that by attaching water-solubilizing tails (such as 8-quinolinesulfonyl,[1] nicotinoyl,[2] isonicotinoyl,[2] 6-carboxypyridine-2-carboxamido,[2,3] etc.) to the molecules of aromatic/heterocyclic sulfonamides of types **1–26** (Chart 1), possessing free amino, hydrazino, imino, or hydroxy groups, it is possible to obtain water-soluble, efficient inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1),[4–8] some of which showed promising antiglaucoma[9] activity via the topical route in experimental animals.

Indeed, CA inhibition in ocular tissues (mainly the ciliary processes) with systemically or topically administered sulfonamide CA inhibitors is followed by an effective reduction of intraocular pressure (IOP) due to the reduced rate of bicarbonate secretion within the aqueous humor.[8–11] Since the systemic inhibitors generally produce undesired side effects due to inhibition of different CA isozymes in other tissues than the eye,[8–11] many efforts have been made in the last 15 years for the development of water-soluble sulfonamide CA inhibitors that should be administered via the topical route.[12–20] Two such drugs are presently available, dorzolamide (**26**)[13,17] and brinzolamide (**27**),[21] both used as hydrochloride salts (Chart 2). The use of such hydrochloride salts is imposed by the need of ensuring a good water solubility to the drugs, but in some cases this represents an undesired complication, since the pH of such solutions becomes acidic enough and consequently produces eye irritation after the topical administration, as already reported for many patients treated with dorzolamide.[22] Moreover, the duration of action of these drugs is generally short (2–3 h), and they must be administered several times a day. It is thus of critical importance to design novel types of water-soluble, topically acting sulfonamide CA inhibitors in order to obtain third-generation drugs (if one considers the systemically administered sulfonamides such as acetazolamide (**28**) or methazolamide (**29**) as the first-generation CA inhibitors and the recently developed dorzolamide (**26**) and brinzolamide (**27**) as the second-generation ones) totally devoid of undesired side effects.

The approach used by us for the design of topically active IOP-lowering agents reported in this paper is based on the interesting findings of Whitesides' group [23,24] regarding the increase of the binding affinity to the enzyme for compounds possessing secondary binding sites adjacent to the sulfonamido one (which is the primary binding moiety, since the ionized sulfonamide is directly coordinated to the Zn(II) ion within the CA active site).[25,26] The above-mentioned findings[23,24] (which generally exploited hydrophobic interactions between hydrophobic patches at the entrance of the hCA II active site and similar moieties present in the inhibitor

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10.1021/jm9901879 CCC: $18.00 © 1999 American Chemical Society
Published on Web 08/10/1999

Chart 1

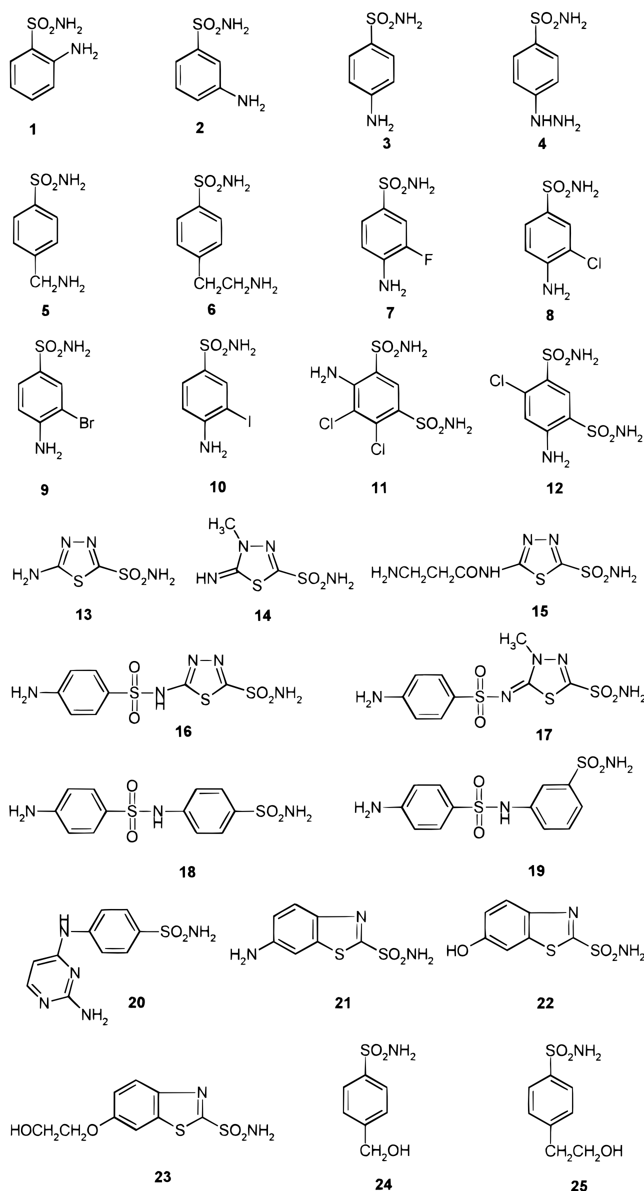
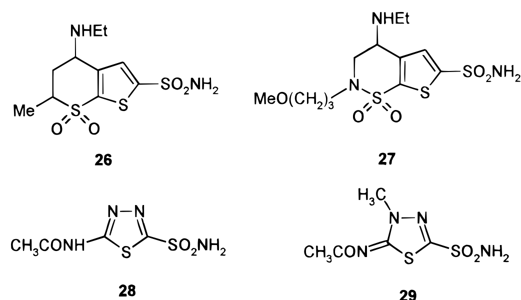


Chart 2



molecule), combined with our strategy of ensuring water solubility (as salts of a strong acid/base for instance),[1−3] might lead to compounds with affinity in the nanomolar range for the relevant CA isozymes involved in aqueous humor secretion (CA II and IV). Obviously, to achieve this goal, we used the opposite of the approach reported by Whitesides' group,[23,24] i.e., taking advantage of the hydrophilic interactions between secondary binding sites and the enzyme, since just hydrophilic inhibitors are of interest for ophthalmologic applications (mention should be made that the above-mentioned researchers[23,24] were not interested—as far as we know—in the antiglaucoma use of their compounds, but their studies greatly contributed to a better understanding of CA inhibition by sulfonamides).[23−26] In this context we[27,28] have recently evidenced by means of X-ray crystallography a cluster of six histidine residues (His 3, His 4, His 10, His 15, His 17, and His 64) at the entrance of the hCA II active site, some of which possess different conformations (as shown by the above-mentioned technique)[27,28] which could easily participate in hydrogen bond formation (as well as other types of interactions) with inhibitors possessing hydrophilic moieties grafted to the aromatic/heterocyclic sulfonamide used as the primary binding group. In fact, in the X-ray structure of the adduct of aminobenzolamide (**16**) with hCA II reported by Liljas' group,[25b] it is possible to evidence that the terminal part of the sulfonamide molecule is placed in such a way to interact favorably with some of the hydrophilic residues belonging to the histidine cluster mentioned above (Figure 1).

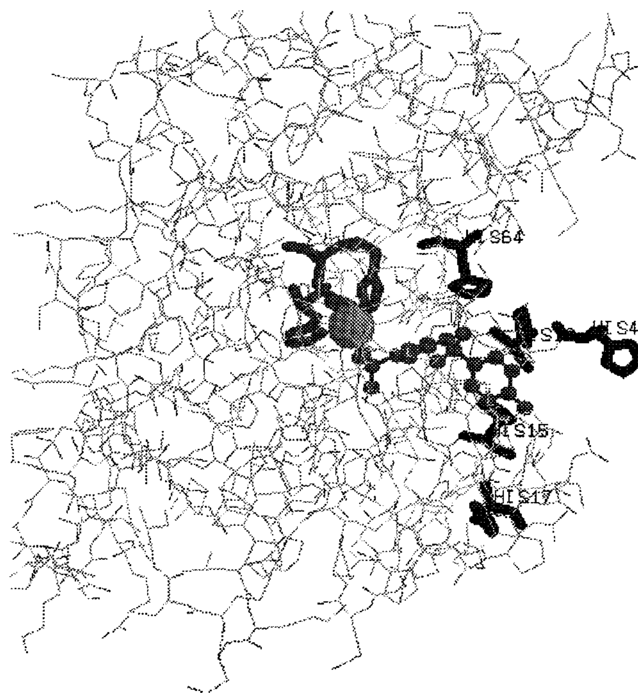


Figure 1. hCA II−aminobenzolamide (**16**) complex. The Zn(II) ion (central sphere) and its three histidine ligands (His 94, 96, and 119), as well as the histidine cluster comprising residues 3 (disordered in this structure), 4, 10, 15, 17, and 64, are shown. The inhibitor molecule (shown in a ball-and-stick form) is coordinated to the Zn(II) ion and interacts with some of the residues of the hydrophilic cluster mentioned above. This figure has been generated from the X-ray coordinates of Liljas group[25b] by using the program Rasmol for Windows. This structure is not deposited in the Brookhaven Protein Database.

In this paper we report the reaction of 26 aromatic/heterocyclic sulfonamides containing a free amino, imino, hydrazino, or hydroxyl group with protected amino acid/dipeptide derivatives, such as Boc-Gly, Boc-Sar, TrS-Crt, or Boc-Gly-Gly (Sar = sarcosine, *N*-Me-Gly; Crt = creatine, *N*-amidinosarcosine; TrS = tritylsulfenyl; Boc = *tert*-butoxycarbonyl), in the presence of carbodiimides, which afforded (after removal of the protecting groups) a series of water-soluble (as hydro-

Chart 3

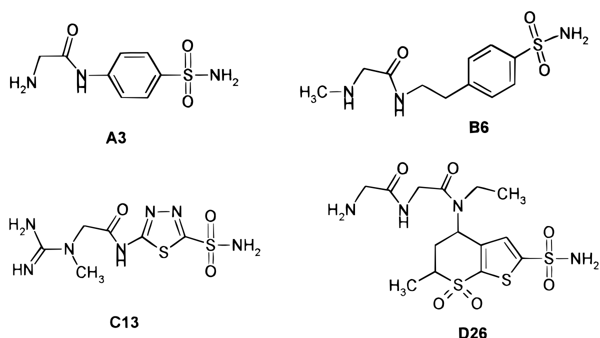
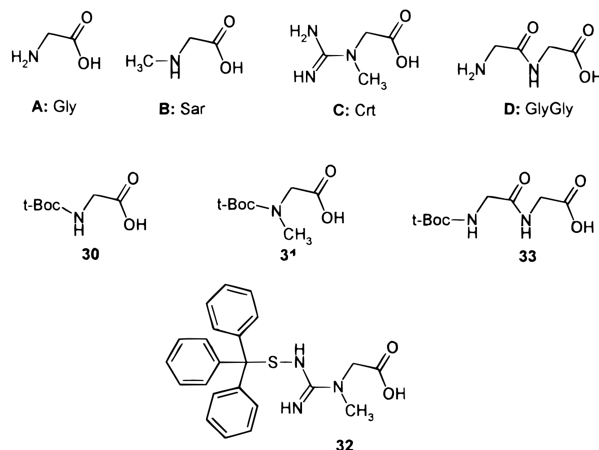


Chart 4



chloride, trifluoroacetate, or triflate salts) sulfonamides with good CA inhibitory properties. Moreover, the clinically used inhibitor dorzolamide (**26**) has been derivatized similarly, at its secondary amino group, and the obtained compounds also possessed good water solubility (as salts of strong acids) and CA inhibitory properties. 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 many compounds against isozymes II and IV, which are those involved in aqueous humor secretion. 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 other heterocyclic sulfonamides devoid of topical activity. Our conclusion is that the aminoacyl/dipeptidyl water-solubilizing tail is critical for the topical activity of these compounds and that antiglaucoma drugs might be obtained by this approach from many other classes of sulfonamides and not only from the thienothiopyransulfonamides and their derivatives. Furthermore, the pH of the solutions of compounds (as salts of strong acids) reported here is around 6.5−7.0, making them much less irritating to the eye as compared to the relatively highly acidic dorzolamide preparations (pH 5.5).

Results

Synthesis. As a large number of derivatives are reported here, each compound will be designated by a letter identifying the amino acid/dipeptide from which it is derived and a figure identifying the sulfonamide of type **1**−**26** at which the aminoacyl/dipeptidyl moiety has been attached. For instance, **A3** is glycylsulfanilamide; **B6** is 4-(sarcosinylamidoethyl)benzenesulfonamide; **C13** is 5-creatinyl-1,3,4-thiadiazole-2-sulfonamide; **D26** is the glycylglycinamido derivative of dorzolamide, etc. (Chart 3).

The new compounds **A(1**−**26)**−**D(1**−**26)** were prepared by reaction of the aromatic/heterocyclic sulfonamides **1**−**26** with the protected amino acid/dipeptide derivatives of type **30**−**33** (originating from the four amino acid/dipeptide derivatives **A**−**D**), followed by removal of the protecting groups in the standard manner (Chart 4).

Nonexceptional routine synthetic procedures were employed for the reaction of amines/imines/alcohols/phenols with protected amino acid/dipeptide derivatives (of type **30**−**33**), followed by the usual deprotection steps (TFA for eliminating the Boc moiety and dioxane-HCl for the tritylsulfenyl one), as reported previously by Whitesides[23,24] or this group[15,16] for structurally related sulfonamides.

CA Inhibitory Activity. Inhibition data against three CA isozymes, hCA I, hCA II, and bCA IV, with compounds **A(1**−**26)**−**D(1**−**26)** as well as the original raw materials and standard sulfonamides of type **1**−**29** are shown in Table 1. The esterase activity of CA isozymes against 4-nitrophenyl acetate as substrate has been used in this assay (see Experimental Section for details). Mention should be made that the Boc- or tritylsulfenyl-protected intermediates prepared here also possessed very good CA inhibitory properties (data not shown).

Transcorneal Penetration of Drugs. Some physicochemical properties of the new compounds, 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 with dorzolamide and some of the new compounds reported here in normotensive albino rabbits is shown in Figure 2.

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. Although several thousand different aromatic/heterocyclic sulfonamide CA inhibitors have been synthesized in the last 45 years in search of diverse pharmacological agents,[4,10] the number of aminoacyl/dipeptidyl derivatives is unexpectedly small. Thus, Antonarolli et al.[29] reported one such compound, **34**

Table 1. CA Inhibition Data with Standard Inhibitors, Parent Sulfonamides **1**−**26**, and New Derivatives **(A**−**D)1**−**26** Reported in the Present Study Against Isozymes I, II, and IV

inhibitor	K_i[a] (nM)			inhibitor	K_i[a] (nM)		
	hCA I[b]	hCA II[b]	bCA IV[c]		hCA I[b]	hCA II[b]	bCA IV[c]
1	45400	295	1310	**B14**	290	6	43
2	25000	240	2200	**B15**	21	3	9
3	28000	300	3000	**B16**	12	1.5	6
4	78500	320	3215	**B17**	11	1.8	7
5	25000	170	2800	**B18**	50	8	62
6	21000	160	2450	**B19**	51	9	61
7	8300	60	180	**B20**	60	19	60
8	9800	110	320	**B21**	10	5	14
9	6500	40	66	**B22**	11	4	30
10	6000	70	125	**B23**	10	3	15
11	6100	28	175	**B24**	1500	57	123
12	8400	75	160	**B25**	1300	50	112
13	8600	60	540	**B26**	78	3	30
14	9300	19	355	**C1**	20300	187	205
15	455	3	125	**C2**	16200	160	177
16	6	2	5	**C3**	14600	111	140
17	1	0.6	0.8	**C4**	20800	190	245
18	42	6	50	**C5**	950	30	71
19	44	9	53	**C6**	775	30	64
20	690	12	154	**C7**	510	10	39
21	70	9	19	**C8**	510	27	60
22	55	8	17	**C9**	525	29	62
23	50	7	15	**C10**	545	33	69
24	24000	125	560	**C11**	525	13	51
25	18000	110	450	**C12**	310	7	42
26	50000	9	45	**C13**	170	5	35
27[d]		3.2	45.3	**C14**	190	6	41
A1	25000	210	245	**C15**	21	2	8
A2	18000	200	240	**C16**	12	1.1	5
A3	15000	124	175	**C17**	11	1.3	6
A4	25500	250	300	**C18**	46	8	51
A5	1400	40	82	**C19**	45	8	57
A6	850	39	76	**C20**	53	15	40
A7	540	12	46	**C21**	10	4	12
A8	600	41	78	**C22**	11	3	15
A9	600	38	73	**C23**	9	3	11
A10	610	45	74	**C24**	1200	41	89
A11	520	13	62	**C25**	1000	40	86
A12	360	10	60	**C26**	75	2	27
A13	350	9	55	**D1**	23500	200	240
A14	335	10	70	**D2**	17000	200	235
A15	21	4	10	**D3**	15000	121	154
A16	14	2	9	**D4**	25200	245	300
A17	15	3	10	**D5**	1360	36	75
A18	62	12	86	**D6**	800	35	70
A19	60	13	79	**D7**	540	11	45
A20	61	23	75	**D8**	550	36	65
A21	17	9	72	**D9**	570	36	70
A22	16	9	69	**D10**	590	40	71
A23	9	8	23	**D11**	500	12	56
A24	1800	73	180	**D12**	360	9	54
A25	1700	64	155	**D13**	320	6	45
A26	98	6	35	**D14**	280	7	44
B1	20300	187	205	**D15**	21	3	12
B2	16600	180	195	**D16**	13	2	6
B3	15000	115	146	**D17**	13	2	9
B4	22000	193	280	**D18**	54	10	66
B5	965	33	74	**D19**	55	10	60
B6	820	32	70	**D20**	60	21	69
B7	500	10	46	**D21**	16	9	64
B8	510	28	62	**D22**	12	8	62
B9	540	30	64	**D23**	9	5	18
B10	560	36	70	**D24**	1500	68	150
B11	520	14	55	**D25**	1500	61	150
B12	350	9	50	**D26**	90	6	31
B13	305	6	39				

[a] Standard error for the determination of K_i values was 10−20% (from 2 different assays). [b] Human (cloned) isozyme. [c] Isolated from bovine lung microsomes. [d] Data from ref 50.

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

compd	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
28 (acetazolamide)[d]	3.2	0.001	0.37	7.0
29 (methazolamide)[d]	12	0.06	1.90	13
26 (dorzolamide)	60[e]	2.0[e]	3.0	5.2
A16	65[f]	0.043	3.1	5.4
B12	54[f]	1.569	4.6	7.9
B16	59[f]	0.983	2.1	6.0
B26	62[f]	1.785	3.9	7.8
C13	73[f]	1.620	4.1	7.9
C15	75[f]	1.944	4.7	8.5
D11	59[f]	2.133	4.8	11.5
D16	51[f]	0.754	2.0	5.0
D23	50[f]	1.850	3.1	6.4

[a] Solubility in pH 7.40 buffer, at 25 °C. [b] Chloroform−buffer partition coefficient. [c] Determined as described.[11,52,53] [d] Data from ref 11. [e] As hydrochloride, at pH 5.8, from ref 17. [f] As hydrochloride salt.

Table 3. Fall of IOP of Normotensive Rabbits (20.2 ± 2.3 mmHg) after Treatment with 1 drop (50 μL) of a 2% Solution of CA Inhibitor (as hydrochloride, trifluoroacetate, or triflate 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
26[b]	5.5	0	2.2 ± 0.20	4.1 ± 0.30	2.7 ± 0.25
A13[b]	5.5	0	2.9 ± 0.25	5.8± 0.45	4.6 ± 0.30
A15[c]	5.5	0	5.0± 0.20	9.3 ± 0.40	8.4 ± 0.35
A16[d]	6.0	0	5.9 ± 0.25	9.5 ± 0.30	9.0 ± 0.25
B12[b]	5.5	0	2.5 ± 0.25	5.2 ± 0.20	4.5 ± 0.12
B14[b]	6.5	0	3.3 ± 0.30	6.2 ± 0.15	5.5 ± 0.30
B16[c]	6.5	0	5.5 ± 0.25	9.9 ± 0.20	9.4 ± 0.30
B18[d]	6.5	0	2.5 ± 0.15	6.3 ± 0.15	5.5 ± 0.25
B26[b]	5.6	0	3.1 ± 0.20	7.0 ± 0.30	5.5 ± 0.30
C7[b]	6.5	0	3.0 ± 0.15	7.2 ± 0.35	5.0 ± 0.20
C13[c]	6.5	0	6.5 ± 0.20	10.1 ± 0.30	9.1 ± 0.20
C14[b]	7.0	0	5.5 ± 0.15	9.2 ± 0.20	9.0 ± 0.30
C15[d]	7.5	0	6.6 ± 0.20	10.7 ± 0.20	9.8 ± 0.30
C26[b]	6.5	0	5.0 ± 0.40	9.1 ± 0.15	7.6 ± 0.25
D11[c]	6.4	0	3.2 ± 0.25	7.0 ± 0.30	4.8 ± 0.35
D13[d]	6.4	0	5.6 ± 0.30	9.0 ± 0.40	8.9 ± 0.30
D16[b]	7.5	0	5.9 ± 0.20	8.8 ± 0.25	9.1 ± 0.35
D23[b]	6.4	0	3.3 ± 0.25	7.0 ± 0.20	5.4 ± 0.40
D26[b]	6.4	0	4.9 ± 0.35	8.7 ± 0.30	6.9 ± 0.30

[a] $\Delta IOP = IOP_{control\ eye} - IOP_{treated\ eye}$; mean ± average spread ($n = 3$). [b] As HCl salt. [c] As TFA salt. [d] As triflate salt.

Table 4. Fall of IOP of Glaucomatous Rabbits (36.3 ± 2.2 mmHg) after Treatment with 1 drop (50 μL) of a 2% Solution of CA Inhibitor (as hydrochloride, trifluoroacetate, or triflate, 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
A16[d]	6.0	0	7.9 ± 0.35	12.5 ± 0.30	14.0 ± 0.20
B16[c]	6.5	0	6.5 ± 0.50	11.3 ± 0.25	13.7 ± 0.15
C13[c]	6.5	0	8.5 ± 0.45	13.4 ± 0.20	14.5 ± 0.40
C15[d]	7.5	0	8.6 ± 0.30	14.2 ± 0.20	14.3 ± 0.35
D13[d]	6.4	0	7.7 ± 0.20	12.0 ± 0.45	13.8 ± 0.35
D16[b]	7.5	0	7.0 ± 0.25	11.8 ± 0.30	13.1 ± 0.30

[a−d] As in Table 3.

(Chart 5); Blackburn's group[30] also synthesized three structurally related derivatives, **35**−**37**, whereas we have recently reported the β-alanylamido-1,3,4-thiadiazole-2-sulfonamide (**15**),[1] possessing very good water solubility (as HCl salt) and strong IOP-lowering properties in normotensive and glaucomatous rabbits. All compounds just mentioned, **15** and **34**−**37**, are 1,3,4-thiadiazole-2-sulfonamide derivatives, as this was the

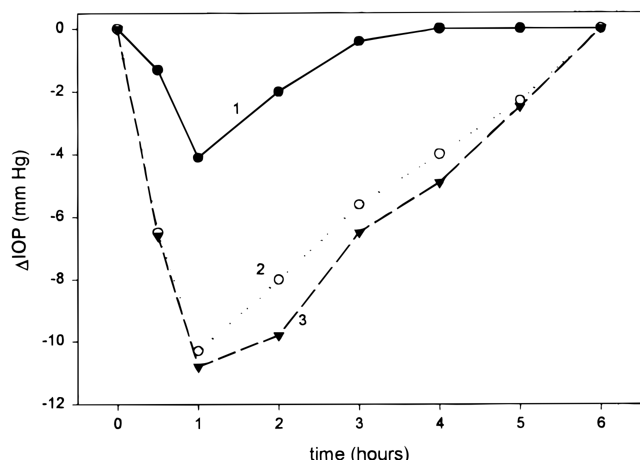


Figure 2. Effect of topically administered sulfonamide inhibitors (2% water solutions) on the IOP of normotensive albino rabbits: curve 1, dorzolamide (**1**) (hydrochloride salt, pH 5.5); curve 2, compound **C13** (trifluoroacetate salt, pH 6.50); curve 3, compound **C15** (triflate salt, pH 7.6).

Table 5. Ocular Tissue Concentrations after 1 and 2 h following Corneal Application of 1 drop (50 μL) of a 2% Solution of **C13** (as trifluoroacetate) and **C15** (as triflate salt) in Normotensive Albino Rabbits

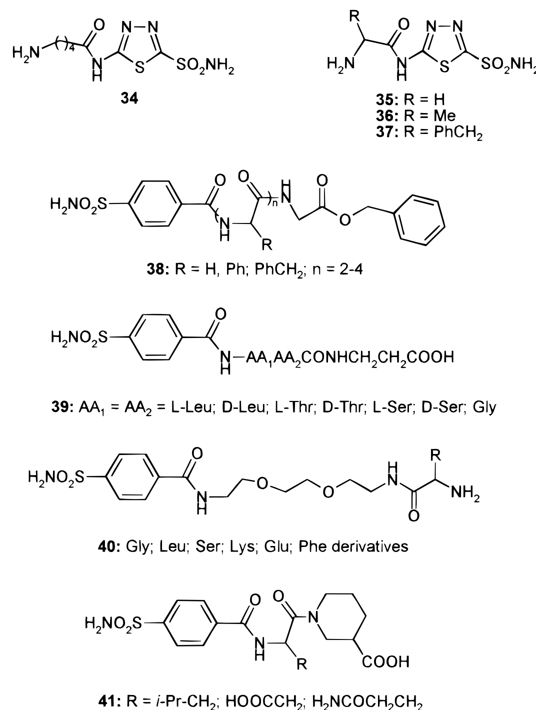
		drug concentration (μM)[a]		
inhibitor	time (h)	cornea	aqueous humor	ciliary process
C13	1	172 ± 9	308 ± 10	54 ± 7
	2	65 ± 8	50 ± 5	18 ± 2
C15	1	184 ± 16	325 ± 24	69 ± 9
	2	73 ± 11	45 ± 6	21 ± 4

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

ring system best studied, due to the fact that the first-generation CA inhibitors derived from it (such as acetazolamide (**28**) or methazolamide (**29**)) were successful drugs, clinically used for more than 40 years.[4,10]

Compounds of a different nature were also reported by Whitesides' (synthesis) and Christianson's (X-ray crystallography) groups.[23,24,26] Generally these were derivatives of 4-carboxybenzenesulfonamide to which oligopeptidyl moieties were attached, of type **38** (eight such derivatives were reported) and **39** (seven such derivatives were reported).[23,24] In another series of such derivatives, oligoethylene glycol units were attached to 4-carboxybenzenesulfonamide and the terminal hydroxy moiety of the tail was derivatized by means of aminoacyl moieties (six derivatives of type **40** were thus obtained).[23,24] Finally, Baldwin's group[31] reported several structurally related inhibitors, of type **41**, obtained again from 4-carboxybenzenesulfonamide, by attaching dipeptidyl moieties incorporating nipecotic acid at its carboxy group. One should notice that almost all these aminoacyl/peptidyl sulfonamide derivatives do not possess free amino groups, as these were derivatized by the COOH moiety of the 4-carboxybenzenesulfonamide used in their preparation (exceptions are the six compounds of type **40**). These free NH_2 groups are of considerable interest for the putative ophthalmologic applications of such inhibitors, since they afford the formation of water-soluble salts with strong acids, which are highly desirable for the topical administration of sulfonamide CA inhibitors (for reasons little explained for the moment, ophthalmologic applications of compounds with weakly acidic pH are preferred over those of alkaline pH).

Chart 5



Obviously, compounds such as **39** or **41** would be highly water-soluble as sodium carboxylate salts, but presumably the pH of such solutions would be in the alkaline range. On the other hand, of all compounds **34**−**41** mentioned above, ophthalmologic applications have been claimed only for those reported by Blackburn's group,[30] but without any quantitative data, and by our own report on compound **15** and some of its derivatives (with detailed ocular pharmacological data).[1] It appeared thus of interest to study in greater detail aminoacyl/dipeptidyl sulfonamide CA inhibitors obtained in such a way as to possess free amino moieties (or their variants, such as *N*-methylamino, *N*-methylguanidino, etc.). Based on previous QSAR studies from this group on several series of CA inhibitors,[32] it emerged that the enhancement of CA inhibitory activity is correlated with increased positive charges on the heterocyclic/aromatic ring incorporated in 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 nitrogen atom, and the long axis of the inhibitor).[32] Examining different potential groups that might be attached to aromatic/heterocyclic sulfonamide CA inhibitors, it appeared of great interest to use glycyl moieties as well as some of their closely related variants, such as sarcosinyl (Sar, *N*-methylglycine) and creatinyl (Crt, *N*-amidinosarcosinyl) as well as glycyl-glycyl (Gly-Gly). Thus, we attached these amino acid/dipeptide derivatives (obviously protected in the appropriate manner) to the amino, hydrazino, imino, or hydroxy groups of sulfonamides **1**−**26**.

Reaction of sulfonamides **1**−**26** with the *N*-protected derivatives **30**−**33** in the presence of EDCI (1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide) or diisopropylcarbodiimide afforded the new derivatives **(A**−**D)1**−**26**, by a procedure relatively similar to that reported by Whitesides' group[23,24] for preparation of the

4-carboxybenzenesulfonamide derivatives **38**−**40**. Mention should be made that the classical *tert*-butoxycarbonyl (*t*-Boc) protecting group was used for the preparation of derivatives of Gly, Sar, and GlyGly, whereas in the case of creatine the guanidino moiety has been protected by using the tritylsulfenyl group, successfully used by us previously for obtaining histamine derivatives with CA activatory properties.[33] Removal of the two protecting groups was achieved then in the standard fashion, i.e., with trifluoroacetic acid (TFA) for *t*-Boc and with HCl-dioxane for TrS.[33]

Salts of the new derivatives were prepared by reacting the free bases mentioned above with a methanolic HCl solution. Similarly were obtained triflate or trifluoroacetate salts, by reaction of the previously mentioned amines with triflic acid or TFA in water as solvent. These salts possessed good water solubility, generally in the range of 2−5% (by weight), i.e., in the range of 50−80 mM (Table 2). Triflates, trifluoroacetates, and hydrochlorides possessed relatively similar water solubilities. The pH of such solution was in the range of 5.5−7.5, making them appropriate for topical application into the eye. As seen from the data of Table 2, some of the newly obtained compounds for which detailed pharmacological data were obtained possessed a relatively moderate lipid solubility, similarly to or slightly less than that of dorzolamide (**26**). In fact, Maren[10] noted in his classical review that one of the conditions needed for a sulfonamide to act as an effective topical IOP-lowering agent is to possess a modest lipid solubility (attributable to its un-ionized form), accompanied by a good water solubility (conferred by the presence of ionizable groups of appropriate pK_a).[10] 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, historically important compounds, acetazolamide (**28**) and methazolamide (**29**), are also shown in Table 2. These two classical high-affinity CA inhibitors (K_i of 8 nM against hCA II for **28** and 7 nM for **29**) possess a too low lipid solubility (acetazolamide) or an acceptable one (methazolamide) correlated with a relatively high water solubility, but only as sodium salts, RSO_2NHNa, and are thus impossible to be adminsitered via the topical route. Both are inactive topically due to poor penetration across the cornea, which is basically due to their very low water solubility (in the neutral form). As seen from the data of Table 2, the compounds reported here possessed excellent water solubility (as HCl salts), balanced by a modest but not insignificant lipid solubility. Their accession rates across the cornea were thus of the same order of magnitude as or slightly better those of dorzolamide.

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; ^{1}H and ^{13}C NMR spectroscopy) that confirmed their structure (see Experimental Section for details) and were assayed for 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 aminoacyl/dipeptidyl moiety attached to these sulfonamides generally led to an increase of the CA inhibitory properties for the obtained compound, as compared to the corresponding parent sulfonamide. Particularly strong inhibitors were those derived from heterocyclic derivatives (1,3,4-thiadiazoles, 1,3,4-thiadiazolines, benzothiazoles, and thienothiopyransulfonamides). Slightly less active were the 1,3-benzenedisulfonamide and 3-fluorosulfanilamide derivatives, together with the pyrimidine-substituted sulfanilamides of type **20** and the sulfanilylsulfanilamides **18** and sulfanilylmetanilamides **19**. The simple aromatic derivatives were (as expected) less active than the previously mentioned heterocyclic sulfonamides. The efficiency of the obtained inhibitor generally varied in the following way, based on the parent sulfonamide from which it was prepared: the derivatives of *p*-hydrazinobenzenesulfonamide **4** < the orthanilamides **1** ≅ the metanilamides **2** < the sulfanilamides **3** < the homosulfanilamides **5** < the *p*-(aminoethyl)benzenesulfonamides **6** ≅ the halogeno-substituted sulfanilamides **7**−**10** ≅ the 1,3-benzenedisulfonamides **11** and **12** ≅ the sulfanilylsulfanilamides **18** and the sulfanilylmetanilamides **19** < the 1,3,4-thiadiazole-2-sulfonamides **13**, **15**, and **16** ≅ the 4-methyl-δ^2-1,3,4-thiadiazoline-2-sulfonamides **14** and **17** ≅ the benzothiazole-2-sulfonamides **21**−**23** ≅ the dorzolamide derivatives **26**. On the basis of the amino acid from which they were obtained, the Gly (**A** type) derivatives were less active than the GlyGly (**D** type) derivatives, which in turn were less active than the sarcosine-based inhibitors (**B** type compounds). The best inhibitors in the whole series were those derived from creatine (**C** type 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 compounds 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).[3−5,11−13]

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: **A13**, **A15**, **A16**, **B12**, **B14**, **B16**, **B18**, **B26**, **C7**, **C13**−**C15**, **C26**, **D11**, **D13**, **D16**, **D23**, and **D26**. The following facts should be noted regarding the IOP-lowering data of Table 3. Some of the new compounds investigated in vivo, such as **A13**, **B12**, **B14**, **B18**, **B26**, **C7**, **D11**, and **D23**, showed IOP-lowering effects generally of the same order of magnitude (or slightly better than) as those of dorzolamide (**26**). Thus, after 0.5 or 1 h after adminsitration of the new drugs, the IOP-lowering values were around 2.5−3.3 and 5.5−7.0 mmHg, respectively (for dorzolamide they are 2.2 and 4.1 mmHg, respectively). An important difference between the two groups of drugs appears at longer periods after administration, since unlike dor-

zolamide, which diminishes its power of action to an IOP lowering of 2.7 mmHg after 90 min, the new compounds mentioned above maintained a more effective IOP lowering, in the range of 4.5−5.5 mmHg, comparable to that observed at 1 h after their administration. A second group of inhibitors, such as **A16**, **B16**, **C13**, **C15**, **D13**, and **D16**, showed much more effective IOP-lowering effects as compared to dorzolamide (**26**), both after 30 min from 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 5.0−6.6 mmHg with the new compounds mentioned above (and only 2.2 mmHg with dorzolamide). At 1 h after administration, the new compounds generally fared at least doubly as well as the clinically used drug **26** (9.0−10.5 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 **26**, for which the pressure decrease amounts to 2.7 mmHg after 90 min). Both hydrochlorides as well as trifluoroacetate or triflate salts of the new derivatives possessed similar IOP-lowering properties.

An important feature of the new class of CA inhibitors reported here is that IOP remained low for longer periods (3−6 h) after their topical administration, as compared to the standard drug dorzolamide (Figure 2). As seen from Figure 2, compounds such as **C13** or **C15** 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 that at 3 or 4 h after administration (when the effects of dorzolamide completely vanished) they still diminished eye pressure appreciably (5.0−6.5 mmHg). IOP generally returned at the baseline values after 5−6 h after administration of the drug. 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 values are much more important as compared to those of normotensive rabbits. Thus, IOP reductions of 6.5−8.6 mmHg were generally observed after 30 min, whereas at longer periods, these amounted to 12.0−14.5 mmHg. No important differences between the different salts 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 potent topical inhibitors in the prepared series, i.e., compounds **C13** and **C15**. 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. On the basis of the inhibition constant of these compounds (5 nM for CA II for **C13** and 2 nM for CA II for **C15**, respectively), the fractional inhibition estimated in these tissues/fluids is 99.5−99.9%, indicating the fact that the IOP decrease is indeed due to CA inhibition.

Conclusions

We report here a general approach for the preparation of water-soluble, topically effective antiglaucoma sulfonamides, by attaching aminoacyl/dipeptidyl water-solubilizing moieties to well-known aromatic/heterocyclic sulfonamides. Ring systems which have been derivatized by the above-mentioned procedures included 2-, 3-, or 4-aminobenzenesulfonamides; 4-(ω-aminoalkyl)-benzenesulfonamides; 3-halogeno-substituted sulfanilamides; 1,3-benzenedisulfonamides; 1,3,4-thiadiazole-2-sulfonamides; and benzothiazole-2-sulfonamides as well as thienothiopyran-2-sulfonamides among others, and they were chosen in such a way as to prove that the proposed approach is a general one. The new compounds formed water-soluble salts by reaction with strong acids, such as hydrochloric, trifluoroacetic, or trifluoromethanesulfonic acids, with protonation of the amino/guanidino nitrogen atom(s). These salts possessed good water solubility, in the range of 2−5%, whereas their lipid solubility and hydrophobicity (log *P*) as well as accession rates across the cornea were those appropriate for acting as efficient topical IOP-lowering agents. The pH of such solutions was in the range of 6.5−7.5. 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 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 and inexpensive antiglaucoma drugs (the presently available topical antiglaucoma sulfonamides dorzolamide and brinzolamide are quite expensive drugs, whereas the patients mostly 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 **1**−**26** used in syntheses were either commercially available compounds (from Sigma, Acros, or Aldrich) or were prepared as described previously: 4-hydrazinobenzenesulfonamide (**4**) by diazotization of sulfanilamide followed by reduction of the diazonium salt with tin(II) chloride;[34] halogenosulfanilamides **7**−**10** by halogenation of sulfanilamide as reported in the literature;[35] compound **15** from 5-amino-1,3,4-thiadiazole-2-sulfonamide (obtained from acetazolamide)[36] by acylation with the phthalimido derivative of β-alanine, followed by hydrazinolysis;[1] imine **14** by deprotection of methazolamide with concentrated hydrochloric acid;[36] aminobenzolamide **16** and the corresponding thiadiazoline **17** as reported in ref 51; sulfanilylsulfanilamide **18** and its *meta* derivative **19** as reported in ref 16. The benzothiazole-2-sulfonamide derivatives **21**−**23** were prepared as described in ref 37, whereas the alcohols **24** and **25** were prepared from the corresponding amines by diazotization followed by hydrolysis of the diazonium salts. Dorzolamide (**26**) was prepared as described in the literature[38] or was obtained from Merck, Sharp and Dohme. Amino acid and dipeptide derivatives; Boc-

On, tritylsulfenyl chloride, EDCI, diisopropylcarbodiimide, TFA, triflic acid, and triethylamine were from Sigma Chemical Co. Acetonitrile, dioxane (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 (A–D)1–26. An amount of 1 mM sulfonamide **1–26** was dissolved/suspended in 25 mL of anhydrous acetonitrile and then treated with the stoichiometric amount (1 mM) of protected amino acid/dipeptide **30–33**. An amount of 190 mg (1 mM) of EDCI·HCl was then added, and the reaction mixture was magnetically stirred at room temperature for 15 min; then 30 μL (2 mM) of triethylamine was added, and stirring was continued for 8–16 h at 4 °C (TLC control). The solvent was evaporated in vacuo and the residue taken up in ethyl acetate (5 mL), poured into a 5% solution of sodium bicarbonate (5 mL), and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate and filtered, and the solvent was removed in vacuo. The obtained oils were directly used in the deprotection step, or the intermediates were recrystallized from ethanol–water or methanol. The removal of the protecting groups was performed as described below. For Boc-protected compounds: the crude intermediate was dissolved in 20 mL of CH_2Cl_2, treated with 4 mL of TFA, and stirred for 10 min at 0 °C. The solvent was removed in vacuo and the residue concentrated from water twice to remove excess TFA, thus giving the amine salt as a colorless syrup. The pure compounds **(A–D)1–26** were obtained by means of preparative HPLC (C_{18} reversed-phase μ-Bondapack or Dynamax-60A (25 × 250 mm) columns; 90% acetonitrile/8% methanol/2% water, 30 mL/min). For the tritylsulfenylated compounds: the crude product was dissolved in 20 mL of dioxane and treated with 25 mL of a 4 M HCl solution in dioxane, followed by heating at 40 °C for 2–3 h (TLC control). The solvent was evaporated in vacuo, the residue taken in 5% solution of sodium bicarbonate (50 mL), and the TrSCl formed in the reaction extracted with 2 × 50 mL of diethyl ether. The aqueous phase was then worked up as described above for the Boc-protected derivatives.

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 and trifluoroacetate salts were similarly obtained from the free bases and the stoichiometric amount of triflic acid/TFA, in water as solvent.

4-Glycinamidobenzenesulfonamide (A3): white crystals, mp 294–6 °C dec; IR (KBr) cm^{-1} 1152 (SO_2^{sym}), 1293 (amide III), 1345 (SO_2^{as}), 1560 (amide II), 1710 (amide I), 3360 (NH, NH_2); ^{1}H NMR (TFA) δ 3.67 (s, 2H, CH_2 of Gly); δ_A 7.18, δ_B 7.75 (AA′BB′system, 4H, J_{AB} = 7.9, ArH from 4-sulfamoylphenyl); 7.59 (br s, 2H, SO_2NH_2) (the H_2N and CONH protons are in fast exchange with the solvent and are not seen in TFA); ^{13}C NMR (TFA) δ 40.8 (s, CH$_2$ of Gly); 129.9; 132.5; 135.9; 142.5; 168.0 (CONH). Anal. Found: C, 42.13; H, 5.02; N, 18.05. $C_8H_{11}N_3O_3S$ Requires: C, 41.91; H, 4.84; N, 18.33.

4-(Sarcosinylamidoethyl)benzenesulfonamide (B6): white crystals, mp 240–2 °C; IR (KBr) cm^{-1} 1158 (SO_2^{sym}), 1295 (amide III), 1355 (SO_2^{as}), 1562 (amide II), 1711 (amide I), 3365 (NH, NH_2); ^{1}H NMR (TFA) δ 3.03 (t, 2H, $^3J_{HH}$ = 6.7, αCH_2); 3.13 (s, 3H, N-Me); 3.70 (s, 2H, CH_2 of Sar); 3.78 (t, 2H, $^3J_{HH}$ = 6.7, βCH_2); δ_A 7.21, δ_B 7.79 (AA′BB′system, 4H, J_{AB} = 8.2, ArH from 4-sulfamoylphenyl); 7.51 (br s, 2H, SO_2NH_2) (the H_2N and CONH protons are in fast exchange with the solvent and are not seen in TFA); ^{13}C NMR (TFA) δ 30.3 (s, CH$_2$ of aminoethyl); 33.7 (s, Me of Crt); 37.5 (s, CH$_2$ of aminoethyl); 41.5 (s, CH$_2$ of Sar); 130.7; 132.8; 134.3; 145.5; 170.2 (CONH). Anal. Found: C, 48.54; H, 6.45; N, 15.33. $C_{11}H_{17}N_3O_3S$ Requires: C, 48.69; H, 6.32; N, 15.49.

5-Creatinyl-1,3,4-thiadiazole-2-sulfonamide (C13): white crystals, mp > 300 °C; IR (KBr) cm^{-1} 1180 (SO_2^{sym}), 1291 (amide III), 1363 (SO_2^{as}), 1564 (amide II), 1715 (amide I), 3060 (NH), 3365 (NH_2); ^{1}H NMR (TFA) δ 3.23 (s, 3H, Me); 3.69 (s, 2H, CH_2 of Crt); 7.25 (br s, 2H, SO_2NH_2); ^{13}C NMR (TFA) δ 33.7 (s, Me of Crt); 40.2 (s, CH$_2$ of Crt); 159.5 (C-2 of thiadiazole); 160.8 (s, NC(=NH)NH$_2$ of Crt), 170.5 (C-5 of thiadiazole); 171.3 (CONH). Anal. Found: C, 24.69; H, 4.01; N, 33.35. $C_6H_{11}N_7O_3S_2$ Requires: C, 24.57; H, 3.78; N, 33.43.

5,6-Dihydro-4-(*N*-glycylglycinamido-*N*-ethyl)-6-methyl-4*H*-thieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-dioxide (D26): white crystals, mp 288–90 °C dec; IR (KBr) cm^{-1} 1133 (SO_2^{sym}), 1292 (amide III), 1345 (SO_2^{as}), 1565 (amide II), 1720 (amide I), 3060 (NH), 3365 (NH_2); ^{1}H NMR (TFA) δ 1.29 (d, 3H, Me); 1.39 (t, 3H, Me from ethyl);: 2.49 (m, 4H, J = 7.0, 2CH_2 of GlyGly); 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); 8.03 (s, 1H, CH, ArH from thienyl); 8.25 (br s, 2H, SO_2NH_2); ^{13}C NMR (TFA) δ 10.0; 11.1; 30.6; 35.8 (s, CH$_2$ of GlyGly); 40.8; 49.3; 51.5; 130.7; 137.5; 141.9; 149.8; 167.1 (CONH); 172.5 (CONH). Anal. Found: C, 38.17; H, 5.23; N, 12.59. $C_{14}H_{22}N_4O_6S_3$ Requires: C, 38.34; H, 5.06; N, 12.78.

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 described by Lindskog et al.[39] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group,[40] and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[41] 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.[42,43] CA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide.[44]

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.[45] 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.[45] 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.[45] 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[46] 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.[47,48] 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 each 30 min for a period of 4−6 h. For all IOP experiments drug was administered to only one eye, leaving the contralateral eye as an untreated control. The ocular hypotensive activity is expressed as the average difference in IOP between the treated and control eye, in this way minimizing the diurnal, seasonal, and interindividual variations commonly observed in the rabbit.[47,48] 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 injection of α-chymotrypsin (Sigma) as described by Sugrue et al.[49] The IOP of operated animals was checked after approximately 4 weeks, and animals with an elevated pressure of 30−36 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.[47,48] The animals were killed with an intracardiac air injection. Aqueous humor (both posterior and anterior chamber fluids) were 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, when ciliary processes were liberated from their attachment to the iris, cut, weighed, and put in 0.5 mL of distilled water. The tissue from 4 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 activities 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.[47,48]

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 the saturated solution; C' = concentration of the saturated solution (mg/mL).[51]

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.[5,51]

Transcorneal Penetration of Drugs. The method of Maren et al.[11] with the modifications of Pierce's group[52,53] (for the HPLC assay of sulfonamides) was been 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 cm². 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 by both the HPLC method of Pierce et al.[52,53] or enzymatically.[5,11] The results of the drug analyses were used to calculate the rate constant of transfer across the cornea (k_{in}). As described by Pierce,[52,53] 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. Thanks are addressed to Drs. M. A. Ilies, B. Iorga, and M. Barboiu for expert technical assistance and to Dr. A. Liljas for providing the X-ray coordinates of the aminobenzolamide−hCA II adduct.

References

(1) This paper is part 77 of the series. Preceding part: Barboiu, M.; Supuran, C. T.; Scozzafava, A.; Menabuoni, L.; Mincione, F.; Briganti, F.; Mincione, G. Carbonic anhydrase inhibitors. Part 76. Synthesis of topically effective intraocular pressure lowering agents derived from 5-(ω-aminoalkylcarboxamido)-1,3,4-thiadiazole-2-sulfonamide. *J. Enzyme Inhib.* **1999**, *14*, in press.

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JM9901879