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Review

Carbonic anhydrases as targets for medicinal chemistry

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Abstract—Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc enzymes acting as efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate. 16 different α-CA isoforms were isolated in mammals, where they play crucial physiological roles. Some of them are cytosolic (CA I, CA II, CA III, CA VII, CA XIII), others are membrane-bound (CA IV, CA IX, CA XII, CA XIV and CA XV), CA VA and CA VB are mitochondrial, and CA VI is secreted in saliva and milk. Three acatalytic forms are also known, the CA related proteins (CARP), CARP VIII, CARP X and CARP XI. Representatives of the β-δ-CA family are highly abundant in plants, diatoms, eubacteria and archaea. The catalytic mechanism of the α-CAs is understood in detail: the active site consists of a Zn(II) ion co-ordinated by three histidine residues and a water molecule/hydroxide ion. The latter is the active species, acting as a potent nucleophile. For β- and γ-CAs, the zinc hydroxide mechanism is valid too, although at least some β-class enzymes do not have water directly coordinated to the metal ion. CAs are inhibited primarily by two classes of compounds: the metal complexing anions and the sulfonamides/sulfamates/sulfamides possessing the general formula $RXSO_2NH_2$ (R = aryl; hetaryl; perhaloalkyl; X = nothing, O or NH). Several important physiological and physio-pathological functions are played by CAs present in organisms all over the phylogenetic tree, related to respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions, such as the gluconeogenesis and ureagenesis among others (in animals), CO_2 fixation (in plants and algae), etc. The presence of these ubiquitous enzymes in so many tissues and in so different isoforms represents an attractive goal for the design of inhibitors with biomedical applications. Indeed, CA inhibitors are clinically used as antiglaucoma drugs, some other compounds being developed as antitumour agents/diagnostic tools for tumours, antiobesity agents, anticonvulsants and antimicrobials/antifungals (inhibitors targeting α- or β-CAs from pathogenic organisms such as *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Plasmodium falciparum*, *Candida albicans*, etc.).

Contents

Keywords: Carbonic anhydrase; Inhibitor; Sulfonamide; Sulfamate; Sulfamide; Drug design; Anticancer drug; Antiobesity agent; Antiglaucoma drug; Antibacterials.

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1. Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc enzymes, present in prokaryotes and eukaryotes, being encoded by four distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, *Bacteria*, algae and cytoplasm of green plants), the β-CAs (predominantly found in *Bacteria*, algae and chloroplasts of both mono- as well as dicotyledons), the γ-CAs (mainly present in *Archaea* and some *Bacteria*) and the δ-CAs, present in some marine diatoms, respectively.[1–8] In mammals, 16 different α-CA isozymes or CA-related proteins (CARP) were described (Table 1), with very different subcellular localization and tissue distribution.[1–8] Basically, there are several cytosolic forms (CA I–III and CA VII), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV and CA XV), one mitochondrial form (CA V), as well as a secreted CA isozyme, CA VI.These enzymes catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity and many other physiologic or pathologic processes.[1–8] As it will be discussed shortly, many of these isozymes are important targets for the design of inhibitors with clinical applications.

In addition to the physiological reaction, the reversible hydration of CO_2 to bicarbonate (reaction 1.1, Fig. 1), α-CAs catalyze a variety of other reactions, such as: the hydration of cyanate to carbamic acid, or of cyanamide to urea (reactions 1.2 and 1.3); the aldehyde hydration to *gem*-diols (reaction 1.4); the hydrolysis of carboxylic esters, or sulfonic acid esters (reactions 1.5 and 1.6), as well as other less investigated hydrolytic processes, such as those described by Eqs. 1.7–1.9 in Figure 1.[9–11] It should be mentioned that the previously reported phosphatase activity of CA III was recently

$$\mathrm{O{=}C{=}O + H_2O \Leftrightarrow HCO_3^- + H^+} \qquad (1.1)$$

$$\mathrm{O{=}C{=}NH + H_2O \Leftrightarrow H_2NCOOH} \qquad (1.2)$$

$$\mathrm{HN{=}C{=}NH + H_2O \Leftrightarrow H_2NCONH_2} \qquad (1.3)$$

$$\mathrm{RCHO + H_2O \Leftrightarrow RCH(OH)_2} \qquad (1.4)$$

$$\mathrm{RCOOAr + H_2O \Leftrightarrow RCOOH + ArOH} \qquad (1.5)$$

$$\mathrm{RSO_3Ar + H_2O \Leftrightarrow RSO_3H + ArOH} \qquad (1.6)$$

$$\mathrm{ArF + H_2O \Leftrightarrow HF + ArOH} \qquad (1.7)$$

(Ar = 2,4-dinitrophenyl)

$$\mathrm{PhCH_2OCOCl + H_2O \Leftrightarrow PhCH_2OH + CO_2 + HCl} \qquad (1.8)$$

$$\mathrm{RSO_2Cl + H_2O \Leftrightarrow RSO_3H + HCl} \qquad (1.9)$$

(R = Me; Ph)

Figure 1. Reactions catalyzed by α-CAs.

Table 1. Higher vertebrate α-CA isozymes, their relative CO_2 hydrase activity, affinity for sulfonamide inhibitors and sub-cellular localization

Isozyme	Catalytic activity (CO_2 hydration)	Affinity for sulfonamides	Sub-cellular localization
CA I	Moderate	Medium	Cytosol
CA II	High	Very high	Cytosol
CA III	Very low	Very low	Cytosol
CA IV	High	High	Membrane-bound
CA VA	Low-moderate[a]	High	Mitochondria
CA VB	High	High	Mitochondria
CA VI	Moderate	High	Secreted into saliva/milk
CA VII	High	Very high	Cytosol
CARP VIII	Acatalytic	[b]	Cytosol
CA IX	Moderate-high	High	Transmembrane
CARP X	Acatalytic	[b]	Secreted
CARP XI	Acatalytic	[b]	Secreted
CA XII	Low	Very high	Transmembrane
CA XIII	Moderate	Medium-high	Cytosol
CA XIV	Moderate	High	Transmembrane
CA XV	Low	Unknown	Membrane-bound

[a] Low at pH 7.4, moderate at pH 8.2 or higher.

[b] The native CARP isozymes do not contain Zn(II), so that their affinity for the sulfonamide inhibitors has not been measured. By site-directed mutagenesis it is possible to modify these proteins and transform them in enzymes with CA-like activity which probably are inhibited by sulfonamides, but no detailed studies on this subject are available presently.

proved to be an artefact.[1] It is unclear at this moment whether other α-CA catalyzed reactions than the CO_2 hydration have physiological significance. The X-ray crystal structure has been determined for six α-CAs at this moment (isozymes CA I–VA, CA XII and CA XIV)[1–6,12,13] as well as for representatives of the β- and γ-CA families.[14–18]

2. Catalytic and inhibition mechanisms of carbonic anhydrases

2.1. α-CAs

The metal ion (which is Zn(II) in all α-CAs investigated up to now) is essential for catalysis.[1–8] X-ray crystallographic data showed that the metal ion is situated at the bottom of a 15 Å deep active site cleft (Fig. 2), being coordinated by three histidine residues (His94, His96 and His119) and a water molecule/ hydroxide ion.[1–8] The zinc-bound water is also engaged in hydrogen bond interactions with the hydroxyl moiety of Thr199, which in turn is bridged to the carboxylate moiety of Glu 106; these interactions enhance the nucleophilicity of the zinc-bound water molecule, and orient the substrate (CO_2) in a favourable location for the nucleophilic attack (Fig. 3).[1–8] The active form of the enzyme is the basic one, with hydroxide bound to Zn(II) (Fig. 3A). This strong nucleophile attacks the CO_2 molecule bound in a hydrophobic pocket in its neighbourhood (the elusive substrate-binding site comprises residues Val121, Val143 and Leu198 in the case of the human isozyme CA II) (Fig. 3B), leading to the formation of bicarbonate coordinated to Zn(II) (Fig. 3C). The bicarbonate ion is then displaced by a water molecule and liberated into solution, leading to the acid form of the enzyme, with water coordinated to Zn(II) (Fig. 3D), which is catalytically inactive.[1–8] In order to regenerate the basic form A, a proton transfer reaction from the active site to the environment takes place, which may be assisted either by active site residues (such as His64—the proton shuttle in isozymes I, II, IV, VI, VII, IX and XII–XIV among others) or by buffers present in the medium. The process may be schematically represented by Eqs. 1.10 and 1.11 below:

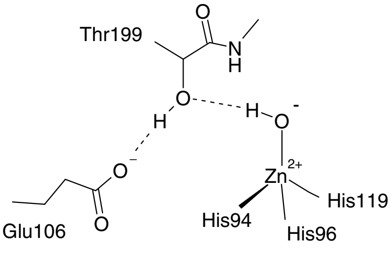


Figure 2. The Zn(II) ion coordination in the hCA II active site, with the three histidine ligands (His94, His96 and His119, isozyme I numbering) and the gate-keeping residues (Thr199 and Glu106) shown.

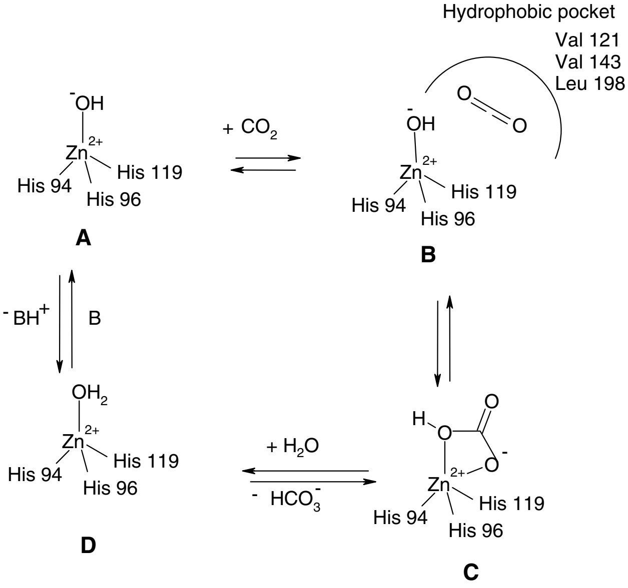


Figure 3. Schematic representation of the catalytic mechanism for the α-CA catalyzed CO_2 hydration. The hydrophobic pocket for the binding of substrate(s) is shown schematically at step (B).

$$EZn^{2+}-OH^{-}+CO_2 \Longleftrightarrow EZn^{2+}-HCO_3^{-}$$

$$\overset{H_2O}{\Longleftrightarrow} EZn^{2+}-OH_2+HCO_3^{-} \tag{1.10}$$

$$EZn^{2+}-OH_2 \Longleftrightarrow EZn^{2+}-HO^{-}+H^{+} \tag{1.11}$$

The rate limiting step in catalysis is the second reaction, that is, the proton transfer that regenerates the zinc-hydroxide species of the enzyme.[1–8,19] In the catalytically very active isozymes, such as CA II, CA IV, CA VII and CA IX, the process is assisted by a histidine residue placed at the entrance of the active site (His64), as well as by a cluster of histidines, which protrudes from the rim of the active site to the surface of the enzyme, assuring thus a very efficient proton transfer process for the most efficient CA isozyme, CA II.[19] This also explains why CA II is one of the most active enzymes known (with a $k_{cat}/K_m = 1.5 \times 10^8\ M^{-1}\ s^{-1}$), approaching the limit of diffusion control, and also has important consequences for the design of inhibitors with clinical applications.[1–8,19]

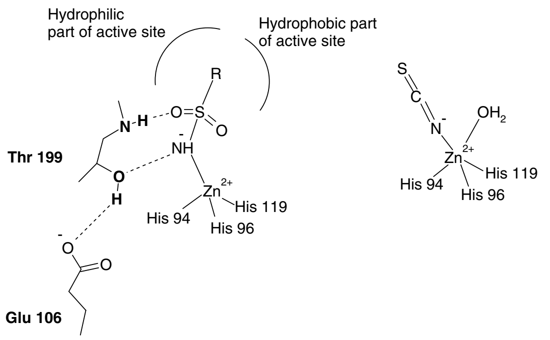
Two main classes of CA inhibitors (CAIs) are known: the metal complexing anions, and the unsubstituted sulfonamides, which bind to the Zn(II) ion of the enzyme either by substituting the non-protein zinc ligand (Eq. 1.12 in Fig. 4) or add to the metal coordination sphere (Eq. 1.13 in Fig. 4), generating trigonal–bipyramidal species.[1–8,20] Sulfonamides, which are the most important CAIs (such as the clinically used derivatives acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, dichlorophenamide **4**, dorzolamide 5 and brinzolamide **6**),[1] bind in a tetrahedral geometry of the Zn(II) ion (Fig. 4), in deprotonated state, with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II) and an extended network of hydrogen bonds, involving residues Thr199 and Glu106, also participating to the anchoring of the inhibitor molecule to the metal ion.

$$\text{E-Zn}^{2+}\text{-OH}_2 + \text{I} \Leftrightarrow \text{E-Zn}^{2+}\text{-I} + \text{H}_2\text{O} \quad \text{(substitution)} \qquad (1.12)$$

Tetrahedral adduct

$$\text{E-Zn}^{2+}\text{-OH}_2 + \text{I} \Leftrightarrow \text{E-Zn}^{2+}\text{-OH}_2\text{(I)} \quad \text{(addition)} \qquad (1.13)$$

Trigonal-bipyramidal adduct



Tetrahedral adduct (sulfonamide)

A

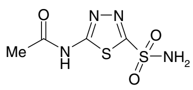
Trigonal-bipyramidal adduct (thiocyanate)

B

Figure 4. α-CA inhibition mechanism by sulfonamide (A) and anionic (B) inhibitors. In the case of sulfonamides, in addition to the Zn(II) coordination, an extended network of hydrogen bonds ensues, involving residues Thr199 and Glu106, whereas the organic part of the inhibitor (R) interacts with hydrophilic and hydrophobic residues of the cavity. For anionic inhibitors such as thiocyanate (B) the interactions between inhibitor and enzyme are much simpler.

The aromatic/heterocyclic part of the inhibitor (R) interacts with hydrophilic and hydrophobic residues of the cavity. Anions may bind either in tetrahedral geometry of the metal ion or as trigonal–bipyramidal adducts, such as for instance the thiocyanate adduct shown in Figure 4B.[1,20] Inhibition data against all catalytically active human isozymes with some of the clinically used compounds (such as the sulfonamides **1**–**6**, or the sulfamate **7**) and the investigational sulfamide derivative **8** are provided in Table 2.

X-ray crystallographic structures are available for many adducts of sulfonamide/sulfamate/sulfamide inhibitors with isozymes CA I, II and IV.[20–26] In all these adducts, the deprotonated sulfonamide/sulfamate/sulfamide is coordinated to the Zn(II) ion of the enzyme, and its NH moiety participates in a hydrogen bond with the Oγ of Thr199, which in turn is engaged in another hydrogen bond to the carboxylate group of Glu106.[20–27] One of the oxygen atoms of the SO_2NH moiety also participates in a hydrogen bond with the backbone NH moiety of Thr199. Examples of various adducts of such inhibitors with CA II are provided for sulfonamide, sulfamate and sulfamide inhibitors (Figs. 5–7).



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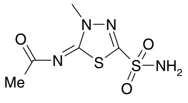

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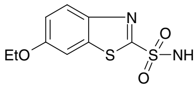

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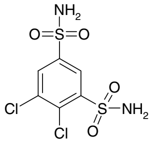

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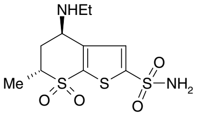

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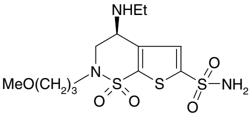

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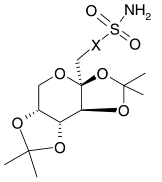


7: X = O
8: X = NH

The different types of interactions by which a sulfonamide CAI achieves very high affinity (in the low nanomolar range) for the CA active site are illustrated in Figures 5–7 for a fluorocontaining sulfonamide inhibitor, **PFMZ**,[21] a steroid sulfamate CA inhibitor, **EMATE**,[26] the antiepileptic drug topiramate **7** and its sulfamide analogue, compound **8**.[27] Thus, potent CAIs may be designed using either sulfonamide, sulfamate or sulfamide zinc-anchoring groups.[21,26,27] For example,

Table 2. Inhibition data with the clinically used sulfonamides **1-6**, the clinically used sulfamate **7** (topiramate) and the topiramate sulfamide analogue **8**, against isozymes I–XIV

Isozyme[c]	K_I (nM)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
hCA I[a]	250	50	25	1200	50,000	45,000	250	3450
hCA II[a]	12	14	8	38	9	3	10	2135
hCA III[a]	3.10^5	1.10^5	5000	nt	8000	nt	nt	nt
hCA IV[a]	74	6200	93	15,000	8500	nt	4900	941
hCA VA[a]	63	65	25	630	42	50	63	32
hCA VB[a]	54	62	19	21	33	30	30	21
hCA VI[a]	11	10	43	79	10	0.9	45	nt
hCA VII[a]	2.5	2.1	0.8	26	3.5	2.8	0.9	35
hCA IX[b]	25	27	34	50	52	37	58[d]	4580
hCA XII[b]	5.7	3.4	22	50	3.5	3.0	3.8	1875
mCA XIII[a]	17	19	nt	23	18	nt	47	30
hCA XIV[a]	41	43	25	345	27	24	1460	25

[a] Full length enzyme.
[b] Catalytic domain.
[c] h, human; m, murine isozyme.
[d] The datum against the full length enzyme is of 1590 nM. nt, not tested (no data available).

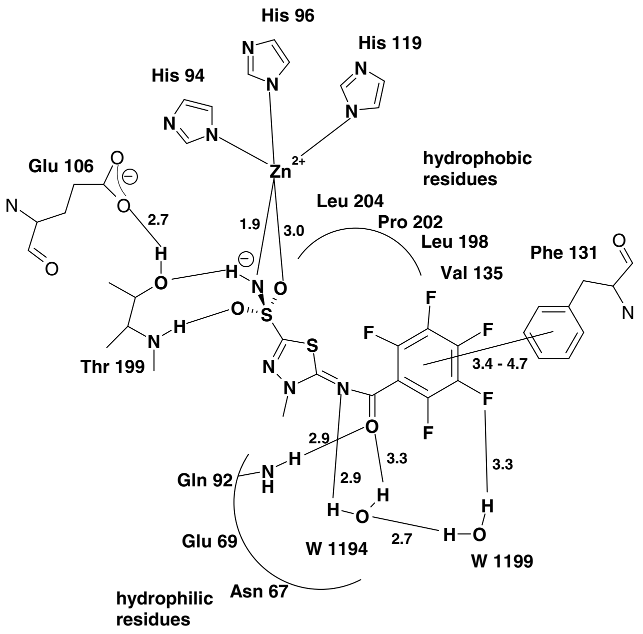


Figure 5. Schematic representation of the pentafluorobenzoyl analogue of methazolamide (**PFMZ**) bound within the hCA II active site (figures represent distances in Å).[21]

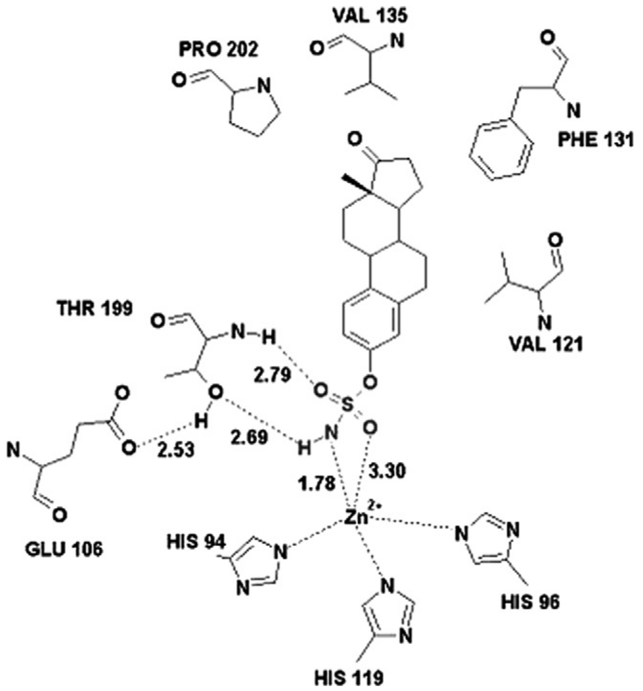


Figure 6. Binding of the sulfamate CA inhibitor **EMATE** to hCA II.[26]

it may be observed that for the sulfonamide compound **PFMZ**, the ionized sulfonamide moiety has replaced the hydroxyl ion coordinated to Zn(II) in the native enzyme (Zn–N distance of 1.95 Å), with the metal ion remaining in its stable tetrahedral geometry, being coordinated in addition to the sulfonamidate nitrogen, by the imidazolic nitrogens of His94, His96 and His119. The proton of the coordinated sulfonamidate nitrogen atom also makes a hydrogen bond with the hydroxyl group of Thr 199, which in turn accepts a hydrogen bond from the carboxylate of Glu106. One of the oxygen atoms of the sulfonamide moiety makes a hydrogen bond with the backbone amide of Thr199, whereas the other one is semi-coordinated to the catalytic Zn(II) ion (O–Zn distance of 3.0 Å). The thiadiazoline ring of the inhibitor lies in the hydrophobic part of the active site cleft, where its ring atoms make van der Waals interactions with the side chains of Leu204, Pro202, Leu198 and Val135 (Figs. 5–7). The carbonyl oxygen of **PFMZ** makes a strong hydrogen bond with the backbone amide nitrogen of Gln92 (of 2.9 Å), an interaction also evidenced for the acetazolamide–hCA II adduct. Besides Gln92, two other residues situated in the hydrophilic half of the CA active site, that is, Glu 69 and Asn67, make van der Waals contacts with the **PFMZ** molecule complexed to hCA II. But the most notable and unprecedented interactions evidenced in this complex regard the hydrogen bond network involving the exocyclic nitrogen atom of the inhibitor, two water molecules (Wat1194 and Wat1199) and a fluorine atom in *meta* belonging to the perfluorobenzoyl tail of **PFMZ** (Fig. 5). Thus, a strong hydrogen bond (of 2.9 Å) is evidenced between the imino nitrogen of **PFMZ** and Wat1194, which in turn makes a hydrogen bond with a second water molecule of the active site, Wat1199 (with a distance of 2.7 Å). The second hydrogen of Wat1194 also participates in a weaker hydrogen bond (3.3 Å) with the carbonyl oxygen of **PFMZ**. The other hydrogen atom of Wat1199 makes a weak hydrogen bond with the fluorine atom in position 3 of the perfluorobenzoyl tail of **PFMZ** (Fig. 5). Finally, a very interesting interaction has been observed between the perfluorophenyl ring of **PFMZ** and the phenyl moiety of Phe131, a residue critical for the binding of inhibitors with long tails to hCA II.[1–3] Indeed, these two rings are almost perfectly parallel, being situated at a distance of 3.4–4.7 Å. This type of stacking interactions has never been observed in a hCA II–sulfonamide adduct. Similar interactions are also observed for the sulfamates (**EMATE** and topiramate **7**) or sulfamide (**8**) type of CA inhibitors (Figs. 6 and 7).[26,27]

Four systemic sulfonamide CAIs have been developed and used clinically mainly as antiglaucoma drugs, for a long time: acetazolamide (**1**), methazolamide (**2**), ethoxzolamide (**3**) and dichlorophenamide (**4**).[1] As seen from data of Table 2, compounds **1**–**8** strongly inhibit most CA isozymes (such as CA I, CA II, CA IV, CA V, CA VII, CA IX and CA XII–XIV), with affinities in the low nanomolar range for many of them. In the last decade, two new drugs have been introduced in clinical practice, as topically acting sulfonamide CAIs, that is, dorzolamide **5** and brinzolamide **6**, which also act as very potent inhibitors of most α-CA isozymes (Table 2). The drug design of sulfonamide CAIs has recently been reviewed.[28]

2.2. β-CAs

Many species belonging to *Bacteria*, some *Archaea* (such as *Methanobacterium thermoautotrophicum*), algae and

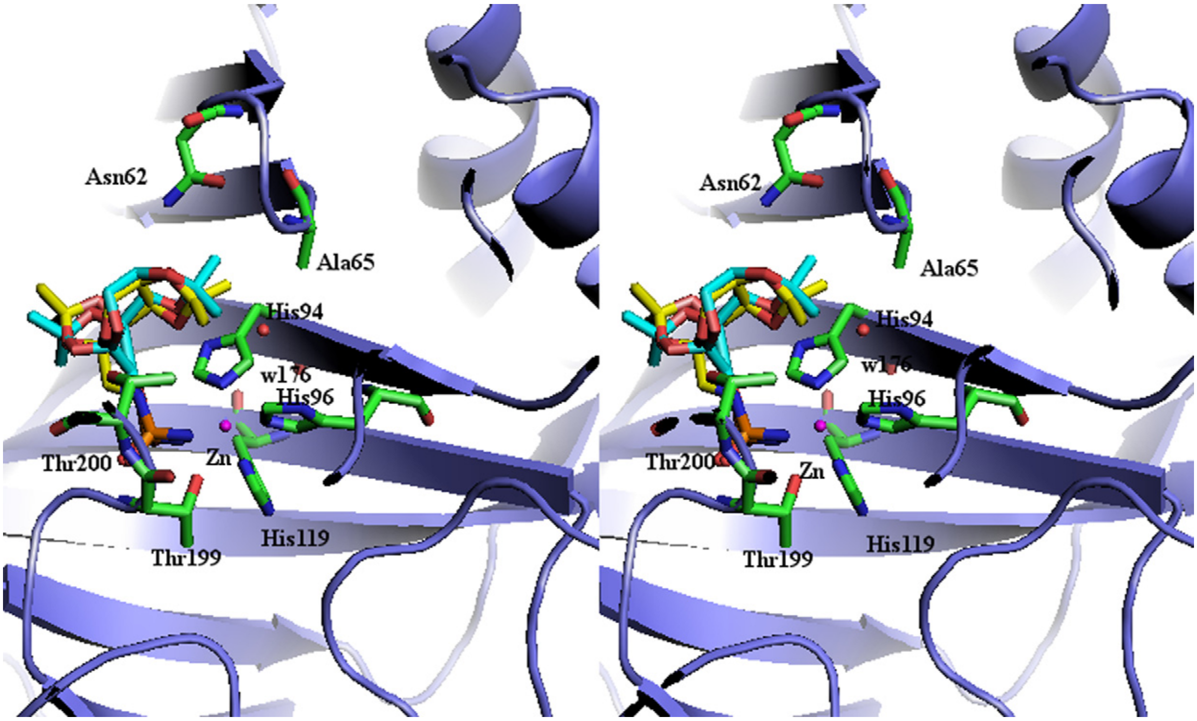


Figure 7. A stereo view of superposition of the hCA II–topiramate **7** (light blue) and hCA II–**8** (yellow) adducts (the Zn(II) ion is the magenta sphere). The protein molecules are superimposed by least square fitting of the first amino acid (His4) and the last (Phe260) amino acid residues (R_{msd} = 0.27). The two zinc-binding groups are highly superposable, whereas the main differences between the two adducts concern the C8 and C9 methyl groups and the corresponding dioxolane rings, which show different orientation in the hCA II–**8** adduct due to steric hindrance and clash with the methyl group of Ala65.[27]

the chloroplasts of superior plants contain CAs belonging to the β-class.[4,14–16] The principal difference between these enzymes and the α-CAs discussed above consists in the fact that usually the β-CAs are oligomers, generally formed of 2–6 monomers of molecular weight of 25–30 kDa. The X-ray structures of four such β-CAs are available at this moment: the enzyme isolated from the red alga *Porphyridium purpureum*, the enzyme from chloroplasts of *Pisum sativum*, another prokaryotic enzyme, this time isolated from *Escherichia coli*, and 'cab', an enzyme isolated from the archaeon *M. thermoautotrophicum*.[4,14–18,29,30]

The *Po. purpureum* CA monomer is composed of two internally repeating structures, being folded as a pair of fundamentally equivalent motifs of an α/β domain and three projecting α-helices. The motif is very distinct from that of either α- or γ-CAs. This homodimeric CA appeared like a tetramer with a pseudo 2-2-2 symmetry.[18] β-CAs are thus very different from the α-class enzymes. The Zn(II) ion is essential for catalysis in both families of enzymes, but its coordination is different and rather variable for the β-CAs: thus, in the prokaryotic β-CAs the Zn(II) ion is coordinated by two cysteinate residues, an imidazole from a His residue and a carboxylate belonging to an Asp residue (Fig. 8A), whereas the chloroplast enzyme has the Zn(II) ion coordinated by the two cysteinates, the imidazole belonging to a His residue and a water molecule (Fig. 8B).[16–18] The polypeptide chain folding and active site architecture is obviously very different from those of the CAs belonging to the α-class.

Since no water is directly coordinated to Zn(II) for some members of the β-CAs (Fig. 8A), the main problem is whether the zinc-hydroxide mechanism presented in this

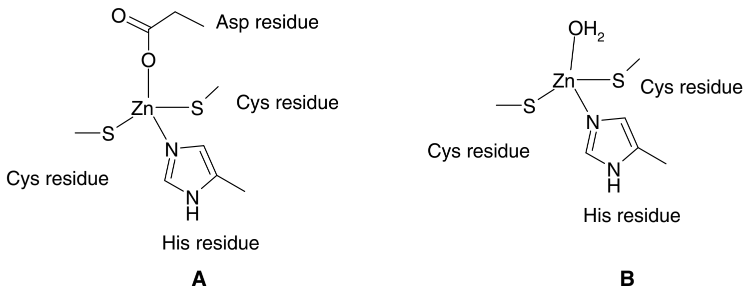


Figure 8. Schematic representation of the Zn(II) coordination sphere in β-CAs: (A) *Porphyridium purpureum*[18] and *Escherichia coli*[14] enzymes; (B) *Pisum sativum* chloroplast and *Methanobacterium thermoautotrophicum* enzyme,[15] as determined by X-ray crystallography.

chapter for the α-CAs is valid also for enzymes belonging to the β-family? A response to this question has been given by Mitsuhashi et al.[18] who have proposed the catalytic mechanism shown in Figure 9.

As there are two symmetrical structural motifs in one monomer of the *Po. purpureum* enzyme, resulting from two homologous repeats which are related to each other by a pseudo twofold axis, there are two Zn(II) ions coordinated by the four amino acids mentioned above. In this case these pairs are: Cys149/Cys403, His205/His459, Cys208/Cys462 and Asp151/Asp405.[18] A water molecule is also present in the neighbourhood of each metal ion, but it is not directly coordinated to it, forming a hydrogen bond with an oxygen belonging to the zinc ligand Asp151/Asp405 (Fig. 9A). It is hypothesized that a proton transfer reaction may occur from this water molecule to the coordinated carboxylate moiety of the aspartate residue, with generation of a hydroxide ion which may be then coordinated to Zn(II) which acquires a trigonal–bipyramidal geometry (Fig. 9B). Thus, the strong nucleophile which may attack CO_2 bound within a hydrophobic pocket of the enzyme is formed (Fig. 9C), with generation of bicarbonate bound to Zn(II) (Fig. 9D). This intermediate is rather similar with the reaction intermediate proposed for the α-CA catalytic cycle (Fig. 3C), except that for the β-class enzyme, the aspartic acid residue originally coordinated to zinc is proposed to participate in a hydrogen bond with the coordinated bicarbonate (Fig. 9D). In the last step, the coordinated bicarbonate is released in solution, together with a proton (no details regarding this proton transfer process are available), the aspartate generated re-coordinates the Zn(II) ion, and the accompanying water molecule forms a hydrogen bond with it. The enzyme is thus ready for another cycle of catalysis.

The structure of the β-CA from the dicotyledonous plant *Pi. sativum* at 1.93 Å resolution has also been reported.[16] The molecule assembles as an octamer with a novel dimer of dimers arrangement. The active site is located at the interface between two monomers, with Cys160, His220 and Cys223 binding the catalytic zinc ion and residues Asp162 (oriented by Arg164), Gly224, Gln151, Val184, Phe179 and Tyr205 interacting with acetic acid. The substrate-binding groups have a one to one correspondence with the functional groups in the α-CA active site, with the corresponding residues being closely superimposable by a mirror plane. Therefore, despite differing folds, α- and β-CAs have converged upon a very similar active site design and are likely to share a common mechanism of action.[16]

Cab exists as a dimer with a subunit fold similar to that observed in plant-type β-CAs. The active site zinc ion was shown to be coordinated by the amino acid residues Cys32, His87, and Cys90, with the tetrahedral coordination completed by a water molecule.[29] The major difference between plant- and cab-type β-CAs is in the organization of the hydrophobic pocket (except for the zinc coordination mentioned above). The structure also revealed a Hepes buffer molecule bound 8 Å away from the active site zinc, which suggests a possible proton transfer pathway from the active site to the solvent.[29] No structural data are available at this moment regarding the binding of inhibitors to this type of CAs, except for the fact that acetate coordinates to the Zn(II) ion of the *Pi. sativum* enzyme.[30]

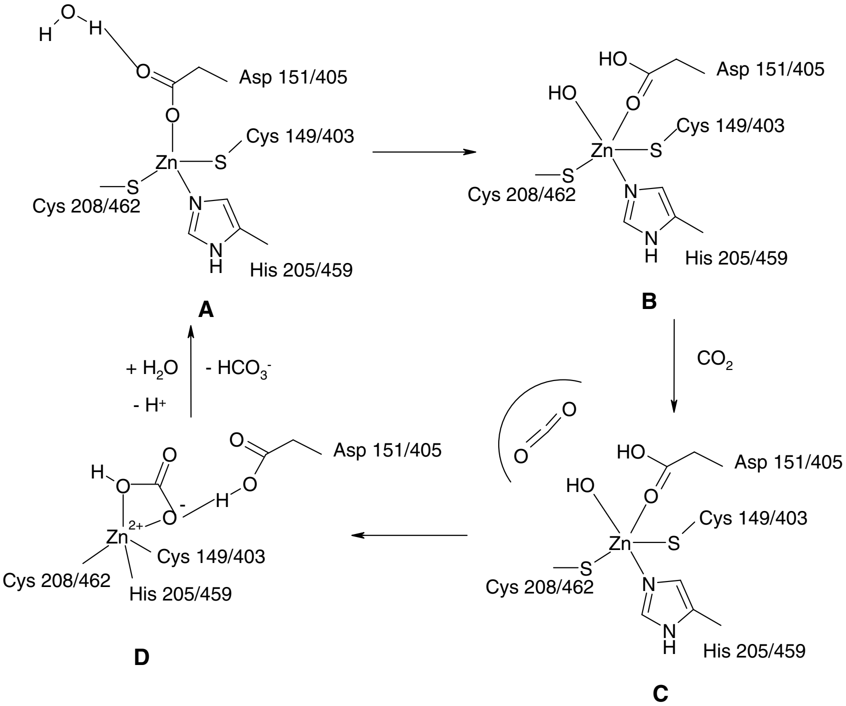


Figure 9. Proposed catalytic mechanism for prokaryotic β-CAs (*Porphyridium purpureum* enzyme numbering).

2.3. γ-CAs

The prototype of the γ-class CAs, 'Cam', has been isolated from the methanogenic archaeon *Methanosarcina thermophila*.[31] The crystal structures of zinc-containing and cobalt-substituted Cam were reported in the unbound form and co-crystallized with sulfate or bicarbonate. Cam has several features that differentiate it from the α- and β-CAs. Thus, the protein fold is composed of a left-handed β-helix motif interrupted by three protruding loops and followed by short and long α-helixes. The Cam monomer self-associates in a homotrimer with the approximate molecular weight of 70 kDa.[31] The Zn(II) ion within the active site is coordinated by three histidine residues, as in α-CAs, but relative to the tetrahedral coordination geometry seen at the active site of α-CAs, the active site of this γ-CA contains additional metal-bound water ligands, so that the overall coordination geometry is trigonal–bipyramidal for the zinc-containing Cam and octahedral for the cobalt-substituted enzyme. Two of the His residues coordinating the metal ion belong to one monomer (monomer A), whereas the third one is from the adjacent monomer (monomer B). Thus, the three active sites are located at the interface between pairs of monomers.[31] The catalytic mechanism of γ-CAs was proposed to be similar to the one presented for the α-class enzymes. Still, the finding that Zn(II) is not tetracoordinated as originally reported but pentacoordinated, with two water molecules bound to the metal ion, demonstrates that much is still to be understood regarding these enzymes. At this moment, the zinc hydroxide mechanism is accepted as being valid for γ-CAs, as it is probable that an equilibrium exists between the trigonal–bipyramidal and the tetrahedral species of the metal ion from the active site of the enzyme.[31]

Ligands bound to the active site were shown to make contacts with the side chain of Glu62 in a manner that suggests this side chain to be probably protonated. In the uncomplexed zinc-containing Cam, the side chains of Glu62 and Glu84 appear to share a proton; additionally, Glu84 exhibits multiple conformations. This suggests that Glu84 may act as a proton shuttle, which is an important aspect of the reaction mechanism of α-CAs, for which a histidine active site residue generally plays this function, usually His64. Anions and sulfonamides were shown to bind to Cam.[32,33]

2.4. δ-CAs

X-ray absorption spectroscopy at the Zn K-edge indicates that the active site of the marine diatom *Thalassiosira weissflogii* CA (TWCA1) is strikingly similar to that of mammalian α-CAs. The zinc has three histidine ligands and a single water molecule, being quite different from the β-CAs of higher plants in which zinc is coordinated by two cysteine thiolates, one histidine and a water molecule.[15] The diatom carbonic anhydrase shows no significant sequence similarity with other carbonic anhydrases and may represent an example of convergent evolution at the molecular level. In the same diatom a rather perplexing discovery has been then made: the first cadmium-containing enzyme, which is a CA-type protein.[7] The marine diatom *T. weissflogii* growing under conditions of low zinc, typical of the marine environment, and in the presence of cadmium salts, led to increased levels of cellular CA activity, although the levels of TWCA1, the major intracellular Zn-requiring isoform of CA in *T. weissflogii*, remained low.[7] ^{109}Cd labelling comigrates with a protein band that showed this CA activity to be distinct from TWCA1 on native PAGE of radiolabelled *T. weissflogii* cell lysates. The levels of the Cd protein were modulated by CO_2 in a manner that was shown to be consistent with a role for this enzyme in carbon acquisition. Purification of the CA-active fraction leads to the isolation of a Cd-containing protein of 43 kDa being clear that *T. weissflogii* expresses a Cd-specific CA, which, particularly under conditions of Zn limitation, can replace the Zn enzyme TWCA1 in its carbon-concentrating mechanism.[7]

3. Distribution of CAs

CAs were shown to be present in a multitude of prokaryotes, where these enzymes play important functions such as respiration, transport of carbon dioxide and photosynthesis.[1–8] The possibility to develop CA inhibitor-based antibiotics/antifungals, by inhibiting bacterial/fungal CAs present in pathogenic species, raised much interest recently.[34–36] Thus, Muhlschlegel's group[34] investigated the ascomycete *Candida albicans* (the most common fungal pathogen in immunocompromised patients) for its ability to change morphology, from yeast to filamentous forms, in response to host environmental cues. Filamentation of this fungus is mediated by second messengers such as cyclic adenosine 3′,5′-monophosphate (cAMP) synthesized by adenylyl cyclase. The distantly related basidiomycete *Cryptococcus neoformans* is an encapsulated yeast that predominantly infects the central nervous system in immunocompromised patients.[34] Similar to the morphological change in *C. albicans*, capsule biosynthesis in *Cr. neoformans* was shown to be dependent upon adenylyl cyclase activity. In a seminal paper, Muhlschlegel's group[34] demonstrated that physiological concentrations of CO_2/bicarbonate (formed due to the reaction catalyzed by CAs present in these organisms) induce filamentation in *C. albicans* by direct stimulation of adenylyl cyclase activity. Furthermore, the CO_2/bicarbonate equilibration by such CAs (belonging to the β-CA family) is essential for pathogenesis of *C. albicans* in niches where the available CO_2 is limited. The adenylyl cyclase from *Cr. neoformans* was also shown to be sensitive to physiological concentrations of CO_2/bicarbonate. Such data were demonstrated by using CA inhibitors, among others. Thus, the link between cAMP signalling and CO_2/bicarbonate sensing is conserved in fungi and revealed CO_2 sensing to be an important mediator of fungal pathogenesis. Novel therapeutic agents, based on inhibition of CAs present in these pathogens, could target such pathways at several levels, in order to control fungal infections.[34]

Helicobacter pylori a Gram-negative neutralophile discovered in the early 1980s was shown to be associated with chronic gastritis, peptic ulcers and more recently, gastric cancer, the second most common tumour in humans.[35,36] Our group cloned and sequenced *H. pylori* α-class carbonic anhydrase (hpCA) from patients with different gastric mucosal lesions, including gastritis, ulcer and cancer.[35,36] Although several polymorphisms were newly identified such as ^{12}Ala, ^{13}Thr, ^{16}Ile and ^{168}Phe, there was no significant relevance of any polymorphism with gastric mucosal lesion types. A library of sulfonamides/sulfamates has been investigated for the inhibition of hpCA, whereas new derivatives have been obtained by attaching 4-*tert*-butyl-phenylcarboxamido-/sulfonamido- tails to benzenesulfonamide/1,3,4-thiadiazole -2-sulfonamide scaffolds. All types of activity for inhibition of hpCA have been detected. Dorzolamide and simple 4-substituted benzenesulfonamides were weak inhibitors (K_Is of 873–4360 nM). Sulfanilamide, orthanilamide, some of their derivatives and indisulam showed better activity (K_Is of 413–640 nM), whereas most of the clinically used inhibitors, such as methazolamide, ethoxzolamide, dichlorophenamide, brinzolamide, topiramate, zonisamide, etc., acted as medium potency inhibitors (K_Is of 105–378 nM). Some potent hpCA inhibitors were detected too (K_Is of 12–84 nM) among acetazolamide, 4-amino-6-chloro -1,3 -benzenedisulfonamide and some newly designed compounds incorporating lipophilic tails. Some of the newly prepared derivatives had selectivity ratios for inhibiting hpCA over hCA II in the range of 1.25–3.48, showing thus some selectivity for inhibiting the bacterial enzyme. Since hpCA is essential for the survival of the pathogen in acid, it might be used as a new pharmacologic tool in the management of drug resistant *H. pylori*.[35,36]

The recent report of parasitic CAs by Krungkrai et al.,[37] who discovered the presence of at least two different α-CAs in *Plasmodium falciparum*, one of the malaria provoking protozoas, opens new vistas to the development of pharmacological agents based on CA inhibitors. Red cells infected by *P. falciparum* ontained CA amounts approximately two times higher than those of normal red cells. The enzyme was then purified to homogeneity, showing a M_r of 32 kDa, being active in monomeric form. The parasite enzyme activity was sensitive to well-known sulfonamide-CA inhibitors such as sulfanilamide and acetazolamide.[37] A series of aromatic sulfonamides, most of which were Schiff's bases derived from sulfanilamide/homosulfanilamide/4-aminoethylbenzenesulfonamide and substituted-aromatic aldehydes, or such ureido-substituted sulfonamides, were investigated for in vitro inhibition of the malarial parasite enzyme (pfCA) and the growth of *P. falciparum*. Several inhibitors with affinity in the micromolar range (K_Is in the range of 0.080–1.230 μM) were detected, whereas the most potent such derivatives were the clinically used sulfonamide CA inhibitor acetazolamide, and 4-(3,4-dichlorophenyl-ureidoethyl)-benzenesulfonamide, which showed an inhibition constant of 80 nM against pfCA, being four times more effective an inhibitor as compared to acetazolamide (K_I of 315 nM). The lipophilic 4-(3, 4-dichlorophenylureido-ethyl)-benzenesulfonamide was also an effective in vitro inhibitor for the growth of *P. falciparum* (IC_{50} of 2 μM), whereas acetazolamide achieved the same level of inhibition at 20 μM. This was the first study proving that antimalarials possessing a novel mechanism of action can be obtained, by inhibiting a critical enzyme for the life cycle of the parasite.[37] Indeed, by inhibiting pfCA, the synthesis of pyrimidines mediated by carbamoylphosphate synthase is impaired in *P. falciparum* but not in the human host. Sulfonamide CA inhibitors have the potential for the development of novel antimalarial drugs.[37]

In higher plants, algae and cyanobacteria, all members of the three CA families were found to be present.[5,38] For example, analysis of the *Arabidopsis* genome revealed that at least 14 different CAs are present in this plant, whereas in the unicellular green alga *Chlamydomonas reinhardtii*, six such enzymes are present.[5,38] In algae, CAs were found in mitochondria, the chloroplast thylakoid, cytoplasm and periplasmic space.[5] In C_3 dicotyledons two types of CAs have been isolated, one in the chloroplast stroma and one in cytoplasm, whereas in C_4 plants these enzymes are present in the mesophyl cells, where they provide bicarbonate to phosphoenolpyruvate (PEP) carboxylase, the first enzyme involved in CO_2 fixation into C_4 acids.[5] In CAM (crassulacean acid metabolism) plants CAs are quite abundant, being probably present in the cytosol, and very abundant in chloroplasts, where they participate in CO_2 fixation, providing bicarbonate to PEP carboxylase.[5] These enzymes are highly abundant in the terrestrial vegetation and seem to be correlated with the content of atmospheric CO_2, and thus with the global warming processes.

In animals, and more specifically mammals, CAs are quite widespread, as mentioned throughout this paper. Since this field has recently been reviewed the reader is invited to consult these particular papers for a detailed overview on the distribution and function of CAs in these organisms.[1,39]

4. Physiological functions of CAs, their inhibition and medicinal chemistry applications

It is not clear whether other reactions catalyzed by CAs (Fig. 1) except for CO_2 hydration/bicarbonate dehydration have physiological relevance.[2,3] Thus, presently, only reaction 1.1 is considered to be the physiological one in which these enzymes are involved.

In prokaryotes, as shown also in the preceding sections, CAs possess two general functions: (i) transport of CO_2/bicarbonate between different tissues of the organism; (ii) provision of CO_2/bicarbonate for enzymatic reactions.[4] In aquatic photosynthetic organisms, an additional role is that of a CO_2-concentrating mechanism, which helps overcome CO_2 limitation in the environment.[5,38] For example, in *Chlamydomonas reinhardtii* this CO_2-concentrating mechanism is maintained by the pH gradient created across the chloroplast thylakoid membranes by photosystem II-mediated electron transport processes.[38] A large number of non-photosynthetic prokaryotes catalyze reactions for which CA could be expected to provide CO_2/bicarbonate in the vicinity of the active site, or to remove such compounds in order to improve the energetics of the reaction.[4] The large number of carboxylation/decarboxylation processes in which prokaryotic CAs may play such an important physiological function has recently been reviewed by Smith and Ferry, in an excellent paper.[4]

In vertebrates, including *Homo sapiens*, the physiological functions of CAs have widely been investigated over the last 70 years, but much is still to be learned about this large family of metalloenzymes.[1,6,15,40] Thus, isozymes I, II and IV are involved in respiration and regulation of the acid/base homeostasis.[1] These complex processes involve both the transport of CO_2/bicarbonate between metabolizing tissues and excretion sites (lungs, kidneys), facilitated CO_2 elimination in capillaries and pulmonary microvasculature, elimination of H^+ ions in the renal tubules and collecting ducts, as well as reabsorption of bicarbonate in the brush border and thick ascending Henle loop in kidneys.[1] Usually, isozymes I, II and IV are involved in these processes. By producing the bicarbonate-rich aqueous humor secretion (mediated by ciliary processes isozymes CA II, CA IV and CA XII) within the eye, CAs are involved in vision, and their misfunctioning leads to high intraocular pressure, and glaucoma.[1] CA II is also involved in the bone development and function, such as the differentiation of osteoclasts, or the provision of acid for bone resorption in osteoclasts. CAs are involved in the secretion of electrolytes in many other tissues/organs, such as: CSF formation, by providing bicarbonate and regulating the pH in the choroid plexus; saliva production in acinar and ductal cells; gastric acid production in the stomach parietal cells; bile production, pancreatic juice production, intestinal ion transport.[1,39] CAs are also involved in gustation and olfaction, protection of gastro-intestinal tract from extreme pH conditions (too acidic or too basic), regulation of pH and bicarbonate concentration in the seminal fluid, muscle functions and adaptation to cellular stress. Some isozymes, such as CA V, are involved in molecular signalling processes, such as insulin secretion signalling in pancreas β cells.[1,39] Isozymes II and VA are involved in important metabolic processes, as they provide bicarbonate for gluconeogenesis, fatty acids de novo biosynthesis or pyrimidine base synthesis.[1] Finally, some isozymes (such as CA IX, CA XII, CARP VIII) are highly abundant in tumours, being involved in oncogenesis and tumour progression.[40–42]

Although the physiological function of some mammal isozymes (CA I, CA III, CA VI, the three CARPs) is still unclear, from the data presented above one may understand the importance of CAs for a host of physiological processes, both in normal and pathological states. This may explain why inhibitors of these enzymes found a place in clinical medicine already in 1954, with acetazolamide (**1**) the first non-mercurial diuretic agent used clinically.[1] Presently, inhibitors of these enzymes are widely used clinically as antiglaucoma agents, diuretics, antiepileptics, in the management of mountain sickness, gastric and duodenal ulcers, neurological disorders, or osteoporosis among others.[1–3] The development of more specific agents is stringently needed, also because of the high number of isozymes present in the human body, as well as the isolation of a large number of new representatives of the CAs in all kingdoms of living organisms. This is possible only by understanding in detail the catalytic and inhibition mechanisms of these enzymes. In fact, presently much research is devoted on at least five fronts in the drug design of pharmacological agents belonging to this class: (i) antiglaucoma drugs with better profiles than dorzolamide and brinzolamide.[1] The target isozymes of such compounds are probably CA II and XII;[1] (ii) anticancer drugs targeting primarily CA IX and/or CA XII, isozymes predominantly present in tumour cells;[40–42] (iii) antiobesity agents, based on the reported such effects of the strong CA inhibitors topiramate[22] and zonisamide,[23] which probably target the mitochondrial isoforms CA VA and/or CA VB; (iv) anticonvulsants (probably targeting CA II, VII, XII and XIV); and (v) antibacterials, antifungals and other types of such agents that target various CAs from pathogenic organisms such as the bacteria *H. pylori*, *Mycobacterium tuberculosis*, etc., the protozoa *P. falciparum* or the fungi *C. albicans*, and *Cr. neoformans* among others.[33–38,43] In fact the first proof-of-concept study regarding the fact that the β-CA from *H. pylori* can be a drug target (similarly with all the α-CAs mentioned in this review) has very recently been reported.[43] The conclusion of this review is that these enzymes and their inhibitors are indeed remarkable: after many years of intense research in this field, they continue to offer interesting opportunities for the development of novel drugs, new diagnostic tools, or for understanding in greater depth fundamental processes of the life sciences. The discovery of many new such CAs in various organisms all over the phylogenetic tree is also a clear signal that these ancient enzymes are involved in critical life processes and that perturbation of their activity may lead to novel ways of controlling widespread diseases.

5. Selectivity issues in the design of CAIs

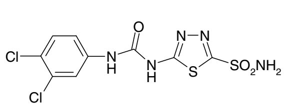
Although many sulfonamide/sulfamate CAIs possess high affinity for the major mammalian isozymes considered to play important physiological functions (such as CA II, CA VA, CA VB, CA VII, CA IX or CA XII—see Table 2 for derivatives **1**–**8**),[1–3,27] the critical challenge for the design of novel pharmacological agents from this class is constituted by the lack of specificity of such compounds towards the different targeted isozymes.[1–3] Among the 16 mammalian isozymes described up to now, the ubiquitous CA II has very high affinities for most such sulfonamide/sulfamate inhibitors (Table 2), and the main challenge consists in finding derivatives that should not act as very strong inhibitors of this isozyme, at the same time preserving good affinity for another target CA, such as for example CA VA, CA VB, CA VII, CA IX, CA XII, etc. The same is true for finding inhibitors with higher affinity to CA I, as compared to the sulfonamide-avide isozyme II, mainly because the physiological function of CA I is still a mystery, although this protein is very abundant in many mammals, including humans.[1,3] Things are relatively simpler for CA III, which is a 'sulfonamide-resistant' isozyme, although it is inhibited well by small compounds such as $CF_3SO_2NH_2$.[1] Some progress has been registered recently in the design of compounds with some selectivity towards various CAs, and these data will be reviewed in this section.

5.1. Isozyme I

The main difference in the active site architecture of isozymes CA I and CA II regards the presence of a higher number of histidine residues in the first isozyme. Thus, in addition to the zinc ligands (His94, His96 and His119), as shown in the introductory section, His64 plays an important role in catalysis. This is the only other histidine residue present in the active site of CA II, whereas in CA I there are three additional such residues, His67, His200 and His243.[19] Another important difference between the two isozymes is that CA II contains a histidine cluster, consisting of residues: His64, His4 (these two residues possess a flexible conformation in the crystal structure), His3, His10, His15 and His17 (prolonging from the middle of the active site to the rim of the cavity, and protruding on the surface of the protein),[19] which is absent in CA I. These two isozymes also possess a different affinity for the two main classes of inhibitors: CA I has larger affinity than CA II for anions (such as cyanide, thiocyanate, cyanate, halides, etc.), whereas CA II has generally a higher affinity for sulfonamides as compared to CA I (see also Table 2).[1,27] As a consequence, it is relatively difficult to obtain sulfonamide inhibitors with higher affinity for CA I than for CA II, although the two isozymes possess significant differences in the active site architecture. The first such compounds were only recently reported by this group,[44–47] and were discovered serendipitously, by screening of a large number of sulfonamides possessing different structural motifs in their molecules. Remarkably, all the compounds possessing higher affinity for CA I as compared to CA II (and CA IV), of types **9**–**16**, contain ureido or thioureido moieties in their molecules. Their inhibition data against the three isozymes mentioned above are shown in Table 3.

Table 3. Inhibition of isozymes I, II and IV with compounds **9**–**21** showing selectivity towards one of these isoforms

Inhibitor	K_I (nM)		
	hCA I	hCA II	bCA IV
9	3	6	8
10	50	53	70
11	7	10	24
12	3	8	20
13	4	10	25
14	8	12	14
16	4	5	11
16a (X = 4-Me)	40	110	120
16b (X = 4-Cl)	60	100	160
17	1100	150	140
18	200	20	10
19	200	10	8
20	620	12	10
21	180	15	12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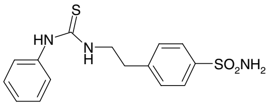
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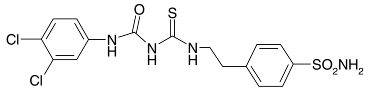
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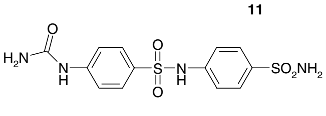
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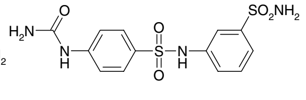
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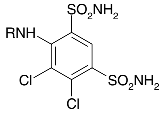
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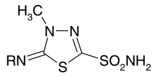
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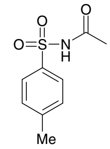
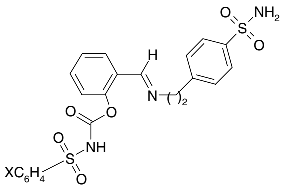
In **14**,**15** R =

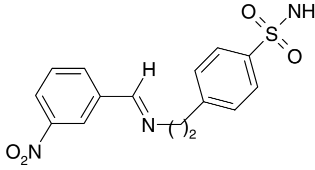



16: X = H, p-Me; o-Me; p-F; p-Cl

Such isozyme I avid inhibitors belong both to the aromatic sulfonamide class, as well as to the heterocyclic sulfonamide class, whereas the ureido/thioureido moieties present in their molecules may be unsubstituted or substituted with bulkier groups (3,4-dichlorophenyl; phenyl; substituted-phenylsulfonyl, etc.). It must also be mentioned that compounds **16** containing arylsulfonylcarbamate moieties instead of the arylsulfonylureido ones were investigated in more details.[44–47] These compounds also inhibit significantly isozymes II and IV, and thus, they are not really isozyme-I specific, but represent anyhow an important step towards the generation of isozyme specific CAIs. It must also be noted that dorzolamide (**5**) has a very low affinity for hCA I, but its deethylated metabolite is a very potent inhibitor of this isozyme.[1,27]

5.2. Isozyme IV

Isozyme CA IV contains only one histidine residue within its active site, His 64, which, as in hCA II, plays a critical role in catalysis, as proton shuttle residue between the active site and the environment.[1,12] The most characteristic feature of the active site of this isozyme is related to the presence of four cysteine residues, which form two disulfide bonds, situated at the entrance within the cavity (Cys 6–Cys 11G, and Cys 23–Cys 203, respectively).[12] These residues occupy practically the same region of the active site as the histidine cluster in hCA II,[19] and it was hypothesized that this might be the most relevant aspect explaining the difference in affinity for sulfonamide inhibitors of these two isozymes.[48] Even so, similarly to CA I, the first compounds with some specificity for CA IV, of type **17**–**21**, were again discovered serendipitously, and they all belong to the same class of Schiff bases of aromatic/heterocyclic sulfonamides.[49–52]



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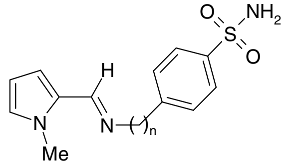
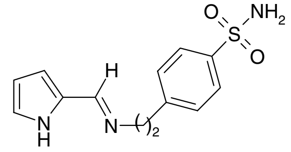


18: n = 1
19: n = 2



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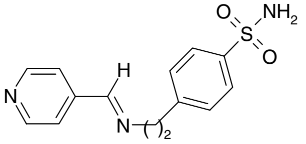

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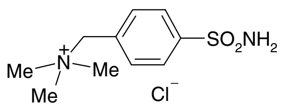


22: QAS

Only Schiff bases of aromatic sulfonamides were investigated in some detail, and it was shown that best CA IV inhibition patterns are connected with the presence of heterocyclic moieties (in the original aldehyde used for the preparation of the Schiff base), or aromatic moieties substituted with electron attracting groups, such as the nitro one.[49–52] Such compounds also appreciably inhibited CA II, and to a smaller extent CA I (Table 3).[49–52]

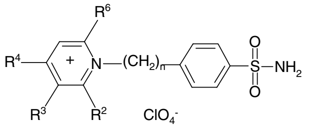
5.3. Isozyme III

Although the structure of this isozyme is relatively similar to that of hCA II, CA III has a CO_2 hydration activity of about 0.3% that of hCA II, as it does not possess a His residue in position 64, but a Lys residue, which is much less effective as a proton shuttle.[1,53] Furthermore, position 198 in CA III is occupied by a Phe, possessing a very bulky side chain, whereas the water bound to Zn(II) has a $\mathrm{p}K_\mathrm{a}$ around 5.5.[1,53] All these particularities may explain the low catalytic activity of CA III, as well as its insensitivity to sulfonamide inhibitors, which do not have space enough to bind in the neighbourhood of the Zn(II) ion, principally due to the steric impairment of Phe198. In fact, only the very small sulfonamide $CF_3SO_2NH_2$ acts as an efficient CA III inhibitor, with an inhibition constant of 0.9 μM (but this compound is a nanomolar inhibitor of CA I, II and IV on the other hand).[1,53] Other sulfonamides (such as acetazolamide, methazolamide, etc.) inhibit CA III, with inhibition constants in the millimolar range (Table 2).[1]

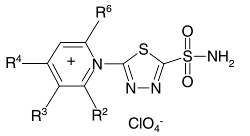
5.4. Selective inhibition of extracellular CAs (CA IV, CA IX, CA XII and CA XIV) with positively charged compounds

At least four CA isozymes (CA IV, CA IX, CA XII and CA XIV) are associated to cell membranes, with the enzyme active site always oriented extracellularly.[1,42] Some of these isozymes were shown to play pivotal physiological roles (such as for example CA IV in the eye, lungs and kidneys, CA IX in the gastric mucosa and many tumour cells; CA XII in tumours and also some normal epithelia)[1,42], whereas the function of other such isozymes (CA XIV) is for the moment less well understood. Due to the extracellular location of these isozymes, it is possible to design membrane-impermeant CAIs, which in this way become specific inhibitors for the membrane-associated CAs. This possibility has been fully explored in this laboratory, by designing positively charged sulfonamides.[48,54–58]

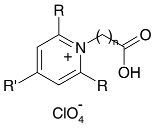
A programme of developing cationic sulfonamides has been initiated in our laboratory, using QAS (**22**) as lead molecule (which is also a relatively weak CAI, with micromolar affinity for CA II).[28] The first such compounds, of types **23**–**26**, were prepared by reaction of aromatic/heterocyclic sulfonamides containing free NH_2 groups with pyrylium salts, affording pyridinium derivatives.[48,54,55] These compounds were moderately active CA II and CA IV inhibitors, with affinities in the 10^{-6}–10^{-7} M range. By using QSAR data of this laboratory,[55] it has been shown that increased CA II and CA IV inhibitory properties of aromatic/heterocyclic sulfonamides are connected with the presence of elongated inhibitor molecules (on the axis passing through the Zn(II) of the enzyme, the sulfonamide nitrogen atom and the long axis of the inhibitor molecule itself). In consequence, such ‘elongated’ molecules have been designed[56–58] by reacting pyrylium salts with amino acids (such as glycine or β-alanine), and coupling of the pyridinium derivatives **27**–**30** with the aromatic/heterocyclic sulfonamides possessing free amino, hydroxy, imino or hydroxyl moieties. The inhibitors obtained in this way, such as for instance **31**–**34**, showed nanomolar affinities both for CA II, as well as CA IV and CA IX, and more importantly, they were unable to cross the plasma membranes in vivo.[48,54–58] In two model systems (human red cells, and perfusion experiments in rats, respectively), this new class of potent, positively charged CAIs was able to discriminate for the membrane-bound versus the cytosolic isozymes, selectively inhibiting only CA IV.[48,54–58] Such data are important both for the specific in vivo inhibition of membrane-associated isozymes, but also for the development of some novel anticancer therapies, since it has been shown that many hypoxic tumour cells predominantly express only some membrane-associated CA isozymes, such as CA IX and CA XII.[42] Indeed, by using such positively charged derivatives of type **25**, Svastova et al.[59] proved that the tumor-associated isozyme CA IX and not lactic acid is the main contributor to acidification of tumours, because its extracellular enzyme domain is highly active, its expression is induced by hypoxia and correlates with poor prognosis. CA IX acidifies pH of the culture medium in hypoxia but not in normoxia, independent of the lactic acid production. Sulfonamide CA IX-selective inhibitors belonging to various classes (among which the positively charged derivatives **24** and **25**) were observed to bind only to hypoxic cells containing CA IX, and to reverse the tumour acidification processes mediated by the enzyme. Since it was previously shown that many sulfonamides possess appreciable tumour cell growth inhibitory properties in vitro and in vivo,[1,28] such findings constituted the proof-of-concept that anticancer therapies based on tumour-associated CA isozyme inhibition can be developed, but also offer interesting tools for investigating hypoxic tumours as well as for their imaging.[59]



23: n = 0
24: n = 1
25: n = 2

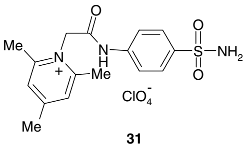


26

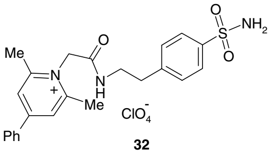


n = 1, 2

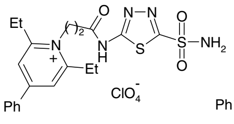
27: R = R' = Me
28: R = Me; R' = Ph
29: R = Et; R' = Ph
30: R = i-Pr; R' = Ph



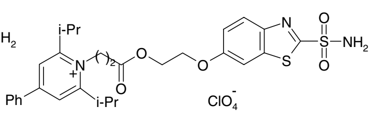
31



32



33



34

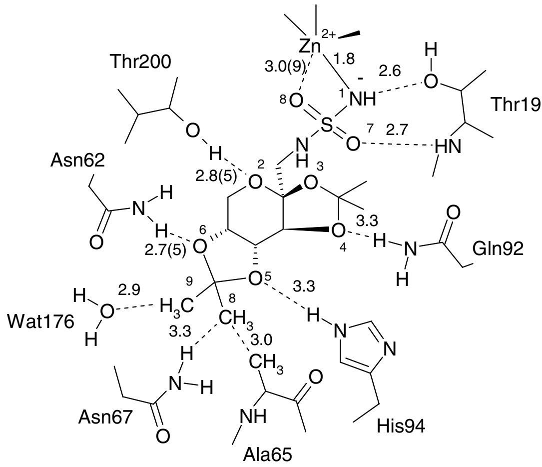


Figure 10. Schematic representation of the interactions of the topiramate sulfamide analogue **8** with the hCA II active site residues. In addition to the favourable interactions in which the inhibitor participates when bound to the hCA II active site, the clash with Ala65 is evidenced, which leads to diminished affinity for CA II and the possibility to design inhibitors with less affinity to this ubiquitous isoform (figures represent distances in Å).[27]

5.5. Clash with Ala65 in CA II active site for designing compounds with low affinity to CA II

The sulfamide analogue **8** of the antiepileptic drug topiramate **7** is a 210 times less potent inhibitor of isozyme CA II as compared to topiramate, but effectively inhibits isozymes CA VA, VB, VII, XIII and XIV (K_Is in the range of 21–35 nM, see Table 2). Its weak binding to CA II was recently shown to be due to a clash between one methyl group of the inhibitor and Ala65, an amino acid residue unique only to the ubiquitous isozyme CA II, and may be exploited for the drug design of compounds with lower affinity for this ubiquitous isozyme.[27] As shown by X-ray crystallography,[27] the sulfamide analogue binds to CA II with the deprotonated sulfamide moiety coordinated to Zn(II), and the organic scaffold making an extended network of hydrogen bonds with Thr199, Gln92, His94, Asn62 and Thr200 (Figs. 7 and 10). In particular, the ionized nitrogen atom of the sulfamide group of **8** is coordinated to the zinc ion at a distance of 1.80 Å, much shorter than the corresponding distance in the topiramate **7** adduct (1.97 Å). The N1 nitrogen is also hydrogen bonded to the hydroxyl group of Thr199 (N–Thr199OG = 2.7 Å), which in turn interacts with the Glu106OE1 atom (2.5 Å). The inhibitor O7 atom is hydrogen bonded to the backbone amide of Thr199 (ThrN–O2 = 2.7 Å), whereas the O8 atom is 3.09 Å away from the catalytic Zn^{2+} ion, being considered as weakly coordinated to the metal ion.[27] All these interactions have also been observed in the adduct of hCA II with topiramate **7**, but the corresponding distances are slightly different.[22] The second NH group of **8**, similarly to the corresponding oxygen atom of topiramate,[22] does not participate in any interaction with the protein or the metal ion. This is another important difference between the sulfamide/sulfamate inhibitors discussed here. The endocyclic sugar oxygen of **8** (O2) makes a hydrogen bond of 2.85 Å with the hydroxyl moiety of Thr200 (the same interaction is present in the topiramate adduct, where the distance is of 2.84 Å).[22] Three oxygen atoms of the dioxolane rings of **8**, that is, O4, O5 and O6, participate in three hydrogen bonds with Gln92, His94 and Asn62, respectively (Fig. 2B). One of them (involving O5) was not observed in the topiramate adduct,[22] whereas the other two are present in both adducts,[22,27] although the distances between the corresponding pairs of atoms are rather different. Thus, the distance between O6 and the amide nitrogen of Asn62 is in the range of 2.7–3.0 Å for the two adducts of hCA II with **8** and **7**, respectively, whereas the interaction with Gln92 is a strong one in the case of topiramate (distance of 2.8 Å) and much weaker in the topiramate sulfamide adduct (distance of 3.3 Å).

However, the most important differences in the structures of the adducts of topiramate **7** and its sulfamide analogue **8** in complex with hCA II regard the C8 and C9 methyl groups of the inhibitors. In the case of the topiramate adduct, the C8 group is in van der Waals contacts (distances of 3.5 Å[22]) with the methyl group of Ala65 and the carboxamide moiety of Asn67. For the topiramate sulfamide **8** adduct the corresponding distances are much shorter (in the range of 3.0–3.3 Å) leading to an important clash between the C8 methyl and the methyl side chain of Ala65. In fact this was the main reason why we experienced difficulties in fitting the electron density of the inhibitor **8** in this region of the active site, which is sterically hindered by the presence of these amino acid side chains (Ala65 and Asn67), and also by the presence of the second methyl group of the inhibitor (C9) and a water molecule with which it is in close contact (Wat176–C9 = 2.9 Å)—Figures 7 and 10.[27] These repulsive interactions were not observed in the hCA II–topiramate **7** adduct,[22] and they constitute the only possible explanation regarding the important differences of activity between the two compounds, with topiramate being approximately 210 times a better hCA II inhibitor as compared to its sulfamide analogue (Table 2).

But how relevant are such findings for the design of better drugs based on CAIs? Obviously, clash interactions between an inhibitor and an amino acid residue within the active site, which may prevent the strong binding of the inhibitor, may be useful to design compounds with selectivity for some isoforms, provided that the interacting residue is present only in isoforms that should be not inhibited. This is especially important for families of enzymes with many isoforms, such as the CAs (in humans 15 CAs are known, 12 of which are catalytically active),[1–3] in which one isozyme is ubiquitous and its inhibition may be deleterious. This happens to be the case with CA II, which is an extremely abundant, house-keeping enzyme in most cells in humans.[1] On the other hand, it is ever more obvious that selective inhibition of other isoforms than CA II, which are involved in specific physiologic/pathologic processes (such as CA IX and XII involved in tumorigenesis,[42] or CA VA and CA VB involved in adipogenesis[60]) or show a restricted localization only in some tissues/organs (such as CA VA, CA VII or CA XIII),[1] may lead to drugs with less severe side effects. A close look at the amino acid sequences of all known human CAs[1] shows that only CA II has Ala in position 65, whereas this amino acid is Ser in CA I, CA IV, CA VB, CA VII, CA IX, CA XII and CA XIII, Thr in CA III, CA VI and CA XIV, and Leu in CA VA. We do not want to imply that just one amino acid substitution may change the binding affinity of an inhibitor for the active site, since it is clear that isozymes possessing Ser65, such as hCA I, hCA IV, hCA IX or hCA XII, are only slightly inhibited by sulfamide **8**, whereas other isoforms possessing the same amino acid, such as CA VB, CA VII or CA XIII, are quite well inhibited by this compound. What we wish to stress is that by resolving the X-ray crystal structure of hCA II in adduct with **8**, we evidenced an amino acid residue which is unique to the hCA II active site and that its clashing with some moieties of an inhibitor may constitute a powerful means for designing more selective CAIs, with weaker binding to this ubiquitous isozyme, but preserving a strong affinity for other isoforms. As far as we know this was the first example in which CA II-sparing inhibitors were evidenced and this fact was explained at molecular level.[27] Such findings may be thus quite useful in designing compounds with less CA II inhibitory activity, but which maintain strong inhibition of clinically relevant isoforms such as CA VA, CA VB, CA IX, CA XII, CA XIII or CA XIV among others.

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