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Carbonic Anhydrase Inhibitors: Metal Complexes of a Sulfanilamide Derived Schiff base and their Interaction with Isozymes I, II and IV

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Metal complexes of aromatic/heterocyclic sulfonamides act as stronger inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) as compared to the uncomplexed sulfonamides from which they are derived. Here we report the synthesis and inhibition studies against the physiologically relevant isozymes CA I, CA II and CA IV, of a series of metal complexes (Co(II), Ni(II) and Cu(II) derivatives) of a Schiff-base ligand, obtained from sulfanilamide and salicylaldehyde. The best activity was observed for the Cu(II) and Co(II) complexes, against CA II and CA IV, for which inhibition constants in the range of 15–39 and 72–108 nM, respectively, were seen. The enhanced efficacy in inhibiting the enzyme may be due to a dual mechanism of action of the metal complexes, which interact with CA both by means of the sulfonamide moieties as well as the metal ions present in their molecule.

Keywords: Carbonic anhydrase; Isozymes I, II, IV; Aromatic sulfonamides; Schiff-base; Metal complexes

INTRODUCTION

The presence of heteroatoms in the molecules of heterocyclic/aromatic sulfonamides acting as inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), such as the clinically used agents acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, dichlorophenamide **4**, dorzolamide **5**, or brinzolamide **6**, makes them attractive ligands for complexation by metal ions.[1–3] Indeed, many metal complexes of these and structurally related heterocyclic sulfonamides have been reported,[4–19] characterized by spectroscopic[4–19] and X-ray crystallographic methods,[3,6,12,13] and investigated for inhibition of different CA isozymes as well as for potential applications as diagnostic tools/pharmacological agents.[1–3] The most interesting fact regarding these metal complexes of sulfonamide CA inhibitors is that they generally act as 10–100 times more potent inhibitors of isozymes CA I,

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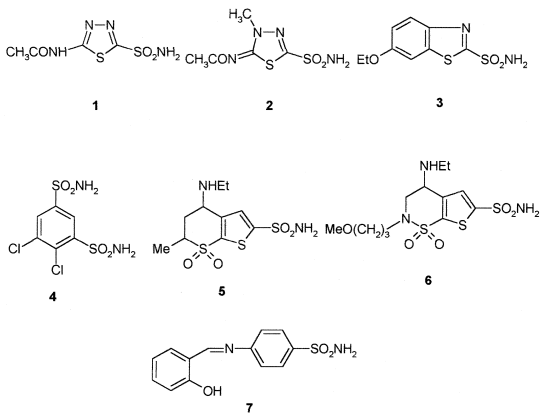
CA II and CA IV, as compared to the parent sulfonamide from which they were obtained, and this has been rationalized from the mechanistic point of view.[4,5] Thus, it is believed that this powerful inhibition is due to a dual mechanism of action, through sulfonamide anions, and metal ions, obtained in dilute solution by dissociation of the coordination compounds. Sulfonamide anions formed in this way then bind to the Zn(II) ion within the enzyme active site, whereas the metal ions block the proton shuttle residues of CA, i.e. His 64 for isozyme II, His 64 and 67 for isozyme I, and probably the entire histidine cluster in the case of isozyme II as well.[1,3–5]



Several interesting applications have been recently reported for some metal complexes of heterocyclic sulfonamides possessing powerful CA inhibitory properties. Thus, zinc or copper complexes of highly lipophilic thiadiazole sulfonamides were shown to act as very efficient intraocular pressure (IOP) lowering agents when administered topically in normotensive or glaucomatous rabbits, although the parent sulfonamides from which the complexes were prepared did not possess topical antiglaucoma activity.[15] It was then showed that many other such metal complexes possess topical antiglaucoma action,[15–18] and this has been explained by changes in the physico-chemical properties of the complex induced by the metal ion, which may become more polar and thus penetrate better through the cornea for inhibiting the ciliary process CAs (isozymes CA II and CA IV).[15–18] Some aluminum sulfonamide complexes were then shown to act as efficient antisecretory agents in dogs.[19] Gastric acid secretion parameters 3 days after treatment with such CA inhibitors were drastically reduced, as compared to the same parameters in animals that did not receive these enzyme inhibitors.[19] It has been proposed that the Zn(II), Mg(II) and Al(III) sulfonamide complexes might constitute a new class of antiulcer agents,[19] acting probably by a double mechanism: neutralization of hydrogen ions by a normal acid-base neutralization reaction (due to the presence of the metal ion derivative), coupled with a strong inhibition of carbonic anhydrase isozymes present in the gastric mucosa (due to both the sulfonamide and metal ion components of the drug), followed by reduction of formation of H^+ ions due to CO_2 hydration.

Taking into account such findings, in this paper we extend our previous studies[20] in the synthesis and evaluation as CA inhibitors of Schiff-base–transition metal complexes, since such derivatives have poorly been investigated previously. We report here the Co(II), Ni(II) and Cu(II) complexes of the Schiff-base **7**,[21] obtained from sulfanilamide and salicylaldehyde. The new complexes reported here were characterized by standard procedures and assayed as inhibitors of the physiologically relevant CA isozymes: hCA I, hCA II and bCA IV.

MATERIALS AND METHODS

Melting points were determined on a Gallenkamp aparatus and are not corrected; IR spectra were obtained in KBr pellets with a Perkin-Elmer PU9800 FTIR spectrometer. Electronic spectra of the metal complexes were obtained on a Hitachi U-2000 double-beam spectrophotometer. Magnetic measurements were done on solid complexes using the Gouy method. Conductances of the metal complexes were determined in DMF on

a YSI-32 model conductometer. Elemental analyses were done by combustion for C, H, N, with an automated Carlo Erba analyzer, or gravimetrically for the metal ions, and were ± 0.4% of the theoretical values. The sulfanilamide Schiff-base **7** has been prepared as reported previously.[21] Sulfonamides used as standards were commercially available from Sigma, Acros or Aldrich. Solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions. Metal salts used for the preparation of the coordination compounds were analytical grade from E. Merck.

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*.[22] (the two plasmids were a gift from Professor Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group,[23] and enzymes were purified by affinity chromatography according to the method of Khalifah *et al*.[24] 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.[25,26] CA IV was isolated from bovine lung microsomes as described by Maren *et al*, and its concentration has been determined by titration with ethoxzolamide.[27]

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC.[28] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25°C. A molar absorption coefficient ϵ of 18,400 $M^{-1}\,cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, under the conditions of the experiments (pH 7.40), as reported in the literature.[29] Non-enzymic 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 means of such results. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constant K_I was determined as described by Pocker and Stone.[29] Enzyme concentrations were 3 nM for CA II, 12 nM for CA I and 39 nM for CA IV (this isozyme has a decreased esterase activity and higher concentrations had to be used for the measurements).[30]

Preparation of Coordination Compounds 8–17

To a hot ethanolic (25 ml) solution of sulfonamide **7** (0.02 moles) an aqueous solution of the corresponding metal(II) salt (0.01 M) was added. The mixture was refluxed for 3 h, the obtained solution filtered and reduced to half of its volume, by evaporation of the solvent *in vacuo*. The concentrated solution was left overnight at room temperature, which led to the formation of a solid product. This solution was filtered, washed with ethanol (2×15 ml) and dried. Recrystallization from 50% aqueous ethanol gave the desired products. Unfortunately only microcrystalline powders could be obtained, which could not be used for X-ray structural determinations. In fact this is the usual technical problem related to the thorough characterization of this type of metal complexes.[20]

RESULTS AND DISCUSSION

The Schiff-base **7**, prepared from sulfanilamide and salicylaldehyde,[21] was shown to behave as a

TABLE I Metal complexes **8**–**17** containing sulfonamide **7** as ligand, their formulas, metal content and elemental analysis data

No. complex	Formula	Analysis (calculated/found)			
		%M*	%C†	%H†	%N†
8 $[CoL_2Cl_2]$	$C_{26}H_{24}Cl_2CoN_4O_6S_2$	8.64/8.50	45.76/45.38	3.54/3.33	8.21/8.12
9 $[CoL_2(NO_3)_2]$	$C_{26}H_{24}CoN_6O_{12}S_2$	8.01/8.40	42.46/42.63	3.29/3.14	11.43/11.21
10 $[CoL_2(AcO)_2]$	$C_{30}H_{30}CoN_4O_{10}S_2$	8.08/7.86	49.38/49.54	4.14/4.10	7.68/7.47
11 $[CoL_2(HSO_4)_2]$	$C_{26}H_{26}CoN_4O_{14}S_4$	7.31/7.80	38.76/39.07	3.25/3.19	6.95/7.04
12 $[NiL_2Cl_2]$	$C_{26}H_{24}Cl_2NiN_4O_6S_2$	8.61/8.85	45.77/45.83	3.55/3.76	8.21/8.09
13 $[NiL_2(NO_3)_2]$	$C_{26}H_{24}NiN_6O_{12}S_2$	7.98/7.75	42.47/42.19	3.29/3.36	11.43/11.37
14 $[NiL_2(AcO)_2]$	$C_{30}H_{30}NiN_4O_{10}S_2$	8.05/8.23	49.40/49.17	4.15/4.36	7.68/7.54
15 $[NiL_2(HSO_4)_2]$	$C_{26}H_{26}NiN_4O_{14}S_4$	7.29/7.76	38.77/39.00	3.25/3.46	6.96/6.61
16 $[CuL_2Cl_2]$	$C_{26}H_{24}Cl_2CuN_4O_6S_2$	9.25/9.33	45.45/45.62	3.52/3.60	8.15/7.89
17 $[CuL_2(NO_3)_2]$	$C_{26}H_{24}CuN_6O_{12}S_2$	8.59/8.47	42.19/42.45	3.27/3.10	11.35/11.03

* By gravimetry.
† By combustion.

moderate–weak CA inhibitor, against isozymes I, II and IV, all of which play critical functions in a host of physiological processes.[1–3] Since metal complexes of heterocyclic/aromatic sulfonamides have recently been shown[4–21] to possess even stronger inhibitory properties, it appeared of interest to synthesize some metal complexes of this Schiff-base, mainly due to its interesting donor system, comprising the nitrogen incorporated in the Schiff-base moiety, as well as the phenolic OH group. Thus, the Cu(II), Co(II) and Ni(II) complexes (Table I) of ligand **7** and different counter-anions have been obtained. It should be noted that similarly to other ligands investigated in previous studies as metal complexing sulfonamides with CA inhibitory properties,[20] the compounds investigated here act as neutral ligands, and not in the deprotonated state (see later in the text). These complexes have purposely been prepared in the absence of deprotonating agents (NaOH, NH_3 or organic amines) so as to favor the participation of the Schiff-base as a neutral ligand in the interaction with the metal ions. Indeed, molar conductivities in DMF solutions measured for 1:2 (M:L) complexes are quite low and are in the range 6.0–11.2 ohm^{-1} cm^2 mol^{-1} (data not shown) which indicates their non-electrolyte nature (Table II). Working in the presence of bases, generally leads to the deprotonation of the sulfonamido moiety of the ligand, which thereafter interacts itself with the metal ion(s) present in the coordination compound.[4–19]

The most important bands in the IR spectra of the free Schiff-base **7** and its complexes are summarized in Table III. IR spectra of the Schiff-base **7** showed the absence of bands at ~1735 and ~3320 cm^{-1} due to the aldehyde ν(CHO) and $\nu(NH_2)$ stretching vibrations respectively, as well as the appearance of a strong new band at 1610 cm^{-1}, assigned to the azomethine ν(CH=N) vibration.[28,31,32] The comparison of the IR spectra of the Schiff-base and its metal derivatives is indicative of the fact that the Schiff-base is coordinated to the metal ions in a bidentate fashion. The band appearing at 1610 cm^{-1} due to the azomethine linkage in the ligand, is shifted to higher frequency by ∽ 10–15 cm^{-1} in the metal

TABLE II Some physico-chemical data for the metal complexes **8**–**17**

No.	M.P. (°C)	B.M. (μ_{eff})	Yield (%)
8	211–2	4.62	73
9	208–11	4.64	75
10	208–10	4.61	71
11	208–9	4.65	75
12	210–2	3.10	69
13	208–10	3.13	73
14	206–8	3.11	72
15	210–12	3.15	70
16	210–2	1.52	71
17	212–14	1.54	72

TABLE III IR and electronic spectroscopic data for the ligand **7** (L) and its metal complexes **8–17**

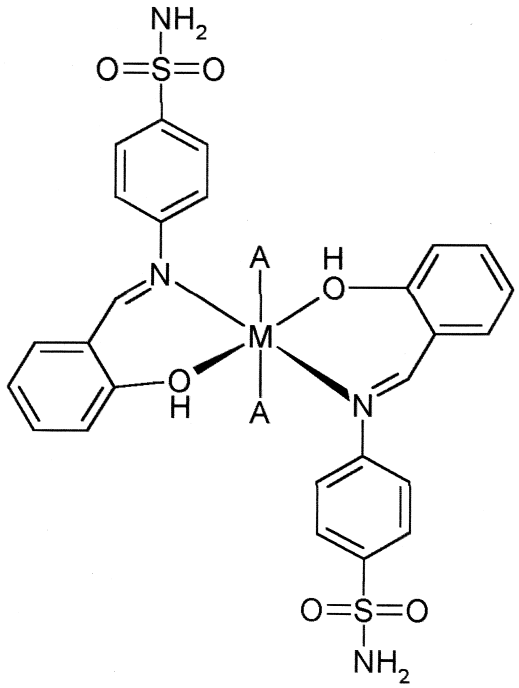
Compound	IR (cm^{-1})*	λ_{max} (cm^{-1})†
7	3350 (OH), 1610(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920.	–
8	3335 (OH), 1620(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 540 (M–N), 445 (M–O), 295 (M–Cl).	20920, 28645
9	3340 (OH), 1620(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 540 (M–N), 440 (M–O).	20915, 28555
10	3340 (OH), 1625(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 540 (M–N), 445 (M–O).	20945, 28610
11	3335 (OH), 1620(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 545 (M–N), 440 (M–O).	20955, 28570
12	3340 (OH), 1625(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 540 (M–N), 445 (M–O), 285 (M–Cl).	16670, 27785
13	3340 (OH), 1625(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 545 (M–N), 440 (M–O).	16675, 27780
14	3335 (OH), 1625(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 540 (M–N), 445 (M–O).	16755, 27810
15	3335 (OH), 1620(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 540 (M–N), 445 (M–O).	16745, 27795
16	3340 (OH), 1625(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 545 (M–N), 440 (M–O), 290 (M–Cl).	16390, 27325
17	3335 (OH), 1620(C=N), 1355 $(SO_2)_{asym}$, 1315, 1175 (SO_2)sym, 1150, 950 (S–N), 920, 540 (M–N), 445 (M–O).	16510, 27250

*In KBr.
†In MgO as standard, by the diffuse reflectance technique.

complexes, indicating participation of the azomethine nitrogen in the complexation.[28,31,32] The band at 3350 cm^{-1} assigned to the hydroxyl ν(OH) group in the spectrum of **7**, did not disappear in the spectra of **8–17**, but is shifted to lower frequency (10–15 cm^{-1}) indicating too coordination of the hydroxyl oxygen to the metal ions.[28,31,32] Further conclusive evidence of the coordination of the Schiff-base **7** with the metal ions was shown by the appearance of weak, low frequency new bands at $\backsim$530 and $\backsim$440 cm^{-1}. These were assigned as being due to the metal–nitrogen ν(M–N) and metal–oxygen ν(M–O) vibrations, respectively.[33] These new bands were only observable in the spectra of the metal complexes and not in the spectrum of the uncomplexed Schiff-base **7**.

The cobalt(II) complexes **8–11** show magnetic moment values of 4.61–4.65 B.M. at room temperature (Table II). These high values of the magnetic moments and the stoichiometries suggest[34,35] an octahedral coordination of Co(II). The electronic spectra of these complexes are also consistent with an octahedral environment around the cobalt(II) ion.[28] The spectra display two bands at ~20,920–20,955 and ~28,570–28,645 cm^{-1} attributed to $^4T_{1g} \rightarrow {}^4T_{1g}$ and $^4T_{1g} \rightarrow {}^4T_{2g}$ transitions, respectively, in a low-spin octahedral geometry[36]. The electronic spectra of the Ni(II) complexes **12–15** exhibited[37,38] absorption bands at ~16,670–16,775 and ~27,810–27,885 cm^{-1} attributable to $^3A_{2g} \rightarrow {}^3T_{1g}$ (F) and $^3A_{2g} \rightarrow {}^3T_{1g}$ (P), transitions, respectively, in an octahedral geometry of the metal ion.[29] The calculated values of the ligand field parameter lie in the range reported for such an octahedral structure (Table II). The values of the magnetic moment (3.10–3.15 B.M.) may be taken as additional evidence[34] for such an octahedral structure. The Cu(II) complexes **16** and **17** exhibit[28] magnetic moments of 1.52–1.55 B.M. at room temperature. The electronic spectra of these complexes display two bands at ~16,390–16,510 and ~27,250–27,325 cm^{-1} assigned to d–d transitions, as well as a charge transfer band,

confirming the octahedral environment around the metal ion.[37] The proposed structure for the new complexes reported here is shown below.



A = Cl, NO_3^-, AcO^-, HSO_4^-

8-17: M = Co(II); Ni(II); Cu(II)

The following observations can be made about inhibition of isozymes CA I, II and IV with the obtained Schiff-base complexes **8**–**17** (Table IV). Thus, Schiff-base **7** behaves as a very weak inhibitor against isozyme hCA I and a weak inhibitor against hCA II, being a slightly better bCA IV inhibitor. Indeed, the inhibition power of this Schiff-base is reduced as compared to that of sulfanilamide against the first two isozymes mentioned above, whereas the Schiff-base is at least seven times more inhibitory against bCA IV as compared to sulfanilamide (Table IV). Metal complexes **8**–**17** on the other hand, act as much more potent inhibitors against all three investigated isozymes, their potency being enhanced as compared to that of the parent sulfonamide **7** as well as sulfanilamide (as standard inhibitor), a feature shared with many metal complexes of sulfonamide CA inhibitors reported up to now.[4–22] Thus, all the prepared complexes showed affinities in the range of 13–39 nM against hCA II, 210–560 nM against hCA I, and 72–108 nM against bCA IV (Table III). The most effective inhibitors were those containing Cu(II), followed by those containing Co(II) ions, which in turn were more effective than those containing Ni(II) ions, but the differences in activity between these compounds is small and the nature of the counterion present in the coordination compound seems also to be of little importance for the biological activity of these derivatives (Table IV).

TABLE IV CA inhibition data with sulfanilamide, the Schiff-base **7** and its metal complexes **8**–**17**, against isozymes I, II and IV, by the esterase method[27]

	K_I (nM)*		
Compound	hCA I†	hCA II†	bCA IV‡
Sulfanilamide	28,000	300	3000
7	35,000	410	420
8	330	24	78
9	320	25	73
10	330	23	73
11	350	22	72
12	540	39	105
13	560	39	102
14	550	37	108
15	550	36	106
16	210	13	83
17	240	15	80

* Errors in the range of ± 10% of the reported value.
† Human (cloned) isozyme.
‡ Isolated from bovine lung microsomes.

In conclusion, in the present paper we report novel Schiff-base metal complexes which possess a highly increased affinity in binding to the physiologically relevant isozymes hCA I, hCA II and bCA IV, as compared to the parent sulfonamides from which they were derived. We also show that the anionic moieties present in these new molecules are rather irrelevant for their biological activity as enzyme inhibitors.

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