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Original article

Carbonic anhydrase inhibitors – Part 78[#]. Synthesis of water-soluble sulfonamides incorporating β-alanyl moieties, possessing long lasting-intraocular pressure lowering properties via the topical route

Claudiu T. Supuran[a]*, Fabrizio Briganti[a], Luca Menabuoni[b], Giovanna Mincione[a], Francesco Mincione[c], Andrea Scozzafava[a]

[a]Università degli Studi, Laboratorio di Chimica Inorganica e Bioinorganica, Via Gino Capponi 7, I-50121, Florence, Italy
[b]Ospedale San Giovanni di Dio, S. O. Oculistica, Via Torregalli 3, I-50123, Florence, Italy
[c]Università degli Studi, Istituto Oculistico, Viale Morgagni 85, I-50134, Florence, Italy

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Abstract – Reaction of 26 aromatic/heterocyclic sulfonamides containing amino, imino, hydrazino or hydroxyl groups with *N*-tert-butyloxycarbonyl-β-alanine (Boc-β-ala; Boc = *t*-butoxycarbonyl) in the presence of carbodiimide derivatives afforded, after removal of the protecting group, a series of water-soluble compounds (as salts of strong acids, such as hydrochloric, trifluoroacetic or trifluoromethane sulfonic). 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. Good inhibition was observed against all three isozymes, but especially against CA II and CA IV (in the nanomolar range), the two isozymes known to play a critical role in aqueous humour secretion within the ciliary processes of the eye. Some of the best inhibitors synthesized were applied as 2% aqueous solutions into the eyes of normotensive or glaucomatous albino rabbits, when strong and long-lasting intraocular pressure (IOP) lowering was observed with many of them. Thus, the amino acyl groups conferring water solubility to these sulfonamide CA inhibitors, coupled with their strong enzyme inhibitory properties and balanced lipid solubility seem to be the key factors for obtaining compounds with effective topical antiglaucoma activity. © 2000 Éditions scientifiques et médicales Elsevier SAS

carbonic anhydrase / sulfonamide / topical action / β-alanine / intraocular pressure lowering

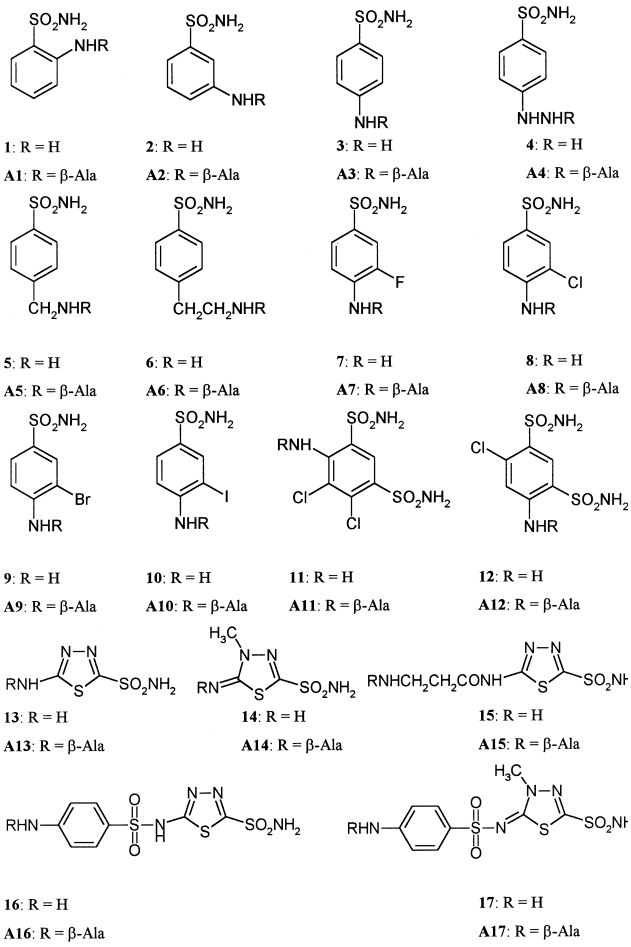
1. Introduction

In previous contributions from this laboratory [1–3] it was shown that by attaching water-solubilizing tails (such as 8-quinoline-sulfonyl- [1], nicotinoyl- [2], isonicotinoyl- [2] or 6-carboxy-pyridine-2-carboxamido- [2, 3] among others) to the molecules of aromatic/heterocyclic sulfonamides of types **1–26**, 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 anti-glaucoma [9] activity following topical administration to experimental animals (*figure 1*).

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 humour [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 could 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. The use of such hydrochlo-

[#] See ref. [1]. This paper is dedicated to the memory of Tom Maren (1917–1999) who contributed important discoveries to the field of carbonic anhydrase inhibitors.

*Correspondence and reprints: cts@bio.chim.unifi.it



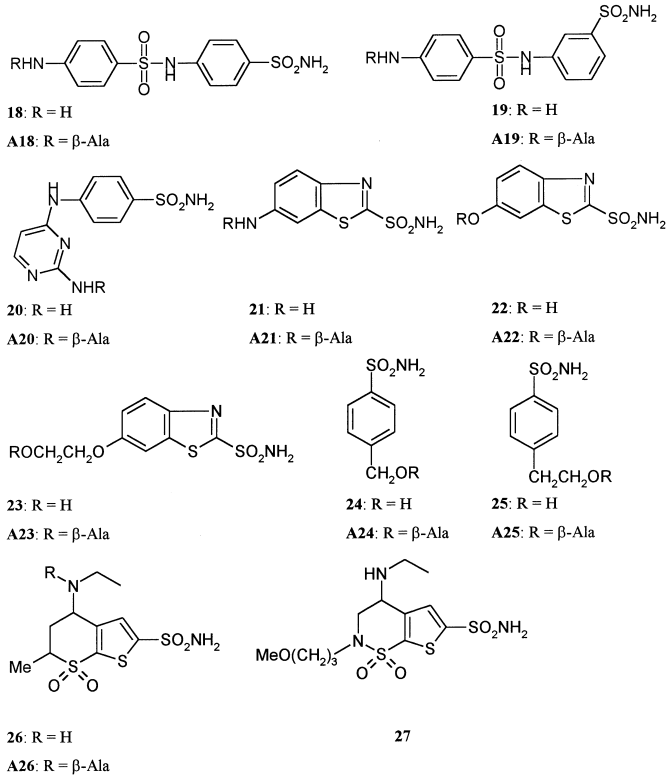


Figure 1. Structure of sulfonamides **1–27** and their β-alanyl derivatives **A1–A26**. In formulas **A1**–**A26**, β-Ala = $H_2NCH_2CH_2CO$-.

ride salts is imposed by the need of assuring a good water solubility to the drugs, but in some cases this represents an undesired complication, since the pH of such solutions becomes acidic, 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 such 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) devoid of severe undesired side effects (*figure 2*).

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 [23–26] 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 co-ordinated to the Zn(II) ion within the CA active site) [27–29]. The above-mentioned findings [23–26] (which generally exploited hydrophobic interactions between hydrophobic patches at the entrance of the hCA II active site and similar moieties present in the

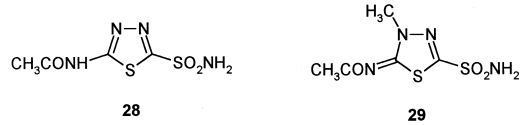


Figure 2. Structure of acetazolamide **28** and methazolamide **29**.

inhibitor 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 humour secretion (CA II and CA IV). Obviously, in order to achieve this goal, we used the opposite of the approach reported by Whitesides' group [23–26], i.e. taking advantage of the hydrophilic interactions between secondary binding sites and the enzyme, since just hydrophilic inhibitors are of interest for ophthalmological applications (mention should be made that the above-mentioned researchers [23–26] 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). In this context we [30, 31] 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) [30, 31] 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 [28] it is possible to evidence that the terminal part of the sulfonamide molecule (i.e., the aminophenyl moiety) is placed in such a way to interact favourably with some of the hydrophilic residues belonging to the histidine cluster mentioned above, pointing towards the entrance of the active site

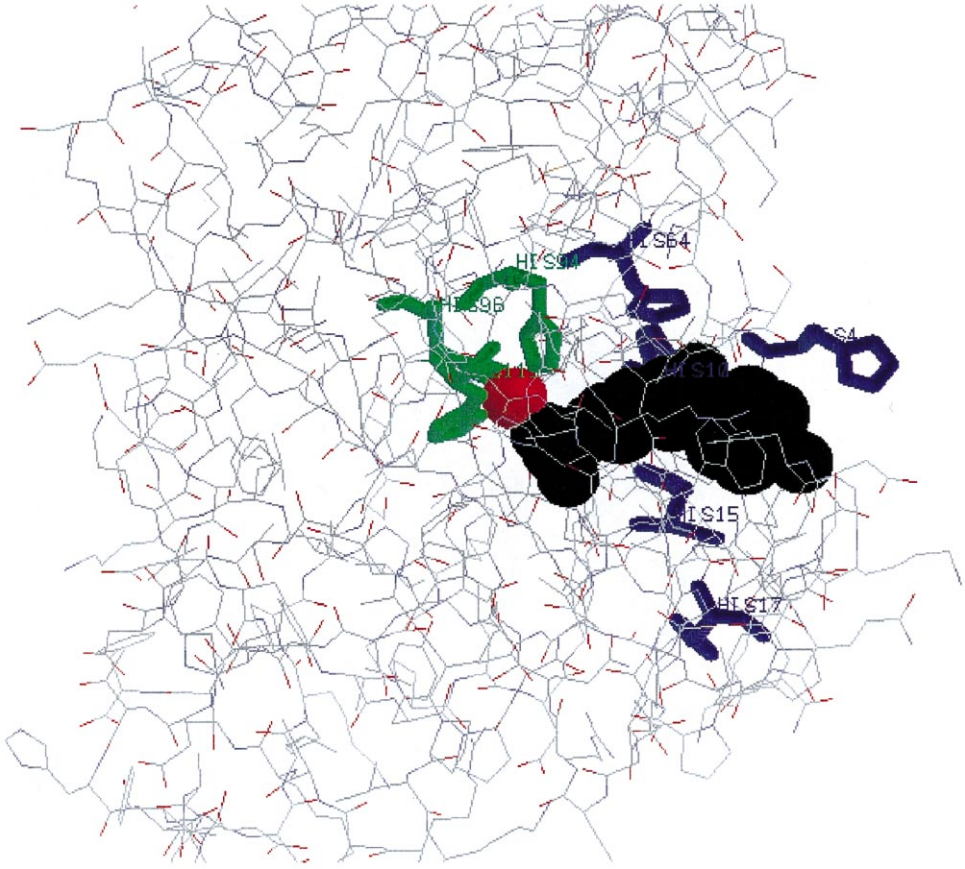


Figure 3. hCA II–aminobenzolamide (**16**) complex. The Zn(II) ion (central red sphere) and its three histidine ligands (His 94, 96 and 119; green), as well as the histidine cluster comprising residues 3 (disordered in this structure), 4, 10, 15, 17 and 64 are evidenced. The inhibitor molecule (shown in the space-fill form, in black) is co-ordinated to the Zn(II) ion and interacts with some of the residues of the hydrophilic cluster mentioned above. The figure has been generated from the X-ray co-ordinates of Liljas group [28] by using the program Rasmol for Windows. This structure is not deposited in the Brookhaven Protein Database.

(*figure 3*). Obviously, compounds with a longer molecule than aminobenzolamide, as some of those reported here, might interact with other amino acid residues of the cluster just mentioned above.

In this paper we report the reaction of 26 aromatic/heterocyclic sulfonamides containing a free amino, imino, hydrazino or hydroxyl group, with the *N*-Boc-protected derivative of β-alanine (Boc-β-Ala, Boc = *t*-butoxycarbonyl) in the presence of carbodiimides, which afforded (after removal of the protecting groups) a series of water-soluble (as hydrochloride, 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 compound also possessed good water solubility (as salt 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 humour secretion. The most active derivatives were assayed in vivo in normotensive and glaucomatous rabbits, for their intraocular pressure (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 amino acyl/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 thienothiopyran-sulfonamides 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).

2. Chemistry

Each new compound reported in the present work will be designated by a letter identifying the amino acid from which it is derived (β-alanine), and a figure identifying the sulfonamide of type **1–26** at which the β-alanyl moiety has been attached. For instance, **A3** is β-alanyl-sulfanilamide; **A6** is 4-(β-alanylamidoethyl)-benzene-sulfonamide; **A13** is 5-(β-alanylamido)-1,3,4-thiadiazole-2-sulfonamide; **A26** is the β-alanylamido derivative of dorzolamide, etc. (*figure 4*).

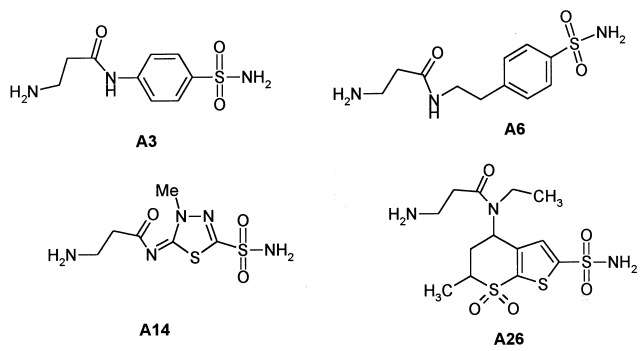


Figure 4. Structures of derivatives **A3**, **A6**, **A14** and **A26.**

The new compounds **A1–A26** were prepared by reaction of the aromatic/heterocyclic sulfonamides **1**–**26** with the *N*-Boc-protected derivative of β-alanine in the presence of carbodiimides, followed by removal of the protecting group in the standard manner (in the presence of trifluoroacetic acid, TFA).

3. Pharmacology

Inhibition data against three CA isozymes, hCA I, hCA II and bCA IV with compounds **A1–A26** as well as with the original raw materials and standard sulfonamides of type **1–29** are shown in *table I*. 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-protected derivatives of compounds **A1–A26** prepared here also possessed very good CA inhibitory properties (data not shown), but since their solubility in water were relatively reduced as compared to those of the corresponding β-alanyl-derivatives, these data are not reported here.

Some physico-chemical properties of the new compounds, 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 active derivatives, such as dorzolamide and some of the new compounds reported in the present study are also shown in *table II*.

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 III* and *IV*, respectively.

The full time dependence of the IOP after dorzolamide and some of the new compounds reported here in normotensive albino rabbits is shown in *figure 5*.

Table I. CA inhibition data with standard inhibitors, the parent sulfonamides **1–26** and the new derivatives **A1–A26** reported in the present study, against isozymes I, II and IV.

Inhibitor	K_I* (nM)		
	hCA I[a]	hCA II[a]	bCA IV[b]
1	45 400	295	1 310
2	25 000	240	2 200
3	28 000	300	3 000
4	78 500	320	3 215
5	25 000	170	2 800
6	21 000	160	2 450
7	8 300	60	180
8	9 800	110	320
9	6 500	40	66
10	6 000	70	125
11	6 100	28	175
12	8 400	75	160
13	8 600	60	540
14	9 300	19	355
15	455	3	125
16	6	2	5
17	1	0.6	0.8
18	42	6	50
19	44	9	53
20	690	12	154
21	70	9	19
22	55	8	17
23	50	7	15
24	24 000	125	560
25	18 000	110	450
26	50 000	9	45
27[c]	–	3.2	45.3
A1	23 000	200	255
A2	17 600	190	233
A3	15 000	115	170
A4	25 000	236	315
A5	1 600	36	77
A6	855	34	71
A7	550	10	42
A8	600	38	72
A9	625	36	70
A10	550	40	69
A11	500	11	54
A12	350	10	50
A13	310	9	42
A14	330	11	46
A15	28	5	18
A16	13	3	12
A17	15	4	16
A18	69	15	80
A19	68	14	75
A20	57	19	82
A21	15	5	12
A22	16	4	14
A23	9	3	13
A24	1 700	70	185
A25	1 700	62	150
A26	94	5	30

* Standard error for the determination of K_I's was of 10–20% (from 2 different assays); [a]Human (cloned) isozyme; [b]Isolated from bovine lung microsomes; [c]Data from ref. [57].

Table II. Solubility, chloroform–buffer partition coefficients and in vitro corneal permeability of some sulfonamide CA inhibitors.

Compound	Solubility[a]	Lpg P[b]	$K_{in} \times 10^3$ (h^{-1})[c]	
	mM		Cornea intact	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
A7	58[f]	1.498	3.6	5.9
A12	53[f]	1.516	3.7	6.3
A13	70[f]	1.059	2.4	5.0
A15	77[f]	2.083	3.7	7.5
A17	49[f]	1.742	2.8	5.5
A26	54[f]	2.125	3.9	7.7

[a]Solubility in pH 7.40 buffer, at 25 °C; [b]Chloroform–buffer partition coefficient; [c]Determined as described in ref. [11, 58, 59]; [d]Data from ref. [11]; [e]As hydrochloride, at pH 5.8, from ref. [17]; [f]As hydrochloride salt.

Ex vivo distribution data of some active compounds in ocular tissues and fluids after topical administration in normotensive rabbits are shown in *table V*.

4. Results and discussion

Although several thousand different aromatic/heterocyclic sulfonamide CA inhibitors have been synthesized in the last 45 years in the search for diverse pharmacological agents [4, 10], the number of amino acyl/oligopeptidyl derivatives is unexpectedly small. Antonarolli et al. [32] reported one such compound, **30**; Blackburn's group [33] also synthesized three structurally related derivatives, **31–33**, whereas we have recently

Table III. Fall of IOP of normotensive rabbits (20.5 ± 2.8 mm Hg), after treatment with one drop (50 μL) of a 2 % solution of CA inhibitor (as hydrochloride, trifluoroacetate or triflate salt, with the pH value shown below) directly into the eye, at 30, 60 and 90 min after administration.

Inhibitor	pH	ΔIOP (mm Hg)*			
		t = 0	t = 30 min	t = 60 min	t = 90*min
26[a]	5.5	0	2.2 ± 0.20	4.1 ± 0.30	2.7 ± 0.25
A7[b]	5.5	0	2.8 ± 0.30	5.3 ± 0.40	4.5 ± 0.20
A12[b]	5.5	0	3.0 ± 0.25	5.8 ± 0.45	4.9 ± 0.30
A13[c]	5.5	0	5.9 ± 0.30	9.7 ± 0.30	9.0 ± 0.35
A15[a]	6.5	0	6.2 ± 0.40	10.9 ± 0.35	9.8 ± 0.35
A17[a]	6.5	0	5.8 ± 0.25	9.8 ± 0.30	9.1 ± 0.35
A26[a]	6.5	0	2.9 ± 0.30	5.7 ± 0.35	4.9 ± 0.30

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

Table IV. Fall of IOP of glaucomatous rabbits (36.7 ± 3.0 mm Hg) after treatment with one drop (50 μL) 2 % of a solution of CA inhibitor (as hydrochloride, trifluoroacetate or triflate, with the pH value shown below) directly into the eye, at 30, 60 and 90 min after administration.

Inhibitor	pH	ΔIOP (mm Hg)*			
		t = 0 min	t = 30 min	t = 60 min	t = 90 min
26[a]	5.5	0	3.6 ± 0.30	6.7 ± 0.35	4.2 ± 0.15
A13[c]	5.5	0	8.0 ± 0.40	12.5 ± 0.30	9.7 ± 0.30
A15[a]	6.5	0	8.2 ± 0.45	12.9 ± 0.40	9.8 ± 0.45
A17[a]	6.5	0	7.8 ± 0.35	11.5 ± 0.30	9.0 ± 0.30

*ΔIOP = $IOP_{control\ eye}$ – $IOP_{treated\ eye}$; Mean ± average spread (n = 3). [a, c]As in *table III*.

Table V. Ocular tissue concentrations (μM) after 1 and 2 h following corneal application of one drop (50 μL) of a 2 % solution of the compounds **A13** (as triflate) and **A15** (as hydrochloride salt) in normotensive albino rabbits.

Inhibitor	Time (h)	Drug concentration (μM)*		
		Cornea	Aqueous humour	Ciliary process
A13	1 h	164 ± 12	295 ± 15	50 ± 3
	2 h	69 ± 7	57 ± 6	18 ± 3
A15	1 h	180 ± 12	329 ± 20	62 ± 7
	2 h	79 ± 10	46 ± 4	25 ± 3

*Mean ± standard deviation (n = 4).

reported the β-alanylamido-1,3,4-thiadiazole-2-sulfonamide **15** [2] possessing very good water solubility (as HCl salt) and strong IOP lowering properties in normotensive and glaucomatous rabbits. All compounds just mentioned, **15** and **30–33** are 1,3,4-thiadiazole-2-sulfonamide derivatives, as this was the 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] (*figure 6*).

Compounds of a different nature were also reported by Whitesides' (synthesis) and Christianson's (X-ray crystallography) groups [23–26, 29]. Generally these were derivatives of 4-carboxy-benzenesulfonamide, to which oligopeptidyl moieties were attached, of type **34** (eight such derivatives were reported) and **35** (seven such derivatives were reported) [23–26]. In another series of such derivatives, oligoethylene glycol units were attached to 4-carboxy-benzenesulfonamide, and the terminal hydroxy moiety of the tail was derivatized by means of amino acyl moieties (six derivatives of type **36** were thus obtained) [26]. Finally, Baldwin's group [34] reported several structurally-related inhibitors, of type **37**, ob-

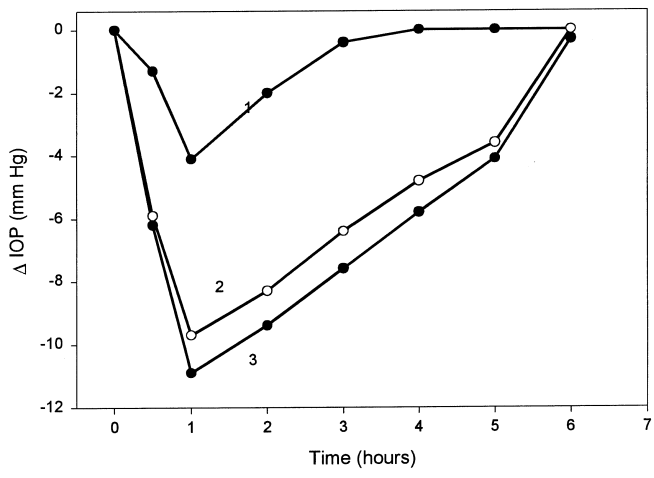


Figure 5. Effect of topically administered sulfonamide inhibitors (2% water solutions) on the IOP of normotensive albino rabbits. Curve 1: dorzolamide **1** (hydrochloride salt, pH 5.5); curve 2: compound **A13** (triflate salt with pH 5.50); curve 3: compound **A15** (hydrochloride salt, pH 6.5).

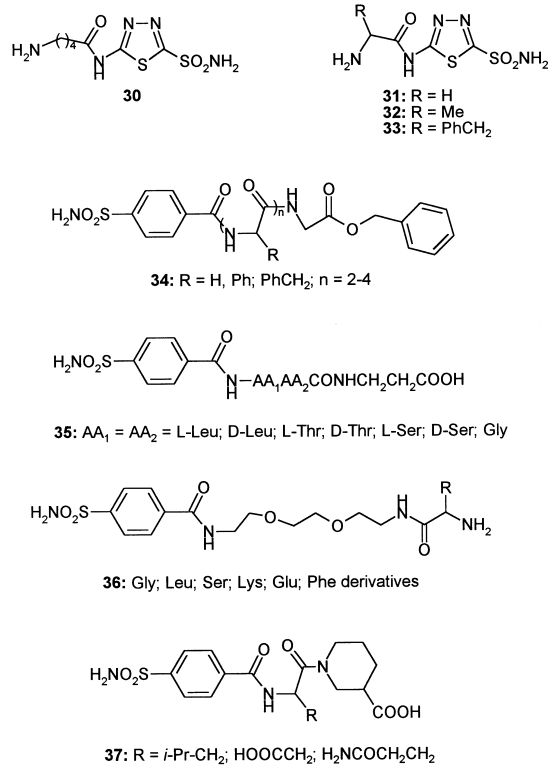


Figure 6. Structures of derivatives **30–37**.

tained again from 4-carboxy-benzenesulfonamide, by attaching dipeptidyl moieties incorporating nipecotic/isonipecotic acid at its carboxy group. One should notice that almost all these amino acyl/peptidyl sulfonamide derivatives do not possess free amino groups, as these were derivatized by the COOH moiety of the 4-carboxy-benzenesulfonamide used in their preparation (exceptions are the 6 compounds of type **36**). These free NH_2 groups are of considerable interest for the putative ophthalmological 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, ophthalmological applications of compounds with weakly acidic pH are preferred to those of alkaline pH). Obviously, compounds such as **35** or **37** 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 **30–37** mentioned above, ophthalmological applications have been claimed only for those reported by Blackburn's group [33], but without any quantitative data, and by our own report on compound **15** and some of its derivatives (with detailed ocular pharmacological data) [2]. It thus appeared of interest to study in greater detail amino acyl/dipeptidyl sulfonamide CA inhibitors obtained in such a way as to possess free amino moieties. Based on previous QSAR studies from this group on several series of CA inhibitors [35–38] 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 in the direction passing through the Zn(II) ion of the enzyme, the sulfonamide nitrogen atom and the long axis of the inhibitor) [35–38]. Examining different potential groups that might be attached to aromatic/heterocyclic sulfonamide CA inhibitors, it appeared of great interest to use β-alanyl, especially considering the fact that in one such derivative previously prepared by us (**15**) the enhancement of activity as compared to the parent sulfonamide (**13**) was quite important [2]. Thus, we attached these amino acyl moiety to the amino-, hydrazino-, imino- or hydroxy groups of sulfonamides **1–26**.

Reaction of sulfonamides **1–26** with the *N*-Boc-protected derivative Boc-β-Ala in the presence of EDCI (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide) or diisopropylcarbodiimide, afforded (after the removal of the Boc group) the new derivatives **A1–A26** by a procedure relatively similar to that reported by Whitesides' [23–26] and Baldwin's [34] groups for preparation of the 4-carboxybenzenesulfonamide derivatives **34–37**. Removal of the protecting group was then achieved in the standard fashion, i.e. with trifluoroacetic acid (TFA) [39].

Salts of the new derivatives were prepared by reacting the free bases obtained as mentioned above with a methanolic HCl solution. Triflate or trifluoroacetate salts were obtained similarly 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 II*). Triflates, trifluoroacetates and hydrochlorides possessed relatively similar water solubilities. The pH of such solutions 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 II*, some of the newly obtained compounds for which detailed pharmacological data were obtained, possessed a relatively moderate lipid solubility, similarly 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 non-ionized form), accompanied by a good water solubility (conferred by the presence of ionizable groups of appropriate pK_a) [10]. In order 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 **27** and methazolamide **28**, are also shown in *table II*. These two classical high affinity CA inhibitors (K_I of 8 nM against hCA II for **27**, and 7 nM for **28**) 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 administered via the topical route. Both are inactive topically due to poor penetrability across the cornea, which is basically due to their very low water solubility (in neutral form). As seen from the data in *table II*, the compounds reported here possessed excellent water solubility (as salts of strong acids), balanced by a modest but not insignificant lipid solubility. Their accession rates across the cornea were thus of the same order of magnitude 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 the inhibition of isozymes hCA I, hCA II and bCA IV (*table I*).

Inhibition data against three CA isozymes, hCA I, hCA II and bCA IV with the new derivatives (*table I*) proved

that the β-alanyl moiety attached to these sulfonamides always 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 thienothiopyran-sulfonamides). Slightly less active were the 1,3-benzene-disulfonamide and 3-fluorosulfanilamide derivatives, together with the pyrimidine-substituted sulfanilamides of type **A20**, and the sulfanilyl-sulfanilamides **A18** and sulfanilyl-metanilamides **A19**. 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*-hydrazino-benzenesulfonamide **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-benzene-disulfonamides **11** and **12** ≅ the sulfanilyl-sulfanilamides **18** and the sulfanilyl-metanilamides **19** < the 1,3,4-thiadiazole-2-sulfonamides **13**, **15** and **16** ≅ 4-methyl-δ^2-1,3,4-thiadiazoline-2-sulfonamide **14** and **17** ≅ the benzothiazole-2-sulfonamides **21–23** ≅ the dorzolamide derivatives **26**.

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.

The promising in vitro CA inhibitory activity, as well as other physico-chemical properties mentioned above for some of the newly prepared compounds, prompted us to investigate their effect in vivo, on the intraocular pressure (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 favourable properties, such as a moderate lipid solubility, good accession rates across the cornea, etc. Such compounds included, among others, **A7**, **A12**, **A13**, **A15**, **A17** and **A26**. The following facts should be noted regarding the IOP lowering data of *table III*. Some of the new compounds investigated in vivo, such as **A7**, **A12** and **A26**, showed IOP lowering effects generally of the same order of magnitude (or slightly better) as those of dorzolamide **26**. Thus, after 0.5–1 h after administration of the new drugs, the IOP lowering values were around 2.8–3.0 mm Hg, and 5.3–5.8 mm Hg, respectively (for dorzolamide they are of 2.2 and 4.1 mm Hg, respectively). 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 mm Hg after 90 min, the new compounds mentioned above maintained a more effective IOP lowering, in the range of 4.5–5.0 mm Hg, comparable to that observed at 1 h after their administration. A second group of inhibitors, such as **A13**, **A15** and **A17**, showed much more effective IOP lowering effects as compared to dorzolamide **26**, 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 5.8–6.2 mm Hg with the new compounds mentioned above (and only of 2.2 mm Hg with dorzolamide). At 1 h after the administration the new compounds generally fared at least 2.5 times as well as the clinically used drug **26** (9.5–10.9 mm Hg for the new derivatives, versus 4.1 mm Hg 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 mm Hg 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 5*). As seen from *figure 5*, compounds such as **A13** or **A15** possessed maximal IOP lowering effects at 1 h after administration, similarly to dorzolamide. In contrast to dorzolamide, these new IOP lowering agents also maintained their effect at 3 or 4 h after administration when they still diminished eye pressure appreciably (5.0–6.5 mm Hg). IOP generally returned to the baseline values after 5–6 h after administration of the drug. Thus, all these derivatives are longer lasting and more effective topical IOP lowering agents as compared to the clinically available drug dorzolamide.

The above findings also apply for the glaucomatous rabbit experiments (*table IV*) but the IOP are much more important as compared to those of normotensive rabbits. Thus, IOP reductions of 7.8–8.2 mm Hg were generally observed after 30 min, whereas at longer periods these amounted to 11.5–12.9 mm Hg. 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).

Table V 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 **A13** and **A15**. 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 humour and ciliary processes. Based on the inhibition constant of these compound (9 nM for CA II for **A13** and 5 nM for CA II for **A15**, respectively), 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.

5. Conclusions

We report here a general approach for the preparation of water-soluble, topically effective antiglaucoma sulfonamides, by attaching β-alanyl 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-benzene-disulfonamides; 1,3,4-thiadiazole-2-sulfonamides; benzothiazole-2-sulfonamides as well as thienothiopyran-2-sulfonamides among others, and 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 acid, with protonation of the β-alanyl amino nitrogen atom. These salts possessed good water solubility, in the range of 2–5%, whereas their lipid solubility, 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 5.5–7.5. 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 pressure in normotensive and glaucomatous rabbits, showing a prolonged duration of action as compared to dorzolamide. The new compounds reported here might lead to the development of more efficient and inexpensive antiglaucoma drugs (the presently available topical antiglaucoma sulfonamides, dorzolamide and brinzolamide are quite expensive drugs, whereas the worst affected patients are generally elderly of more than 60 years).

6. Experimental protocols

6.1. Chemistry

Melting points: heating plate microscope (not corrected); IR spectra: KBr pellets, 400–4 000 cm^{-1} Perkin-Elmer 16PC FTIR spectrometer; ^{1}H-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 were performed on a reversed-phase C_{18} Bondapack column with a Beckman EM-1760 instrument. Sulfonamides **1–26** used in synthesis were either commercially available compounds (from Sigma, Acros or Aldrich) or were prepared as described previously: 4-hydrazino-benzenesulfonamide **4** by diazotization of sulfanilamide followed by reduction of the diazonium salt with tin(II) chloride [40]; halogenosulfanilamides **7**–**10** by halogenation of sulfanilamide as reported in the literature [41]; compound **15** from 5-amino-1,3,4-thiadiazole-2-sulfonamide (obtained from acetazolamide) [42] by acylation with the phthalimido-derivative of β-alanine, followed by hydrazinolysis [2], whereas imine **14** by deprotection of methazolamide with concentrated hydrochloric acid [42]. Aminobenzolamide **16** and the corresponding thiadiazoline **17** as reported in ref. [43], whereas the sulfanilyl-sulfanilamide **18** and its *meta*-derivative **19** as reported in ref. [16]. The benzothiazole-2-sulfonamide derivatives **21–23** were prepared as described in ref. [44], whereas the alcohols **24** and **25** from the corresponding amines by diazotization followed by hydrolysis of the diazonium salts. Dorzolamide **26** was prepared as described in the literature [45] or was obtained from Merck, Sharp and Dohme. *N*-Boc-β-alanine; Boc-On, EDCI, diisopropyl carbodiimide, trifluoroacetic acid (TFA), triflic acid and triethylamine were from Sigma Chemical Co. Acetonitrile, dioxane (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.

6.1.1. General procedure for the preparation of compounds **A1–A26**

An amount of 1 mM sulfonamide **1–26** was dissolved/suspended in 25 mL of anhydrous acetonitrile and then treated with the stoichiometric amount (190 mg, 1 mM) of *N*-Boc-β-Ala. 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 were added and stirring

was continued for 8–16 h at 4 °C (TLC control). The solvent was evaporated in vacuo and the residue taken up in ethyl acetate (5 mL), poured into a 5% solution of sodium bicarbonate (5 mL) and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate and filtered, and the solvent removed in vacuo. The obtained oils were either directly used in the deprotection step, or the intermediates were recrystallized from ethanol/water or methanol. The removal of the protecting group was performed as described below. The crude intermediate was dissolved in 20 mL of CH_2Cl_2, treated with 4 mL of trifluoroacetic acid (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, giving thus the amine salt as a colourless syrup. The pure compounds **A1**–**A26** 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).

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 analysed 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, using water as solvent.

6.1.1.1. 4-β-Alanylamido-benzenesulfonamide **A3**

White crystals, m.p. 290–292 °C (dec.); IR (KBr), cm^{-1}: 1 156 (SO_2^{sym}), 1 297 (amide III), 1 350 (SO_2^{as}), 1 560 (amide II), 1 710 (amide I), 3 360 (NH, NH_2); ^{1}H-NMR (TFA), δ, ppm: 2.77–2.88 (m, 2H, C$\underline{H}_2$ of β-Ala); 3.11–3.27 (m, 2H, C$\underline{H}_2$ of β-Ala); δ_A 7.18, δ_B 7.75 (AA′BB′ system, 4H, J_{AB} = 8.0, ArH from 4-sulfamoylphenyl); 7.54 (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, ppm: 37.5 (s, NH$\underline{C}H_2$ of β-Ala); 40.9 (s, $CH_2\underline{C}H_2CO$ of β-Ala); 129.9, 132.7, 135.5, 142.3, 175.2 ($\underline{C}ONH$); Anal. found: C, 44.54; H, 5.36; N, 17.13%; $C_9H_{13}N_3O_3S$ requires: C, 44.43; H, 5.39; N, 17.27%.

6.1.1.2. 4-(β-Alanylamidoethyl)-benzenesulfonamide **A6**

White crystals, m.p. 254–245 °C; IR (KBr), cm^{-1}: 1 159 (SO_2^{sym}), 1 295 (amide III), 1 361 (SO_2^{as}), 1 568 (amide II), 1 714 (amide I), 3 360 (NH, NH_2); ^{1}H-NMR (TFA), δ, ppm: 2.77–2.88 (m, 2H, C$\underline{H}_2$ of β-Ala); 3.03 (t, 2H, $^3J_{HH}$ = 6.7, αCH_2 of aminoethylbenzenesulfonamide); 3.11–3.27 (m, 2H, C$\underline{H}_2$ of β-Ala); 3.78 (t, 2H, $^3J_{HH}$ = 6.7, βCH_2 of aminoethylbenzenesulfonamide); δ_A 7.15, δ_B 7.78 (AA′BB′ system, 4H, J_{AB} = 8.1, ArH from 4-sulfamoylphenyl); 7.58 (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, ppm: 30.3 (s, $\underline{C}H_2$ of aminoethyl-benzenesulfonamide); 37.3 (s, $\underline{C}H_2$ of aminoethylbenzenesulfonamide); 37.9 (s, NH$\underline{C}H_2$ of β-Ala); 41.4 (s, $CH_2\underline{C}H_2CO$ of β-Ala); 130.4, 132.9, 134.6, 145.4, 175.2 ($\underline{C}ONH$); Anal. found: C, 48.76; H, 6.31; N, 15.40%; $C_{11}H_{17}N_3O_3S$ requires: C, 48.69; H, 6.32; N, 15.49%.

6.1.1.3. 5-β-Alanyl-β-alanylamido-1,3,4-thiadiazol-2-sulfonamide **A15**

White crystals, m.p. > 300 °C; IR (KBr), cm^{-1}: 1 178 (SO_2^{sym}), 1 296 (amide III), 1 369 (SO_2^{as}), 1 565 (amide II), 1 715 (amide I), 3 065 (NH), 3 365 (NH_2); ^{1}H-NMR (TFA), δ, ppm: 2.77–2.93 (m, 4H, 2 CH_2 of two β-Ala); 3.10–3.31 (m, 4H, 2 C$\underline{H}_2$ of two β-Ala); 7.28 (br s, 2H, SO_2NH_2); ^{13}C-NMR (TFA), δ, ppm: 37.7 (s, NH$\underline{C}H_2$ of β-Ala); 41.5 (s, $CH_2\underline{C}H_2CO$ of β-Ala); 159.5 (C-2 of thiadiazole); 170.5 (C-5 of thiadiazole); 175.4 and 178.3 (CONH); Anal. found: C, 29.69; H, 4.47; N, 26.05%; $C_8H_{14}N_6O_4S_2$ requires: C, 29.81; H, 4.38; N, 26.07%.

6.1.1.4. 5,6-Dihydro-4-(N-β-alanylamido-N-ethyl)-6-methyl-4H-thieno-[2,3-b]thiopyran-2-sulfonamide-7,7-dioxide **A26**

White crystals, m.p. 281–283 °C (dec.); IR (KBr), cm^{-1}: 1 133 (SO_2^{sym}), 1 292 (amide III), 1 345 (SO_2^{as}), 1 565 (amide II), 1 720 (amide I), 3 060 (NH), 3 365 (NH_2); ^{1}H-NMR (TFA), δ, ppm: 1.29 (d, 3H, Me); 1.39 (t, 3H, Me from ethyl); 2.55 (m, 1H, CH); 2.80 (m, 1H, CH); 2.77–2.88 (m, 2H, C$\underline{H}_2$ of β-Ala); 3.11–3.27 (m, 2H, C$\underline{H}_2$ of β-Ala); 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, ppm: 10.0, 11.1, 30.6, 37.7 (s, NH$\underline{C}H_2$ of β-Ala); 40.8, 41.5 (s, $CH_2\underline{C}H_2CO$ of β-Ala); 49.3, 51.5, 130.7, 137.5, 141.9, 149.8, 167.1 (CONH); 172.5 (CONH); Anal. found: C, 39.55; H, 5.21; N, 10.51%; $C_{13}H_{21}N_3O_5S_3$ requires: C, 39.48; H, 5.35; N, 10.62%.

6.2. Pharmacology

6.2.1. Enzyme preparations and inhibition assay

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the pACA/hCA I and pACA/hCA II plasmids described by Lindskog et al. [46] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group [47], and

enzymes were purified by affinity chromatography according to the method of Khalifah et al. [48]. 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 [49, 50]. CA IV was isolated from bovine lung microsomes as described by Maren et al. and its concentration was determined by titration with ethoxzolamide [51].

Initial rates of 4-nitrophenyl acetate hydrolysis catalysed by different CA isozymes were monitored spectrophotometrically at 400 nm with a Cary 3 instrument interfaced with an IBM compatible PC [52]. Solutions of substrates 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 [53]. 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 pre-incubated 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 [52]. 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 [53] and higher concentrations had to be used for the measurements).

6.2.2. 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, USA) as described by Maren's group [54, 55]. 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 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 inter-individual variations commonly observed in the rabbit [54, 55]. All data are expressed as mean ± SE, using a one-tailed *t*-test. Ocular hypertension was elicited in the right eye of albino rabbits by the injection of α-chymotrypsin (from Sigma) as described by Melena et al. [56]. The IOP of operated animals was checked after approximately 4 weeks, and animals with an elevated pressure of 30–36 mm Hg were used at least 1 month after the injection of α-chymotrypsin.

6.2.3. Drug distribution in ocular fluids and tissues

The general procedure of Maren's group was followed [54, 55]. The animals were killed with an intracardiac air injection. Aqueous humour (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 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 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 ref. [54, 55].

6.2.4. 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 magnetically stirring 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 and C′ = concentration of the saturated solution (mg/mL) [43].

6.2.5. 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, 43].

6.2.6. Transcorneal penetration of drugs

The method of Maren et al. [11] with the modifications of Pierce's group [58, 59] (for the HPLC assay of sulfonamides) have 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 cm^2. Concentrations of drug of 40–2 000 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. [58, 59], 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 in refs. [58, 59], this value was determined by using the formula:

$$k_{in} \cdot (\times 10^3\ hr^{-1}) = [drug]_{endo}/[drug]_{epi} \times 60/t \times 1\,000$$

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

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