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Carbonic Anhydrase Inhibitors With Strong Topical Antiglaucoma Properties Incorporating a 4-(2-amino-pyrimidin-4-yl-amino)-benzenesulfonamide Scaffold

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Reaction of 4-(2-amino-pyrimidin-4-yl-amino)-benzenesulfonamide with alkyl/aryl-sulfonyl halides, acyl halides or arysulfonyl isocyanates afforded a series of derivatives which were tested for inhibition of three carbonic anhydrase (CA) isozymes. These compounds were designed in such a way as to (i) strongly inhibit several CA isozymes involved in aqueous humor secretion within the eye (such as CA II and CA IV), and (ii) to possess a pharmacological profile that allows easy penetration through the cornea, when administered as eye drops in solution or suspension, constituting thus a valuable therapeutic approach for glaucoma. Several of the obtained inhibitors showed low nanomolar affinities for the two isozymes involved in aqueous humor secretion, CA II and CA IV. Furthermore, in normotensive and hypertensive rabbits, some of them showed an effective and prolonged intraocular pressure (IOP) lowering when administered topically, as 2% suspensions/solutions.

Keywords: Carbonic anhydrase; Sulfonamide; Intraocular pressure; Glaucoma

INTRODUCTION

Sulfonamides possessing carbonic anhydrase (CA, EC 4.2.1.1) inhibitory properties such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** or dichlorophenamide **DCP** have been used for more than 40 years as systemic eye pressure lowering drugs in the treatment of open-angle glaucoma as well as other diseases associated with acid/base secretory disequilibria.[1–3] Their ocular effects are due to inhibition of at least two CA isozymes present within ciliary processes of the eye, i.e., CA II and CA IV, followed by a diminished secretion of bicarbonate and a 25–30% reduction of aqueous humor secretion.[1–3] The main drawback of such agents is constituted by side effects such as augmented diuresis, fatigue, paresthesias, anorexia, etc., due to CA inhibition in other tissues/organs than the target one (CA, in the form of 14 isozymes, is ubiquitous in vertebrates).[1–4]

The above-mentioned side effects are absent where the inhibitor has topical activity, and is applied directly into the eye. This route, discovered in 1983 by Maren's group[5] was shortly followed by the development of the first agents of this type, dorzolamide **DZA** (clinically launched in 1995)[6] followed soon thereafter (in 1999), by the structurally-related brinzolamide **BRZ**.[7] These topically acting antiglaucoma sulfonamides incorporate secondary amine moieties in their molecule, as the required water solubility needed for effective topical action is achieved by using their hydrochloride salts (in the case of **DZA**; **BRZ** is used as a suspension, as free base).[6,7] Still, this represents an undesired problem, since the pH of such solutions is rather acidic (pH 5.5), and consequently produces eye irritation after the topical administration of the drug, as already reported for many patients treated with dorzolamide (the use of brinzolamide is rather

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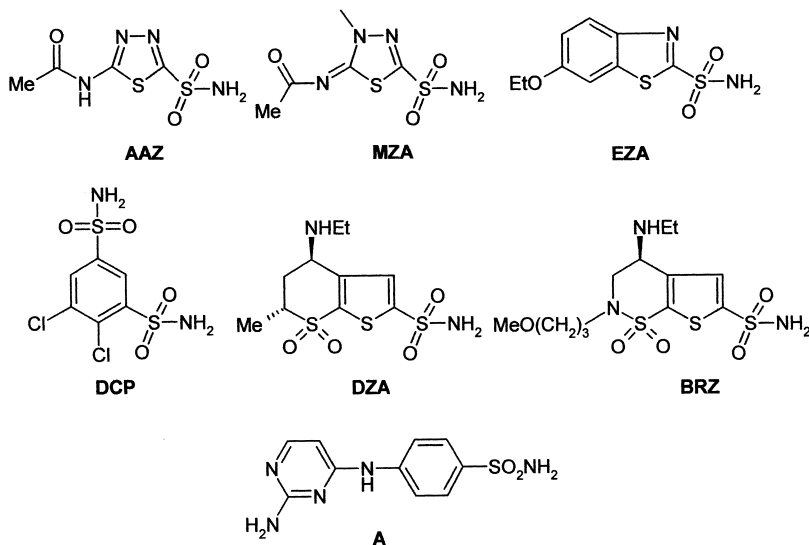
recent, and few studies of the side effects of this drug are available at present).[1,6,7] In fact, the most common adverse effects after topical dorzolamide treatment are local burning and stinging of the eyes, reddening of the eyes, blurred vision and bitter taste,[1,6,7] but more serious side effects, such as contact allergy, nephrolithiasis, anorexia, depression and dementia as well as irreversible corneal decompensation in patients already presenting corneal problems, were also reported with dorzolamide.[8–11]

The clinical importance of the two existing topical antiglaucoma sulfonamides, but also their imperfections due to many undesired topical side effects, fostered much research in the synthesis and evaluation of novel generation of such derivatives.[12–20] The major side effects of dorzolamide mentioned above might be avoided for compounds that should not be administered as hydrochloride salts, but this generally leads to a drastic diminution of water solubility of such a sulfonamide.[1,2,12] Here we propose another approach for obtaining high affinity sulfonamide CA inhibitors with potent topical antiglaucoma properties, when administered as solutions or suspensions directly into the rabbit eye, using a 4-(2-amino-pyrimidin-4-yl-amino)-benzenesulfonamide scaffold to which alkyl/arylsulfonylamido-, alkyl/arylcarboxamido- or arylsulfonylureido moieties were attached at the aminopyrimidine moiety. Clearly we have used the "tail" approach reported earlier by this group[1,16] for the design of these new pharmacologically active enzyme inhibitors.

MATERIALS AND METHODS

General

Melting points were recorded with a heating plate microscope and are not corrected. IR spectra were recorded in KBr pellets with a Carl Zeiss IR-80 instrument. ^{1}H-NMR spectra were recorded in DMSO-d_6 or TFA as solvents, with a Bruker CPX200 or Varian 300 instrument. Chemical shifts are reported as δ values, relative to Me$_4$Si as internal standard. Elemental analyses were done by combustion for C, H, N with an automated Carlo Erba analyzer, and were ±0.4% of the theoretical values. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Analytical and preparative HPLC was performed on a reversed-phase C$_{18}$ Bondapack column, with a Beckman EM-1760 instrument. Sulfonamide **A**, sulfonyl halides, acyl halides and arylsulfonylisocyanates used in synthesis were either commercially available compounds (from Sigma-Aldrich, Milan, Italy, or Acros, Milan, Italy) or were prepared as described previously.[13,15,16] Dorzolamide was from Merck and Co. (Trusopt[R] eye drops), whereas brinzolamide was from Alcon Laboratories (Azopt[R] eye drops). Acetone, acetonitrile, DMF and other solvents (E. Merck) used in the synthesis/chromatography were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions.

General Procedure for the Preparation of Sulfonamides 1–40

Sulfonamide **A**, 5 mmol, was dissolved/suspended in 50 ml of anhydrous acetonitrile and cooled to 2–5°C in a salt-ice bath and then 5 mmol of sulfonyl/acyl chloride were added in small portions, concomitantly with the stoichiometrc amount of triethylamine, maintaining the temperature under 10°C. The reaction mixture was then stirred at room temperature for 5–10 h (TLC control), adjusted to pH 2 with 5N HCl, and the precipitated sulfonamides

were filtered and recrystallized from aqueous ethanol. Yields were in the range 75–85%.

General Procedure for the Preparation of Sulfonamides 41–45

Sulfonamide **A**, 10 mmol, was suspended in 50 ml of anhydrous acetonitrile and treated with a solution obtained from 10 mmol of arylsulfonylisocyanates dissolved in 10 ml of the same solvent. A small volume (100 μl) of triethylamine was added as catalyst. The reaction was performed by magnetically stirring the mixture at room temperature for 2–6 h (TLC control). 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, when 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 80–90%.

4-(2-Methylsulfonylamino-pyrimidin-4-yl-amino)-benzenesulfonamide 1

As white crystals, m.p. 254°–5°C (MeOH). IR (KBr), cm^{-1}: 1135 and 1160 (SO$_2^{sym}$), 1330 and 1374 (SO$_2^{as}$); ^{1}H-NMR (d$_6$-DMSO), δ, ppm; J, Hz: 2.76 (s, 3H, Me), 6.19 (d, 1H, H6-pyrimidine, 2.9), 6.52 (m, 1H, H5-pyrimidine, 2.9), 7.10 (br s, 1H, NH), 7.23 (s, 2H, SO$_2$NH$_2$), 7.78 (d, 2H, AA′BB′, 8.9), 7.91 (d, 2H, AA′BB′, 8.9), 10.23 (s, 1H, SO$_2$NH); ^{13}C-NMR (d$_6$-DMSO), δ, ppm: 25.50 (Me), 110.34 (C5-pyrimidine), 112.67 (C6-pyrimidine), 119.20 (C2/C3-Ph), 125.71 (C4-pyrimidine), 126.51 (C3/C2-Ph), 137.92 (C1/C4-Ph), 142.39 (C4/C1-Ph), 161.33 (C2-pyrimidine). Anal. (C$_{11}$H$_{13}$N$_5$O$_4$S$_2$) C, H, N, S.

4-(2-Pentafluorophenylsulfonylamino-pyrimidin-4-yl-amino)-benzenesulfonamide 30

As white crystals, m.p. 213°–5°C (EtOH–water, 2:1). IR (KBr), cm^{-1}: 1143 and 1160 (SO$_2^{sym}$), 1330 and 1358 (SO$_2^{as}$); ^{1}H-NMR (d$_6$-DMSO), δ, ppm; J, Hz: 6.21 (d, 1H, H6-pyrimidine, 2.9), 6.54 (m, 1H, H5-pyrimidine, 2.9), 7.11 (br s, 1H, NH), 7.15 (s, 2H, SO$_2$NH$_2$), 7.78 (d, 2H, AA′BB′, 8.9), 7.91 (d, 2H, AA′BB′, 8.9), 10.53 (s, 1H, SO$_2$NH); ^{13}C-NMR (d$_6$-DMSO), δ, ppm: 110.30 (C5-pyrimidine), 112.69 (C6-pyrimidine), 118.84 (C2/C3-Ph), 125.63 (C4-pyrimidine), 126.42 (C3/C2-Ph), 137.70 (C1/C4-Ph), 142.12 (C4/C1-Ph), 161.40 (C2-pyrimidine); ^{19}F-NMR (DMSO-d$_6$), δ, ppm: −134.0 (2,6-F$_2$), −143.7 (4-F), −158.9 (3,5-F$_2$). Anal. (C$_{16}$H$_{10}$F$_5$N$_5$O$_4$S$_2$) C, H, N, S.

4-(2-Nicotinoylamido-pyrimidin-4-yl-amino)-benzenesulfonamide 39

As white crystals, m.p. 238°–9°C (EtOH). IR (KBr), cm^{-1}: 1160 (SO$_2^{sym}$), 1290 (amide III), 1330 (SO$_2^{as}$), 1560 (amide II), 1690 (amide I); ^{1}H-NMR (d$_6$-DMSO), δ, ppm; J, Hz: 6.21 (d, 1H, H6-pyrimidine, 2.9), 6.54 (m, 1H, H5-pyrimidine, 2.9), 7.05–7.70 (m, 7H, ArH from nicotinoyl + NH + SO$_2$NH$_2$), 7.78 (d, 2H, AA′BB′, 8.9), 7.91 (d, 2H, AA′BB′, 8.9), 8.75 (s, 1H, CONH); ^{13}C-NMR (d$_6$-DMSO), δ, ppm: 110.30 (C5-pyrimidine), 112.69 (C6-pyrimidine), 118.84 (C2/C3-Ph), 121.34 (C2/C6 nicotinoyl), 125.63 (C4-pyrimidine), 126.42 (C3/C2-Ph), 127.13 (C5 nicotinoyl), 137.70 (C1/C4-Ph), 139.27 (C4-nicotinoyl), 142.12 (C4/C1-Ph), 144.50 (C3-nicotinoyl), 161.40 (C2-pyrimidine), 173.45 (CONH). Anal. (C$_{16}$H$_{14}$N$_6$O$_3$S) C, H, N, S.

4-[2-(4-Tosylureido)-pyrimidin-4-yl-amino]-benzenesulfonamide 42

As white crystals, m.p. 290°–1°C (EtOH–water, 1:1). IR (KBr), cm^{-1}: 1131 and 1160 (SO$_2^{sym}$), 1287 (amide III), 1330 and 1380 (SO$_2^{as}$), 1579 (amide II), 1710 (amide I); ^{1}H-NMR (d$_6$-DMSO), δ, ppm; J, Hz: 2.63 (s, 3H, CH_3C$_6$H$_4$), 6.20 (d, 1H, H6-pyrimidine, 2.9), 6.52 (m, 1H, H5-pyrimidine, 2.9), 7.10 (br s, 1H, NH), 7.23 (s, 2H, SO$_2$NH$_2$), 7.65 (d, ^{3}J$_{HH}$ = 8.1, 2H, *H*ortho of CH$_3$C$_6$H$_4$), 7.78 (d, 2H, AA′BB′, 8.9), 7.91 (d, 2H, AA′BB′, 8.9), 7.99 (d, ^{3}J$_{HH}$ = 8.1, 2H, *H*meta of CH$_3$C$_6$H$_4$), 8.21 (br s, 2H, NHCONH); ^{13}C-NMR (d$_6$-DMSO), δ, ppm: 26.1 (s, *C*H$_3$C$_6$H$_4$), 110.34 (C5-pyrimidine), 112.67 (C6-pyrimidine), 119.20 (C2/C3-Ph), 125.71 (C4-pyrimidine), 126.51 (C3/C2-Ph), 130.9 (s, *C*meta of CH$_3$C$_6$H$_4$), 132.4 (s, NHCONH), 135.0 (s, *C*ortho of CH$_3$C$_6$H$_4$), 137.92 (C1/C4-Ph), 142.39 (C4/C1-Ph), 145.0 (s, *C*ipso of CH$_3$C$_6$H$_4$), 148.6 (s, *C*para of CH$_3$C$_6$H$_4$), 161.33 (C2-pyrimidine) Anal. (C$_{18}$H$_{18}$N$_6$O$_5$S$_2$) C, H, N, S.

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.*[26] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group,[27] and enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[28] 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.[29,30] CA IV was isolated from bovine lung microsomes as described by Maren *et al.*, and its

concentration has been determined by titration with ethoxzolamide.[31]

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.[32] 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, under the conditions of the experiments (pH 7.40), as reported in the literature.[32] Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constant K_I was determined as described by Pocker and Stone.[32] 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[33] 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*, being maintained on a 12 h:12 h light/dark cycle in a temperature controlled room, at 22°–26°C. Solutions/suspensions of inhibitors (2%, by weight, as hydrochlorides, or sodium carboxylates) were obtained in distilled deionized water. The pH of these solutions was in the range of 5.5–7.4.

IOP was measured using a Digilab 30R pneuma-tonometer (BioRad, Cambridge, MA, USA) as described by Maren's group.[34,35] The pressure readings were matched with two-point standard pressure measurements at least twice each day using a Digilab Calibration verifier. All IOP measurements were done by the same investigator with the same tonometer. One drop of 0.2% oxybuprocaine hydrochloride (novesine, Sandoz) diluted 1:1 with saline was instilled in each eye immediately before each set of pressure measurements. IOP was measured three times at each time interval, and the means reported. IOP was measured first immediately before drug administration, then at 30 min after the instillation of the pharmacological agent, and then each 30 min for a period of 2 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.[34,35] All data are expressed as mean ± SE. 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.*[36] The IOP of operated animals was checked after approximately four weeks, and animals with an elevated pressure of 30–36 mm Hg were used at least one month after the injection of α-chymotrypsin.

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 (λ_{max} and ε were the same in methanol and buffer, within the limits of the experimental error). 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).[37]

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.[37]

Transcorneal Penetration of Drugs

The method of Maren *et al.*[5] with the modifications of Pierce's group[38,39] (for the HPLC assay of sulfonamides) have been used. Excised rabbit corneas with

TABLE I   Compounds of type **1–45** prepared in this study, with their CA inhibition data against hCA I, hCA II, and bCA IV. Inhibition data for standard sulfonamides are also included

1-35   **36-41**   **42-45**
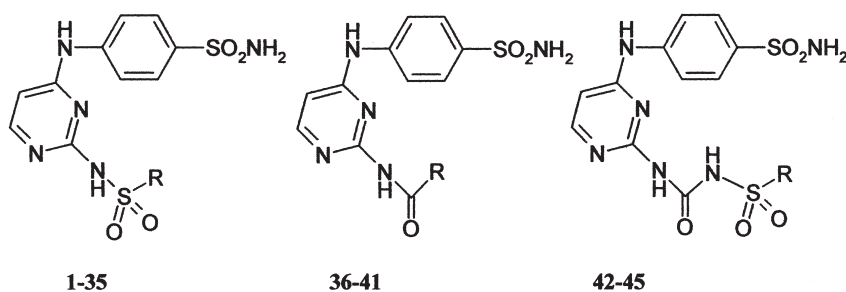
No.	R	K_I (nM)			M.p. (°C)
		hCA I*	hCA II*	BCA IV†	
1	Me	530	11	54	254–5
2	Et	510	11	50	237–9
3	n-Pr	500	10	52	215–6
4	i-Pr	550	12	59	198–200
5	CF_3	460	10	45	201–3
6	CCl_3	480	11	43	216–8
7	C_4F_9	410	9	42	137–8
8	C_8F_{17}	450	9	58	135–7
9	Me_2N	540	10	130	268–70
10	$PhCH_2$	350	5	62	241–3
11	Ph	310	4	60	270–1
12	$4\text{-Me-}C_6H_4$	250	7	63	249–50
13	$4\text{-F-}C_6H_4$	260	4	50	240–2
14	$4\text{-Cl-}C_6H_4$	250	4	50	246–7
15	$4\text{-Br-}C_6H_4$	310	3	43	250–2
16	$4\text{-I-}C_6H_4$	270	3	42	248–9
17	$4\text{-MeO}C_6H_4$	300	5	46	233–5
18	$4\text{-AcNH}C_6H_4$	290	3	38	275–7
19	$4\text{-Ac}C_6H_4$	310	3	36	219–21
20	$4\text{-}H_2N\text{-}C_6H_4$	240	2	35	279–80
21	$3\text{-}H_2N\text{-}4\text{-MeO-}C_6H_3$	260	3	37	231–3
22	$2,4,6\text{-}Me_3C_6H_2$	500	8	120	187–8
23	$2,4,6\text{-i-}Pr_3C_6H_2$	450	13	135	162–4
24	$2\text{-}HO_2CC_6H_4$	190	5	35	208–9
25	$3\text{-}HO_2CC_6H_4$	400	4	39	269–71
26	$4\text{-}HO_2CC_6H_4$	410	4	36	273–5
27	$4\text{-Cl-}3\text{-}O_2N\text{-}C_6H_3$	250	3	42	228–30
28	$3,5\text{-}Cl_2C_6H_3$	300	3	40	215–7
29	$2\text{-HO-}3,5\text{-}Cl_2\text{-}C_6H_2$	310	3	47	266–8
30	C_6F_5	325	1	24	213–5
31	$3\text{-}CF_3C_6H_4\text{-}$	300	1	24	174–6
32	1-Naphthyl	350	6	48	192–3
33	2-Naphthyl	380	7	56	198–9
34	Quinolin-8-yl	360	8	59	210–1
35	Thiophen-2-yl	120	6	43	237–8
36	C_8F_{17}	410	9	50	141–3
37	$2,4\text{-}Cl_2C_6H_3$	380	8	67	246–8
38	C_6F_5	310	5	42	176–8
39	3-Pyridyl	290	4	37	238–9
40	4-Pyridyl	320	6	45	261–3
41	2-Pyridyl	300	6	44	215–6
42	$4\text{-Me-}C_6H_4$	3	4	10	288–9
43	$2\text{-Me-}C_6H_4$	5	6	13	254–6
44	$4\text{-F-}C_6H_4$	2	4	7	271–2
45	$4\text{-Cl-}C_6H_4$	2	3	6	268–9
A	–	690	12	154	
DZA		50000	9	45	
BRZ		nt	3	45	

*Human (cloned) isozymes.
†From bovine lung microsomes, nt = not tested.

TABLE II Solubility, chloroform−buffer partition coefficients and *in vitro* corneal permeability of some sulfonamide CA inhibitors

			$k_{in} \times 10^3$ (h^{-1})‡	
Compound	Solubility*(mM)	P†	Cornea intact	No epithelium
AAZ	3.2¶	0.001	0.37	7.0
EZA	0.04	25	40	49
DZA	60§	2.00	3.0	5.2
10	2.0	2.41	4.3	16
11	2.8	1.85	3.6	9.4
15	2.4	1.56	2.8	7.5
30	27	1.95	3.9	9.8
31	15	1.63	4.0	9.6
39	54§	1.27	2.7	7.6
42	0.05	0.024	0.8	1.5
45	0.10	0.058	0.9	1.7

* Solubility in pH 7.40 buffer, at 25°C.
† Chloroform−buffer partition coefficient.
‡ Determined as described in reference5.
¶ Data from references [13,14].
§ As hydrochloride, at pH 5.5 for **DZA** and 6.5 for **39**.

either intact or denuded epithelium were used in these experiments. The pH was 7.4 and the exposed area was $1.2\,cm^2$. Concentrations of drug of 40– 2000 μM were placed in the epithelial chamber and samples of fluid were collected from the endothelial chamber at different intervals, up to 4 h. Both chambers contained 6 ml. Drugs present in these fluids were assayed both by the HPLC method of Pierce *et al.*[38,39] or enzymatically.[37] The results of the drug analyses were used to calculate the rate constant of transfer across the cornea (k_{in}). As described by Pierce[38,39], this value was determined by using the formula:

$$k_{in}(\times 10^3\,hr^{-1}) = [drug]_{endo}/[drug]_{epi} \times 60/t \times 1000$$

where $[drug]_{endo}$ = concentration of drug on endo- thelial side; $[drug]_{epi}$ = concentration of drug on epithelial side; t = time (in min).

Drug Distribution in Ocular Fluids and Tissues

The general procedure of Maren's group was followed.[34,35] The animals were killed with an intracardiac injection. Aqueous humor (both pos- terior 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 activity of the free enzyme and in the presence of the inhibitor was determined as described above. A calibration curve was used in order to determine the fractional inhibition in the different tissues, as described in references 34,35.

RESULTS

Synthesis

Compounds **1**–**45** prepared by attaching alkyl/ar- ylsulfonylamido-, alkyl/arylcarboxamido- or aryl- sulfonylureido moieties to 4-(2-amino-pyrimidin-4- yl-amino)-benzenesulfonamide (**A**) are shown in Table I. A large series of such derivatives was prepared, as it is well established that the nature of the "tail" moiety strongly influences the physico- chemical, biological and pharmacological properties of such CA inhibitors.[1,12−21]

Carbonic Anhydrase Inhibitory Activity

Inhibition data against three CA isozymes, known to play a crucial role in aqueous humor secretion (CA II and CA IV) or in the manifestation of side effects after the treatment with sulfonamide CA inhibitors (CA I), with the compounds prepared in this study are shown in Table I.

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

TABLE III Fall of IOP of normotensive rabbits (21 ± 2 mm Hg), after treatment with one drop (50 μl) 2% water suspension/solution (for dorzolamide, **DZA** and **39**) of CA inhibitor directly into the eye, at 30, 60, 90 and 120 minutes after administration

Inhibitor	pH	ΔIOP (mm Hg)*				
		t = 0	t = 30 min	t = 60 min	t = 90 min	t = 120 min
DZA†	5.5	0	2.0 ± 0.2	4 ± 0.3	3 ± 0.25	1.2 ± 0.3
BRZ	5.5	0	2.9 ± 0.1	3.2 ± 0.3	6.3 ± 0.4	7.1 ± 0.20
10	7.5	0	9.1 ± 0.3	2 ± 0.25	1 ± 0.2	0
11	7.5	0	6.4 ± 0.2	12.1 ± 0.5	9.1 ± 0.2	7.3 ± 0.4
15	7.5	0	9.0 ± 0.4	8.1 ± 0.3	7.3 ± 0.2	5.8 ± 0.3
18	7.5	0	10.2 ± 0.3	8.6 ± 0.4	6.5 ± 0.2	5.4 ± 0.2
30	7.5	0	9.3 ± 0.2	9.7 ± 0.5	9.5 ± 0.4	6.1 ± 0.2
31	7.5	0	6.0 ± 0.2	9.5 ± 0.4	9.9 ± 0.3	10.1 ± 0.2
39†	6.5	0	8.1 ± 0.3	9.2 ± 0.5	6.5 ± 0.3	5.8 ± 0.3
42	7.5	0	0	0	0.5	0

* $\Delta IOP = IOP_{control\ eye} - IOP_{treated\ eye}$ Mean ± SE ($n = 3$).
† As HCl salt, in solution.

Intraocular Pressure Lowering

In vivo IOP lowering data with some of the most active CA inhibitors reported here, in normotensive and hypertensive rabbits after topical administration of the drug, are shown in Tables III and IV, respectively.

Distribution of Drugs in Ocular Fluids and Tissues

Ex vivo distribution data of some active compound in ocular tissues and fluids after the topical administration in normotensive rabbits are shown in Table V.

DISCUSSION

The drug design of compounds **1**–**45** reported here has been done by considering a finding that emerged after several QSAR studies on aromatic/heterocyclic sulfonamides possessing CA inhibitory properties[22,23] as well as topical activity as antiglaucoma agents.[24] Thus, it has been observed that the enhancement of CA inhibitory activity for a series of aromatic/heterocyclic sulfonamides 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).[22–24] The recent finding[19] that the pyrimidinyl-substituted, commercially available sulfonamide **A**, and especially some of its derivatives, show good CA inhibitory properties, prompted us to use this scaffold for generating novel structures possessing the desired properties: an elongated molecule that would presumably lead to powerful CA inhibitory properties, accompanied by desired physico-chemical features that might be fine-tuned by choosing different substituents of the primary amino moiety to be derivatized. In previous papers[13,16–23] we showed that introduction of alkyl/arylsulfonyl- or alkyl/arylcarboxyl moieties in the molecules of aromatic sulfonamides possessing free amino, hydrazino or hydroxy moieties, is correlated with a strong increase in the CA inhibitory properties of the obtained derivatives. Thus, we decided to derivatize sulfonamide **A** by using the same approach. It was also observed during these studies that the reactivity of the secondary amino group of **A** is very weak, and that practically only compounds derivatized at the primary amino group are obtained by reacting **A** with sulfonyl halides, acyl

TABLE IV Fall of IOP of hypertensive rabbits (35 ± 2 mm Hg) after treatment with one drop (50 μl) 2% suspension/solution of sulfonamide directly into the eye, at 30, 60, 90 and 120 minutes after administration

Inhibitor	pH	ΔIOP (mm Hg)*				
		t = 0	t = 30 min	t = 60 min	t = 90 min	t = 120 min
DZA†	5.5	0	3.6 ± 0.20	6.7 ± 0.30	4.2 ± 0.15	3.5 ± 0.30
30†	7.5	0	11.9 ± 0.3	12.6 ± 0.4	11.5 ± 0.4	9.7 ± 0.4
31†	7.5	0	9.5 ± 0.5	11.4 ± 0.5	15.3 ± 0.2	14.7 ± 0.3
39‡	6.5	0	9.6 ± 0.3	12.1 ± 0.2	14.5 ± 0.3	13.1 ± 0.5

* $\Delta IOP = IOP_{control\ eye} - IOP_{treated\ eye}$ Mean ± SE ($n = 3$).
† Solution.
‡ Suspension.

TABLE V   Ocular tissue concentrations (μM) after one and two
hours, following corneal application of one drop ($50\,\mu$l) of 2%
solution of the sulfonamide carbonic anhydrase inhibitors **DZA**
(dorzolamide. HCl); **31** and **39** (as hydrochloride salt) in
normotensive albino rabbits

| Inhibitor | Time (h) | Drug concentration (μM)* | | |
		Cornea	Aqueous humor	Ciliary process
DZA	1	105 ± 5	32 ± 3	15 ± 3
(.HCl)	2	39 ± 4	21 ± 2	6 ± 1
31	1	165 ± 5	270 ± 10	67 ± 3
	2	95 ± 6	86 ± 3	32 ± 1
39	1	153 ± 6	275 ± 15	54 ± 5
(.HCl)	2	87 ± 8	79 ± 8	33 ± 3

*Mean $\pm$ standard error ($n = 3$).

halides or arylsulfonyl isocyanates under normal
conditions (i.e., one equivalent of **A** and acylating/
sulfonylating agent in the presence of base—Et$_3$N,
pyridine or sodium bicarbonate—at room tempera-
ture). The new compounds **1**–**35**, **36**–**40** and **41**–**45**
were obtained by the above-mentioned reactions,
respectively (Table I). The syntheses were effected in
very good yields, without obtaining undesired side
products, by using the previously reported pro-
cedures for derivatization of aromatic sulfonamides
with sulfonyl halides (for **1**–**35**),[13,18,22,23] acyl halides
(for **36**–**40**),[16,20] and arylsulfonyl isocyanates (for
41–**45**),[25] respectively.

The new compounds **1**–**45** have been tested as
inhibitors of three, physiologically relevant CA
isozymes, i.e., hCA I, hCA II and bCA IV (Table I).
It may be seen that introducing arylsulfonyl-,
arylcarbonyl or arylsulfonylureido moieties on the
free NH$_2$ group of **A**, generally led to an increase of
the CA inhibitory properties of the obtained
derivatives. Thus, the largest majority of these new
sulfonamides possessed affinities in the 1–9 nM
range against hCA II (compared to 12 nM for the
parent sulfonamide **A**), 6–70 nM against bCA IV
(compared to 154 nM for the parent sulfonamide **A**),
and 2–410 nM against hCA I (compared to 690 nM
for the parent sulfonamide **A**), respectively. Several
of the new inhibitors, such as those incorporating
alkylsulfonamido- (**1**–**6**), dimethylureido (**9**) or
bulky arylsulfonylamido (**23**) moieties, were slightly
less active CA inhibitors as compared to the
previously mentioned derivatives. The derivatives
incorporating arylsulfonylureido moieties (**41**–**45**)
possessed excellent CA inhibitory properties against
all isozymes, with inhibition constants of 2–5 nM
against the sulfonamide-resistant isozyme hCA I, of
3–6 nM against the hCA II, and 6–13 nM against
bCA IV. Many sulfonamides reported here possessed
quite similar potency as CA inhibitors to the
clinically used drugs dorzolamide **DZA** and brinzo-
lamide **BRZ** against isozymes hCA II and bCA IV,

whereas they were much better hCA I inhibitors as
compared to dorzolamide.

IOP lowering data in normotensive rabbits with
several of the most active *in vitro* CA inhibitors
reported here, such as compounds **10**, **11**, **15**, **18**, **30**,
31, **39** and **42** (Table III), indicate that the derivatives
incorporating 4-(2-amino-pyrimidin-4-yl-amino)-
benzenesulfonamide scaffolds may act as efficient
antiglaucoma agents in experimental animals. Thus,
except for the derivatives incorporating arylsulfony-
lureido moieties (**41**–**45**) which do not possess IOP
lowering properties (although they were among the
most potent *in vitro* inhibitors) —a phenomenon that
will be explained below—both sulfonamides of type
1–**35** as well as carboxamides of type **36**–**40** were
effective IOP lowering agents after topical adminis-
tration into the eye, as 2% suspensions/solutions.
Thus, at 30 minutes after administration, the IOP
lowering amounted to 6.0–10.2 mm Hg with the new
derivatives investigated here, versus 2–2.9 mm Hg
with the two clinical drugs **DZA** and **BRZ**. After one
hour, IOP lowering with the new derivatives was in
the range of 8.1–12 mm Hg, versus 3.2–4 mm Hg
with the standard drugs. A compound faring not too
well (2 mm Hg IOP lowering), such as **10** was also
included in these experiments in order to demon-
strate how the physico-chemical properties are of
crucial importance for the strong IOP lowering effect
of this class of pharmacological agents. In fact, as will
be discussed shortly, this compound is too lipophilic,
being washed away from the ciliary processes, and
passing into the blood which contains high amounts
of CAs. This is the common fate of all too lipophilic
CA inhibitors, such as ethoxzolamide **EZA** for
example (Table II). After 90 and 120 minutes post-
administration, dorzolamide shows a very weak IOP
lowering (of 3 and 1.2 mm Hg, respectively), whereas
brinzolamide is more effective (6.3 and 7.1 mm Hg,
respectively—this is the peak of IOP lowering
achieved with **BRZ**). With the new compounds
investigated here, the IOP lowering after 90 minutes
was in the range of 6.5–9.9 mm Hg, whereas at
120 minutes of 5.4–10.1 mm Hg. The IOP returned to
baseline after 5–6 h with these new compounds
(especially **11**, **30**, **31** and **39**), whereas the same effect
was seen 4 h after BRZ (data not shown). It may be
clearly observed that some of the new derivatives
reported in the present paper are much more
effective IOP lowering agents than the two clinically
available drugs, both in terms of peak IOP lowering,
as well as duration of action, which is prolonged for
some of them.

At this point it is interesting to observe how the
moiety derivatizing the primary amino group of **A** in
the new sulfonamides investigated here influences
the physico-chemical and antiglaucoma properties of
these new compounds (Table II). Thus, all the
sulfonamides investigated *in vivo* were quite power-

ful CA inhibitors (K_I-s against hCA II in the 1–6 nM range), similarly to dorzolamide and brinzolamide. Only one compound (the isonicotinoyl derivative **39**) was soluble enough to be administered as a solution (at pH 6.5), similarly to **DZA** (administered at pH 5.5, which may be quite irritating to the eye tissues). The fluoro-containing derivatives **30** and **31** had an intermediate solubility (15–27 mM) between **39** and the other, poorly water soluble compounds, such as **10**, **11**, **15**, etc (solubility in the range of 2.0–2.8 mM, at pH 7.5). Thus, all other derivatives investigated here (except **39** and **DZA**) were administered as suspensions, similarly to brinzolamide **BRZ**. Another factor that varied much in the series of sulfonamides reported here was the lipophilicity. Thus, it may be observed that the very lipophilic drug ethoxzolamide has a very high corneal accession rate, and although it is a very potent CA inhibitor (K_I of 1 nM against hCA II) it is totally devoid of IOP lowering effects, due to the fact that it is washed away from the ciliary processes by the blood, which contains high amounts of CAs, as shown in the classical work of Maren's group.[4,5] Conversely, too hydrophilic compounds (such as acetazolamide **AAZ**) are also ineffective, due to the fact that an insufficient amount of inhibitor arrives at the ciliary processes enzymes. Thus, effective topical IOP lowering is best achieved by compounds possessing a balanced hydro- and liposolubility, and this is very well exemplified by some of the derivatives investigated here. The arylsulfonylureido derivatives **41**–**45** were among the most effective *in vitro* CA inhibitors in this series, but they were devoid of IOP lowering properties, due to their very low lipophilicity (Log P in the range of 0.024–0.058) and water solubility. Conversely, the phenylmethylsulfonyl derivative **10** was one of the most lipophilic among the investigated compounds, which may account for the strong IOP lowering observed at 30 minutes after administration (−9.1 mm Hg), which vanished rapidly (−2 mm Hg after one hour), due to the fact that the compound shares the same fate as the much more lipophilic drug ethoxzolamide. The rest of the investigated derivatives (such as **11**, **15**, **30**, **31** and **39**) possessed a decreased lipophilicity compared to **10** Log P in the range of 1.2–1.9, which is quite similar to that of dorzolamide. This leads to good accession rates through the cornea and inhibition of ciliary process CAs, which ultimately provoked a prolonged IOP lowering after topical administration of such sulfonamides.

The same strong IOP lowering has been observed with some of the new compounds (such as **30**, **31** and **39**) in hypertensive rabbits (Table IV), but the effect is much more pronounced. Thus, after 30 minutes, the IOP lowering was in the range of 9.5–11.9 mm Hg, after 60 minutes of 11.4–12.6 mm Hg, after 90 minutes of 11.5–15.3 mm Hg, and after 120 minutes of 9.7–14.7 mm Hg. Pressure returned to baseline after 5–6 h (data not shown).

Table V shows *ex vivo* data obtained in normotensive rabbits after the topical administration of SOME of the most active topical inhibitors in the prepared series, i.e., compounds **31** and **39**, as compared to the standard drug dorzolamide. It can be observed that at one and two hours after topical administration of the drug, high levels of inhibitors were found in the cornea, aqueous humor and ciliary processes. Based on the inhibition constant of these compound (in the low nanomolar range, see Table I), the fractional inhibition estimated in these tissues/fluids is 99.5–99.9%, indicating that the IOP decrease is indeed due to CA inhibition. Furthermore, as seen from the data of Table V, the new compounds reported here, such as **31** and **39**, tend to concentrate in the aqueous humor (concentrations of around 270–275 µM were detected after one hour after administration), whereas dorzolamide reaches much lower concentrations there (32 µM after one hour). High concentrations of the inhibitor were maintained at 2 h after administration too. Concentrations of the new compounds **31**, **39** in the cornea and ciliary processes are also enhanced as compared to those of dorzolamide. Thus, it may be concluded 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 and under investigation in this laboratory.

CONCLUSIONS

We report here a novel class of topically acting antiglaucoma sulfonamides which incorporate 4-(2-amino-pyrimidin-4-yl-amino)-benzenesulfonamide scaffolds. These were obtained by derivatizing the primary amino group of sulfonamide **A** by reaction with sulfonyl halides, acyl halides or arylsulfonylisocyanates. Some of these new CA inhibitors, possessed affinities in the low nanomolar range for isozymes hCA I, hCA II and bCA IV, acting as effective enzyme inhibitors *in vitro*. Many of the newly reported derivatives showed good water solubility, hydrophobicity (Log P) as well as accession rates across the cornea, appropriate for acting as efficient topical IOP lowering agents. Several of the most active *in vitro* inhibitors strongly lowered IOP pressure in normotensive and hypertensive rabbits, showing a prolonged duration of action as compared to dorzolamide or brinzolamide. The new compounds reported here might lead to the development

of more efficient antiglaucoma drugs from the class of the sulfonamide CA inhibitors, with less side effects as compared to the presently available drugs.

Acknowledgements

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