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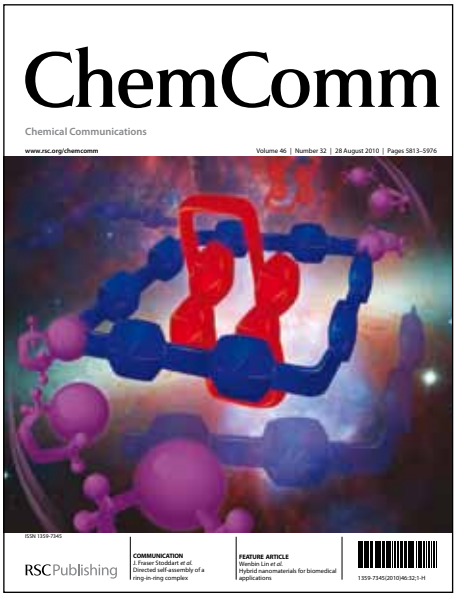

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ARTICLE TYPE

Hydroxamate represents a versatile zinc binding group for the development of new carbonic anhydrase inhibitors†

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Hydroxamates (R-CONHOH) were scarcely investigated as carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs). An inhibition/structural study of PhCONHOH is reported against all human isoforms. Comparing aliphatic (R=Me and CF3) and aromatic (R=Ph) hydroxamates as CAIs, we prove that CONHOH is a versatile zinc binding group. Depending on the nature of the R moiety, it can adopt different coordination modes to the catalytic ion within the CA active site.

Human α-carbonic anhydrases (CA, EC 4.2.1.1) occur as 15 isoforms, which differ by their tissue distribution, cellular localization and kinetic properties.[1,2] All these enzymes catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton, a very simple reaction, which is however critically important for many physiological processes. Indeed, defective activities or expression levels of several human (h) CAs have been associated with various human diseases, such as glaucoma, obesity, cancer, epilepsy, etc.[1,3] For this reason, last years have seen an increasing interest in the utilization of α-CA isozymes as targets for the design of inhibitors and activators with biomedical applications.[1,4,5] α-CA inhibitors (CAIs) found their first applications as diuretics, antiglaucoma agents, and antiepileptics, while recently they are becoming to be considered for the development of antiobesity drugs, and diagnostic and therapeutic tools for cancer treatment.[6,7] However, none of the currently clinically used CAIs shows selectivity for a specific isozyme, thus there is a constant need of improving the selectivity profile of such molecules, in order to avoid side effects due to the simultaneous inhibition of different isoforms.[1,8]

Derivatized sulfonamides of type R-SO_2NH_2 represent the class of CAIs mostly utilized and best characterized both from a structural and functional viewpoint. Indeed, a large number of crystallographic studies are available on the adducts that such molecules form with several CA isozymes, such as CA I, II, IV, VII, IX and XIII among others.[2] Such studies have clarified the main factors responsible for the binding of the sulfonamide moiety to the CA active site and have provided an explanation for the characteristic properties of this metal-anchoring group. In particular, in all the studied adducts the binding of the sulfonamide derivatives is principally driven by the coordination of the deprotonated sulfonamide nitrogen to the catalytic Zn^{2+} ion, and by two H-bonds of the sulfonamide moiety with residue Thr199, which is conserved among all isoforms. Additional hydrophobic and/or hydrophilic interactions can be established by the R moiety with active site residues (Figure 1).

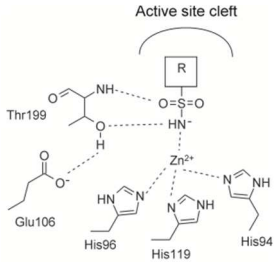

Figure 1 Schematic illustration of the key interactions between a generic sulfonamide inhibitor and the CA active site.

These studies highlighted that the sulfonamide group is an ideal ligand of the CA active site since it combines the negative charge of the deprotonated nitrogen with the positively charged zinc ion, and at the same time, it has no affinity for other metalloenzymes possessing the same zinc coordination, such as for example the matrix metalloproteinases (MMPs).[9] However, unfortunately the presence of such a good zinc binding group (ZBG) in these derivatives presents an important drawback, since, although these molecules generally behave as very potent CAIs, they do not show selectivity for the different isoforms. Indeed, due to the predominant role played by the sulfonamide moiety in the interaction with the enzyme, any change in the thermodynamics of binding, caused by the nature of the R substituent, has generally a rather marginal effect on the enzyme-inhibitor affinity.[10] Consequently, much efforts were dedicated in last years to the development of new ZBGs that, although presenting lower affinity for the CA active site, would be able to be more selective toward the different isoforms. In this context we recently demonstrated that the introduction in the model compound benzene-sulfonamide **1** of hydroxy- (**2**) and methoxy-moieties (**3**) (Figure 2) at the sulfonamide nitrogen generates interesting lead compounds for the development of more selective CAIs.[10] In this paper, we are continuing such studies evaluating the effect of the substitution in the model compound **1** of the sulfonamide moiety with a hydroxamate group (see compound **4**). Hydroxamate derivatives are potent inhibitors of several zinc enzymes among which the pharmaceutically significant MMPs;[11-13] nevertheless their interaction with the different α-CA isozymes

has been so far only poorly investigated,[14-18] and a systematic comparative study with sulfonamide CAIs is completely missing. Such a study has been undertaken in this paper where N-(Hydroxy)-benzamide **4** has been assayed for the inhibition of all catalytically active human CA isoforms (CA I-XIV) and compared to compounds **1-3** containing the same R moiety. Furthermore, the X-ray structure of the complex of **4** with hCA II has been solved, allowing us to unravel interesting aspects related to the inhibition mechanism of hydroxamates, as well as to the design of more isoform-selective CAIs.

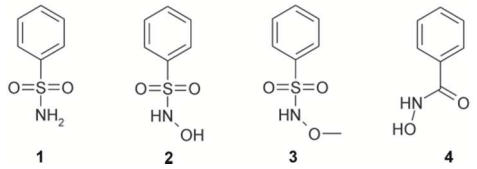

Figure 2 Chemical structures of compounds **1- 4**

Data of Table 1 show the inhibition of all catalytically active hCA isoforms and two MMPs (MMP-2 and MMP-8) with compounds **1-4**. It may be noted that hydroxamate **4** inhibited all 12 CA isoforms, with inhibition constants in the range of 0.94 – 179 μM, being thus less effective as CAI compared to the sulfonamide **1**, but showing an activity comparable to that of the N-substituted sulfonamides **2** and **3**. However, there are several notable features of the inhibition profile of compound **4** with respect to the other three compounds. In particular, the dominant, offtarget isoform hCA II was the least inhibited by **4** (K_I of 179 μM) whereas sulfonamide **1** potently inhibits this isoform, and derivatives **2** and **3** also show good inhibition. The other highly abundant cytosolic isoform hCA I was also relatively resistant to inhibition by **4** but is highly inhibited by **1** and **2**. The other interesting aspect of the hydroxamate **4** was that it strongly inhibited two transmembrane isoforms, hCA XII and XIV, with inhibition constants in the range of 0.94 – 9.51 μM. As many transmembrane isoforms are important drug targets for the development of antiglaucoma or anticancer therapies, this class of underexplored CAIs may constitute an interesting starting point for compounds with increased selectivities for such isoforms over the cytosolic, highly abundant offtarget ones hCA I and II. It should be also noted that many of the CA isoforms investigated here were modestly inhibited by **4**, with K_Is in the range of 23.0 – 84.7 μM. Overall, the inhibition profile of the hydroxamate **4** is very different from those of the unsubstituted or N-substituted sulfonamides **1-3**, which constitutes an important finding in the search of isoform-selective CAIs. It should be also noted that only N-hydroxybenzenesulfonamide **2** and phenyl hydroxamate **4** were also MMP inhibitors, whereas **1** and **3** did not inhibit significantly these enzymes. Interestingly, phenyl hydroxamate is a rather poor MMP inhibitor (K_Is in the range of 215-326 μM), being much more effective as a CAI. N-Hydroxy benzenesulfonamide **2** is a more efficient MMP inhibitor compared to **4** (Table 1).

The binding mode of N-(Hydroxy)-benzamide **4** to the CA active site was investigated by means of X-ray crystallographic studies on the adduct that this molecule forms which the best characterized and easily crystallizable CA isoform, namely hCA II. Crystals of the complex were obtained as described in the **ESI** and the three-dimensional structure analyzed by difference Fourier techniques, the crystals being isomorphous to those of the native protein.[19] The statistics for data collection and refinement are summarized in Table S1.

Table 1: Inhibition of CA isozymes I-XIV (of human = h, and murine = m origin) and MMP*s*-2/8 with compounds **1-4**.

	1	**2**	**3**	**4**
		K_I (μM)[a]		
hCA I	0.086[b]	2.73[b]	>1000[b]	83.1
hCA II	0.101[b]	5.47[b]	8.96[b]	179
hCA III	2.26[b]	4.42[b]	5.72[b]	44.8
hCA IV	7.96[b]	24.6[b]	39.5[b]	84.7
hCA VA	1.68[b]	5.28[b]	6.70[b]	67.4
hCA VB	8.82[b]	67.9[b]	10.7[b]	53.6
hCA VI	0.097[b]	86.2[b]	27.0[b]	78.1
hCA VII	0.095[b]	8.73[b]	9.56[b]	70.7
hCA IX[c]	0.097[b]	60.3[b]	64.3[b]	45.9
hCA XII[c]	0.090[b]	1.53[b]	8.32[b]	9.51
mCA XIII	0.100[b]	4.97[b]	6.21[b]	23.0
hCA XIV	0.092[b]	1.66[b]	1.57[b]	0.94
MMP-2	>1000	70.0	>1000	215
MMP-8	>1000	74.14	>1000	326

[a]Errors in the range of ± 5 % of the reported data from three different assays. [b]Data from ref. 10. [c]Catalytic domain.

The inspection of the initially calculated electron density maps in the active-site region showed clear evidence of one inhibitor molecule bound in the hCA II active site cavity (Figure 3A).

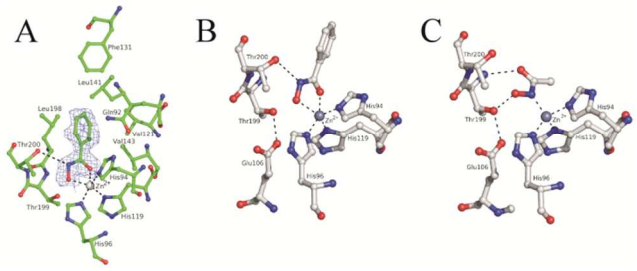

Figure 3 (**A**) Active site region in the hCA II–**4** complex. The simulated annealing omit 2|Fo|-|Fc| electron density map, contoured at 1.0 σ, associated to the inhibitor molecule is also shown. (**B**) Zn^{2+} coordination geometry of N-(Hydroxy)-benzamide **4.** (**C**) Zn^{2+} coordination geometry of acetohydroxamic acid (PDB code 1AM6).[18]

The binding of the inhibitor molecule did not cause any significant change in the overall protein structure as judged by a r.m.s. deviation of only 0.25 Å for the superposition of the Cα atoms of native protein with those of the complexed one. N-(Hydroxy)-benzamide **4** binds to the hCA II active site with the CO and OH groups which simultaneously coordinate to the zinc ion to form an energetically favorable 5-membered chelate complex (Figure 3A, and 3B). Inhibitor binding is also stabilized by several other interactions with enzyme active site residues; in particular, the nitrogen atom forms a hydrogen bond with the Thr200OG1 atom (N----Thr200OG1=3.12 Å), while the phenyl ring, whose position is rather well superimposable to that of the phenyl ring in the hCA II/**2** adduct (Figure 4), is involved in a number of van der Waals interactions with the side chains of residues Gln92, Val121, Phe131, Leu141, Val143, Leu198 and Thr200 (Figure 3A). Although, several controversial data have been reported on the putative deprotonation site of hydroxamates

of type R-CONHOH, highlighting that the solvent and the environment can play a key role in favoring the N-deprotonation or the O-deprotonation,[20-22] the observation that compound **4** coordinates to the catalytic zinc ion through its CO and OH groups suggests that in this case the O-deprotonated form is the most probable. For this reason the deprotonated oxygen atom cannot form a hydrogen bond with the Thr199OG1 atom, although being at a distance of only 2.74 Å from it. Indeed, the Thr199OG1 atom is known to be involved in the classical H-bond with Glu106[1,23] and thus it does not have further hydrogens to donate to the hydroxamate functionality (Figure 3B).

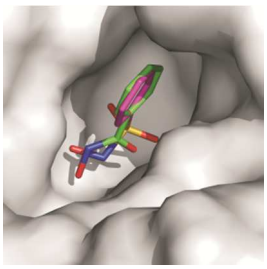

Figure 4 Superposition of compounds **4** (green) and **2** (magenta) (PDB code 3T5U)[10] when bound to the hCA II active site.

It is worth noting that the Zn^{2+} coordination observed in the adduct here reported is identical to that described for the majority of the MMP/hydroxamate complexes so far structurally characterized,[24] but is completely different from that observed in other CA/aliphatic hydroxamate adducts studied earlier.[18] Only two such compounds have been so far structurally characterized in their adducts with hCA II, namely acetohydroxamic and trifluoroacetohydroxamic acids. In these studies it has surprisingly been reported that the acetohydroxamic acid coordinates to the Zn^{2+} ion in a tetrahedral coordination by means of the nitrogen atom, thus suggesting in this case the occurrence of an N-deprotonation (Figure 3C).[18] The same coordination was observed for the trifluoroacetohydroxamic acid.[18] However, in the last case a weakly polar C-F→Zn^{2+} interaction was also present. The observation that hydroxamate derivatives of type R-CONHOH can adopt such completely different coordination modes to the CA catalytic zinc ion, depending on the nature of the R substituent, strongly suggests that this ZBG is very versatile and can represent an interesting alternative to the classical sulfonamides for the development of more selective CAIs.

In conclusion, we prove here that the hydroxamates, an underexplored class of CAIs, may constitute interesting leads for the development of compounds with enhanced selectivity for pharmaceutically relevant CA isoforms, such as the transmembrane ones CA XII and XIV. The very simple model compound phenyl hydroxamate was investigated as inhibitor of 12 catalytically active hCA isoforms. The compound, unlike the unsubstituted or N-substituted sulfonamides, selectively inhibited the transmembrane isoforms being less effective against the cytosolic or mitochondrial ones. The X-ray crystal structure of the compound bound to hCA II also afforded interesting hints regarding the versatility of the hydroxamate as a ZBG for designing CAIs. Indeed, we showed that depending on the nature of the R moiety, this ZBG can adopt different coordination modes to the catalytic zinc ion within the CA active site. These findings suggest that the enzyme-inhibitor interaction of this new CAI class can be largely modulated by exploring different substitution patterns at the R group, thus providing interesting hints for the development of new CAIs of the non-sulfonamide type with pharmaceutical applications in the treatment of various diseases.

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† Electronic Supplementary Information (ESI) available: Experimental details and Table of Crystallographic data. See DOI: 10.1039/b000000x/

Acknowledgments: This research was financed in part from an FP7 EU project (Metoxia).