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Bioorganic & Medicinal Chemistry Letters 15 (2005) 3096–3101
Bioorganic & Medicinal Chemistry Letters

Carbonic anhydrase inhibitors. Inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, IX, and XII with Schiff's bases incorporating chromone and aromatic sulfonamide moieties, and their zinc complexes

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Received 1 February 2005; revised 31 March 2005; accepted 12 April 2005

Abstract—A series of Schiff's bases was prepared by reaction of 3-formyl-chromone or 6-methyl-3-formyl-chromone with aromatic sulfonamides, such as sulfanilamide, homosulfanilamide, 4-aminoethyl-benzenesulfonamide, a pyrimidinyl-substituted sulfanilamide derivative, sulfaguanidine and 4-amino-6-trifluoromethyl-benzene-1,3-disulfonamide. The zinc complexes of these sulfonamides have also been obtained. The new derivatives and their Zn(II) complexes were investigated for the inhibition of four physiologically relevant isozymes of carbonic anhydrase (CA, EC 4.2.1.1): the cytosolic isoforms I and II, as well as the tumor-associated, transmembrane isozymes CA IX and XII. Except for the sulfaguanidine-derived compounds which were devoid of activity against all isozymes, the other sulfonamides and their metal complexes showed interesting inhibitory activity. Against isozyme CA I, the inhibition constants were in the range of 13–100 nM, against isozyme CA II in the range of 1.9–102 nM, against isozyme CA IX in the range of 6.3–48 nM, and against CA XII in the range of 5.9–50 nM. Generally, the formyl-chromone derived compounds were better CA inhibitors as compared to the corresponding 6-methyl-chromone derivatives, and for the simple, benzenesulfonamide derivatives activity increased with an increase of the spacer from sulfanilamide to homosulfanilamide and 4-aminoethylbenzenesulfonamide derivatives, respectively. Some of these compounds may show applications for the development of therapies targeting hypoxic tumors in which CA IX and XII are often highly overexpressed.

1. Introduction

Schiff's bases of aromatic/heterocyclic sulfonamides have previously been investigated[1–7] as inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1). The 15 CA isozymes presently known in humans are involved in a multitude of physiologic and pathologic processes, and their inhibition may be thus exploited clinically for the treatment of glaucoma (case in which CA II and CA XII are targeted by sulfonamide or sulfamate inhibitors),[8–11] obesity (when presumably CA II, CA Va, and CA Vb are targeted),[12–14] as anticonvulsant drugs in the treatment of epilepsy or diverse neuromuscular disorders (when CA VII and probably also CA II are targeted),[15–20] as well as for the management (imaging and treatment) of a multitude of tumors, in which the overexpressed, transmembrane isozymes CA IX and XII are targeted by the CA inhibitor (CAI),[21–28] to mention only the most important applications of this class of pharmacological agents, some of which were used clinically (mainly as diuretics) for more than 50 years.[29,30] Among these clinically used inhibitors are the heterocyclic sulfonamides acetazolamide **AZA**, methazolamide **MZA**, and ethoxzolamide, **EZA**, as well as the aromatic derivatives dichlorophenamide **DCP** and indisulam (E7070) **IND**.[30–32]

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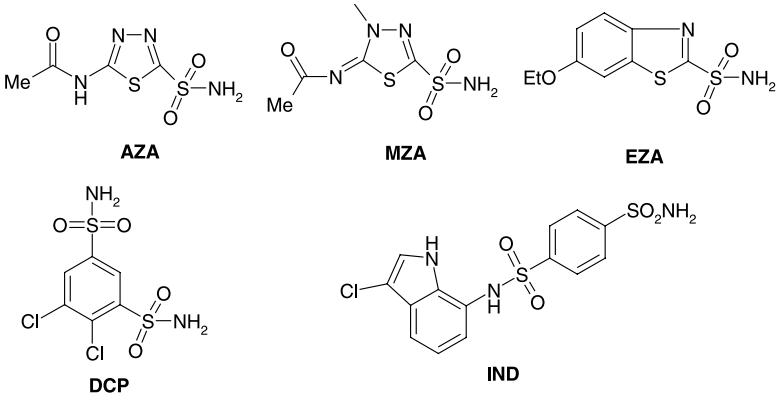
doi:10.1016/j.bmcl.2005.04.055

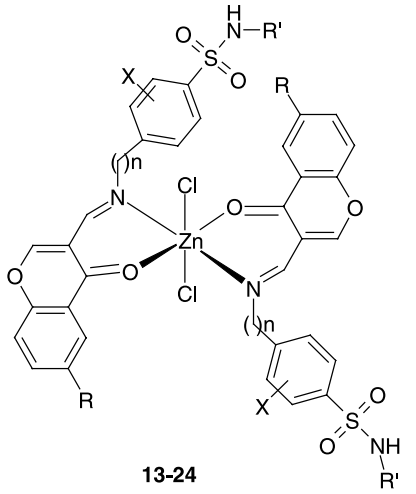


Very recently, our and Pastorekova's groups[21] showed that the acidification of the tumor microenvironment is produced by the enzymatic activity of CA IX (which catalyzes with high efficiency the CO_2 hydration reaction with formation of bicarbonate and H^+ ions) and that this process may be reverted by inhibiting this enzyme with sulfonamide or sulfamate potent CA IX-targeted inhibitors. Returning of the tumor pH to a normal value may be important for the clinical outcome as well as for the response to radio- or chemotherapy (as hypoxic, acidic tumors are generally non-responsive to these clinical interventions),[33–35] and thus, the search for potent, eventually selective CAIs targeting the tumor-associated isozymes CA IX and XII is pursued in many laboratories. We have reported the first inhibition studies of the tumor-associated isozymes IX and XII,[9,24,26,36,37] as well as some of the most potent and selective inhibitors targeting the first such isozyme, CA IX (the best studied one at the moment).[38] Continuing the work in the design of CAIs targeting the tumor-associated isozymes CA IX and XII, we report here the synthesis and inhibition studies of these isozymes as well as the ubiquitous, cytosolic ones CA I and II, with a series of Schiff's bases obtained by reaction of 3-formyl-chromones with aromatic/heterocyclic sulfonamides. The Zn(II) complexes of these Schiff's bases have also been prepared and investigated for the interaction with the above-mentioned four CA isozymes.

2. Chemistry

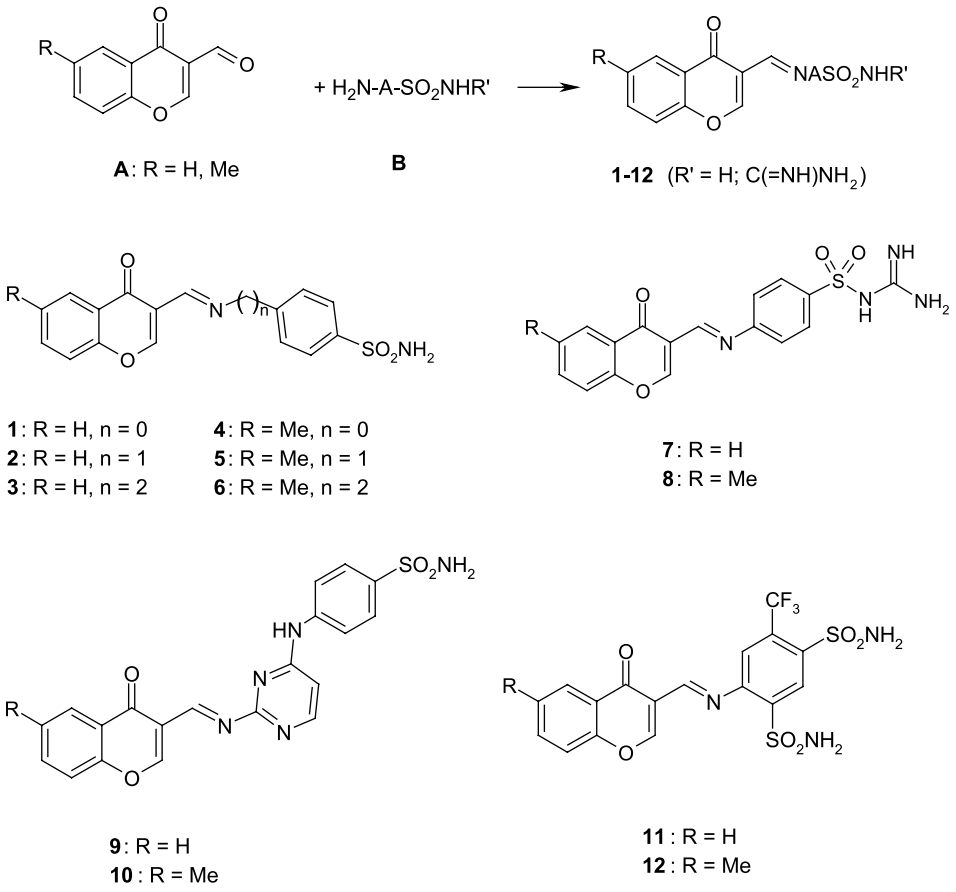
It has been shown earlier that Schiff's bases of aromatic or heterocyclic sulfonamides lead to potent inhibitors of several physiologically relevant CA isozymes, such as CA I, II, and IV.[1–7] However, this class of derivatives has never been investigated up to now for its interaction with the tumor-associated isozymes CA IX and XII. Thus, we decided to synthesize a new class of Schiff's bases of such sulfonamides, also incorporating in their molecules chromone moieties. 3-Formyl-chromone, 3-formyl-6-methylchromone (**A**) and the aromatic sulfonamide derivatives **B** used in the synthesis, as well as the clinically used sulfonamide CAIs were commercially available from Sigma–Aldrich (Milan, Italy).

Condensation of formyl-chromones **A** with a series of aromatic sulfonamides **B**, including among others sulfanilamide, homosulfanilamide, 4-aminoethyl-benzenesulfonamide, sulfaguanidine, 4-(2-amino-pyrimidin-4-yl)-amino-benzenesulfonamide, and 4-amino-6-trifluoromethyl-benzene-1,3-disulfonamide led to the Schiff's bases **1**–**12**, by the procedure previously reported by this group[1–7] (Scheme 1). It has also been reported that metal complexes of sulfonamides act as very potent CAIs, by this and other groups.[39–44] Thus, the Zn(II) complexes of sulfonamides **1**–**12** have also been prepared thereafter, by reacting Schiff's bases **1**–**12** with $ZnCl_2$ at reflux for 8 h, leading to complexes possessing the general formula $[ZnL_2]Cl_2$ and the structures shown below (where L stands for the Schiff's base ligand of type **1**–**12**), by the general procedure reported earlier.[5–7] The Zn(II) complex of **1** is numbered as derivative **13**, of **2** as derivative **14**, and so on, so that the Zn(II) complex of **12** is derivative **24**.[45]



(For the nature of R, R', n and X see Scheme 1)

hCA I and hCA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II as previously described.[8,9] The enzymes were purified by affinity chromatography according to the method of Khalifah et al.[47] 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,



Scheme 1.

and 29.3 kDa for CA II, respectively.[8,9] The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[22]) was amplified by using PCR and specific primers for the glutathione S-transferase (GST)-Gene Fusion Vector pGEX-3X. The obtained fusion construct was inserted in the pGEX-3X vector and then expressed in *E. coli* BL21 Codon Plus bacterial strain. The GST part of the fusion protein was cleaved with thrombin, and the obtained CA IX was purified by sulfonamide affinity chromatography,[47] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[46] Similarly was obtained hCA XII, by the method recently published by this group.[9]

3. CA inhibition data

Inhibition data against isozymes hCA I, II, IX, and XII with Schiff's bases **1**–**12**, their zinc complexes **13**–**24** as well as standard sulfonamide CAIs are presented in Table 1.

The following should be noted regarding CA inhibition data of Table 1: (i) against the slow cytosolic isozymes hCA I, the new derivatives reported here (except the sulfaguanidine-derived Schiff's bases **7** and **8** and their Zn(II) complexes **19** and **20**, which are not inhibitory against anyone of the four investigated isozymes) showed good inhibitory properties, with inhibition constants in the range of 12–100 nM. SAR is quite obvious: for the simple benzenesulfonamide derivatives, hCA I inhibitory properties increase with *n* (from 0 to 2) both for the formyl-chromone as well as 6-methyl-formyl-chromone derived Schiff's bases **1**–**6** and their corresponding Zn(II) complexes **13**–**18**. The derivatives incorporating the pyrimidinyl-sulfanilamide moiety **9** and **10** (and their corresponding Zn(II) complexes **21** and **22**) also act as effective hCA I inhibitors, whereas the benzene-1,3-disulfonamide derivatives **11** and **12** (as well as their corresponding complexes **23** and **24**) are less effective inhibitors. Thus, the nature of the sulfonamide head to which the azomethine moiety has been attached is the principal factor influencing activity in this class of CAIs, as already established earlier.[1–7] However, the nature of the moiety originating from the aldehyde used to prepare the Schiff's bases is also important in inducing stronger inhibitory properties, as these Schiff's bases are much more inhibitory as compared to the simple, corresponding sulfonamides **B** from which they were prepared (data not shown, see Refs. 1–7 for relevant examples). The Zn(II) complexes were more inhibitory than the corresponding uncomplexed sulfonamides, a phenomenon explained earlier by this group;[39–42] (ii) against the major, rapid cytosolic isozyme hCA II, again the new compounds reported here showed quite effective inhibitory properties (except the sulfaguanidine

Table 1. Inhibition of isozymes hCA I, II, IX, and XII with Schiff's bases **1**–**12**, their zinc complexes **13**–**24** and standard CAIs

Inhibitor	K_I* (nM)			
	hCA I[a]	hCA II[a]	hCA IX[b]	hCA XII[b]
AZA	250	12	25	5.7
MZA	280	14	27	3.4
EZA	25	8	34	22
DCP	1200	38	50	50
IND	31	15	24	3.4
1	100	28	37	39
2	29	14	32	38
3	35	21	26	33
4	62	35	48	52
5	44	55	45	50
6	30	60	37	44
7	>1000	>1000	>1000	>1000
8	>1000	>1000	>1000	>1000
9	37	33	21	16
10	32	41	24	19
11	94	102	30	27
12	85	133	32	30
13	54	3.2	10	17
14	12	1.9	8.1	15
15	15	2.7	7.3	11
16	36	4.9	15	29
17	21	5.3	14	30
18	14	7.8	12	27
19	>1000	>1000	>1000	>1000
20	>1000	>1000	>1000	>1000
21	13	3.6	6.3	5.9
22	10	5.5	6.8	7.4
23	62	14	7.5	8.5
24	51	18	8.2	12

* Errors in the range of 5–10% of the shown data from three different assays.

[a] Human recombinant isozymes.

[b] Catalytic domain of the human recombinant isozyme, CO_2 hydrase assay method.[46]

derivatives mentioned above), with K_Is in the range of 1.9–133 nM. Thus, the formyl-chromone derived Schiff's bases incorporating sulfanilamide and its congeners, of types **1**–**3** showed hCA II inhibitory properties in the same range as the clinically used sulfonamides **AZA**, **MZA**, **DCP**, and **IND** (K_Is in the range of 14–28 nM), whereas the corresponding 6-methyl-chromone derivatives **4**–**6** at least two times less inhibitory (K_Is in the range of 35–60 nM). Clearly, the presence of the additional methyl moiety in **4**–**6** as compared to the corresponding derivative **1**–**3** may interfere with the proper binding of the inhibitor within the enzyme active site (a situation not observed for the inhibition of hCA I with these derivatives, see Table 1). Again the pyrimidinyl-sulfanilamides **9** and **10** showed a rather effective hCA II inhibitory activity (K_Is in the range of 33–41 nM), which for the benzene-1,3-disulfonamide derived Schiff's bases **11** and **12** is much reduced (K_Is in the range of 102–133 nM). All the Zn(II) complexes (except those incorporating sulfaguanidine-derived ligands, **19** and **20**) were more inhibitory than the corresponding sulfonamide ligands, due to the dual inhibition of the enzyme by metal ions and sulfonamide anions formed in diluted solution due to the dissociation of the complex, as demonstrated earlier.[39–42] Indeed, these derivatives showed K_Is in the range of 1.9–18 nM; (iii) the tumor-associated isozyme hCA IX was also inhibited by the new sulfonamides reported here (except the sulfaguanidines **7** and **8** and their corresponding Zn(II) complexes **19** and **20**), with inhibition constants in the range of 6.3–48 nM. SAR is here quite different from what observed for the cytosolic isozyme discussed above. Thus, the heads inducing the best hCA IX inhibitory properties were those incorporating the pyrimidinyl-sulfanilamide and benzene-1,3-disulfonamide moieties. Indeed, compounds **9**–**12** were the best inhibitors (K_Is in the range of 21–32 nM), followed then by the simple benzenesulfonamide derivatives **1**–**6** (K_Is in the range of 26–48 nM). For these last derivatives, again the formyl-chromone Schiff's bases led to best inhibitors as compared to the corresponding 6-methyl-chromone ones, and the increase of *n* leads to a slight increase in the hCA IX inhibitory properties. The Zn(II) complexes were more inhibitory than the corresponding ligands from which they were prepared, with K_Is in the range of 6.8–15 nM. This is the first report of hCA IX inhibition with metal complexes of sulfonamides, which up to now were investigated only for their interaction with isozymes I, II, and IV;[39–42] (iv) the inhibitory data of the second tumor-associated isozyme hCA XII with the compounds reported here parallels quite well the interaction of these CAIs with hCA IX. Thus, again the best inhibitors were those incorporating pyrimidinyl-sulfanilamide and benzene-1,3-disulfonamide moieties of types **9**–**12** (K_Is in the range of 16–30 nM). The Schiff's bases derived from sulfanilamide and its congeners of type **1**–**6** were also inhibitory: those derived from formyl-chromone, **1**–**3** (K_Is of 33–39 nM) were better as compared to the corresponding derivatives of 6-methyl-chromone **4**–**6** (K_Is in the range of 44–52 nM). Thus, inhibitory power slightly increased with the increase of *n*, as for hCA I, II, and IX inhibition data with this class of sulfonamides. The sulfaguanidine derivatives (**7** and **8**) and their Zn(II) complexes (**19** and **20**) were not inhibitory against this isozyme too. The Zn(II) complexes were more inhibitory as compared to the parent ligands from which they were prepared (K_Is in the range of 5.9–30 nM); (v) this class of sulfonamides and their Zn(II) complexes do not show specificity for any of the investigated isozymes, although some of them act as better inhibitors of the tumor-associated isozymes hCA IX and XII over the cytosolic ones hCA I and II (e.g., compounds **9**–**12**, **23**, and **24**).

4. Conclusions

A series of Schiff's bases was prepared by reaction of 3-formyl-chromone or 6-methyl-3-formyl-chromone with aromatic sulfonamides, such as sulfanilamide, homosulfanilamide, 4-aminoethyl-benzenesulfonamide, a pyrimidinyl-substituted sulfanilamide derivative, sulfaguanidine, and 4-amino-6-trifluoromethyl-benzene-1,3-disulfonamide. The zinc complexes of these sulfonamides have also been obtained. The new derivatives and their Zn(II) complexes were investigated for the inhibition of four physiologically relevant isozymes of carbonic anhydrase: the cytosolic isoforms I and II, as well as the tumor-associated, transmembrane isozymes

CA IX and XII. Except for the sulfaguanidine-derived compounds which were devoid of activity against all isozymes, the other sulfonamides and their metal complexes showed interesting inhibitory activity. Against isozyme CA I, the inhibition constants were in the range of 13–100 nM, against isozyme CA II in the range of 1.9–102 nM, against isozyme CA IX in the range of 6.3–48 nM, and against CA XII in the range of 5.9–50 nM. Generally, the formyl-chromone derived compounds were better CA inhibitors as compared to the corresponding 6-methyl-chromone derivatives, and for the simple benzenesulfonamide derivatives activity increased with an increase of the spacer from sulfanilamide to homosulfanilamide and 4-aminoethylbenzenesulfonamide derivatives, respectively. Some of these compounds may show applications for the development of therapies targeting hypoxic tumors in which CA IX and XII are often highly overexpressed.

Acknowledgments

This research was financed in part by a grant of the sixth Framework Programme of the European Union (EUROXY project) and by a grant from Miroglio Vestebene S.p.A (Alba, Italy).

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45. To a hot stirred solution of 3-formyl-6-methylchromone (1.88 g, 0.01 mol) in methanol (40 mL) was added sulfanilamide (1.72 g, 0.01 mol) in the presence of catalytic amount of concd H_2SO_4. The reaction mixture was refluxed for 2 h and the reaction was monitored through TLC. After completion of the reaction, precipitates appeared on cooling in an ice-bath, which were filtered and recrystallized from aqueous-ethanol (1:3) affording TLC-pure products in good yields. Mp 164 °C, ^{1}H NMR: 1.54 (s, 3H, Me); 4.26 (s, 1H, CH═ from chromone); 7.15 (m, AA′BB′, 4H, ArH from 1,4-phenylene); 7.17–7.49 (m, 3H, ArH from chromone); 7.65 (br s, 2H, SO_2NH_2); 8.13 (s, 1H, CH from azomethine).
46. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573, An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. 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 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The cloned enzymes were obtained as reported earlier by this group.[8,9,24]
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