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**Letter to the Editor**

# Carbonic anhydrase inhibitors – Part 52†. Metal complexes of heterocyclic sulfonamides: A new class of strong topical intraocular pressure-lowering agents in rabbits

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**Abstract** – A new sulfonamide possessing strong carbonic anhydrase (CA) inhibitory properties has been prepared by reaction of 5-amino-1,3,4-thiadiazole-2-sulfonamide with adamantyl-1-carboxylic acid chloride. Metal complexes of the adamantyl derivative, containing di- and trivalent cations, were also obtained and characterized by standard procedures. Although the parent sulfonamide does not possess topical pressure-lowering effects in rabbits when applied as a 2% solution directly into the eye, some of its metal complexes, such as the Zn(II) and Cu(II) derivatives, lower intraocular pressure (IOP) in experimental animals better than dorzolamide, the topical sulfonamide with strong antiglaucoma action, recently introduced in clinical medicine. We propose here that the whole class of metal complexes of heterocyclic sulfonamides (which generally possess much stronger CA inhibitory properties as compared to the original sulfonamides from which they were prepared) might be used for lowering IOP with enhanced efficiencies as compared to those of the simple sulfonamides. Thus, metal complexes of sulfonamides possessing themselves topical antiglaucoma properties might lead to more effective pharmacological agents of this type. © Elsevier, Paris

**topical sulfonamide / carbonic anhydrase / metal complex / intraocular pressure / antiglaucoma drug**

## 1. Introduction

Sulfonamides possessing carbonic anhydrase (CA, EC 4.2.1.1) inhibitory properties [2] such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3** and dichlorophenamide **4** have been used for more than 40 years as pressure lowering systemic drugs in the treatment of open-angle glaucoma [3, 4]. Their effect is due to inhibition of at least two CA isozymes present within cilliary processes of the eye, i.e., CA II and CA IV, which is followed by lowered bicarbonate formation and reduction of aqueous humor secretion [5–7]. Their main drawback is constituted by side effects such as fatigue, augmented diuresis, paresthesias, etc, due to CA inhibition in other tissues/organs than the target one, i.e., the eye [8] (see *figure 1*).

The above-mentioned side effects are absent in the case in which the inhibitor has topical activity, and is applied directly into the eye. This route has been demonstrated only in 1983 by Maren's group [9] and was followed by the development of the first clinical agent of this type, dorzolamide **5** [10, 11]. Dorzolamide (Trusopt®) has been introduced in clinical use in 1995 in USA and Europe and it constituted the beginning of a radically new treatment of glaucoma, devoid of the severe side effects observed with the systemic inhibitors [4–6]. The success of topical antiglaucoma CA inhibitors fostered much research in the synthesis and clinical evaluation of other types of such compounds [12–15].

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†Part 51: see [1].
*Abbreviations:* CA, carbonic anhydrase; bCA, bovine carbonic anhydrase; hCA, human carbonic anhydrase; IOP, intraocular pressure

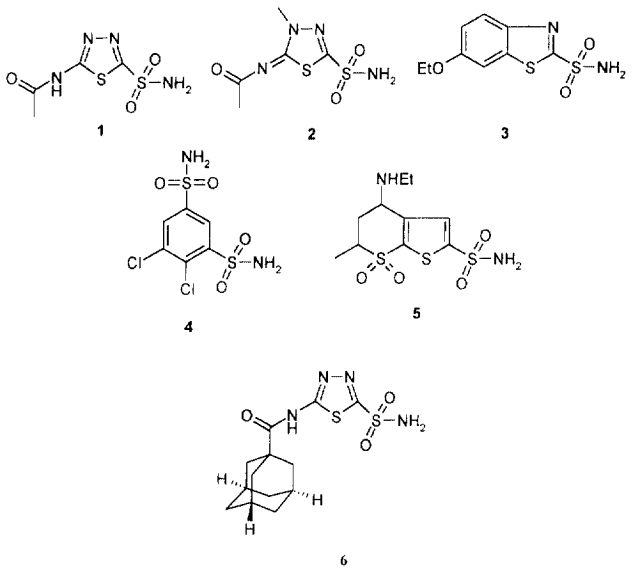

**Figure 1.** Structure of compounds **1–6**.

On the other hand, metal complexes of heterocyclic sulfonamides of type **1–5** have been recently prepared by Borras' and our groups [16–20], and it was proved that they possess much stronger CA inhibitory properties than the sulfonamides from which they were prepared [18–22]. Although the mechanism of CA inhibition of the metal complexes is presently unknown, it was hypothesized that their increased inhibitory power might be due to two processes, occurring separately or in concert, i.e., (i) dissociation of the complex inhibitor in sulfonamide anions and metal ions (in diluted solution), which in turn both interact thereafter with the enzyme, at different binding sites, and (ii) direct interaction of the undissociated complex with the enzyme, and more specifically with the hydrophilic patch at the entrance of hCA II active site [23], this being the isozyme most susceptible to inhibition with this class of compounds [2, 22]. Whether initially the first mechanism of action mentioned above was favored by us [22], recent evidences suggest that the undissociated complex is the inhibitory species, at least for some isozymes [24]. Since metal complexes are much more inhibitory than the parent sulfonamide from which they were prepared, it appeared of interest to test whether this property might be useful for their use in lowering IOP in experimental animals and whence as a possible glaucoma therapy. Here we report the first study regarding the possible applications of metal complexes of heterocyclic sulfonamides as IOP lowering agents. A new sulfonamide, **6** (see *figure 1*), possessing strong CA inhibitory properties was prepared by reaction of adamantyl-1-carboxylic acid chloride with 5-amino-1,3,4-thiadiazole-2-sulfonamide. The metal complexes containing the anion of the above sulfonamide and di- and trivalent cations such as Zn(II); Cu(II); Ni(II); Co(II); Fe(III) and Al(III) were then prepared and characterized by standard procedures. All these new derivatives showed strong inhibitory activity against three CA isozymes, hCA I, hCA II and bCA IV. Although the original sulfonamide did not show topical IOP lowering properties, some of its metal complexes, such as the Zn(II) and Cu(II) derivatives, lowered IOP in rabbits when applied directly into the eye as a 2% solution, much stronger than the clinically used compound dorzolamide.

## 2. Results and discussion

### *2.1. Chemistry*

Reaction of adamantyl-1-carboxylic acid chloride with 5-amino-1,3,4-thiadiazole-2-sulfonamide in the presence of triethylamine afforded 5-(adamantyl-1-carboxamido)-1,3,4-thiadiazole-2-sulfonamide **6** by the procedure previously described from this laboratory [25]. Metal complexes of the new sulfonamide were prepared from the sodium salt of **6** and salts of di- and trivalent transition and main group metal ions [26]. The prepared new complexes of type **7–12** as well as their CA inhibitory properties are shown in *table I*. Mention should be made that metal ions which were previously shown [18–22] to lead to powerful complex CA inhibitors were included in the study, such as Zn(II), Cu(II), Co(II), Ni(II), Fe(III) and Al(III). On the other hand, many such ions are known to play a critical biological role as constituents of metallo-enzymes or non-catalytic metallo-proteins [27, 28] or to be present in pharmacological agents (Zn(II) and Al(III) in antacids [29, 30]; iron and cobalt compounds in anti-anemia drugs [31]).

The new compounds **6–12** have been characterized by elemental analysis and physico-chemical methods (UV, IR and $^1$H-NMR spectroscopy, magnetic, thermogravimetric and conductimetric data) which confirmed the proposed formulas (see Experimental protocols for details).

Spectroscopic, thermogravimetric and magnetic measurements for compound **6** and its metal complexes **7–12** (*table II*) proved that the ligand (the sulfonamide anion, obtained in situ by deprotonation of **6** with one equivalent of base) interacts with metal ions in a bidentate fashion, by means of the sulfonamide nitrogen atom and the endocyclic N-3, similarly with other thiadiazole-sulfonamides investigated

**Table I.** CA inhibition data with standard inhibitors **1–5**, the new sulfonamide **6** and its metal complexes **7–12**. LH stands for sulfonamide **6**, whereas L for the sulfonamide-deprotonated species of **6**.

| Compound | Inhibitor | $K_I$ (nM) | | |
|---|---|---|---|---|
| | | hCA I[a] | hCA II[a] | bCA IV[b] |
| **1** | Acetazolamide | 900 | 12 | 220 |
| **2** | Methazolamide | 780 | 14 | 240 |
| **3** | Ethoxzolamide | 25 | 8 | 13 |
| **4** | Dichlorophenamide | 1200 | 38 | 380 |
| **5** | Dorzolamide | > 50 000 | 9 | 43 |
| **6**[c] | LH | 850 | 10 | 65 |
| **7** | $[ZnL_2]$ | 640 | 2 | 18 |
| **8** | $[CuL_2(OH_2)_2]$ | 320 | 0.5 | 12 |
| **9** | $[CoL_2(OH_2)_2]$ | 380 | 4 | 16 |
| **10** | $[NiL_2(OH_2)_2]$ | 450 | 5 | 15 |
| **11** | $[FeL_3]$ | 490 | 4 | 10 |
| **12** | $[AlL_3]$ | 650 | 7 | 12 |

[a]Human (cloned) isozymes; [b]from bovine lung microsomes; [c]elemental analysis data (metal, C, H, N) for compounds **6–12** were within ± 0.4% of the theoretical values calculated for the proposed formulas.

earlier [16–23]. This is confirmed both by the stoichiometry of the obtained complexes as well as by spectral changes in the spectra of complexes as compared to the spectrum of **6** (or its sodium salt). Thus, in the IR spectra the following features should be noted: (i) the intense sulfonamide vibrations, at 1160 and 1350 $cm^{-1}$ in **6** are shifted with 20–40 $cm^{-1}$ towards lower wavenumbers in complexes **7–12**, due to the involvement of this moiety in the interaction with the metal ions [16–23]; (ii) the strong amide band is present at 1680 $cm^{-1}$ in all compounds **6–12**, proving that this moiety does not interact with the metal ions, (iii) the thiadiazole C=N stretching vibration from 1640 $cm^{-1}$ in sulfonamide **6** undergoes a shift in the spectra of complexes **7–12**, where appears at 1610–1624 $cm^{-1}$ for reasons identical to those mentioned at (i); (iv) vibrations in the region 320–480 $cm^{-1}$ were identified in the spectra of the complex

**Table II.** IR and electronic spectral data, as well as magnetic moments at room temperature, and thermogravimetric (TG) analysis data for sulfonamide **6** and its metal complexes **7–12**.

| Compound | IR spectra ($cm^{-1}$)[a] | | | UV spectra[b] $\lambda_{max}$, nm (log ε) | $\mu_{eff}$ (BM)[c] | TG analysis[d] (calc./found) |
|---|---|---|---|---|---|---|
| | $SO_2^s$ | $SO_2^{as}$ | CONH | | | |
| **6** | 1160 | 1350 | 1640 | 255 (4.20); 319 (4.67) | [e] | [f] |
| **7** | 1140 | 1320 | 1624 | 245 (3.97); 315 (4.18) | [e] | [f] |
| **8** | 1135 | 1320 | 1618 | 245 (4.17); 315 (4.26) | 1.90 | 4.60/4.50[g] |
| **9** | 1120 | 1320 | 1620 | 245 (4.02); 315 (4.10) | 5.24 | 4.62/4.41[g] |
| **10** | 1130 | 1310 | 1610 | 245 (3.99); 315 (4.29) | 3.40 | 4.63/4.38[g] |
| **11** | 1140 | 1330 | 1610 | 245 (4.11); 315 (4.25) | 5.76 | [f] |
| **12** | 1135 | 1325 | 1615 | 245 (4.13); 315 (4.23) | [e] | [f] |

[a]In KBr pellets; [b]in DMSO; [c]at room temperature; [d]weight loss (%) in the temperature range 120–180 °C; [e]diamagnetic; [f]no weight loss under 280–300 °C; [g]corresponding to two coordinated water molecules.

derivatives, which are absent in the spectrum of the ligand, and were assigned as due to M–N (or M–O) vibrations [16–22] (data not shown).

Complexes **7–12** possessed UV spectra highly similar to those of the sodium salt of the ligand **6** (*table II*), proving the presence of sulfonamide anionic moieties in their molecule. Thus, the ligand **6** has two strong absorption maxima, one at 255 nm and the other one at 319 nm, similarly to other 1,3,4-thiadiazole-2-sulfonamide derivatives previously investigated [15, 25, 26], whereas the sodium salt and its metal complexes show a hypsochromic shift of these bands, which appear at 245 and 315 nm, respectively. Correlated with the magnetic moment measurements (characteristic for the respective metal ions in the indicated geometries) and the presence of water molecules (evidenced by means of thermogravimetric analysis) in some of these derivatives (*table II*), the structures shown below were proposed for the new derivatives **7–12**. They all involve bidentate coordination by the donor system mentioned above, and Zn(II) ions in tetrahedral geometry, whereas all other metal ions are coordinated in octahedral surrounding. Two axial water molecules are also present in the Cu(II); Co(II) and Ni(II) complexes. Conductimetric measurements (in DMSO solution, 1 mM, at 25 °C) showed the complexes **7–12** to be non-electrolytes, proving that they are not dissociated in solution in sulfonamide anions and metal ions (data not shown; see *figure 2*).

## 2.2. *Pharmacology*

The new compounds **6–12** and standard CA inhibitors **1–5** were assayed for CA inhibition against three isozymes, hCA I, hCA II and bCA IV (*table I*). As seen from the above data, the new sulfonamide **6** behaves as a strong inhibitor against all these isozymes. It is more inhibitory than acetazolamide, methazolamide and dichlorophenamide, having a potency similar to that of dorzolamide or ethoxzolamide. The metal complexes **7–12** are much more inhibitory than **6**, and than all other simple sulfonamides assayed. They behave similarly to the metal complexes of acetazolamide, methazolamide or dorzolamide previously reported by this group, which were all more inhibitory than the parent sulfonamide from which were prepared [16–22, 24, 26]. Particularly strong inhibition was observed for the Zn(II) and Cu(II) derivatives **7** and **8**, especially against CA II and CA IV, the isozymes critical for aqueous humor formation.

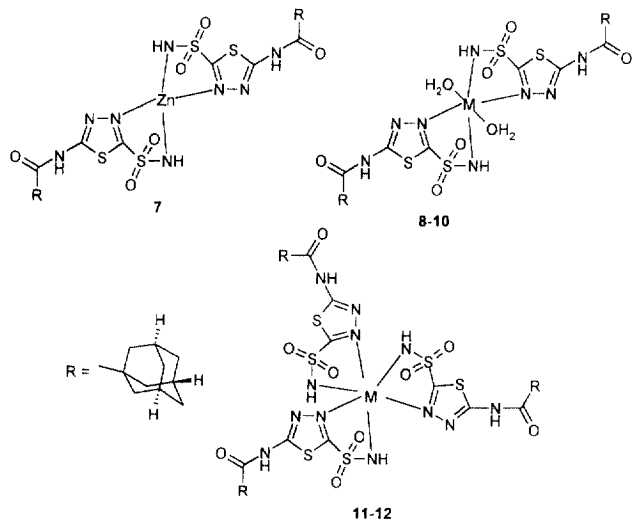


**Figure 2.** Structure of compounds **7–12**.

In vivo IOP lowering experiments were done in rabbits with three of the new compounds, i.e., the sulfonamide **6** and its Zn(II) and Cu(II) complexes **7** and **8**, which were among the strongest CA II and CA IV inhibitors in the obtained series. Some of the IOP lowering data at half an hour and one hour after the instillation of one drop of 2% solution of inhibitor within the rabbit eye are shown in *table III*, with dorzolamide (at the same concentration) as standard. In *figure 3* the time dependence of IOP lowering with dorzolamide **5** and the two complexes **7** and **8** is presented.

From the above data it is obvious that in contrast to dorzolamide **5**, the new thiadiazole sulfonamide **6** does not possess at all topical activity in lowering IOP in rabbits, similarly to acetazolamide or methazolamide with which **6** is structurally related. The exciting finding is that its metal complexes, among which the strong CA inhibitors **7** and **8**, containing Zn(II) and Cu(II) ions, respectively, strongly reduce IOP in

**Table III.** IOP lowering following topical application of CA inhibitors, half an hour and one hour after instillation into the eye of a drop (50 μL) of 2% solution of inhibitor.

| Inhibitor | Δ IOP ± SE[a] (mm Hg) | |
|---|---|---|
| | 1/2 h | 1 h |
| Dorzolamide **5** | 2.2 ± 0.10 | 4.1 ± 0.15 |
| **6** | 0 ± 0.10 | 0 ± 0.09 |
| **7** | 8.0 ± 0.14 | 8.1 ± 0.21 |
| **8** | 2.0 ± 0.09 | 5.0 ± 0.12 |

[a]Δ IOP = $IOP_{control\ eye} - IOP_{treated\ eye}$ ($N = 3$).

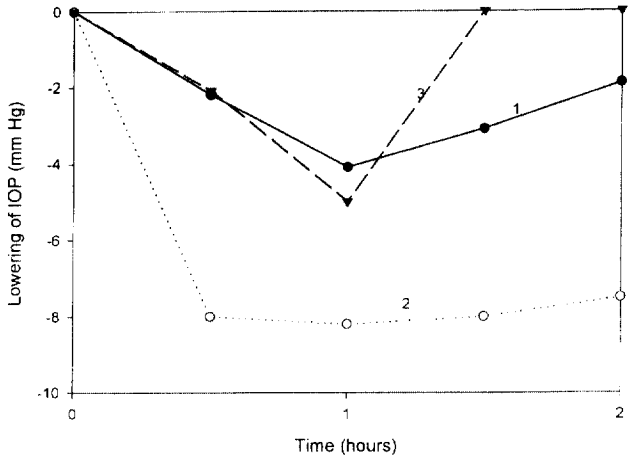


**Figure 3.** Time dependence of IOP lowering with dorzolamide (curve 1); the zinc complex **7** (curve 2) and the copper complex **8** (curve 3), after topical administration of one drop of 2% solution (in DMSO–water 2:3, v/v) of inhibitor in rabbit.

the same animal model. The Zn(II) complex **7** is much more active as compared to the Cu(II) derivative **8**, and especially dorzolamide **5**, the clinically used compound. What is even more fascinating is the fact that the decreased pressure after administration of the zinc complex remains at low values for a much longer time as compared to dorzolamide (*figure 3*) which might have importance for the pharmacology of a putative drug of the class described by us here. For the copper complex (which is slightly more active than dorzolamide at half an hour and one hour after administration), the return to the initial IOP is more rapid as compared to dorzolamide, and obviously the zinc complex. This feature is important, since it proves that the nature of the metal ion contained in the coordination compound is of paramount importance for the IOP lowering effects. The above data might be important for the design of better antiglaucoma drugs. Thus, dorzolamide is administered three times a day, due to its specific pharmacology (see its IOP lowering effects in *figure 3*) [4–6]. A drug of the type described by us, such as the zinc complex **7**, would need to be administered in much lower doses and at larger time intervals, due to the increased inhibitory effect and duration of action of IOP lowering properties (*figure 3*).

In *table IV* the drug distribution in ocular fluids and tissues is shown, after the topical administration of the Zn(II) complex **7**.

It is seen from the above data that one and two hours after topical administration of the drug, high levels of **7** were found in the cornea, aqueous humor and ciliary processes. Based on the inhibition constant of this compound (2 nM for CA II, and 18 nM for CA IV), the fractional inhibition estimated in these tissues/fluids is of 98–99.9% [41–43], proving the fact that the IOP decrease is indeed due to CA inhibition.

**Table IV.** Ocular tissue concentrations (μM) after one and two hours, following corneal application of one drop (50 μL) of 2% solution of the Zn(II) complex **7** in albino rabbits.

| Time (h) | Drug concentration (μM)[a] | | |
|---|---|---|---|
| | Cornea | Aqueous humor | Ciliary process |
| 1 h | 153 ± 7 | 276 ± 12 | 49 ± 3 |
| 2 h | 34 ± 5 | 32 ± 3 | 5.1 ± 0.8 |

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

A last remark should be made about the possible mechanism of action of the new class of IOP lowering agents. Obviously, their activity is due to inhibition of CA isozymes present in the cilliary processes within the eye, similarly to other topically active sulfonamides [2–6]. Still the fact that the sulfonamide from which the complexes were prepared is *inactive* via the topical route, whereas the metal complexes possess very efficient such properties, which are much better as compared to those of the recently introduced drug dorzolamide, proves that the presence of metal ions in the molecules of these CA inhibitors is critical for this action, conferring them completely new properties. One should mention that preliminary results from this laboratory indicate that the metal complexes of topically active sulfonamides show even increased IOP lowering effects than the complexes prepared in the present study [Supuran et al., unpublished results]. Our hypothesis is that the presence of the metal ion in the molecules of these complex inhibitors has as a consequence a dramatic change in their physicochemical properties as compared to those of the parent sulfonamide. This phenomenon is certainly governed by the strong polarization induced by the metal ions. In this way, it is quite probable that the right balance between the lipo- and hydrosolubility of these compounds is achieved, which has been considered to be the critical factor for not observing topical activity in the classical CA inhibitors, such as acetazolamide, methazolamide and ethoxzolamide, which were either too lipophilic or too hydrosoluble [2, 3]. Obviously, by choosing different metal ions and diverse sulfonamides, much larger possibilities arise to fine tune these pharmacological properties which strongly influence the value of a drug.

In conclusion we describe here a novel class of IOP lowering agents, i.e., the metal complexes of sulfonamide CA inhibitors. These derivatives possess very strong and longer acting such properties as compared to the drug dorzolamide, and might constitute the basis for a new generation of antiglaucoma drugs.

## 3. Experimental protocols

### *3.1. Chemistry*

Melting points were recorded with a heating plate microscope and are not corrected. IR spectra were recorded in KBr pellets with a Carl Zeiss IR-80 instrument. $^1$H-NMR spectra were recorded in DMSO-$d_6$ as solvent, with a Bruker CPX200 instrument. Chemical shifts are reported as δ values, relative to $Me_4Si$ as internal standard. Magnetic susceptibility measurements were carried out at room temperature with a fully automated AZTEC DSM8 pendulum-type susceptometer. Mercury(II) tetrakis-(thiocyanato)cobaltate(II) was used as a susceptibility standard. Corrections for the diamagnetism were estimated from Pascal's constants [32]. Conductimetric measurements were done at room temperature (1 mM concentration of complex) in DMSO solution with a Fisher conductimeter. Elemental analyses were done by combustion for C, H, N with an automated Carlo Erba analyzer, and gravimetrically for the metal ions, and were ± 0.4% of the theoretical values. Thermogravimetric measurements were done in air, at a heating rate of 10 °C/min, with a Perkin Elmer 3600 thermobalance.

Sulfonamides used as standards in the enzymatic assay (except for **5**), acetazolamide and adamantane-1-carboxylic acid used for the preparation of compound **6**, solvents as well as inorganic reagents were from Sigma, Merck and Carlo Erba. 5-Amino-1,3,4-thiadiazole-2-sulfonamide was prepared from acetazolamide by literature procedures [25], by desacetylation with concentrated hydrochloric acid, followed by neutralization with sodium bicarbonate of the corresponding hydrochloride. Dorzolamide hydrochloride **5** was from Merck, Sharp and Dohme or was prepared as described by Ponticello et al. [10, 11].

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 Forsman et al. [33] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group [34], and enzymes were purified by affinity chromatography according to the method of Khalifah et al. [35]. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1}$ $cm^{-1}$ for CA I and 54 $mM^{-1}$ $cm^{-1}$ for CA II, respectively, based on $M_r$ = 28.85 kDa for CA I, and 29.3 kDa for CA II, respectively [36, 37]. CA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide [38].

#### *3.1.1. Synthesis of 5-(adamantyl-1-carboxamido)-1,3,4-thiadiazole-2-sulfonamide* **6**

An amount of 1.80 g (10 mmol) of 5-amino-1,3,4-thiadiazole-2-sulfonamide was suspended in 50 mL of anhydrous acetonitrile and 1.40 mL of triethylamine were added. The mixture was magnetically stirred at 4 °C for 20 minutes, then 10 mmol of adamantyl-1-carboxylic acid chloride (obtained from the corresponding carboxylic acid and $SOCl_2$) were added dropwise for 20 min, and stirring was continued for other 5 hours, maintaining the temperature under 5 °C. The precipitated crystals were filtered and recrystallized from ethanol. Yield of 83% (2.85 g), white crystals, m.p. 237–238 °C; IR (KBr), $cm^{-1}$: 750, 920, 1160, 1350, 1500, 1640, 1680, 3200; UV spectrum, $\lambda_{max}$, nm (log ε): 255 (4.20); 319 (4.67) $^1$H-NMR (DMSO-$d_6$), δ, ppm: 1.75–2.00 (m, 15H, from adamantyl); 3.80 (br s, 2H, $SO_2NH_2$); 8.30 (s, 1H, CONH). Anal. ($C_{13}H_{18}N_4S_2O_3$) C, H, N.

#### *3.1.2. General procedure for the preparation of compounds* **7–12**

An amount of 6 mmol of sodium salt of **6** was prepared by reacting the sulfonamide with the required amount of an alcoholic 1 N NaOH solution, in ethanol as solvent. To this solution was added the metal salt (Zn(II), Cu(II), Co(II), Ni(II) chlorides, Fe(III) perchlorate and Al(III) nitrate); solution, working in molar ratios $RSO_2NH^-$: $M^{n+}$ of 2:1 for the divalent cations and 3:1 for the trivalent cations, respectively. The aqueous–alcoholic reaction mixture was heated on a steam bath for one hour and after being cooled at 0 °C the precipitated complexes were filtered and thoroughly washed with alcohol–water 1:1 (v/v) and air dried. Yields were in the range of 85–90%. The obtained powders of compounds **7–12** (white for the Zn(II) and Al(III) complexes; green for the Cu(II) and Ni(II) derivatives, pink for the Co(II) and brown for the Fe(III) complexes, respectively) melt with decomposition at temperatures higher than 300 °C, and are poorly soluble in water and alcohol, but have good solubilities in DMSO, DMF as well as mixtures of DMSO–water, DMF–water.

### *3.2. Pharmacology*

#### *3.2.1. Carbonic anhydrase inhibition*

Initial rates of 4-nitrophenylacetate hydrolysis catalysed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC [39]. Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2 x $10^{-2}$ and 1 x $10^{-6}$ 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 [39]. 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 [2]) 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 [39]. Enzyme concentrations were 3.3 nM for CA II, 10 nM for CA I and 34 nM for CA IV (this isozyme has a decreased esterase activity [40] and higher concentrations had to be used for the measurements).

#### *3.2.2. Measurement of tonometric IOP*

Adult male New Zealand albino rabbits weighing 2–3 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 light–12 h dark cycle in a temperature-controlled room, at 22–26 °C. Solutions of inhibitors (2%, by weight) were obtained in DMSO–water (2:3, v/v) due to the lower water solubility of some of these derivatives. Control experiments with DMSO (at the same concentration as that used for obtaining the inhibitors solutions showed that it does not possess IOP lowering or increasing effects.

IOP was measured using a Digilab 30R pneumatonometer (BioRad, Cambridge, MA, USA) as described by Maren's group [41–43]. 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 several hours. 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 [41–43]. All data are expressed as mean ± SE, using a one-tailed $t$ test.

*3.2.3. Drug distribution in ocular fluids and tissues*

The general procedure of Maren's group has been followed [41–43]. The animals were killed with an intracardiac injection. Aqueous humor (both posterior and anterior chamber fluids) was withdrawn. Then, the cornea and anterior uvea (iris plus attached ciliary body) were dissected, rinsed well with water, blotted, weighed and put into 1–2 mL of water. For isolation of the ciliary processes, intact anterior uvea rings were placed on a parafilm covered piece of polystyrene foam in a Petri dish. The tissue has been wetted with normal saline and dissected under a microscope, when cilliary 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 minutes (in order to denaturate 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 has been used in order to determine the fractional inhibition in the different tissues, as described by Maren's group [41–43].

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