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Carbonic anhydrase inhibitors. Part 37.# Novel classes of isozyme I and II inhibitors and their mechanism of action. Kinetic and spectroscopic investigations on native and cobalt-substituted enzymes

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Summary — The interaction of Zn(II)- and Co(II)-carbonic anhydrase (CA) with a series of compounds possessing moieties resembling the aromatic sulfonamides, such as sulfamide, sulfamic acid, *N*-substituted aromatic sulfonamides, sulfenamides, sulfinic and seleninic acids, was investigated using kinetic and spectroscopic techniques. All these compounds inhibit the hydrasic and esterasic activity of the enzyme. Their binding within the active site of isozymes I and II is discussed on the basis of modifications of electronic and ^{1}H-NMR spectra of their adducts with the Co(II) enzyme. Some of these compounds represent novel classes of CA inhibitors, possessing equal or stronger potencies than the prototypical inhibitors, the unsubstituted sulfonamides. Qualitative structure–activity correlations are discussed.

carbonic anhydrase / sulfonamide–anion inhibitors / electronic spectroscopy / NMR spectroscopy / inhibition mechanism

Introduction

Sulfonamides are well-known inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) which have been extensively investigated for their applications in the treatment of diverse diseases in clinical medicine [1–3]. The other important class of CA inhibitors is represented by metal-complexing inorganic anions [4], such as cyanide, cyanate, thiocyanate, and hydrosulfide, which were first studied by the discoverers of this enzyme, Meldrum and Roughton [5]. Inhibitors of both types bind to the Zn(II) ion within the enzyme active site as anions, by substituting the metal-bound solvent molecule or increasing the coordination number of the metal [6, 7]. Much information regarding the mechanism of inhibition by anions and sulfonamides was obtained working on Co(II)-substituted CAs which, catalytically, are still fully active derivatives [6–10]. The Co(II) ion is a good spectroscopic probe [6–8], and Co(II)CA has very characteristic electronic, NMR and EPR spectra, which are highly sensitive to the environment around the metal ion, constituting an easy approach for studying the interaction of this enzyme with inhibitors or substrates [6–11].

Besides the theoretical interest in understanding the mechanisms of inhibition in CAs at the molecular level, the inhibitors also have considerable practical applications in the treatment of a variety of disorders [1–3, 12]. Thus, recently a new sulfonamide inhibitor was introduced in clinical medicine as a successful topical antiglaucoma drug [12]. Moreover, the generally accepted theory that only aromatic/heterocyclic unsubstituted sulfonamides may act as inhibitors [2] has also been shown to be incorrect, because recently aliphatic fluorosulfonamides such as $CF_3SO_2NH_2$ and some of its congeners [13], as well as sulfamates of type $ArOCH_2CH_2OSO_2NH_2$ [14] were shown to act as very potent inhibitors. It was also proved [15] that *N*-substituted sulfonamides of the type Ar-SO_2NHX (where X is generally a small and compact group, such as hydroxy, methoxy, etc) inhibit isozyme CA II, but no direct proof for the mechanism of inhibition with these types of compounds was obtained. On the other hand, at least nine CA isozymes are presently known in vertebrates [16] but the physiological function is not very well understood for many of them, such as CA I, CA III and CA VII–IX [3, 16]. Thus,

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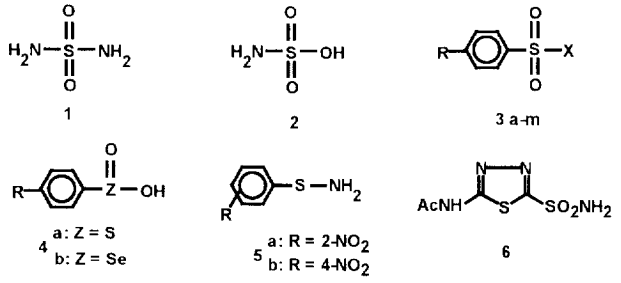
isozyme-specific inhibitors [17] are needed in order to assess the physiological role for many of these isozymes. We want to report here a study on the interaction between red cell isozymes CA I and CA II and a series of compounds possessing structural elements in common with the two main classes of CA inhibitors, ie, the inorganic anions and the sulfonamides of types **1**–**5**.



The investigated derivatives include simple compounds such as sulfamide **1** (the most simple sulfonamide-like substance), sulfamic acid **2**, a series of *N*-substituted sulfonamide derivatives **3** (some of which contain moieties present in inorganic anion inhibitors of CA, such as NCS, NO and N_3), and also the S(IV), Se(IV) and S(II) derivatives **4** and **5**. Inhibition constants have been measured both for CO_2 hydration reaction and 4-nitrophenyl acetate hydrolysis on the native isozymes I and II, whereas electronic absorption and ^{1}H-NMR spectra for the adducts of some of these derivatives with Co(II)-substituted CA II have been recorded, leading to the conclusion that these new types of inhibitors are bound to the metal ion in a variety of ways not evidenced previously. In addition to evidencing novel classes of inhibitors, this study also proves their mechanism of action and might be useful to develop more specific and potent inhibitors for the newly isolated isozymes. It should be stressed that this study evidences the first uncharged inhibitors ever reported for CAs, as well as the fact *N,N*-disubstituted sulfonamides may possess such an action. Moreover, this is the largest series of *N*-modified sulfonamides investigated for its interaction with the enzyme, and, in addition to giving the possibility of designing more specific inhibitors for the different isozymes, such derivatives might be useful for obtaining prodrug forms [18] of this type of pharmacological agent.

Experimental protocols

Melting points were determined with a heating plate microscope and are not corrected. IR spectra were recorded in KBr pellets with C Zeiss Jena or Perkin-Elmer 890 spectrophotometers. ^{1}H-NMR spectra (in solvents specified in each case) were obtained with a Varian EM360L spectrometer. Chemical shifts are expressed as δ values, relative to Me_4Si as internal standard. Elemental analysis was done by microcombustion (C, H, N) with an automated Carlo Erba analyzer. Values were ±0.4% of the theoretical data calculated for the proposed formulas.

Bovine CA II, buffers (Tris, Hepes, phosphate, etc), 4-nitrophenyl acetate, sulfonyl and sulfenyl chlorides, pyridine-2,6-dicarboxylic acid, tosylimidazole **3g** and halazone **3k** (4-(*N,N*-dichlorosulfamyl)benzoic acid) were from Sigma; acetonitrile, sulfamide **1**, sulfamic acid **2**, 4-toluenesulfonamide **3a**, tosic acid **3o**, 4-toluenesulfinic acid (sodium salt) **4a**, benzeneseleninic acid **4b**, chloramine B **3j** and chloramine T **3k**, were from Acros, and were used without further purification. Other compounds of type **3** were prepared as described earlier from tosyl chloride and the corresponding nucleophile [15, 19]. Sulfenamides **5** were prepared from the coresponding sulfenyl chlorides and ammonia and recrystallized from water. Nucleophiles used to prepare derivatives **3** were commercial compounds from Merck, Aldrich or Acros and were used without additional purification, except for *O*-methylhydroxylamine, which was a gift from MD Banciu (Polytechnic University Bucharest, Romania).

Human CA I cDNAs was expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/HCA I and pACA/HCA II [20] (the two plasmids were a gift from S Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group [21], and enzymes were purified by affinity chromatography according to the method of Khalifah et al [22]. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1}\cdot cm^{-1}$ for CA I and 54 $mM^{-1}\cdot cm^{-1}$ for CA II, respectively, based on M_r = 28.85 kDa for CA I, and 29.3 kDa for CA II, respectively [23, 24].

All buffers used in the kinetic measurements were brought to an ionic strength μ = 0.1, by addition of K_2SO_4. Cobalt(II)-CA II was prepared by the method of Hunt et al [25], by removing zinc from the native enzyme in the presence of 50 mM pyridine-2,6-dicarboxylic acid, followed by dialysis against metal-free Tris-H_2SO_4 buffer, and addition of the stoichiometric amount of Co(II) salt.

Initial rates of 4-nitrophenyl acetate hydrolysis were monitored spectrophotometrically, at 400 nm and 25 °C, with a Cary 3 apparatus interfaced with an IBM-compatible PC, accordingly to the method of Pocker and Stone [26]. Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 10^{-2} and 10^{-4} M. A molar absorption coefficient ε = 18 400 $M^{-1}\cdot cm^{-2}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.80), as reported by Pocker and Stone [26]. Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor, and the values reported throughout the paper are the averages of such results.

K_I's for the hydrase activity were obtained using Maren's micromethod [27] in the conditions of the EI (enzyme–inhibitor) technique, in barbital buffer (pH 7.2).

The ^{1}H-NMR spectra were recorded with a Bruker MSL 200 instrument, by using the modified DEFT [28] or WEFT [29] pulse sequences in order to suppress water and other diamagnetic signals. Chemical shifts were measured from H_2O or HDO signals and reported from Me_4Si assumed to be at –4.8 ppm from the water signal. All spectra were recorded by using 0.2–0.5 mM of CoCA II, at pH values and molarities of inhibitor indicated in the text. D_2O solutions were prepared by exchanging the aqueous solution with D_2O. The pH-metric recordings were uncorrected for isotopic effects.

General procedure for the preparation of compounds ***3***

Tosyl chloride (5 mmol) was dissolved in 25 mL of anhydrous tetrahydrofuran and the corresponding amount (5 mmol) of nucleophile (hydroxylamine, *O*-methylhydroxylamine, potassium thiocyanate, sodium azide, imidazole, hydrazine, phenylhydrazine, cyanamide, *O*-carboxymethylhydroxylamine and sodium hydrosulfide, respectively) dissolved in the minimum amount of water or alcohol/water was added, together with the calculated amount of potassium carbonate (2.5 mmol) dissolved in 4 mL water. The reaction mixture was stirred at room temperature for 5 h, then left overnight, the organic solvents were evaporated under reduced pressure and the *N*-modified sulfonamide was extracted in chloroform and recrystallized from solvents specified in each case. Yields were of 40–65%.

General procedure for the preparation of sulfenamides ***5***

The *o*- or *p*-nitrobenzenesulfenyl chloride (1.2 g) was dissolved in 10 mL cold concentrated (32%) ammonia solution and the mixture was energetically stirred at 4 °C for 4 h. The excess ammonia was evaporated, the reaction mixture brought to pH 7 with hydrochloric acid, and the precipitated sulfenamides were recrystallized from boiling water. Yields were in the range of 30–35%.

N-Hydroxy-4-toluenesulfonamide ***3b***
White crystals, mp 149 °C (from MeOH); lit mp 148 °C (from benzene) [30]. IR (KBr), cm^{-1}: 550, 570, 735, 825, 905, 1000, 1085, 1170, 1315, 1390, 1595, 3210, 3375 br; ^{1}H-NMR (TFA (trifluoroacetic acid)), δ, ppm: 2.50 (s, 3H, Me), 7.10–7.90 (m, AB, 4H, ArH), 9.20 (s, 1H, NH), 10.10 (s, 1H, OH). Anal $C_7H_9NO_3S$ (C, H, N).

N-Methoxy-4-toluenesulfonamide ***3c***
White crystals, mp 111 °C (from MeOH); lit mp 113 °C (from CCl_4) [30, 31]. IR (KBr), cm^{-1}: 550, 565, 738, 830, 910, 1050, 1095, 1180, 1325, 1400, 1597, 2940, 3220; ^{1}H-NMR ($CDCl_3$), δ, ppm: 2.40 (s, 3H, 4-Me), 3.75 (s, 3H, OMe), 7.10 (br s, ^{1}H, NH), 7.20–7.90 (m, AB, 4H, ArH). Anal $C_8H_{11}NO_3S$ (C, H, N).

Compound ***3d***
This derivative was prepared by oxidation of **3b** with excess H_2O_2 at room temperature for 24 h, until the NH and OH signals at 9.20 and 10.10 ppm, respectively, in the ^{1}H-NMR spectrum of the raw material disappeared. Greyish crystals, mp 123–125 °C (from MeOH). IR (KBr), cm^{-1}: 553, 570, 739, 844, 915, 1000, 1080, 1173, 1315, 1388, 1590, 3230; ^{1}H-NMR (TFA), δ, ppm: 2.50 (s, 3H, Me), 7.10–7.90 (m, AB, 4H, ArH). Anal $C_7H_7NO_3S$ (C, H, N).

Tosylisothiocyanate ***3e***
Pale yellow crystals, mp 132 °C, lit mp 132 °C [32]. IR (KBr), cm^{-1}: 525, 710, 985, 1095, 1139, 1364, 1490, 1570, 1610, 1905; ^{1}H-NMR (AcOH-d_4), δ, ppm: 2.50 (s, 3H, Me), 7.30–7.90 (m, AB, 4H, ArH). Anal $C_8H_7NO_2S_2$ (C, H, N).

Tosylazide ***3f***
Colorless oil which becomes crystalline by refrigeration, mp 22 °C, lit mp 22 °C [33]. IR (KBr), cm^{-1}: 525, 715, 980, 1095, 1135, 1360, 1490, 1570, 1610, 2340; ^{1}H-NMR (AcOH-d_4), δ, ppm: 2.50 (s, 3H, Me), 7.30–7.90 (m, AB, 4H, ArH). Anal $C_7H_8N_3O_2S$ (C, H, N).

Tosylhydrazide ***3h***
Colorless crystals, mp 109 °C (from MeOH), lit mp 109–110 °C [34]. IR (KBr), cm^{-1}: 510, 705, 980, 1090, 1115, 1350, 1490, 1570, 1610, 3000 br, 3260 br; ^{1}H-NMR (AcOH-d_4), δ, ppm: 2.50 (s, 3H, Me), 7.30–8.10 (m, AB, 4H, ArH), 12.00 (s, 3H, $NHNH_2$). Anal $C_7H_{10}N_2O_2S$ (C, H, N).

N^2-Phenyltosylhydrazide ***3i***
White crystals, mp 160–162 °C (from MeOH), lit mp 163 °C (from benzene) [35]. IR (KBr), cm^{-1}: 500, 605, 660, 770, 820, 1090, 1160, 1315, 1490, 1595, 3240, 3330 br; ^{1}H-NMR (TFA), δ, ppm: 2.50 (s, 3H, Me), 7.10–7.80 (m, AB, 4H, ArH), 10.05 (s, 2H, NHNH). Anal $C_{13}H_{14}N_2O_2S$ (C, H, N).

Tosylcyanamide ***3m***
Colorless crystals, mp 247–249 °C (from EtOH/water 1:1, v/v); lit mp 246–248 °C [19]. IR(KBr), cm^{-1}: 510, 705, 980, 1090, 1115, 1350, 1490, 1570, 1610, 2175, 3250 br; ^{1}H-NMR (AcOH-d_4), δ, ppm: 2.50 (s, 3H, Me), 7.30–8.10 (m, AB, 4H, ArH), 11.20 (s, 1H, NH). Anal $C_8H_8N_2O_2S$ (C, H, N).

N-Carboxymethoxy-4-toluenesulfonamide ***3n***
Tan crystals, mp 215 °C (from EtOH) lit mp 212–214 °C [36]. IR (KBr), cm^{-1}: 550, 569, 743, 837, 915, 1050, 1090, 1180, 1325, 1400, 1597, 1645, 2975, 3220; ^{1}H-NMR ($CDCl_3$), δ, ppm: 2.45 (s, 3H, 4-Me), 3.90 (s, 2H, OCH_2), 7.10 (br s, 1H, NH), 7.20–7.90 (m, AB, 4H, ArH), 9.85 (s, 1H, COOH). Anal $C_9H_{11}NO_4S$ (C, H, N).

Toluenethiosulfonic acid, sodium salt ***3p***
Colorless crystals, mp 320–322 °C (dec) (from water); lit mp not reported [37]. IR (KBr), cm^{-1}: 528, 730, 958, 1090, 1145, 1375, 1498, 1570, 1605, 2550. ^{1}H-NMR (AcOH-d_4), δ, ppm: 2.50 (s, 3H, Me), 7.30–7.90 (m, AB, 4H, ArH). Anal $C_7H_7O_2S_2Na$ (C, H, N).

2-Nitrobenzenesulfenamide ***5a***
Yellow crystals, mp 115–117 °C (from water); lit mp 117 °C [38]. IR (KBr), cm^{-1}: 545, 710, 960, 1085, 1490, 1585, 1605, 3340; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.10 (s, 2H, NH_2), 7.30–7.60 (m, 4H, ArH). Anal $C_6H_8N_2O_2S$ (C, H, N).

4-Nitrobenzenesulfenamide ***5b***
Yellow crystals, mp 155–156 °C (from water); lit mp 155 °C [38]. IR (KBr), cm^{-1}: 550, 735, 824, 963, 1078, 1495, 1585, 1605, 3330; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.15 (s, 2H, NH_2), 7.30–7.70 (m, AB, 4H, ArH). Anal $C_6H_8N_2O_2S$ (C, H, N).

Results

Although CA inhibition by sulfamide **1** and sulfamic acid **2** was previously reported by Mann and Keilin [4], no exact values of inhibition parameters are available, even if these two compounds represent the simplest structures containing the SO_2NH_2 moiety, which is responsible for binding to the Zn(II) ion. The two compounds differ considerably with respect to their acid-base properties, the pK_a for sulfamide being around 12.5 [39, 40], whereas that of sulfamic acid is 1.2 [40, 41]. Thus, the last compound can also be considered as an anion-like inhibitor, because in the range of pH values in which CA inhibition was

studied, the sulfamate anion is the most representative species. The series of derivatives **3** included compounds possessing modified sulfonamide groups, ie, derivatives of 4-substituted benzenesulfonamides. The modifications of the sulfonamido moiety consisted of substituting one or both hydrogen atoms of the NH_2 group with moieties such as chloro, hydroxy, methoxy, amino, phenylamino, etc. More substantial changes of the sulfonamido moiety were also envisaged, and compounds such as tosyl isothiocyanate, tosyl azide, tosyl imidazole or tosyl cyanamide were considered (see table I). Some of these derivatives possess structural features in common with both sulfonamides and inorganic anions with CA inhibitory properties. Again benzenesulfonamides were the prototypical compounds for considering derivatives **4** and **5** as putative CA inhibitors. Sulfinic acid **4a** was also included in the present study because it is structurally related to sulfonic acids. The latter substances caused much controversy after the claims of Merz's group [42] who suggested that they act as potent CA inhibitors (in the micromolar range). Indeed it has been subsequently shown that inhibition occurs only at millimolar concentrations [15, 43]. The Se(IV) derivative **4b** is the obvious analogue of the sulfinic acid **4a**, whereas the sulfenamides **5** contain the S-NH_2 moiety which can be considered as an unoxidized precursor of SO_2NH_2. No such derivatives have been investigated previously as CA inhibitors.

CA I and II inhibition constants with derivatives **1–6** are reported in tables I and II, respectively (acetazolamide **6**, the prototypical and very strong CA inhibitor, was included as standard) for both CO_2 hydration and 4-nitrophenyl acetate hydrolysis reactions.

Table I. CA I and II inhibition with derivatives **1–6** for CO_2 hydration reaction.

Compound	*R*	*X*	*K_I (μM)*	
			CA I[a]	*CA II*[b]
1	–	–	310	1130
2	–	–	21	390
3a	Me	NH_2	50	11
3b	Me	NHOH	41	9
3c	Me	NHOMe	220	173
3d	Me	NO	35	24
3e	Me	NCS	30	18
3f	Me	N_3	27	45
3g	Me	Imidazol-1-yl	160	34
3h	Me	$NHNH_2$	70	53
3i	Me	NHNHPh	>1000	119
3j[c]	H	NHCl	25	2.8
3k[c]	Me	NHCl	19	2.1
3l	COOH	NCl_2	12	3.6
3m[c]	Me	N=C=NH	210	125
3n	Me	$NHOCH_2COOH$	150	85
3o[c]	Me	OH	130	460
3p	Me	SH	5	10
4a[c]	Me	–	120	310
4b[c]	H	–	325	1000
5a	2-NO_2	–	440	125
5b	4-NO_2	–	460	39
6 (acetazolamide)	–	–	2	0.07

[a]Human CA I (80 nM) in veronal buffer, pH 7.4, in the conditions of the E-I technique of Maren's micromethod and water saturated with CO_2 [27]. [b]Bovine CA II (15 nM), in veronal buffer (pH 7.4), in the conditions of the E-I technique of Maren's micromethod and water saturated with CO_2 [27]. [c]As sodium salts.

Table II. CA I and II inhibition with derivatives **1–6** for 4-nitrophenyl acetate hydrolysis reaction.

Compound	*R*	*X*	*K_I (μM)*	
			CA I[a]	*CA II*[b]
1	–	–	35	82
2	–	–	21	97
3a	Me	NH_2	96	78
3b	Me	NHOH	120	102
3c	Me	NHOMe	451	392
3d	Me	NO	115	89
3e	Me	NCS	77	124
3f	Me	N_3	52	67
3g	Me	Imidazol-1-yl	225	95
3h	Me	$NHNH_2$	180	114
3i	Me	NHNHPh	1100	437
3j[c]	H	NHCl	19	2.4
3k[c]	Me	NHCl	21	2.0
3l	COOH	NCl_2	32	5.2
3m[c]	Me	N=C=NH	1000	2500
3n	Me	$NHOCH_2COOH$	530	155
3o[c]	Me	OH	240	3400
3p	Me	SH	20	140
4a[c]	Me	–	625	1980
4b[c]	H	–	130	845
5a	2-NO_2	–	840	245
5b	4-NO_2	–	751	20
6 (acetazolamide)	–	–	18	7

[a]Human CA I (12 μM) in Tris-H_2SO_4 buffer (pH 7.8), at 0.1 mM concentration of substrate [25]. [b]Bovine CA II (3 μM), in Tris-H_2SO_4 buffer (pH 7.8), at 0.1 mM concentration of substrate [25]. [c]As sodium salts.

In order to get more insight into the manner of interaction of these new classes of inhibitors with the enzyme itself, kinetic measurements for the esterase activity and electronic absorption and ^{1}H-NMR spectra of the Co(II)-substituted enzyme have been recorded.

Kinetic data for 4-nitrophenyl acetate hydrolysis in the absence and presence of some inhibitors of type **1–5** are shown in table III and figure 1.

Electronic spectral data for adducts of inhibitors **1–6** with Co(II)-substituted CA II are shown in table IV and figures 2 and 3, whereas ^{1}H-NMR data at 200 MHz for adducts of Co(II)CA II with some of the investigated inhibitors (more precisely, derivatives **3b–h**) are reported in table V.

Discussion

Inhibition studies with inhibitors **1–5** were performed with two CA isozymes, ie, CA I and CA II, for two reactions catalyzed by them, CO_2 hydration and ester hydrolysis (tables I and II).

For CO_2 hydration catalyzed by isozyme CA I, the following features were observed for inhibitors **1–5**. Sulfamic acid **2** (but not sulfamide **1**) is generally a stronger inhibitor than the series of *N*-modified sulfonamides **3**, except for the chloro-substituted

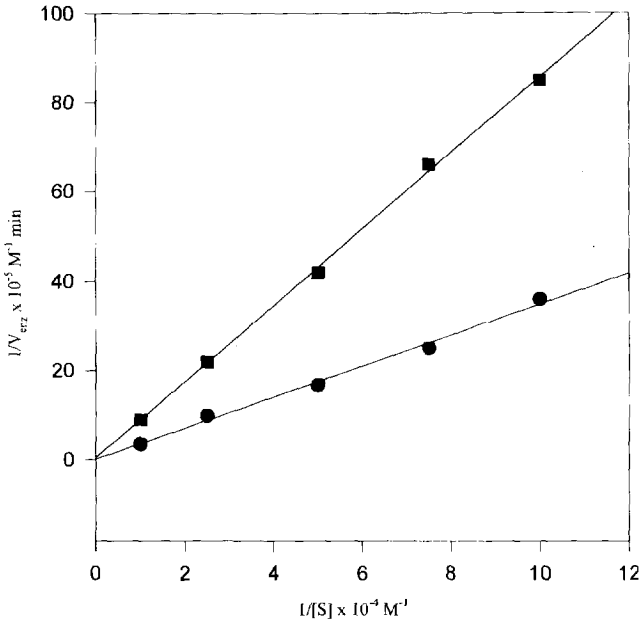


Fig 1. Lineweaver–Burk plots for 4-nitrophenyl acetate hydrolysis reaction in the presence of bovine CA II (circles) and bovine CA II + sulfamide **1** (squares). Enzyme concentration was 3.0 μM, Tris buffer 10 mM, ionic strength 0.1 (K_2SO_4), pH 7.80. Inhibitor concentration was 0.40 mM.

Table III. Kinetic parameters for 4-nitrophenyl acetate hydrolysis at 25 °C in the presence of CA II and derivatives of type **1–5** (all data represent mean ± standard deviation from duplicate determinations).

System	K_m *(mM)*	V_{max} x 10^6 *(M•min^{-1})*	k_{enz} *(min^{-1})*
CA II[a] (pH 7.8)	2.92 ± 0.44	84.0 ± 4.1	28 ± 1.3
CA II[a] + **1**[b]	2.25 ± 0.48	20.5 ± 8.2	6.8 ± 2.7
CA II[a] + **2**[b]	2.74 ± 0.39	24.6 ± 5.4	8.2 ± 1.8
CA II[a] + **3a**[b]	2.69 ± 0.40	19.1 ± 4.9	6.4 ± 1.6
CA II[a] + **3e**[b]	2.86 ± 0.35	47.1 ± 6.5	15.7 ± 2.2
CA II[a] + **3j**[c]	2.34 ± 0.57	4.9 ± 2.0	1.6 ± 0.7
CA II[a] + **3m**[d]	2.61 ± 0.32	65.9 ± 5.8	21.9 ± 1.9
CA II[a] + **4a**[b]	2.57 ± 0.40	56.1 ± 7.8	18.7 ± 2.6
CA II[a] + **4**[b]	2.85 ± 0.28	43.5 ± 5.6	14.5 ± 1.8
CA II[a] + **5b**[e]	2.54 ± 0.47	10.8 ± 2.6	3.6 ± 0.8

[a][CA II] = 3.0 μM; [b][inhibitor] = 1 mM; [c][inhibitor] = 1 μM; [d][inhibitor] = 2 mM; [e][inhibitor] = 10 μM. In all cases pH was maintained constant at 7.80 (Tris-HCl buffer 10 mM, ionic strength at 0.1, with K_2SO_4).

Table IV. Spectral data (in the range 400–750 nm) for adducts of bovine Co(II)CA II with inhibitors **1–5** as well as acetazolamide and thiocyanate (for comparison).

Adduct	*pH*	*Band position (nm) and molar absorptivity (M^{-1} cm^{-1})*
Pure enzyme	6.0	520 (180), 550 (250), 616.5 (135), 640 (100)
Pure enzyme	8.0	520 (280), 550 (380), 616.5 (280), 640 (260)
Acetazolamide **6**[a]	8.0	518 (390), 549 (220), 574 (530), 595 sh (500)
Thiocyanate[a]	8.1	465 (100), 529 sh (90), 571 (100), 689 (9)
1	7.2	518 (210), 550 (270), 600 (215)
2	7.2	545 (300), 550 (330)
3a	7.2	518 (320), 550 (210), 574 (380), 600 sh (380)
3b	8.0	507 (270), 574 (310), 600 sh (325)
3c	7.2	515 (325), 549 sh (270), 591 (320)
3c	8.0	515 (230), 552 sh (210), 574 (230), 598 (250)
3d	7.2	507 (355), 550 sh (375), 568 sh (360), 598 (340)
3e	7.2	518 (375), 551 sh (370), 600 (250)
3f	7.2	519 (370), 550 sh (380), 600 (245)
3g	7.2	505 (170), 549 sh (220), 609 (120), 641 (90)
3h	6.5	502 (200), 552 sh (190), 592 (200)
3i	9.0	507 (100), 543 (105), 609 (80), 636 sh (60)
3j	7.2	518 (250), 562 (260), 600 sh (225)
3k	7.2	515 (200), 570 (310), 600 sh (230)
3l	7.2	522 (200), 550 (210), 610 (100), 635 (80)
3m	7.2	520 (300), 550 (210), 575 sh (340), 595 sh (350)
3n	7.2	515 (310), 550 sh (220), 595 (280)
3o	7.2	545 (335), 550 (380), 595 sh (230)
3p	7.2	540 (330), 552 (375)
4a	7.2	470 (100), 530 sh (100), 570 (100), 670 (10)
4b	7.2	470 (100), 525 sh (100), 570 (90), 670 (10)
$HSeO_3^-$	7.2	470 (50), 530 (55), 570 (50)
5a	7.2	520 (240), 610 (175), 635 sh (155)
5b	7.2	520 (250), 610 (175), 635 sh (150)

Enzyme concentrations were in the range 0.1–0.4 mM, at pH values specified in each case. Inhibitors concentrations were in the range of 0.1–2 mM. [a]From reference [7].

sulfonamides **3j–l** and *para*-toluenethiosulfonate **3p**, which also behave as strong inhibitors (it is well known [1–3] that CA I does not have a very strong affinity for sulfonamide inhibitors (unlike CA II), but is more susceptible to inhibition by inorganic anions, such as cyanide, cyanate and thiocyanate [3, 6]). Thus, this discovery is quite exciting, especially after comparing the K_I's of the thiosulfonate **3p** and acetazolamide **6** (one of the strongest inhibitors known [1–3]). Practically, the two compounds have similar affinities for this isozyme (compare also with data for CA II in tables I and II, and see discussion below). For the series of derivatives **3**, bulky substituents at the sulfonamido moiety led to inactive inhibitors (eg, **3g**, **3i**, **3m** and **3n**), whereas moieties present in inorganic CA I inhibitors (such as NO, NCS and N_3^-) led to compounds with appreciable inhibitory properties. It should be noted the great difference between tosylate and thiotosylate in inhibiting CA I, as well as the fact that only four compounds, **1**, **2**, **3f** and **3p**, were better inhibitors of CA I than of CA II. Sulfenamides **5** together with compounds **4** were also poor inhibitors.

For 4-nitrophenyl acetate hydrolysis in the presence of CA I, the behavior of inhibitors **1–6** was the following. Sulfamide **1** and sulfamic acid **2** behave as strong inhibitors, with inhibition constants of the

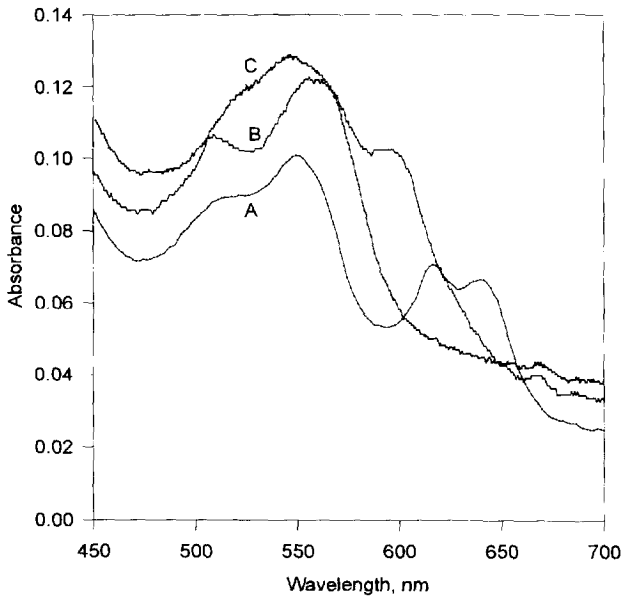


Fig 2. Electronic spectra of Co(II)CA II (**A**) and its adducts with sulfamide **1** (**B**) and sulfamic acid **2** (**C**). In all experiments [CoCA] = 0.4 mM, pH 7.2; [**1**] = 2.5 mM; [**2**] = 2.1 mM.

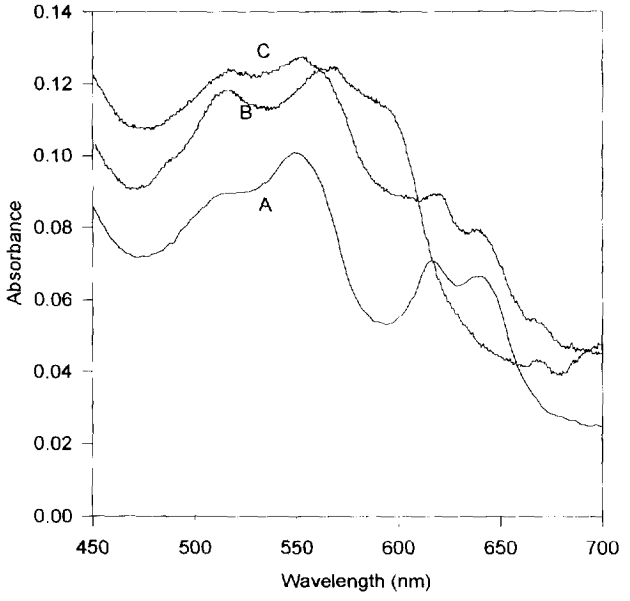


Fig 3. Electronic spectra of Co(II)CA II (**A**) and its adducts with chloramine T **3j** (**B**) and halazone **3k** (**C**). In all experiments [CoCA] = 0.4 mM, pH 7.2. Inhibitor concentrations were: [**3j**] = 0.20 mM; [**3k**] = 0.18 mM.

Table V. ^{1}H-NMR spectra at 200 MHz for adducts of bovine CA II with some inhibitors of type **3**.

Adduct	*NH His*	*H δ2 His 119*	*NH His 119*	*NH His*	*Downfield methyl*	*Upfield methyl*
Pure enzyme	62.1	60.3[a]	54.2	54.2	<6	–3
+ **3b**, pH 7	58.9	51.4[a]	57.8	63.8	<10	–5.8
+**3c**, pH 8	60.8, 63.1	48.4[a]	54.0, 56	67.6, 66	14.6	–12.2
+**3c**, pH 5	63.4	48.4[a]	67.6	67.6	<10	–7.9
+**3d**, pH 7	60.7	53.7[a]	56.0	62.9	10.1	–5.1
+**3e**, pH 7	60.5	53.8[a]	56.4	63.2	10.2	–6.3
+**3g**, pH 7	61.8	50.0[a]	59.1	63.9	10.3	–14.6
+**3h**, pH 6	62.1	67.6[b]	70.0	[c]	15.9	–7.9
+**3i**, pH 8	62.3	54.3[b]	70.2	[c]	17.8	–13.4

Chemical shifts of the signals are expressed in ppm, relative to tetramethyl silane as standard. Enzyme concentrations were 0.2–0.4 mM; inhibitor concentrations: 0.5–1.1 mM. [a]Signal not exchangeable in D_2O; [b]integrated as for two protons, one of which does not exchange in D_2O; [c]probably under the signal of 67.6.

same order of magnitude with that of acetazolamide, together with the chloro-substituted sulfonamides **3j–l** and the thiosulfonate **3p**. The compounds with bulkier substituents at the modified sulfonamido moiety, the sulfenamides **5** and derivatives **4** were much more ineffective in inhibiting this isozyme (in contrast to CA II, see below).

The following features can be observed for CA II inhibition with derivatives **1–5**, for the CO_2 hydration reaction. A sulfonamide-like affinity, with K_I's in the micromolar range, is shown by the majority of derivatives **3** (exceptions are **3c**, **3i** and **3m**), and the sulfenamides **5**, whereas compounds **1** and **4b** show a much weaker affinity (of the anion type) with K_I's in the millimolar range. Sulfamic acid **2**, 4-toluensulfonic acid **3o** and 4-toluenesulfinic acid **4a** possess an intermediate behavior (K_Is between 300 and 500 μM). The strongest inhibitor in the investigated series is *not* the unsubstituted sulfonamide **3a** (4-toluenesulfonamide), but the *N*-chloro-derivatives **3j** and **3k** (which are five times stronger inhibitors) and the *N,N*-dichloro-substituted sulfonamide **3l**. This is the first example of compounds in a homologous series in which stronger inhibitory activity than that of unsubstituted sulfonamides was detected. It should also be noted that *N*-hydroxy-4-toluenesulfonamide **3b** has the same potency as the unsubstituted sulfonamide **3a**. This type of substitution has been studied previously by Blackburn's group on Cd(II)CA II by means of ^{111}Cd-NMR spectroscopy [44], and it was shown that the *N*-hydroxy sulfonamide directly binds to the metal ion within the enzyme active site. The series investigated by this Blackburn's group [44] included only NHOH- and NHOMe-modified sulfonamides, since halogeno- or nitrogen-containing moieties were not considered. Of course, all these aromatic derivatives are weaker inhibitors than the heterocyclic sulfonamide acetazolamide **6**, but this type of behavior is well studied [1–3], so that 4-toluenesulfonamide **1a** (not acetazolamide) is the real standard for comparing the biological activity of these inhibitors.

For the esterase activity the strongest inhibitors against CA II are again the *N*-chlorosulfonamides **3j–l**, but with a factor of 15–39 times that of the unsubstituted sulfonamide **3a**. Another interesting aspect is the fact that sulfamide **1** and sulfamic acid **2** have *comparable* inhibitory properties with 4-toluenesulfonamide for the esterasic activity, whereas they were much weaker inhibitors for the hydrase activity. The weakest inhibitors are the sulfonic and sulfinic acids **3m**, **4a**, and benzeneseleninic acid **4b**. Sulfenamides **5** are also unexpectedly strong inhibitors for the esterase activity. It is also important to underline that acetazolamide **6** is a weaker inhibitor than the chloro-substituted aromatic sulfonamides **3j–l**. The data shown above present for the first time (as far as we know) important differences of behavior between the two major CA isozymes towards a large series of inhibitors and might constitute the starting point for developing CA I-specific inhibitors. For instance, we predict that a thiosulfonate possessing a heterocyclic moiety instead of the aromatic one of derivative **3p** would probably inhibit CA I much better, as in the case of the corresponding sulfonamides, with the heterocyclic derivatives 10–100 more active than the aromatic ones [1–3].

In order to assess the inhibition mechanism with these new types of inhibitors, kinetic and spectroscopic determinations were performed. The kinetic data (table III) show that all these inhibitors are non-competitive with 4-nitrophenyl acetate as substrate. The Michaelis constants are the same (around 2.6 ± 0.3 mM, in the limits of the experimental error) with and without inhibitors. Such behavior is similar to that of the simple inorganic anions or unsubstituted sulfonamides, previously investigated by Pocker and Stone who suggested that the substrate and the inhibitors bind to different sites of the active cavity [25]. Presumably, these inhibitors are also non-competitive with CO_2 as substrate [1, 4].

The electronic spectral data (table IV) indicate that sulfamic acid **2** and the majority of derivatives **3** bind to the Co(II) ion in the enzyme active site giving rise to a pseudo-tetrahedral geometry, similar to the unsubstituted sulfonamides, of which acetazolamide **6** is a well-known example (and a clinically employed drug [1–3]). Such adducts are characterized by intense spectra with molar absorbances above 300 M^{-1} cm^{-1} [7–9, 45, 46], and the pseudo-tetrahedral geometry has been confirmed by X-ray crystallographic data for some of these complexes [47]. Probably the binding to the metal ion occurs as $H_2N\text{-}SO_3^-$ anion for **2** and $ArSO_2N^-X$ anions for **3a–c**, **3i**, **3j** and **3l**. Compounds **3d–f** are **3k** are unable to ionize, since they do not possess a proton directly bound to the sulfonamido moiety, and presumably they coordinate the metal ion by means of the terminal atom (oxygen for **3d**, sulfur for **3e**, the endocyclic nitrogen of the imidazole moiety for **3f** and probably chlorine for **3k**). Regarding the last adduct, it is important to mention that the original spectrum (presented in table IV) is completely changed after 5 min. The new spectrum recorded after this time is similar to that of **3b**, probably due to hydrolysis of the dichlorosulfonamide to an *N*-hydroxy derivative. On the other hand, the spectra of adducts with the chloramines **3i** and **3j** do not change over time, proving that no hydrolysis or oxidation of the enzyme (or metal ion, in the case of Co(II)CA) occurs during the experiments. Co(II) is pentacoordinated in the adducts with inhibitors **4a** and **4b**, as well as with hydrogen selenite. These spectra are very similar to those of the previously

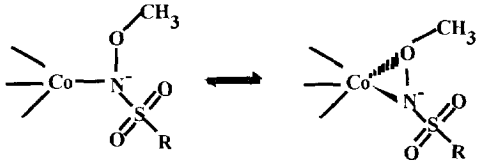
investigated thiocyanate adduct [7, 12]. Binding is probably done through the ionized oxygen atom of these anions, and a water molecule is also directly bound to Co(II). It should be mentioned that hydrogen selenite is a very weak inhibitor, with a K_I for the esterasic activity of 50 mM, making it the weakest in the series studied here. On the other hand, the benzeneseleninate (the anion of **4b**) is 60 times stronger than $HSeO_3^-$ as CA inhibitor (see tables I and II).

Some of the derivatives **1–5** possess electronic spectra, which indicate the existence of equilibria between tetra- and pentacoordinated species, as pointed out by Bertini et al [7–11] for anions such as chloride, azide, etc. These inhibitors are sulfamide **1**, and the derivatives **3f–h** and **5**.

The use of ^{1}H-NMR spectroscopy for elucidating the coordination of Co(II)-substituted CA isozymes with different anion inhibitors is well known [7, 45, 46]. Thus, it was shown that inhibitors that bind in a pseudo-tetrahedral geometry to Co(II), such as cyanate or hydroxide in the non-inhibited enzyme, give rise to spectra with three or four isotropically shifted signals, assigned to *meta*-like protons belonging to the three histidines coordinated to the metal ion. Three of these signals are exchangeable and the non-exchangeable one is assigned to H δ2 of His-119 (which unlike His 94 and His 96 is coordinated by N δ1, and not by N ε2) [8, 9, 46]. Furthermore, in tetracoordinated complexes a small broadening of these signals is observed [46]. In pentacoordinated complexes, such as the thiocyanate adduct, these signals spread to a much greater extent, due to the fact that the larger magnetic anisotropy gives rise to pseudo-contact contributions to the isotropic shifts [46].

As seen from the data in table V, spectra attributable to typical pseudo-tetrahedral species were observed in the case of adducts with derivatives **3b** and **3c** at pH 5.5 and **3d–f**, whereas inhibitors **3g** and **3h** showed spectra which are intermediate between tetra- and pentacoordination of the metal ion. These data fully agree with the analysis of the electronic spectra (table IV).

The most interesting behavior is seen in the case of the *N*-methoxysulfonamide **3c** at high pH values (pH 8.0 or higher). It has to be noted that at this pH **3c** is ionized (pK_a of the SO_2NHOCH_3 moiety in this compound is around 7.7 [3, 15]). The spectra of this adduct at pH 8 shows seven isotropically shifted signals (table V), which are probably due to two different coordination modes of the inhibitor to the metal ion, one of which corresponds to pentacoordinated Co(II) species (it should be noted that in the non-inhibited enzyme, pentacoordinated Co(II) is observed only at acidic pH values [7, 8]). At this pH, **3c** should



R = 4-Me-C_6H_4

Scheme 1.

be 50% as anion and 50% as free acid, and an equilibrium of the type shown in scheme 1 is then proposed to occur at binding. This would explain why all signals (except for that of H δ2 of His-119) appear as doublets (one accounting for the species with pentacoordinated Co(II), and the other for that of the tetrahedral species; scheme 1). At more acidic pH values, the binding of this inhibitor is similar to that of other *N*-modified sulfonamides studied here, ie, only by the nitrogen atom of the sulfonamido moiety. This is the first spectroscopic evidence for such a type of binding for a sulfonamide to the CA active site.

In conclusion, this study evidenced that effective inhibitors as potent as the unsubstituted sulfonamides can be designed from diverse classes of compounds. Some of these substances possess moieties which make them intermediate between inorganic anions and unsubstituted sulfonamides, filling a gap between the two main classes of CA inhibitors. Moreover, the binding of some of these inhibitors to the metal ion seems to involve coordination in the neutral state, although the great majority of previously studied inhibitors were bound as anions. This is the first time that the binding of neutral molecules was evidenced for any CA isozyme. Net discriminations were evidenced between the two CA isozymes investigated for binding of some of these inhibitors, leading to the possibility of designing tight-binding CA I inhibitors that would inhibit to a lesser extent CA II, the isozyme with the greatest affinity to sulfonamides. We note that exploration of diverse classes of novel types of inhibitors might evidence the specificity of the different isozymes to inhibition, helping in this way to establish their generally unknown physiological role.

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