Hydroxamates as Carbonic Anhydrase Inhibitors

Claudiu T. Supuran

Abstract The 14 different mammalian carbonic anhydrase (CA, EC 4.2.1.1) isozymes as well as many such enzymes isolated up to now in other organisms, play important physiological functions such as pH regulation, signaling, biosynthetic reactions, electrolyte secretion, etc. Unsubstituted sulfonamides act as high affinity inhibitors for the first type of these enzymes, whereas hydroxamates were also shown to strongly inhibit many of them. The investigated hydroxamates as CA inhibitors (CAIs) include *N*-hydroxyurea, the aliphatic/aromatic hydroxamates of the type RCONHOH (R = Me, CF_3 and Ph), as well as sulfonylated amino acid hydroxamates of the type RSO_2NX-AA-CONHOH (X = H; benzyl; substituted benzyl; AA = amino acid moiety, such as those of Gly, Ala, Val, Leu). The most salient feature of the hydroxamates as CAIs regards their high versatility as zinc-binding groups (ZBGs). Indeed, depending on the nature of the R moiety of a hydroxamate of the type RCONHOH, the hydroxamate moiety can adopt different coordination modes to the catalytic zinc ion within the CA active site: monodentate through the deprotonated N atom; bidentate trough the NH and OH groups, or bidentate through the OH and O atoms (deprotonation at the OH moiety). These findings suggest that the enzyme-inhibitor interaction of the hydroxamate 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

Keywords Carbonic anhydrases · Carbonic anhydrase inhibitors · Hydroxamates · Hydroxyureas

C. T. Supuran (✉)
Dipartimento di Scienze Farmaceutiche, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Florence, Italy
e-mail: claudiu.supuran@unifi.it

S. P. Gupta (ed.), *Hydroxamic Acids*, DOI: 10.1007/978-3-642-38111-9_3,

Abbreviations

CA	Carbonic anhydrase
CAI	CA inhibitor
ChC	*Clostridium histolyticum* collagenase
MMPI	Matrix metalloproteinase inhibitor
MMP	Matrix metalloproteinase
ZBG	Zinc-binding group

Contents

1 Introduction on Carbonic Anhydrases

The ubiquitous enzyme carbonic anhydrase (CA, EC 4.2.1.1), is present in prokaryotes and eukaryotes as five genetically different families of enzymes, the α-CAs (mainly in vertebrates and in some green bacteria, fungi, and plants), β-CAs (mainly in bacteria, fungi, algae, and green plants), γ-CAs (in *Archaea* and mitochondria of plants) and the δ- and ζ-CA families, present in diatoms (Supuran 2008, 2010, 2011; Neri and Supuran 2011; Alterio et al. 2012). In higher vertebrates, 16 different CA isozymes were described up to now, which are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions, such as the lipogenesis, gluconeogenesis, and ureagenesis among others (Supuran 2008, 2010, 2011; Neri and Supuran 2011; Alterio et al. 2012). Some of these isozymes are cytosolic (such as CA I, CA II, CA III, CA VII, CA VIII, CA X, CA XI, and CA XIII), others are membrane-bound (CA IV, CA IX, CA XII, CA XIV, and XA XV), CA VA, and CA VB are present only in mitochondria, whereas CA VI is secreted in saliva; several isoforms are acatalytic (CA VIII, CA X, and CA XI) (Supuran 2008, 2010, 2011, 2012; Neri and Supuran 2011; Alterio et al. 2012).

In addition to the physiological reaction, the reversible hydration of carbon dioxide to bicarbonate, CAs also catalyze a variety of other reactions, such as the aldehyde hydration; the hydrolysis of carboxylic acid esters, as well as esters of sulfonic or phosphoric acids (Alterio et al. 2012; Supuran 2012). On the other

hand, CAs do not possess at all peptidase activity, which in turn is the only reaction catalyzed by the proteases of the matrix metalloproteinase (MMP) family with very great efficiency (Alterio et al. 2012). We mention this class of enzymes, the MMPs here, because the hydroxamates, compounds constituting the main topic of this chapter, are the most important class of MMP inhibitors (Nagase and Woessner 1999; Lovejoy et al. 1999; Borkakoti 2000; Borkakoti et al. 1994; Bottomley et al. 1998).

Specific inhibitors of both these types of zinc enzymes are well-known, and some of them were clinically used for more than 60 years (the sulfonamide CAIs) (Supuran 2008, 2010, 2011). Inhibition of CAs by aromatic/heterocyclic sulfonamides has been and it is successfully used in the treatment of a variety of diseases such as glaucoma, epilepsy, obesity, congestive heart failure, mountain sickness, gastric and duodenal ulcers, tumors, or as diuretic agents (Supuran 2008, 2010, 2011). MMPs on the other hand became targets for the drug design more recently, with several hydroxamate drugs in clinical use in the last decade (Whittaker et al. 1999; Supuran and Scozzafava 2002).

CAs and MMPs possess very similar metal coordination spheres within their catalytic sites, consisting of a Zn(II) ion coordinated by three histidines, with the fourth ligand being a water molecule/hydroxide ion, which is the nucleophile intervening in the catalytic cycle of both enzymes (Fig. 1) (Christianson and Ippolito 1991; Coleman 1998; Supuran and Winum 2009).

The main structural difference between these two types of enzymes regards the residues with which the zinc-bound water molecule/hydroxide ion interacts: in CAs, the non-protein zinc ligand forms a hydrogen bond with the hydroxyl moiety of Thr199 (hCA II numbering), which in turn is hydrogen-bonded to the carboxylate of Glu106, leading thus to a dramatic enhance of nucleophilicity of the water molecule/hydroxide ion (Scozzafava and Supuran 2000a; Supuran and Winum 2009). In the case of MMPs, the zinc-bound water molecule interacts with the carboxylate moiety of a conserved glutamate residue (Glu198 in MMP-8), probably forming two hydrogen bonds with it (Scozzafava and Supuran 2000a).

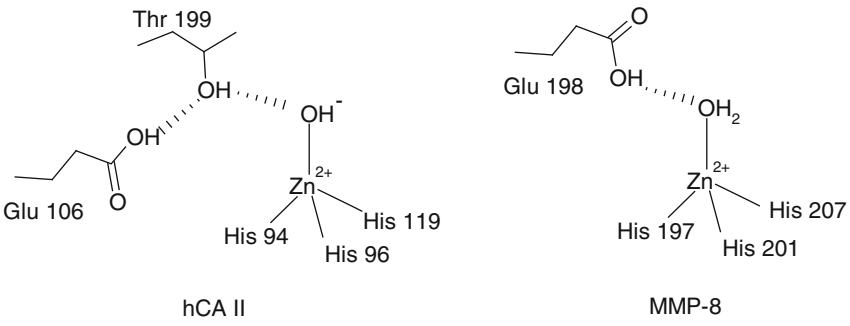


Fig. 1 Active site coordination of the Zn(II) ion in human CA isozyme II (hCA II) and human collagenase 2 (MMP-8). The non-protein zinc ligand of hCA II is a hydroxide ion (as shown above) or a water molecule, depending on the pH of the solution (Scozzafava and Supuran 2000a)

Thus, a very effective nucleophile is formed again, which will attack the amide scissile bond of the peptide substrate. The principal difference between the enzymatic mechanisms of CAs and MMPs consists in the fact that the nucleophilic adduct formed after the attack of the zinc-bound nucleophile to the substrate is the reaction product in the case of CAs (the bicarbonate ion), whereas the nucleophilic adduct is only a reaction intermediate in the case of the MMPs (Scozzafava and Supuran 2000a). This is also of crucial importance for the interaction of these enzymes with their inhibitors.

Inhibition of both CAs as well as MMPs is correlated with the coordination of the inhibitor molecule (in neutral or ionized state) to the catalytic metal ion, with or without substitution of the metal-bound water molecule (Supuran 2008; Scozzafava and Supuran 2000a). Thus, CA and MMP inhibitors (abbreviated as CAIs and MMPIs, respectively) must contain a zinc-binding group (ZBG) attached to a scaffold that will interact with other binding regions of the enzymes (Supuran and Winum 2009). In the case of CAIs, unsubstituted aromatic/heterocyclic sulfonamides as well as *N*-hydroxy-sulfonamides proved to be very effective inhibitors, with affinities in the low nanomolar range for isozymes such as CA I, CA II, CA IV, etc. These derivatives bind monodentately, as anions (RSO_2NH^-) to the Zn(II) ion within CA active site, interacting also with several other active site residues, by means of hydrogen bonds or hydrophobic interactions (Supuran 2008, 2010, 2011). It has been observed that CAIs possessing elongated molecules, able to interact with amino acid residues situated at the edge of the active site entrance (and obviously, with the zinc ion, as mentioned above), are among the most efficient ones (Scozzafava et al. 1999).

Depending on the ZBG contained in their molecule, MMPIs belong to several chemical classes, such as the carboxylates, the hydroxamates, the thiols, the phosphorus-based ligands, or the sulfodiimines, among others (Whittaker et al. 1999; Supuran and Scozzafava 2002). The strongest and most investigated class of MMPIs is constituted by the hydroxamates, as mentioned above (Whittaker et al. 1999; Supuran and Scozzafava 2002). The interaction of the catalytic domain of several MMPs with some inhibitors has been investigated by means of X-ray crystallography (Fig. 2) (Whittaker et al. 1999; Supuran and Scozzafava 2002).

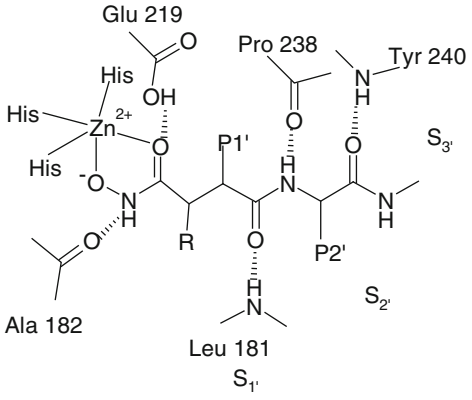


Fig. 2 Schematic representation for the binding of a succinate hydroxamate inhibitor to MMP-7, as determined by X-ray crystallography. The Zn(II)-ligand and hydrogen bond interactions in the enzyme-inhibitor adduct are evidenced (Supuran and Scozzafava 2002)

As seen in the above figure, hydroxamates bind bidentately to the catalytic Zn(II) ion of the MMP, which acquires a distorted trigonal bipyramidal geometry in this way (Whittaker et al. 1999; Supuran and Scozzafava 2002). The hydroxamate anion forms a short and strong hydrogen bond with the carboxylate moiety of Glu219, which is orientated toward the unprimed binding regions, whereas the NH hydroxamate participates in a hydrogen bond with the carbonyl oxygen of Ala182. Thus, several strong interactions are achieved at the zinc site, without any significant unfavorable contacts (Whittaker et al. 1999; Supuran and Scozzafava 2002). However, we will not insist on the MMP inhibition of the hydroxamates as this topic is treated in other chapters of the book.

However, investigating hydroxamates which act as strong MMPIs, also as CAIs, was quite logical considering the similar active site architecture of the two classes of such enzymes. Such studies started some years ago with the report of Christianson's group of the X-ray crystal structure of two aliphatic hydroxamates complexed to CA II (Scolnick et al. 1997). They were followed by the report of *N*-hydroxyurea (the simplest hydroxamate) as CAI against all the catalytically active mammalian isoforms (Supuran and Scozzafava 2003). The X-ray structure of this compound complexed to CA II has also been reported subsequently (Temperini et al. 2006). A rather large number of amino acid hydroxamates have been thereafter designed and investigated as CAIs (in addition to their MMP inhibition studies) (Scozzafava and Supuran 2000a, b, c), and more recently, aromatic hydroxamates have also been investigated as CAIs (Di Fiore et al. 2012).

2 *N*-Hydroxyurea as CA Inhibitor

The idea to investigate *N*-hydroxyurea as CAI started after the discovery that urea acts in such a way (Briganti et al. 1999). Indeed, our group discovered that cyanamide acts as a suicide inhibitor of CA, being transformed to urea (actually ureate) which thereafter coordinated to the Zn(II) from the CA active site. We thus investigated whether *N*-hydroxyurea, the simplest stable hydroxamate (H_2N-CO-NHOH) may inhibit CAs and discovered that it inhibits several mammalian CA isoforms with inhibition constants in the micromolar–submicromolar range (Supuran and Scozzafava 2003). Hydroxyurea acts as a weak, non-competitive inhibitor of both CA I and II isozymes, for their 4-nitrophenyl acetate esterase activity. The spectrum of the adduct of hydroxyurea with the Co(II)-substituted CA II was similar to the spectra of tetrahedral such adducts (e.g., with sulfamide, acetazolamide, or cyanamide), proving a direct interaction of the inhibitor molecule with the metal center of the enzyme, whose geometry remains tetrahedral. Based on the X-ray crystal structure of the adducts of hCA II with ureate and hydroxamate inhibitors, the hypothetical binding of hydroxyurea was proposed to be achieved in deprotonated state, with the nitrogen atom coordinated to Zn(II), and the OH group of the inhibitor making a hydrogen bond with Thr199. This binding was thereafter confirmed when the X-ray crystal structure of the adduct of

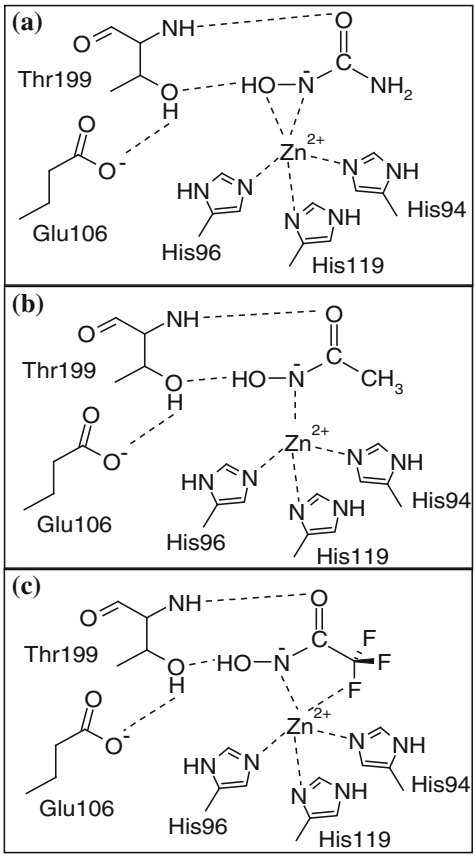


Fig. 3 Binding of *N*-hydroxyurea (**a**), methyl hydroxamate (**b**) and trifluoromethyl hydroxamate (**c**) to hCA II, as determined by X-ray crystallography

CA II complexed with *N*-hydroxyurea has been reported (Fig. 3a). The crystallographic structure of the human (h) hCA II/N-hydroxyurea adduct showed that this molecule binds to the Zn^{2+} ion of hCA II active site in a bidentate mode, by means of the oxygen and nitrogen atoms of the NHOH moiety. Additional hydrogen bonds involving the hydroxyl and the carbonyl moieties of the inhibitor and the enzyme residue Thr199 were also observed (Fig. 3a).[91] Worth noting is that the related acetohydroxamic and trifluoroacetohydroxamic acids, although containing the same hydroxamate functionality, were demonstrated to adopt a very different binding mode (see next section).

3 Aliphatic Hydroxamates

Christianson's group investigated two simple hydroxamates (methyl- and trifluoromethyl hydroxamates) as CAIs, and reported the X-ray structure of such an adduct (hCA II–$CF_3CONHOH$) (Scolnick et al. 1997). The second compound

binds to hCA II with an affinity of 3.8 μM but its interaction with the Zn(II) ion of CA active site is very different from that of the classical sulfonamide inhibitors. Thus, the ionized nitrogen atom of the hydroxamate moiety of both compounds, was directly coordinated to Zn(II), whereas a fluorine atom of the trifluoromethyl moiety also participated in the interaction with the metal ion (Fig. 3b, c). In addition, hydrogen bonds between the hydroxamate OH and active site residue Thr199 were also evidenced, which further stabilize the E–I adduct (Fig. 3b, c).

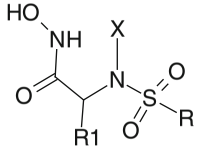
It may be thus observed a highly versatile behavior of the hydroxamate ZBG in the three simple "aliphatic" CAIs investigated so far by means of X-ray crystallography.

4 Amino Acid Hydroxamates

Based on the data presented above for *N*-hydroxyurea and aliphatic hydroxamates acting as CAIs, a large series of sulfonylated amino acid hydroxamates were investigated as CAIs (Scozzafava and Supuran 2000a). The following types of compounds were included in the study: (i) sulfonylated amino acid hydroxamates possessing an unsubstituted RSO_2NH-amino acyl moiety. The amino acid hydroxamates included in the study were the Gly, Ala, Val, and Leu derivatives (Table 1); (ii) sulfonylated amino acid hydroxamates possessing a substituted RSO_2NX-amino acyl moiety, where X is generally a benzyl or a 2- or 4-substituted benzyl group (the same amino acid hydroxamates as above were included in the study, Table 1). For all these types of compounds, three different examples have been employed (R moieties) from the large series of available aliphatic, aromatic, and heterocyclic derivatives reported previously by our group. They included the perfluorobutyl, the pentafluorophenyl, and the 4-methoxyphenyl sulfonyl moieties, and were chosen in such a way as to include a very potent, a slightly weaker, and an even weaker MMPI. Anyhow, all these three groups incorporated in amino acid hydroxamates, generally led to potent MMPIs, with affinities (for the most active derivatives) in the (low) nanomolar range (5–15 nM) for the different MMPs as well as for the *Clostridium histolyticum* collagenase (ChC) (Van Wart and Steinbrink 1981; Scozzafava and Supuran 2000a).

The inhibition data with compounds **1–39**, presented in Table 1 led to the following observations: (i) sulfonyl amino acyl hydroxamates possessing moieties of the type RSO_2NH-amino acyl (such as **1–3**; **7–9**; **13–15;** and **19–21**) generally acted as efficient CAIs, and were relatively weak MMP and ChC inhibitors. Thus, for CAs, these inhibitors generally showed affinities in the range of 7–50 nM (hCA I); 8–45 nM (hCA II); and 10–40 nM (bCA IV), whereas for the different MMPs investigated here and ChC, their affinities were in the range of 40–>200 nM. For the three types of investigated derivatives, the most active were the pentafluorophenylsulfonyl derivatives, followed by the corresponding perfluorobutyl ones, whereas the least active were the corresponding 4-methoxyphenyl-substituted compounds. For CA inhibition, best activity was observed for the Gly derivatives,

Table 1 Inhibition of MMPs, ChC and CAs with the hydroxamates **1–39**



No.	R1	R	X	MMP-1[b]	MMP-2[b]	MMP-8[b]	K_i[a](nM) MMP-9[b]	ChC[c]	hCA I[d]	hCA II[d]	bCA IV[d]
1	H	*n*-C_4F_9	H	>200	75	130	125	80	18	15	16
2	H	C_6F_5	H	145	44	125	100	54	7	8	10
3	H	4-MeO-C_6H_4	H	>200	110	155	143	120	30	32	29
4	H	*n*-C_4F_9	$C_6H_5CH_2$	30	3.9	5.3	5	13	105	85	100
5	H	C_6F_5	$C_6H_5CH_2$	7	1.5	1.1	1.2	6	90	36	42
6	H	4-MeO-C_6H_4	$C_6H_5CH_2$	60	18	31	42	27	>200	120	145
7	Me	*n*-C_4F_9	H	>200	69	118	121	79	21	16	17
8	Me	C_6F_5	H	150	40	116	96	45	7	8	10
9	Me	4-MeO-C_6H_4	H	>200	87	125	137	130	32	35	30
10	Me	*n*-C_4F_9	$C_6H_5CH_2$	26	3.2	4.9	4.3	12	121	92	107
11	Me	C_6F_5	$C_6H_5CH_2$	7	0.9	1.1	1.4	6	84	38	43
12	Me	4-MeO-C_6H_4	$C_6H_5CH_2$	58	15	19	35	20	195	120	136
13	iPr	*n*-C_4F_9	H	>200	66	111	120	78	29	15	20
14	iPr	C_6F_5	H	139	41	104	89	40	8	11	13
15	iPr	4-MeO-C_6H_4	H	>200	82	126	126	103	33	39	38
16	iPr	*n*-C_4F_9	$C_6H_5CH_2$	21	2.4	4.2	4.3	10	140	108	124
17	iPr	C_6F_5	$C_6H_5CH_2$	7	0.8	1.0	1.2	5	88	45	48
18	iPr	4-MeO-C_6H_4	$C_6H_5CH_2$	43	11	13	27	17	>200	>200	185
19	iBu	*n*-C_4F_9	H	>200	62	108	36	69	36	18	30
20	iBu	C_6F_5	H	155	39	101	122	38	10	11	19
21	iBu	4-MeO-C_6H_4	H	>200	84	123	78	95	50	5	39

(continued)

Table 1 (continued)

No.	R1	R	X	MMP-1[b]	MMP-2[b]	MMP-8[b]	K_i[a](nM) MMP-9[b]	ChC[c]	hCA I[d]	hCA II[d]	bCA IV[d]
22	iBu	*n*-C_4F_9	$C_6H_5CH_2$	16	1.9	3.3	4.0	8	180	116	139
23	iBu	C_6F_5	$C_6H_5CH_2$	6	0.8	0.6	1.1	5	100	56	75
24	iBu	4-MeO-C_6H_4	$C_6H_5CH_2$	44	10	9	1.3	13	>200	>200	190
25	H	*n*-C_4F_9	2-$O_2NC_6H_4CH_2$	25	3.7	5.5	4.6	13	>200	100	120
26	H	C_6F_5	2-$O_2NC_6H_4CH_2$	6	1.4	1.0	1.3	6	127	74	49
27	H	4-MeO-C_6H_4	2-$O_2NC_6H_4CH_2$	54	15	27	39	24	>200	170	140
28	H	*n*-C_4F_9	4-$O_2NC_6H_4CH_2$	62	1.5	2.4	2.0	12	>200	118	136
29	H	C_6F_5	4-$O_2NC_6H_4CH_2$	3	0.7	0.1	0.6	5	150	107	130
30	H	4-MeO-C_6H_4	4-$O_2NC_6H_4CH_2$	28	18	21	31	20	>200	>200	>200
31	Me	*n*-C_4F_9	2-$O_2NC_6H_4CH_2$	24	2.9	5.1	4.4	10	>200	>200	>200
32	Me	C_6F_5	2-$O_2NC_6H_4CH_2$	7	0.8	1.1	1.0	5	>200	160	>200
33	Me	4-MeO-C_6H_4	2-$O_2NC_6H_4CH_2$	39	13	20	24	19	>200	>200	>200
34	Me	*n*-C_4F_9	4-$O_2NC_6H_4CH_2$	60	1.4	2.3	1.5	11	>200	>200	>200
35	Me	C_6F_5	4-$O_2NC_6H_4CH_2$	4	0.7	0.3	0.6	5	>200	>200	>200
36	Me	4-MeO-C_6H_4	4-$O_2NC_6H_4CH_2$	25	15	18	28	21	>200	>200	>200
37	Me	*n*-C_4F_9	2-$ClC_6H_4CH_2$	37	3.7	6.4	5.0	11	>200	>200	>200
38	Me	C_6F_5	2-$ClC_6H_4CH_2$	10	1.5	1.3	1.7	5	>200	>200	>200
39	Me	4-MeO-C_6H_4	2-$ClC_6H_4CH_2$	53	12	27	31	22	>200	>200	>200

[a] K_I-s values were obtained from Easson–Stedman plots using a linear regression program, from at least three different assays. Standard errors were of 5–10 % (Scozzafava and Supuran 2000a)

[b] With the thioester substrate Ac-ProLeuGly-S-LeuLeuGlyOEt, spectrophotometrically (Scozzafava and Supuran 2000a)

[c] With FALGPA as substrate, spectrophotometrically (Scozzafava and Supuran 2000a)

[d] With 4-NPA as substrate, by the esterase method, spectrophotometrically (Scozzafava and Supuran 2000a)

followed by the corresponding Ala derivatives, which in turn were more active than the corresponding Val and Leu derivatives. Just the opposite was generally true for MMP and ChC inhibition, with the bulkier Val and Leu derivatives generally more inhibitory than the corresponding Ala and Gly derivatives (Table 1); (ii) sulfonyl amino acyl hydroxamates possessing RSO_2N(benzyl/substituted benzyl)-amino acyl moieties (such as **10–12**; **16–18**; **22–241;** and **28–45**) were weak or very weak CA inhibitors, but showed excellent MMP and ChC inhibitory properties. Thus, these compounds were generally 4–8 times weaker CA inhibitors as compared to the corresponding unsubstituted compounds mentioned above, whereas their affinities for MMPs were very much enhanced as compared to those of the corresponding unsubstituted compounds. It was in fact reported that the benzyl moiety of this type of hydroxamate inhibitors fits well within the $S_{2'}$ site of the protease, contributing substantially to the formation of strong E–I adducts. Obviously the different MMPs possess quite diverse affinities for these derivatives, with important differences between the deep pocket (MMP-2; MMP-8; and MMP-9) and the short pocket enzymes (MMP-1). Thus, as already shown previously by us for some structurally related derivatives, the deep pocket enzymes MMP-2, MMP-8, and MMP-9 are much more susceptible to be inhibited by this class of hydroxamates (K_I-s in the range of 0.6–20 nM) than collagenase 1, MMP-1 (K_I-s in the range of 7–60 nM). Again the pentafluorophenylsulfonyl derivatives were the most active inhibitors, followed by the corresponding perfluorobutyl ones, whereas the least active were the corresponding 4-methoxyphenyl-substituted compounds. The Leu derivatives were generally more active than the corresponding Val derivatives, which in turn were more inhibitory than the Ala and Gly derivatives; (iii) further substitution (with nitro or chloro moieties, in position 2 or 4) of the $P_{2'}$ benzyl moiety, such as in compounds **25–39** lead to a slight enhancement of the MMP inhibitory properties, to an enhancement of the ChC inhibitory effects, and to a drastic reduction of the CA inhibitory properties of the corresponding compounds (Table 1) (Scozzafava and Supuran 2000a). A putative binding mode for these compounds to the CAs was also proposed and is shown in Fig. 4, but this binding mode has not yet been confirmed by means of X-ray crystallography. An interesting QSAR study of these compounds has also bee reported by Gupta et al. (2003).

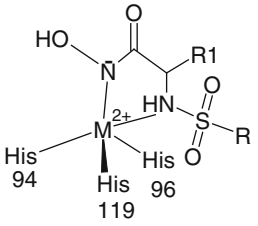


Fig. 4 Proposed binding of a sulfonylated amino acid hydroxamate (as monoanion) to the metal ion within the active site of CA (M = Zn(II) for the native enzyme or Co(II) for the cobalt-substituted one) (Scozzafava and Supuran 2000a)

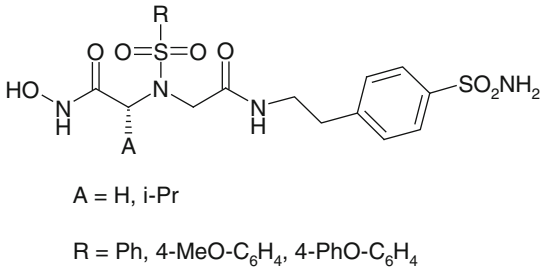


Fig. 5 Dual CA/MMP inhibitors of the hydroxamate type (Santos et al. 2007; Marques et al. 2008)

The data of Table 1 proved that potent CAIs can be obtained from the class of investigated sulfonylated amino acid hydroxamates (Scozzafava and Supuran 2000a). Although it was noted that in addition to MMPs, hydroxamates also inhibit other metalloproteinases, such as leucine aminopeptidase, neprilysin, thermolysis, and tumor necrosis factor-alpha among others (Supuran and Winum 2009), affinities as high as for the CAs (in the nanomolar range) were not evidenced up to now (Scozzafava and Supuran 2000a). Thus, such results are quite promising for the eventual design of novel types of potent CAIs, or of compounds with a dual activity, both as CAIs and MMPIs (Fig. 5). In fact, such derivatives were subsequently reported (Santos et al. 2007; Marques et al. 2008).

Compounds **40** reported using this dual drug approach (Santos et al. 2007; Marques et al. 2008) are iminodiacetic derivatives which possess the following derivatization type in their scaffolds: (i) one of the COOH moieties of the iminodiacetic moiety has been transformed to the hydroxamate ZBG; (ii) the other COOH moiety has been derivatized by transforming it to the amide, by reaction with 4-aminoethyl-benzenesulfonamide, a well-known CAI (Supuran 2008). This part of the molecule should interact only with the CAs, whereas the hydroxamate part may interact both with CAs as well as MMPs (Santos et al. 2007; Marques et al. 2008); (iii) the central imino moiety has been transformed to a secondary aromatic sulfonamide, and (iv) an isopropyl moiety may be present or absent on one of the CH_2 spacers coming from the iminodiacetic moiety.

Some of these compounds were low nanomolar CAIs and MMPs against several such enzymes (isoforms) involved in tumorigenesis (e.g., CA IX, CA XII, MMP-8, MMP-9, MMP-13, etc.) (Santos et al. 2007; Marques et al. 2008).

5 Aromatic Hydroxamates

The simple compound phenylhydroxamate, PhCONHOH **41**, has recently investigated by means of kinetic and X-ray crystallographic techniques, for its interaction with all mammalian CA isoforms, CA I–XIV (Di Fiore et al. 2012). Hydroxamate **41** inhibited all CA isoforms, with inhibition constants in the range of 0.94–179 μM, being thus less effective as CAI compared to the sulfonamides,

which usually are micro–nano molar CAIs (Supuran 2008). However, there were several notable features of the inhibition profile of compound **41**. Thus, the dominant, offtarget isoform hCA II was the least inhibited by **41** (K_I of 179 μM). The other highly abundant cytosolic isoform hCA I was also relatively resistant to inhibition by **41** (K_I of 83.1 μM). An interesting aspect of the hydroxamate **41** was that it strongly inhibited two transmembrane isoforms, hCA XII and XIV, with inhibition constants in the range of 0.94–9.51 K_I of 179 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 investigated CA isoforms (e.g., hCA III, IV, VA, VB, VI, VII, and XIII) were modestly inhibited by **41**, with K_Is in the range of 23.0–84.7 K_I of 179 μM. Hydroxamate **41** inhibited most efficiently hCA XIV (Di Fiore et al. 2012).

But the most interesting facts emerged when the X-ray crystal structure of **41** complexed to hCA II has been resolved (Di Fiore et al. 2012). The binding of the inhibitor molecule did not cause any significant change in the overall protein structure. Hydroxamate **41** 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 (Fig. 6a, b). The inhibitor binding was 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), whereas the phenyl ring, whose position is rather well superimposable to that of the phenyl ring in the hCA II/acetohydroxamic acid adduct (Fig. 6c), was involved in a number of van der Waals interactions with the side chains of residues Gln92, Val121, Phe131, Leu141, Val143, Leu198, and Thr200 (Fig. 6a). Although, several controversial data have been reported on the

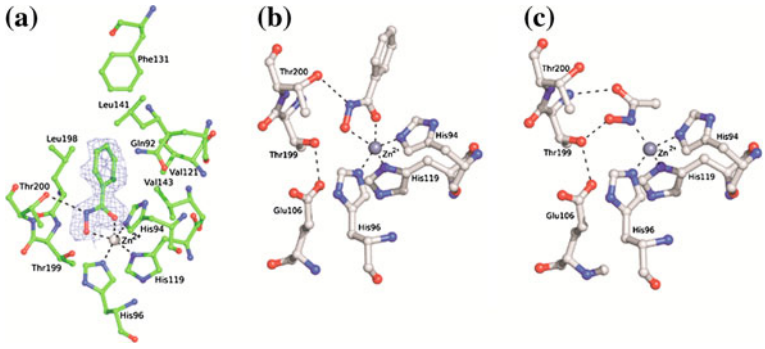


Fig. 6 (**a**) Active site region in the hCA II–**41** complex. The simulated annealing omit 2|Fo|-|Fc| electron density map inhibitor, contoured at 1.0 sigma, and associated to the inhibitor molecule is also shown. (**b**) Zn^{2+} coordination geometry of *N*-(Hydroxy)-benzamide **41**. (**c**) Zn^{2+} coordination geometry of acetohydroxamic acid (PDB code 1AM6) (Di Fiore et al. 2012)

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, the observation that compound **41** 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 (Di Fiore et al. 2012). 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 involve in the classical H-bond with Glu106[1,23] and thus it does not have further hydrogens to donate to the hydroxamate functionality (Fig. 6b). It is worth noting that the zinc ion coordination observed in the adduct here reported is identical to that described for the majority of the MMP/hydroxamate complexes so far structurally characterized (Whittaker et al. 1999), but is completely different from that observed in other CA/aliphatic hydroxamate adducts studied earlier (Scolnick et al. 1997) and discussed above. 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.

6 Conclusion

As MMPs, the main class of metalloenzymes interacting with the hydroxamates, CAs is also zinc enzymes possessing at the active center a Zn(II) ion coordinated by three His residues and a water molecule/hydroxide ion. The latter one is crucial for catalysis and is many times replaced by inhibitors binding to the metal center. The 14 different mammalian CAs as well as many such enzymes isolated up to now in other organisms, play important physiological functions such as pH regulation, signaling, biosynthetic reactions, electrolyte secretion, etc. Unsubstituted sulfonamides act as high affinity inhibitors for the first type of these enzymes, whereas hydroxamates were also shown to strongly inhibit some of them. The investigated hydroxamates as CA inhibitors include *N*-hydroxyurea, the aliphatic/aromatic hydroxamates of the type RCONHOH (R = Me, CF_3 and Ph), as well as sulfonylated amino acid hydroxamates of the type RSO_2NX-AA-CONHOH (X = H; benzyl; substituted benzyl; AA = amino acid moiety, such as those of Gly, Ala, Val, Leu). The most salient feature of the hydroxamates as CAIs regards their very high versatility as ZBGs. Indeed, depending on the nature of the R moiety of a hydroxamate of the type RCONHOH, this ZBG can adopt different coordination modes to the catalytic zinc ion within the CA active site: monodentate through the deprotonated N atom; bidentate trough the NH and OH atoms, or bidentate through the OH and O atoms (deprotonation at the OH moiety). These findings suggest that the enzyme-inhibitor interaction of the hydroxamate 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.

Acknowledgments Work from my laboratory was supported by several FP7 EU projects (Metoxia and Dynano)

References

Alterio V, Di Fiore A, D'Ambrosio K et al (2012) Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? Chem Rev 112:4421–4468

Borkakoti N (2000) Structural studies of matrix metalloproteinases. J Mol Med 78:261–268

Borkakoti N, Winkler FK, Williams DH et al (1994) Structure of the catalytic domain of human fibroblast collagenase complexed with an inhibitor. Nat Struct Biol 1:106–110

Bottomley KM, Johnson WH, Walter DS (1998) Matrix metalloproteinase inhibitors in arthritis. J Enz Inhib 13:79–102

Briganti F, Mangani S, Scozzafava A et al (1999) Carbonic anhydrase catalyzes cyanamide hydration to urea: is it mimicking the physiological reaction? J Biol Inorg Chem 4:528–536

Christianson DW, Ippolito JA (1991) Structure-function relationship between the carbonic anhydrases and the zinc proteases. In: Botrè F, Gros G, Storey BT (eds) Carbonic Anhydrase. VCH, New York, pp 95–110

Coleman JE (1998) Zinc enzymes. Curr Opin Chem Biol 2:222–234

Di Fiore A, Maresca A, Supuran CT, De Simone G (2012) Hydroxamate represents a versatile zinc binding group for the development of new carbonic anhydrase inhibitors. Chem Commun 48:8838–8840

Gupta SP, Maheswaran V, Pande V, Kumar D (2003) A comparative QSAR study on carbonic anhydrase and matrix metalloproteinase inhibition by sulfonylated amino acid hydroxamates. J Enzyme Inhib Med Chem 18:7–13

Lovejoy B, Welch AR, Carr S et al (1999) Crystal structures of MMP-1 and -13 reveal the structural basis for selectivity of collagenase inhibitors. Nat Struct Biol 6:217–221

Marques SM, Nuti E, Rossello A et al (2008) Dual inhibitors of matrix metalloproteinases and carbonic anhydrases: iminodiacetyl-based hydroxamate-benzenesulfonamide conjugates. J Med Chem 51:7968–7979

Nagase H, Woessner JF (1999) Matrix metalloproteinases. J Biol Chem 274:21491–21494

Neri D, Supuran CT (2011) Interfering with pH regulation in tumours as a therapeutic strategy. Nat Rev Drug Discov 10:767–777

Santos MA, Marques S, Vullo D et al (2007) Carbonic anhydrase inhibitors. Inhibition of cytosolic/tumor-associated isoforms I, II and IX with iminodiacetic carboxylates/hydroxamates also incorporating benzenesulfonamide moieties. Bioorg Med Chem Let 17:1538–1543

Scolnick LR, Clements AM, Liao J et al (1997) Novel binding mode of hydroxamate inhibitors to human carbonic anhydrase II. J Am Chem Soc 119:850–851

Scozzafava A, Menabuoni L, Mincione F et al (1999) Carbonic anhydrase inhibitors: synthesis of water-soluble, topically effective intraocular pressure lowering aromatic/heterocyclic sulfonamides containing cationic or anionic moieties: is the tail more important than the ring? J Med Chem 42:2641–2650

Scozzafava A, Supuran CT (2000a) Carbonic anhydrase and matrix metalloproteinase inhibitors. Sulfonylated amino acid hydroxamates with MMP inhibitory properties act as efficient inhibitors of carbonic anhydrase isozymes I, II and IV, and N-hydroxysulfonamides inhibit both these zinc enzymes. J Med Chem 43:3677–3687

Scozzafava A, Supuran CT (2000b) Protease inhibitors. Part 5. Alkyl/arylsulfonyl- and arylsulfonylureido-/arylureido-glycine hydroxamate inhibitors of *Clostridium histolyticum* collagenase. Eur J Med Chem 35:299–307

Scozzafava A, Supuran CT (2000c) Protease inhibitors. Part 9. Synthesis of *Clostridium histolyticum* collagenase inhibitors incorporating sulfonyl-L-alanine hydroxamate moieties. Bioorg Med Chem Lett 10:499–502

Scozzafava A, Supuran CT (2003) Hydroxyurea is a carbonic anhydrase inhibitor. Bioorg Med Chem 11:2241–2246

Supuran CT (2008) Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 7:168–181

Supuran CT (2010) Carbonic anhydrase inhibitors. Bioorg Med Chem Lett 20:3467–3474

Supuran CT (2011) Carbonic anhydrase inhibitors and activators for novel therapeutic applications. Future Med Chem 3:1165–1180

Supuran CT (2012) Structure-based drug discovery of carbonic anhydrase inhibitors. J Enzyme Inhib Med Chem 27:759–772

Supuran CT, Scozzafava A (2002) Matrix metalloproteinases (MMPs). In proteinase and peptidase inhibition: recent potential targets for drug development, Smith HJ, Simons C, Eds., Taylor & Francis, London, pp 35–61

Supuran CT, Winum JY (2009) Introduction to zinc enzymes as drug targets. In: Supuran CT, Winum JY (eds) Drug Design of Zinc-Enzyme Inhibitors: Functional, Structural, and Disease Applications. Wiley, Hoboken, pp 3–12

Temperini C, Innocenti A, Scozzafava A, Supuran CT (2006) N-Hydroxyurea—a versatile zinc binding function in the design of metalloenzyme inhibitors. Bioorg Med Chem Lett 16:4316–4320

Van Wart HE, Steinbrink DR (1981) A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase. Anal Biochem 113:156–165

Whittaker M, Floyd CD, Brown P, Gearing AJH (1999) Design and therapeutic application of matrix metalloproteinase inhibitors. Chem Rev 99:2735–2776