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Original article

Carbonic anhydrase inhibitors. Part 60#. The topical intraocular pressure-lowering properties of metal complexes of a heterocyclic sulfonamide: influence of the metal ion upon biological activity

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Abstract – Metal complexes of a sulfonamide possessing strong carbonic anhydrase (CA) inhibitory properties have been obtained from the sodium salt of the sulfonamide or from the free sulfonamide in the presence of ammonia, and the following metal ions: Mg(II); Zn(II); Mn(II); Cu(II); Co(II); Ni(II); Be(II); Cd(II); Pb(II); Al(III); Fe(III) and La(III). The original sulfonamide, 5-(3,4-dichlorophenylureido)-1,3,4-thiadiazole-2-sulfonamide and its complexes were assayed for in vitro inhibition of three CA isozymes, CA I, II and IV, some of which play a critical role in ocular fluid secretion. All these compounds (the sulfonamide and its metal complexes) behave as very powerful inhibitors against the three investigated CA isozymes. The parent sulfonamide possesses strong topical pressure lowering effects in rabbits when applied as a 1% solution directly into the eye, but some of its metal complexes, such as the Mg(II); Zn(II); Mn(II) and Cu(II) derivatives, lower intraocular pressure (IOP) in experimental animals much better. Ex vivo data showed a 98.5–99.9% inhibition of CA II and IV in ocular fluids and tissues of the rabbits treated with these agents, proving that the IOP lowering properties are due to CA inhibition. The influence of the different metal ions upon the efficiency of the obtained complexes as pressure lowering drugs are discussed, considering the possibility to design in this way more selective pharmacological agents from this class. © 1999 Éditions scientifiques et médicales Elsevier SAS

topical sulfonamide / carbonic anhydrase / metal complex / intraocular pressure lowering drug

1. Introduction

1,3,4-Thiadiazole-2-sulfonamide derivatives [2–7] played a critical role in the development of several important classes of pharmacological agents, such as the diuretics with saluretic action [8, 9], benzothiadiazine [10] and high-ceiling diuretics [11], or the antiglaucoma drugs with carbonic anhydrase (CA) inhibitory action, among others [12, 13]. The prototype of all these drugs was constituted by acetazolamide **1a**, the first non-mercurial diuretic [2, 8], used for more than 45 years in clinical medicine as a diuretic [8], antiglaucoma [8, 12], antiepileptic [14] and antiulcer compound [15]. It is still used nowadays, mainly as a diagnostic tool in NMR imaging [16, 17], and in many physiological studies [18–20]. The major biological action of acetazolamide and related heterocyclic/aromatic sulfonamides is connected with the powerful inhibition of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), of which at least eight isozymes are presently known in higher vertebrates [6–8, 12].

Many structural variants were derived using acetazolamide **1a** as lead molecule, such as 5-aryl/alkylsulfonylamido-1,3,4-thiadiazole-2-sulfonamides **2** [21, 22] (of which benzolamide **2a** is the most important representative [23], whereas other derivatives of this series, of type **2b**, might be used for developing diagnos-

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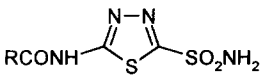
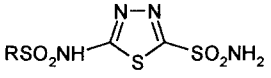
See ref. [1]
Abbreviations: CA, carbonic anhydrase; bCA, bovine carbonic anhydrase; hCA, human carbonic anhydrase, IOP, intraocular pressure.

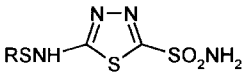
tic tools in PET (positron emission tomography) [22]); sulfenamido-sulfonamides of type **3** [24], some of which could display omeprazole-like activation in acidic media in vivo [24]; Schiff bases of type **4** which showed increased affinity for the membrane-associated isozyme CA IV [25, 26], as well as ureido/thioureido-derivatives of type **5** and **6**, which were recently shown to possess very strong affinities for the physiologically relevant isozymes CA I, II and IV [27, 28].



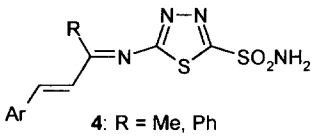
1 **a**: R = Me; **b**: R = 1-adamantyl



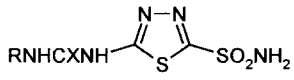
2 **a**: R = Ph; **b**: 4-substituted-phenyl



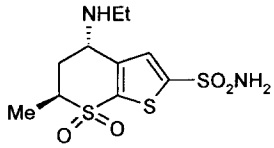
3 R = 2- or 4-$O_2NC_6H_4$



4: R = Me, Ph; Ar = Ph; substituted-phenyl

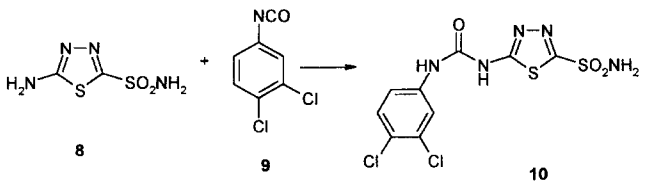


5: X = O; **6**: X = S; R = alkyl, aryl



7

Metal complexes of sulfonamides of type **1** and **2**, containing a large number of main group or transition metal ions, were shown to possess very strong CA inhibitory properties [29–35], which were explained mechanistically as being due to a dual process: binding of the undissociated complex to the histidine cluster of isozyme II [36] as well as inhibition due to the dissociation of the complex in dilute solution in the assay system [37]. Such dissociation processes lead to the formation of sulfonamide anions which bind thereafter to the Zn(II) ion within the CA active site, and metal ions which probably bind to critical histidine residues or the histidine cluster itself in isozymes in which this is present (CA II and CA III) [36, 37]. The result of these phenomena is that, generally, metal complexes of heterocyclic sulfonamides are 10–100 times more active as CA inhibitors than the sulfonamides from which they were obtained, assuring affinities for the receptor in the 10^{10}–10^{12} range [29–35, 37]. Recently we have reported [38] the unexpected finding that metal complexes of a sulfonamide structurally related to acetazolamide **1b**, which does not possess IOP (intraocular pressure) lowering effects when applied directly into the rabbit eye, were extremely potent topical IOP lowering agents. In the above mentioned study [38], only the Zn(II) and Cu(II) complexes of 5-adamantylcarboxamido-1,3,4-thiadiazole-2-sulfonamide **1b** were tested for their antiglaucoma action, showing much better properties than dorzolamide **7**, the recently introduced into the clinic sulfonamide with antiglaucoma action [11, 12]. It appeared thus of great interest to prepare metal complexes of topically active sulfonamides too, and test whether such compounds possess increased IOP lowering properties as compared to that of the parent sulfonamide. The influence of different metal ions has not been previously investigated in much detail, although we hypothesized this factor as being extremely important for this type of biological action [38]. In this paper we report the synthesis of coordination compounds of a sulfonamide of type **5**, previously reported by this group [28], ie., 5-(3,4-dichlorophenylureido)-1,3,4-thiadiazole-2-sulfonamide, containing a large number of di- and trivalent metal ions, such as: Mg(II), Zn(II), Mn(II), Cu(II), Co(II), Ni(II), Be(II), Cd(II), Pb(II), Al(III), Fe(III) and La(III). The 20 newly obtained complexes were characterized by standard procedures in order to assign their structures, and were assayed as CA inhibitors against three isozymes, CA I, II and IV. In vivo studies in rabbits allowed us to determine the influence of diverse metal ions upon the IOP lowering properties of the new complexes in order to detect the best candidates for the development of novel types of antiglaucoma drugs from this class.



8 **9** **10**

Scheme 1.

2. Results

2.1. Chemistry

Reaction of 5-amino-1,3,4-thiadiazole-2-sulfonamide **8** with 3,4-dichlorophenyl-isocyanate **9** in anhydrous tetrahydrofurane afforded 5-(3,4-dichlorophenylureido)-1,3,4-thiadiazole-2-sulfonamide **10** by the procedure previously described from this laboratory [28] (*scheme 1*).

Metal complexes of the new sulfonamide were prepared from the sodium salt of **10** and salts of di- and trivalent transition and main group metal ions [29–35, 37, 38] (reactions 1 and 2). In a variant of these syntheses, the

Table I. Metal complexes **11–30** containing the conjugate base of 5-(3,4-dichlorophenylureido)-1,3,4-thiadiazole-2-sulfonamide (L) as ligand and their proposed formulas.

No.	Complex	Formula*
11	$[BeL_2]$	$[Be(C_9H_6N_5O_3S_2Cl_2)_2]$
12	$[MgL_2].3\ H_2O$	$[Mg(C_9H_6N_5O_3S_2Cl_2)_2]\ .\ 3\ H_2O$
13	$[ZnL_2]$	$[Zn(C_9H_6N_5O_3S_2Cl_2)_2]$
14	$[ZnL_2(NH_3)_2]$	$[Zn(C_9H_6N_5O_3S_2Cl_2)_2(NH_3)_2]$
15	$[CdL_2(OH_2)_2]$	$[Cd(C_9H_6N_5O_3S_2Cl_2)_2(OH_2)_2]$
16	$[CdL_2(NH_3)_2]$	$[Cd(C_9H_6N_5O_3S_2Cl_2)_2(NH_3)_2]$
17	$[MnL_2(OH_2)_2]$	$[Mn(C_9H_6N_5O_3S_2Cl_2)_2(OH_2)_2]$
18	$[MnL_2(NH_3)_2]$	$[Mn(C_9H_6N_5O_3S_2Cl_2)_2(NH_3)_2]$
19	$[Co_2L_2(OH_2)_2(OH)_2]$	$[Co_2(C_9H_6N_5O_3S_2Cl_2)_2(OH_2)_2(OH)_2]$
20	$[CoL_2(NH_3)_2]$	$[Co(C_9H_6N_5O_3S_2Cl_2)_2(NH_3)_2]$
21	$[NiL_2(OH_2)_2]$	$[Ni(C_9H_6N_5O_3S_2Cl_2)_2(OH_2)_2]$
22	$[NiL_2(NH_3)_2]$	$[Ni(C_9H_6N_5O_3S_2Cl_2)_2(NH_3)_2]$
23	$K[Cu_2L_4(OH)]$	$K[Cu_2(C_9H_5O_3S_2Cl_2)_2(NH_3)_2]$
24	$[CuL_2(NH_3)_2]$	$[Cu(C_9H_6N_5O_3S_2Cl_2)_2(NH_3)_2]$
25	$[PbL_2(OH_2)_2]$	$[Pb(C_9H_6N_5O_3S_2Cl_2)_2(OH_2)_2]$
26	$[PbL_2(NH_3)_2]$	$[Pb(C_9H_6N_5O_3S_2Cl_2)_2(NH_3)_2]$
27	$[FeL_3]$	$[Fe(C_9H_6N_5O_3S_2Cl_2)_3]$
28	$[AlL_3]$	$[Al(C_9H_6N_5O_3S_2Cl_2)_3]$
29	$[LaL_2(OH_2)_5].\ 3H_2O$	$[La(C_9H_6N_5O_3S_2Cl_2)_2(OH_2)_5]\ .\ 3\ H_2O$
30	$[LaL_2(NH_3)_2(OH_2)_3]$	$[La(C_9H_6N_5O_3S_2Cl_2)_2(NH_3)_2(OH_2)_3$

*± 0.4% of the calculated value for C, H, N (by combustion) and the metal ion (by gravimetry or volumetry, respectively).

free sulfonamide **10**, aqueous ammonia and metal salts were used for preparing complexes also containing ammonia molecules as ligands, in addition to the sulfonamidate anions [30] (reactions 3 and 4 below).

$$R\text{-}SO_2\text{-}NH_2 + NaOH \rightarrow R\text{-}SO_2\text{-}NH^- + Na^+ \quad (1)$$

$$n\ R\text{-}SO_2\text{-}NH^- + M^{n+} + x\ H_2O \rightarrow [M(R\text{-}SO_2\text{-}NH)_n(OH_2)_x] \quad (2)$$

$$R\text{-}SO_2\text{-}NH_2 + NH_3 \rightarrow R\text{-}SO_2\text{-}NH^- + NH_4^+ \quad (3)$$

$$n\ R\text{-}SO_2\text{-}NH^- + M^{n+} + x\ NH_3 \rightarrow [M(R\text{-}SO_2\text{-}NH)_n(NH_3)_x] \quad (4)$$

The prepared new complexes of type **11–30** as well as their proposed formulas are shown in *table I*. Mention should be made that metal ions which were previously shown [29–35] 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).

The new compounds **11–30** 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 (*tables II–V*).

2.2. Pharmacology

The new compounds **11–30** and standard CA inhibitors were assayed for CA inhibition against three isozymes, hCA I, hCA II and bCA IV (*table VI*). The sulfonamide **10** and its metal complexes behave as strong inhibitors against all these isozymes.

IOP pressure measurements after the topical administration of one drop (50 μL) of a 1% solution of inhibitors of type **10–30** or a 2% solution of the clinical drug dorzolamide **7** (much less effective than the compounds described by us here) are shown in *table VII*.

The amount of CA inhibitor present in ocular fluids and tissues after the topical administration of one of the new compounds reported here is shown in *table VIII*.

3. Discussion

3.1. Chemistry

By using different experimental conditions, generally two series of metal complexes could be prepared from the conjugate base of sulfonamide **10** (abbreviated as L in formulas of complexes **11–30**) and different main group and transition di- and trivalent cations: (i) complexes containing only L (and eventually water in the coordination sphere) and (ii) complexes containing ammonia, L and eventually water in the coordination sphere of the cation. For the Be(II) and Mg(II) ions, only complexes of the first type were obtained, even when deprotonating **10** in the presence of ammonia, whereas for other cations, both types of derivatives were synthesized (*table I*). Elemental analysis data (obtained by combustion for C, H, N, and gravimetrically or volumetrically for the metal

Table II. IR, solution electronic spectral, and thermogravimetric (TG) analysis data for sulfonamide **10** and its metal complexes **11–30.**

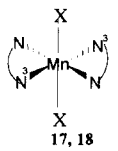
Compound	IR spectra[a], (cm^{-1})			UV spectra[b]	TG analysis[c]
	SO_2^s	SO_2^{as}	ν(C=N) ν(MX)	λ_{max}, nm (lgε)	(calc./found)
10	1190	1340	1650	274 (3.96); 302 (3.12)	d
11	1180	1300	1655	267 (4.15); 293 (3.52)	d
12	1190	1310	1650	268 (4.19); 295 (4.20)	6.65/6.79[e]
13	1150	1320	1620	265 (4.02); 295 (4.10)	d
14	1170	1330	1635	265 (3.99); 295 (4.09)	4.08/4.04[f]
15	1140	1330	1620	265 (4.01); 295 (4.23)	4.07/3.97[f]
16	1170	1330	1640	265 (4.13); 295 (4.17)	3.86/4.02[f]
17	1160	1350	1640	265 (4.20); 293 (4.67)	4.36/4.51[f]
18	1180	1340	1650	265 (3.97); 295 (4.18)	4.13/4.10[f]
19	1190	1350	1650	261 (4.27); 292 (4.12)	2.47/2.38[f]
20	1130	1330	1670	265 (4.02); 295 (4.10)	4.11/4.33[f]
21	1145	1345	1660	265 (3.99); 295 (4.24)	4.34/4.50[f]
22	1140	1340	1670	265 (4.05); 295 (4.25)	4.11/4.23[f]
23	1140	1300	1620	265 (4.18); 295 (4.13)	d
24	1140	1310	1630	265 (4.20); 299 (4.07)	4.08/4.00[f]
25	1140	1320	1620	265 (3.93); 295 (4.14)	3.68/3.80[f]
26	1130	1330	1630	265 (4.17); 295 (4.21)	3.48/3.66[f]
27	1170	1350	1650	265 (4.52); 295 (4.30)	d
28	1160	1340	1640	265 (4.59); 295 (4.29)	d
29	1140	1330	1640	265 (4.61); 295 (4.25)	
30	1140	1350	1640	265 (4.63); 295 (4.23)	9.15/9.38[h]

[a]In KBr pellets, [b]In DMSO, [c]Weight loss (%) in the temperature range of 100–180 °C, [d]No weight loss under 280–300 °C, [e]Corresponding to three uncoordinated water molecules, lost between 100–110 °C, [f]Corresponding to two coordinated ammonia/water molecules, lost between 150–180 °C, [g]Corresponding to five coordinated water molecules, lost in one step, between 170–180 °C, [h]Corresponding to three coordinated water molecules and two coordinated ammonia molecules, lost in one step, between 145–180 °C.

ions) were within ± 0.4% of the theoretical data, calculated for the proposed formulae (data not shown).

IR and solution electronic spectroscopic data allowed us to determine the coordination mode of the sulfonamidate anion of **10** in the new complexes **11–30** reported here. Thus, similarly to other 1,3,4-thiadiazole-2-sulfonamide derivatives (such as **1a** and **1b** [31–35, 38] or **2a** [39]) for which metal complexes have been prepared and characterized by X-ray crystallography and spectroscopic methods, the donor system of sulfonamide **10** is constituted by the sulfonamidic nitrogen and the endocyclic N-3 atom of the 1,3,4-thiadiazole ring (shown schematically as "N" and "N^3", respectively, in the structures proposed for the new complexes). The conjugate base of **10** acts in this way as a bidentate ligand, similarly to acetazolamide, benzolamide or other such derivatives previously investigated [30–39]. Several spectral changes in the spectra of complexes as compared to the corresponding spectrum of **10** (or its sodium salt) confirm the above assumptions. Thus, in the IR spectra the following features should be noted: (i) the intense sulfonamide vibrations, at 1 190 and 1 340 cm^{-1} in the spectrum of **10** are generally shifted with 20–80 cm^{-1}

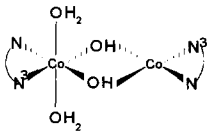
Table III. Electronic spectroscopic data for the Mn(II) complexes **17** (X = H_2O) and **18** (X = NH_3), by the diffuse reflectance technique in MgO as standard, and the proposed structures.



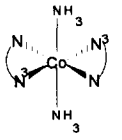
17, 18

Complex (colour)	Abs. maxima λ (nm.)	Wavenumber ν (cm^{-1})	Assignements
17 (white)	391	25 575	$^6A_{1g} \rightarrow {}^4T_{2g}$ (D)
	412	24 270	$^6A_{1g} \rightarrow {}^4A_{1g}$, E_g (G)
	508	19 685	$^6A_{1g} \rightarrow {}^4T_{2g}$
	618	16 180	$^6A_{1g} \rightarrow {}^4T_{1g}$
18 (white)	380	26 315	$^6A_{1g} \rightarrow {}^4T_{2g}$ (D)
	412	24 270	$^6A_{1g} \rightarrow {}^4A_{1g}$, E_g (G)
	508	19 685	$^6A_{1g} \rightarrow {}^4T_{2g}$
	618	16 180	$^6A_{1g} \rightarrow {}^4T_{1g}$

Table IV. Electronic spectroscopic data for complexes **19–22** and **27** by the diffuse reflectance technique in MgO as standard, and the proposed structures.



19



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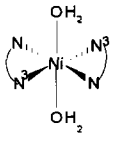
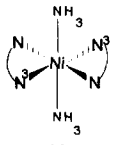
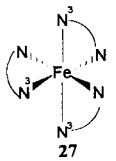


21



22



27

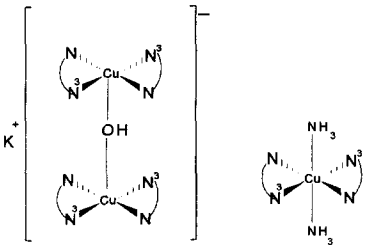
Complex (colour)	Abs. maxima λ (nm)	Wavenumber ν (cm^{-1})	Assignements
19 (greyish-green)	380	26 315	(π→π*)
	497	20 120	$^4T_{1g}\rightarrow{}^4T_{1g}$(P) (Oh)
	600	16 670	$^4A_2\rightarrow{}^4T_1$ (P) (Td)
	652	15 340	$^4A_2\rightarrow{}^4T_1$ (P) (Td)
	1 091	9 165	[$^4T_{1g}\rightarrow{}^4T_{1g}$(P) (Oh)] + [$^4A_2\rightarrow{}^4T_1$ (F) (Td)]
20 (pink)	380	26 313	(π→π*)
	525	19 050	$^4T_{1g}\rightarrow{}^4T_{1g}$(P) (Oh)
	585	17 100	$^4T_{1g}\rightarrow{}^4T_{1g}$(P) (Oh)
	1 050	9 502	$^4T_{1g}\rightarrow{}^4T_{2g}$ (Oh)
21 (magenta)	380	26 315	(π→π*) ($^3A_{2g}\rightarrow{}^3T_{1g}$(P))
	580	17 240	$^3A_{2g}\rightarrow{}^3E_g(D_{4h})$
	646	15 480	$^3A_{2g}\rightarrow{}^3A_{2g}(D_{4h})$
	1 081	9 250	$^3A_{2g}\rightarrow{}^3T_{2g}$
22 (blue)	380	26 315	(π→π*) + ($^3A_{2g}\rightarrow{}^3T_{1g}$(P))
	415	24 096	(π→π*)
	570	17 543	$^3A_{2g}\rightarrow{}^3T_{1g}$
	904	11 060	$^3A_{2g}\rightarrow{}^{1E}_{g}$
	1134	9 080	$^3A_{2g}\rightarrow{}^3T_{2g}$
27 (yellow)	380	26 315	π→π* + CT#
	460	21 740	$^6A_{1g}\rightarrow{}^4T_{2g}$ (Oh high spin.)
	566	18 050	$^6A_{1g}\rightarrow{}^4T_{1g}$(Oh high spin.)

#CT = charge transfer band.

towards lower wavenumbers in complexes **11–30**, due to the involvement of this moiety in the interaction with the metal ions [29–39] (*table II*); (ii) the strong amide (urea) band present at 1 730 cm^{-1} in the spectrum of the ligand and its sodium salt is unchanged in the spectra of all compounds **11–30**, proving that this moiety does not interact with the metal ions, (iii) the thiadiazole C=N stretching vibration from 1 650 cm^{-1} in sulfonamide **10** undergoes shifts in the spectra of complexes **11–30**, where generally it appears at lower wavenumbers, at 1 620–1 640 cm^{-1} for reasons identical to those mentioned in (i). Still, in some cases, shifts towards higher wavenumbers (1 660–1 670 cm^{-1}) were also evidenced [29–39] (*table II*); (iv) vibrations in the region 320–480 cm^{-1} were identified in the spectra of the complex derivatives, which are absent in the spectrum of the ligand, and were assigned as due to M-N (or M-O) vibrations [29–39] (*table II*).

Complexes **11–30** possessed UV spectra highly similar to those of the sodium salt of the ligand **10** (*table II*), proving the presence of sulfonamide anionic moieties in their molecule. Thus, the ligand **10** has two strong absorption maxima, one at 274 nm and the other one at 302 nm, similarly to other 1,3,4-thiadiazole-2-sulfo-

Table V. Room temperature magnetic moment and electronic spectroscopic data of the Cu(II) complexes **23** and **24** by the diffuse reflectance technique in MgO as standard, and the proposed structures.



23

24

Complex (colour)	Magnetic moment BM	Wavenumber ν (cm^{-1})	Assignements
23 (grey-blue)	0.689	23 696	$\pi \rightarrow \pi^*$
		16 129	$d_{yz},d_{xz} \rightarrow d_{x-y}$
		13 157	$CT^{\#} + d_{yz},d_{xz} \rightarrow d_z$
		10 060	$d_{yz},d_{xz} \rightarrow d_{xy}$
24 (green-blue)	1.47	25 000	$CT + \pi \rightarrow \pi^*$
		16 900	$d_{yz} \approx d_{xz} \rightarrow d_{x-y}$
		14 285	$d_{yz} \approx d_{xz} \rightarrow d_z$
		11 300	$d_{yz} \approx d_{xz} \rightarrow d_{xy}$

$^{\#}$CT = charge transfer band.

namide derivatives previously investigated [9, 24, 25, 29], whereas the sodium salt and its metal complexes show a hypsochromic shift of these bands, which generally appear at 265 and 295 nm, respectively (*table II*).

TG analysis showed the presence of lattice water molecules in compounds **12** and **29**, as well as coordinated water or ammonia molecules in many of the prepared complexes. In the first case, the lattice water was lost in one step between 90–130 °C, whereas the coordinated water/ammonia molecules were also lost in one step, but at higher temperatures (160–200 °C) (*table II*).

Diffuse reflectance electronic spectroscopic and magnetic data (for paramagnetic transition metal ions) helped us to establish the geometry of the cations in the prepared complexes (*tables III–V*) [40–43]. Thus, octahedral structures were proposed for the majority of the prepared complexes [such as the Cd(II), Mn(II), Ni(II), Co(II), Cu(II) (the ammonia containing complex), Zn(II) (the ammonia containing complex), Pb(II); Fe(III) and Al(III) derivatives], whereas tetrahedral structures were detected for the Be(II), Mg(II), one Zn(II) complex (and probably a tetrahedral Co(II) ion is also present in the binuclear cobalt derivative **19**), whereas La(III) is in its preferred nine-coordinated geometry [44]. The binuclear Cu(II) complex **23** probably contains Cu(II) in trigonal bipyramidal geometry [42], which is confirmed by the low magnetic moment and the absence of an EPR signal at room temperature (data not shown). The other Cu(II) complex, **24**, shows a typically octahedral EPR spectrum (data not shown) [42]. For all these derivatives, the expected transitions were evidenced in the electronic spectra, which were assigned in detail in *tables III–V*, and which are in agreement with literature data for similar systems [29–34, 37–44]. Conductimetric data have shown all complexes except **23** to be non-electrolytes, with molar conductibilities in the range of 0.5–3.6 $\Omega^{-1}.cm^2.mol^{-1}$ (data not shown). The Cu(II) complex **23** is a 1:1 electrolyte, with the molar conductibility of 118 $\Omega^{-1}.cm^2.mol^{-1}$.

3.2. Pharmacology

As seen from the data of *table VI*, the sulfonamide **10** and its metal complexes **11–30** behave as very strong inhibitors against all the three investigated isozymes, hCA I, hCA II and bCA IV. The original sulfonamide **10** is already more inhibitory than acetazolamide, methazolamide and benzolamide, having a potency similar to that of dorzolamide or ethoxzolamide against the red cell isozyme hCA II and the membrane-bound isozyme CA IV. The remarkable finding (which was previously re-

Table VI. CA inhibition data with standard inhibitors, sulfonamide **10** and its metal complexes **11–30**.

Inhibitor		K_I (nM)		
		hCA I[a]	hCA II[a]	bCA IV[b]
1a	Acetazolamide	900	12	220
1b		850	10	65
	Methazolamide	780	14	240
	Ethoxzolamide	25	8	13
2a	Benzolamide	15	9	12
7	Dorzolamide	50 000	9	43
10		3.5	6.0	8.2
11		3.3	5.6	7.1
12		2.0	4.0	6.4
13		2.2	4.8	6.6
14		3.0	4.5	5.7
15		2.4	4.5	4.9
16		1.5	3.7	5.5
17		1.0	2.4	3.9
18		2.0	2.5	2.8
19		3.2	0.5	1.2
20		3.8	0.4	1.9
21		3.0	0.5	1.5
22		3.9	1.4	1.8
23		1.5	0.3	1.2
24		0.9	0.1	1.0
25		1.4	1.2	1.8
26		1.2	0.5	1.2
27		2.8	1.4	1.6
28		2.4	1.5	1.5
29		0.9	0.4	0.6
30		0.9	0.7	1.2

[a]Human (cloned) isozymes, [b]From bovine lung microsomes.

ported by us [29] for the whole class of ureas/thioureas of type **5**) was that these compounds have a very high affinity for the slow red cell isozyme, hCA I, which is generally less susceptible to inhibition by this class of compounds [6–8]. The metal complexes **11–30**, being still more inhibitory than **10**, and than all other simple sulfonamides assayed, preserve this feature mentioned above, inhibiting in a high degree hCA I too. Thus, 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 they were prepared [29–35, 37–39]. Particularly strong inhibition was observed for the Zn(II), Co(II), Mn(II), Cu(II) and La(III) derivatives of sulfonamide **10**. The presence of ammonia in the molecules of these derivatives seems to not have influenced too much their biological activity, since complexes with or without this ligand have a similar CA inhibitory action (*table VI*).

In vivo IOP data in rabbits, after the topical administration of different inhibitors prepared in the present study, are shown in *table VII*. As can be seen from *table VII* important reductions of IOP have been observed with many such compounds, the most active being the Zn(II) and Cu(II) derivatives, as already discovered previously by this group [38]. Generally, the potency varied in the following way: Zn(II) complexes > Cu(II) complex > Mg (II) complex ≅ ligand sulfonamide (**10**) ≅ Cd(II) complexes ≅ La(III) complex > Mn(II) complex > Co(II) complex > Ni(II) complex > Al(III) complex > dorzolamide.

From data of *table VIII* it is also possible to see the amount of CA inhibitor present in ocular fluids and tissues after the topical administration of the Zn(II) complex of sulfonamide **10** (compound **13**). It is seen from these data that 1 and 2 h after topical administration of the drug, high levels of **13** were found in the cornea, aqueous humour and ciliary processes. Based on the

Table VII. IOP reduction following topical application of CA inhibitors, at 30 min, 1 h and 1.5 h after instillation into the eye of a drop (50 μL) of a 1 % solution of inhibitor **10–30**, or a 2 % solution of the standard inhibitor dorzolamide **7**. The pH of the inhibitor solution is also shown. All solutions were obtained in 2:3 (v/v) DMSO-water.

Inhibitor	pH	Δ IOP ± SE [a] (mm Hg)		
		1/2 h	1 h	1.5 h
Dorzolamide **7**	5.5	2.2 ± 0.10	4.1 ± 0.15	3.6 ± 0.10
10	6.8	3.1 ± 0.10	9.0 ± 0.09	10.5 ± 0.10
12	7.2	3.4 ± 0.15	9.3 ± 0.20	10.4 ± 0.12
13	7.3	5.5 ± 0.10	11.2 ± 0.10	15.0 ± 0.14
14	7.0	4.1 ± 0.20	9.8 ± 0.10	10.1 ± 0.08
15	6.9	3.0 ± 0.12	8.1 ± 0.20	10.5 ± 0.10
16	7.0	2.5 ± 0.10	7.5 ± 0.17	9.0 ± 0.13
17	7.2	2.1 ± 0.08	7.0 ± 0.16	8.5 ± 0.12
19	7.1	1.4 ± 0.12	3.6 ± 0.10	7.4 ± 0.10
20	6.9	1.5 ± 0.10	3.2 ± 0.07	6.8 ± 0.09
21	7.2	2.0 ± 0.10	4.0 ± 0.07	4.5 ± 0.10
22	7.2	1.7 ± 0.05	3.3 ± 0.10	4.4 ± 0.06
24	7.0	3.5 ± 0.10	9.2 ± 0.10	11.0 ± 0.13
28	6.8	1.1 ± 0.10	5.0 ± 0.06	5.5 ± 0.10
29	6.9	3.0 ± 0.05	9.7 ± 0.10	10.4 ± 0.12

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

inhibition constant of this compound (4.8 nM for CA II, and 6.6 nM for CA IV), the fractional inhibition estimated in these tissues/fluids is of 98.5–99.9% [45–47], proving the fact that the IOP decrease is indeed due to CA inhibition.

One should note that the parent ligand, sulfonamide **10**, is a very potent IOP lowering agent, and this feature is maintained for all its metal complexes. They all act better than the clinical drug dorzolamide. Still, the presence of some metal ions (such as Zn(II), Cu(II), Mg(II) or Cd(II) among others) seem to induce much stronger IOP lowering properties, whereas other metal ions (such as Al(III) or Ni(II)) seem to be less beneficial for this purpose.

In conclusion we report here the first study relating the influence of metal ions upon the IOP lowering properties of the metal complexes containing a sulfonamide with strong CA inhibitory properties as ligand. The most beneficial metal ion for the biological activity of such compounds seems to be Zn(II). Correlated with the general lack of toxicity of Zn(II) compounds, some of the complexes reported here might be good candidates for developing novel types of antiglaucoma drugs.

Table VIII. Ocular tissue concentrations (μM) after 1 and 2 h, following corneal application of one drop (50 μL) of a 2 % solution of the Zn(II) complex **13** and the parent compound **10**, in albino rabbits.

Time (h)	Inhibitior	Drug concentration (μM)*		
		Cornea	Aqueous humor	Ciliary process
1 h	**10**	140 ± 8	266 ± 10	40 ± 5
2 h	**10**	54 ± 5	41 ± 3	9 ± 0.5
1 h	**13**	150 ± 4	279 ± 10	43 ± 5
2 h	**13**	76 ± 5	59 ± 5	12 ± 0.5

*Mean ± standard deviation (n = 3).

4. Experimental protocols

4.1. 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 susceptibility standard. Corrections for the diamagnetism were estimated from Pascal's constants [48]. 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 analyser, and gravimetrically or volumetrically 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 **7**), acetazolamide, solvents, as well as inorganic reagents, were from Sigma, E. Merck and Carlo Erba. 5-(3,4-dichlorophenylureido)-1,3,4-thiadiazole-2-sulfonamide **10** was prepared as described previously [29]. Dorzolamide hydrochloride **5** was from Merck, Sharp and Dohme.

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. [49] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group [50], and enzymes were purified by affinity chromatography according to the method of Khalifah et al. [51]. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1}.cm^{-1}$ for hCA I and 54 $mM^{-1}.cm^{-1}$ for hCA II, respectively, based on M_r = 28.85 kDa for hCA I, and 29.3 kDa for hCA II, respectively [52, 53]. bCA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide [54].

4.1.1. General procedure for the preparation of compounds **11–30**

Method A: an amount of 6 mmol of sodium salt **10** 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 (Be(II), Mg(II), Zn(II), Pb(II), Mn(II), Cu(II), Co(II), Ni(II) chlorides, Fe(III) perchlorate and La(III) 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 1 h and after being cooled to 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 complexes 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.

Method B: as above, except that the sulfonamide **10** suspended in 10 mL of methanol or ethanol was treated with an excess (5 mL) of aqueous 25% solution of ammonia, instead of the alcoholic NaOH solution. The followed procedure was then identical to the one described above.

4.2. Pharmacology

4.2.1. Carbonic anhydrase inhibition

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 [55]. Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25 °C. A molar absorption coefficient ε of 18 400 $M^{-1}.cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature [55]. 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 [6–8]) 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 [55]. Enzyme concentrations were 3.3 nM for hCA II, 10 nM for hCA I and 34 nM for bCA IV (this isozyme has a decreased esterase activity [56] and higher concentrations had to be used for the measurements).

4.2.2. 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:12 h light/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 inhibitor 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 [45–47]. The pressure readings were

matched with two-point standard pressure measurements at least twice each day using a Digilab Calibration verifier. The same investigator did all IOP measurements 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 every 30 min for a period of several hours. For all IOP experiments drugs were 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 [45–47]. All data are expressed as mean ± SE, using a one-tailed t test.

4.2.3. Drug distribution in ocular fluids and tissues

The general procedure of Maren's group has been followed [45–47]. The animals were killed with an intracardiac 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 cilliary processes were liberated from their attachment to the iris, cut, weighed and put into 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, 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 [45–47].

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