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Review Article

Carbonic Anhydrases: Current State of the Art, Therapeutic Applications and Future Prospects

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Carbonic anhydrases (CAs, EC 4.2.1.1) are wide-spread enzymes, present in mammals in at least 14 different isoforms. Some of these isozymes are cytosolic (CA I, CA II, CA III, CA VII, CA XIII), others are membrane-bound (CA IV, CA IX, CA XII and CA XIV), CA V is mitochondrial and CA VI is secreted in the saliva and milk. Three cytosolic acatalytic forms are also known (CARP VIII, CARP X and CARP XI). The catalytically active isoforms, which play important physiological and patho-physiological functions, are strongly inhibited by aromatic and heterocyclic sulfonamides. The catalytic and inhibition mechanisms of these enzymes are understood in great detail, and this greatly helped the design of potent inhibitors, some of which possess important clinical applications. The use of such CA inhibitors (CAIs) as antiglaucoma drugs are discussed in detail, together with the recent developments that led to isozyme-specific and organ-selective inhibitors. A recent discovery is connected with the involvement of CAs and their sulfonamide inhibitors in cancer: many potent CAIs were shown to inhibit the growth of several tumor cell lines *in vitro* and *in vivo*, thus constituting interesting leads for developing novel antitumor therapies. Future prospects for drug design of inhibitors of these ubiquitous enzymes are dealt with. Although activation of CAs has been a controversial issue for some time, recent kinetic, spectroscopic and X-ray crystallographic experiments offered an explanation of this phenomenon, based on the catalytic mechanism. It has been demonstrated recently, that molecules that act as carbonic anhydrase activators (CAAs) bind at the entrance of the enzyme active site participating in facilitated proton transfer processes between the active site and the reaction medium. In addition to CA II-activator adducts, X-ray crystallographic studies have been also reported for ternary complexes of this isozyme with activators and anion (azide) inhibitors. Structure–activity correlations for diverse classes of activators is discussed for the isozymes for which the phenomenon has been studied, i.e., CA I, II, III and IV. The possible physiological relevance of CA activation/inhibition is also addressed, together with recent pharmacological/biomedical applications of such compounds in different fields of life sciences.

Keywords: Carbonic anhydrase; Isozymes; Inhibitors; Sulfonamide; Clinical application

INTRODUCTION

Among the metalloenzymes extensively studied in the past, the carbonic anhydrases (CAs, EC 4.2.1.1) occupy a special place for several reasons: (i) these enzymes are ubiquitous in all kingdoms, starting with *Archaea*, *Eubacteria*, algae and green plants, and ending with superior animals, including vertebrates;[1–4] (ii) their physiological function is essential for these organisms, as CAs catalyze a fundamental physiological reaction, the interconversion between carbon dioxide and bicarbonate.[1–4] This reaction is critical for respiration and transport of CO_2 between metabolizing tissues and excretion sites, secretion of electrolytes in a variety of tissues and organs, pH regulation and homeostasis, CO_2 fixation (for algae and green plants), several metabolic biosynthetic pathways, such as gluconeogenesis, lipogenesis and ureagenesis, bone resorption, calcification, tumorigenicity

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TABLE I 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	low	medium	cytosol
CA II	high	very high	cytosol
CA III	very low	very low	cytosol
CA IV	high	high	plasma membrane
CA V	moderate-high[#]	high	mitochondria
CA VI	moderate	medium-low	secreted into saliva
CA VII	high	very high	cytosol
CARP VIII	acatalytic	*	cytosol
CA IX	high	high	plasma membrane
CARP X	acatalytic	*	cytosol
CARP XI	acatalytic	*	cytosol
CA XII	low	low	plasma membrane
CA XIII	low	high	cytosol
CA XIV	high	high	plasma membrane

[#]Moderate at pH 7.4, high at pH 8.2 or higher. *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 studies on this subject are available at present.

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(in vertebrates), etc.;[1–3] (iii) inhibition (but also activation) of these enzymes may be exploited clinically in the treatment or prevention of a variety of disorders.[1–3] In consequence, CA inhibitors (CAIs) and to a lesser extent up to now, CA activators (CAAs) possess a variety of applications in therapy.[1–4] In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) have been described (Table I), with very different subcellular localization and tissue distribution.[1–3] Among the active enzymes, there are several cytosolic forms (CA I–III, CA VII, CA XIII), four membrane-bound isozymes (CA IV, CA IX, CA XII and CA XIV), a mitochondrial form (CA V), as well as a secreted CA isozyme (CA VI).[1,2] 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*, Figure 1), CAs catalyze a variety of other reactions, such as: the hydration of cyanate to carbamic acid, or of cyanamide to urea (reactions *2* and *3*); aldehyde hydration to *gem*-diols (reaction *4*); hydrolysis of carboxylic, or sulfonic esters (reactions *5*, *6*), as well as other less investigated hydrolytic processes, such as those described by Equations (*7*–*9*) in Figure 1.[2,4–6] It should be mentioned that the previously reported phosphatase activity of CA III was recently proven to be an artefact.[7] It is unclear at this moment whether CA catalyzed reactions other 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–V and CA XII),[8,9] as well as for the representatives of the β- and γ-CA families.[10]

$O=C=O + H_2O \Leftrightarrow HCO_3^- + H^+$	(*1*)
$O=C=NH + H_2O \Leftrightarrow H_2NCOOH$	(*2*)
$HN=C=NH + H_2O \Leftrightarrow H_2NCONH_2$	(*3*)
$RCHO + H_2O \Leftrightarrow RCH(OH)_2$	(*4*)
$RCOOAr + H_2O \Leftrightarrow RCOOH + ArOH$	(*5*)
$RSO_3Ar + H_2O \Leftrightarrow RSO_3H + ArOH$	(*6*)
$ArF + H_2O \Leftrightarrow HF + ArOH$	(*7*)
(Ar = 2,4-dinitrophenyl)	
$PhCH_2OCOCl + H_2O \Leftrightarrow PhCH_2OH + CO_2 + HCl$	(*8*)
$RSO_2Cl + H_2O \Leftrightarrow RSO_3H + HCl$	(*9*)
(R = Me; Ph)	

FIGURE 1 Reactions catalyzed by α-CAs.

METAL ION, ITS LIGANDS, CATALYTIC AND INHIBITION MECHANISMS OF CAs

The Zn(II) ion of CAs is essential for catalysis.[1–4,7] X-ray crystallographic data has shown that for α-CAs, the metal ion is situated at the bottom of a 15 Å deep active site cleft (Figure 2), being coordinated by three histidine residues (His 94, His 96 and His 119) and a water molecule/hydroxide ion.[1–4,7,11] The zinc-bound water is also engaged in hydrogen bond interactions with the hydroxyl moiety of Thr 199, 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 favorable location for the nucleophilic attack (Figure 3).[1–4,7,11–13] The active form of the enzyme is the basic one, with hydroxide bound to Zn(II) (Figure 3A).[13] This strong nucleophile attacks the CO_2 molecule bound in a hydrophobic pocket in its neighbourhood (the elusive substrate-binding site comprises residues Val 131, Val 143 and Leu 198 in the case of the human

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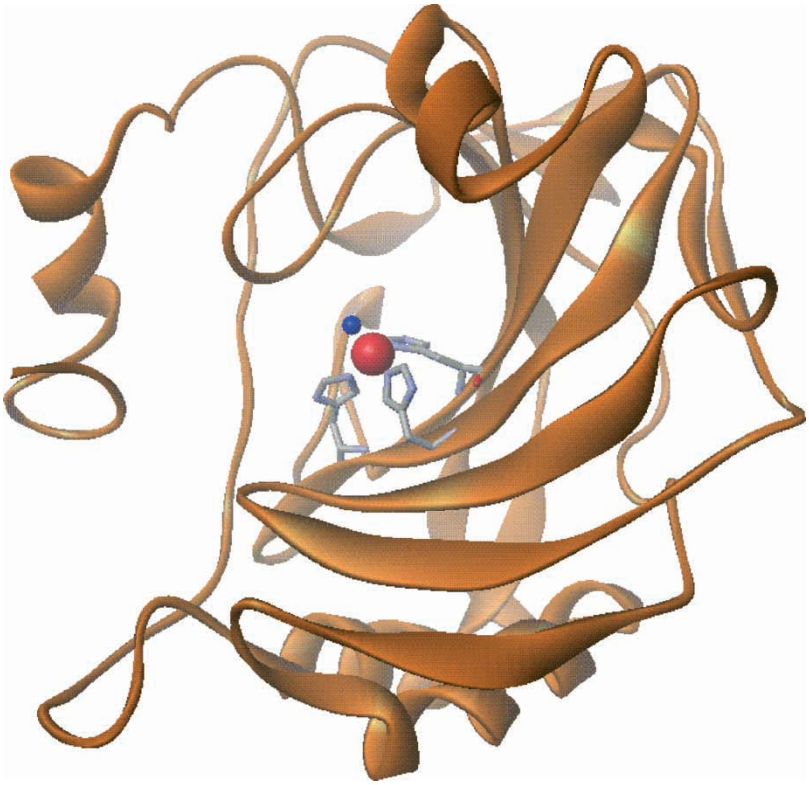

FIGURE 2 hCA II, with active site details: the zinc(II) ion (red sphere) coordinated by His 94, His 96 and His 119, and its fourth ligand, a water molecule/hydoxide ion (blue sphere) are seen.

isozyme CA II[12]) (Figure 3B), leading to the formation of bicarbonate coordinated to Zn(II) (Figure 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) (Figure 3D), which is catalytically inactive.[2,7,11] 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 His 64—the proton shuttle in isozymes CA I, II and IV) or by buffers present in the medium. The process may be schematically represented by Equations (*10*) and (*11*) below:

$$EZn^{2+}\text{–}OH^{-} + CO_2 \Leftrightarrow EZn^{2+}\text{–}HCO_3^{-} \Leftrightarrow EZn^{2+}\text{–}OH_2 + HCO_3^{-} \quad (10)$$

$$EZn^{2+}\text{–}OH_2 \Leftrightarrow EZn^{2+}\text{–}HO^{-} + H^{+} \quad (11)$$

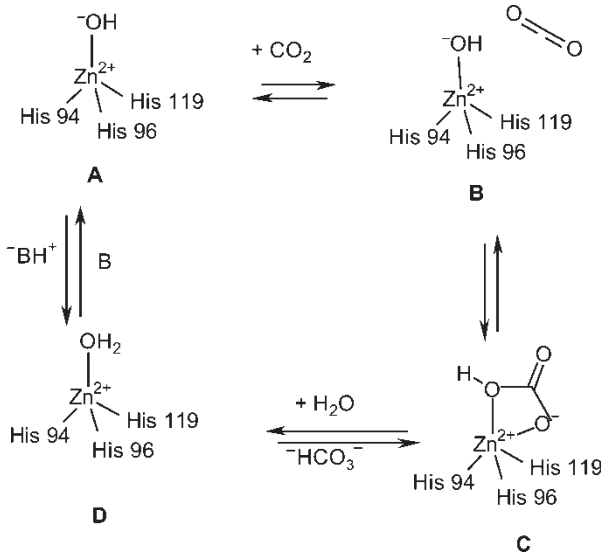

FIGURE 3 Schematic representation of the catalytic mechanism for the catalyzed CO_2 hydration by α-CAs.

The rate limiting step in catalysis is the second reaction, i.e., the proton transfer that regenerates the zinc-hydroxide species of the enzyme.[13] 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 (His 64), 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 tranfer process.[11] 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,[7–13] and also has important consequences for the design of inhibitors with clinical applications.

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

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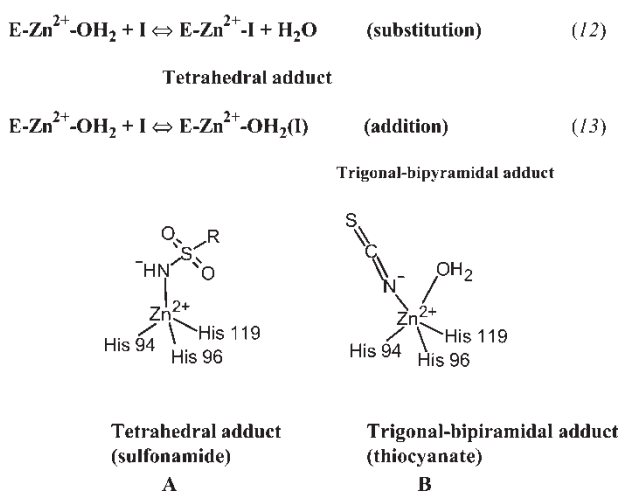

FIGURE 4 CA inhibition mechanism by sulfonamide and anionic inhibitors.

the non-protein zinc ligand (Equation *12* in Figure 4) or add to the metal coordination sphere (Equation *13* in Figure 4), generating trigonal-bipyramidal species.[2,7,14,15] Sulfonamides, which are the most important CAIs, bind in a tetrahedral geometry of the Zn(II) ion (Figure 4A), in the deprotonated state, with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II); 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.[16]

X-ray crystallographic structures are available for many adducts of sulfonamide inhibitors with isozymes CA I, II and IV.[2,7,16–18] In all these adducts, the deprotonated sulfonamide is coordinated to the Zn(II) ion of the enzyme, and its NH moiety participates in a hydrogen bond with the Oγ of Thr 199, which in turn is engaged in another hydrogen bond to the carboxylate group of Glu 106.[16–18] One of the oxygen atoms of the SO_2NH moiety also participates in a hydrogen bond with the backbone NH moiety of Thr 199.[16–18] In Figure 5, the crystal structures of the hCA II adducts with the simplest compound incorporating a sulfamoyl moiety (sulfamide) is shown. The monoanion of sulfamide $NHSO_2NH_2^-$ was shown to bind to the Zn(II) ion within the enzyme active site.[17] This structure provides some close insights into why this functional group (the sulfonamide one) appears to have unique properties for CA inhibition: (i) it exhibits a negatively charged, most likely mono-protonated nitrogen coordinated to the Zn(II) ion; (ii) simultaneously, this group forms a hydrogen bond as donor to the oxygen Oγ of the adjacent Thr 199, and (iii) a hydrogen bond is formed between one of the SO_2 oxygens to the backbone NH of Thr 199. Thus, the basic structural elements explaining the strong

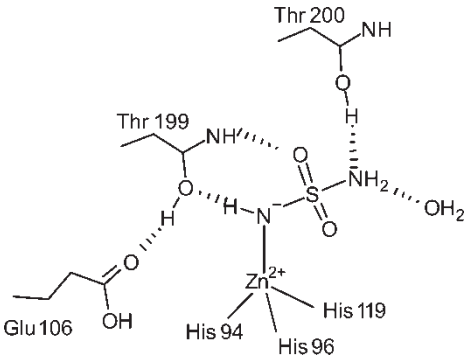

FIGURE 5 Schematic binding of sulfamide (as monoanion) to hCA II, as determined by X-ray crystallography.[16]

affinity of the sulfonamide moiety for the Zn(II) ion of CAs were defined in all details by using this simple compound as prototypical CAI, without the need to analyze the interactions of the organic scaffold usually present in other inhibitors (generally belonging to the aromatic/heterocyclic sulfonamide class).[17]

The physiological function of the major red cell isozyme, CA I (present in concentrations of up to 150 μM in the blood)[1,2] is still unknown. Recently, the X-ray crystal structure of a natural mutant of CA I, i.e., CA I Michigan 1 was reported (this isozyme was isolated from three generations of a family of European Caucasians[19]). CA I Michigan 1 differs from wild type CA I in a single amino acid residue present into the active site cavity, i.e., Arg 67 instead of His 67.[19] This amino acid residue is located in an important region of the catalytic site, which is involved both in shuttling protons from the active cavity to the environment and also in the binding of aromatic/heterocyclic sulfonamides, the classical, clinically important CAIs. The structure of the native mutant enzyme has been determined, as well as its adduct with a second zinc ion, which revealed the presence of a second metal ion binding site within the active cavity. Arg 67 appeared to promote the binding of this second zinc ion to His 64, His 200 and itself (through one of the guanidino nitrogen atoms).[19] This second metal ion bound into the active cavity is involved in the previously observed activation mechanism for substrate specific α- and β-naphthyl acetates hydrolyses.[19] Furthermore, this is the first example of a Zn(II) enzyme containing an arginine residue in the metal ion coordination sphere, as well as the first CA isozyme that binds two metal ions within its active site.[19] The crystal structures of sulfanilamide **1** (4-aminobenzenesulfonamide) complexed to native hCA I Michigan 1 variant and to its $(Zn)_2$ adduct have also been reported[20] (Figure 6). Comparisons among these structures and the corresponding sulfonamide adduct of hCA I showed significant differences in the orientation of the inhibitor molecule and in its interactions with active site residues such as His 200, Thr 199, Leu 198, Gln 92,

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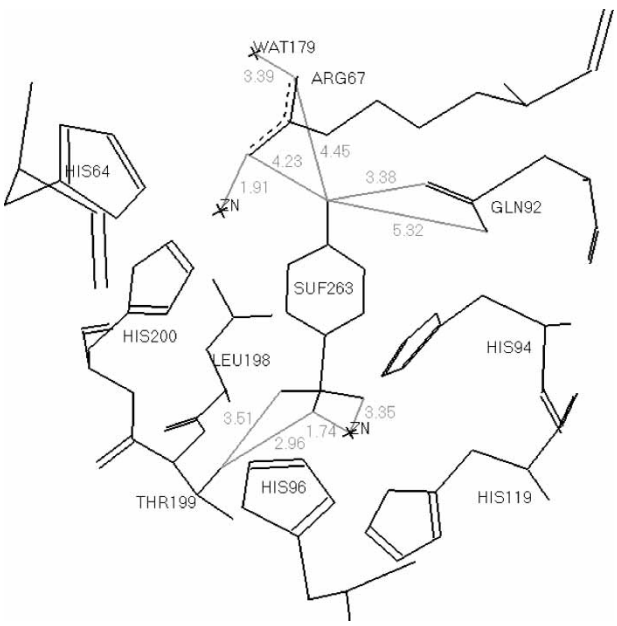

FIGURE 6 Model of sulfanilamide binding within the active site of the natural mutant, blood isozyme hCA I Michigan 1 containing two zinc ions (only molecule A shown), as determined by X-ray crystallography.[18,19]

and Arg/His 67 which are known to play important roles in substrate or inhibitor binding and recognition.[1,2,19,20] In CA I Michigan 1 it was observed that there was a lengthening of the Zn–N1 sulfanilamide bond distance and a corresponding shortening of the distance between the sulfamido group and Thr 199 with respect to wild type CA I. When the second Zn(II) ion was present in the active site, the *p*-amino group and the aromatic ring of the inhibitor molecule appeared tilted towards Gln 92 and Arg 67, moving away from residues His 200 and Leu 198. The structural differences in inhibitor binding between the CA I isozyme and the CA I Michigan 1 variant showed that even a point mutation within the active site of a CA isozymes may have relevant consequences for the binding of inhibitors.[20] This work opens new ways for the design of isozyme-specific CAIs.

Many bacteria, some archaeas (such as *Methanobacterium thermoautotrophicum*), algae and the chloroplasts of superior plants contain CAs belonging to the β-class.[10,21–24] The principal difference between these enzymes and the α-CAs discussed above resides 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 structure of four such β-CAs are available at present: the enzyme isolated from the red alga *Porphyridium purpureum*,[10] the enzyme from chloroplasts of *Pisum sativum*,[23] another prokariotic enzyme, this time isolated from *Escherichia coli*[24] and "cab", an enzyme isolated from the archaeon *Methanobacterium thermoautotrophicum*.[25] The *Porphyridium 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.[10] 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 (Figure 7A), 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 (Figure 7B).[23–25] 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 (Figure 7A), the main problem is whether the zinc-hydroxide mechanism presented in this here for the α-CAs is valid also for enzymes belonging to the β-family. A solution has been given by Mitsuhashi *et al.*[10] who have proposed the catalytic mechanism shown in Figure 8.

As there are two symmetrical structural motifs in one monomer of the *Porphyridium purpureum* enzyme, resulting from two homologous repeats which are related to each other by a pseudo

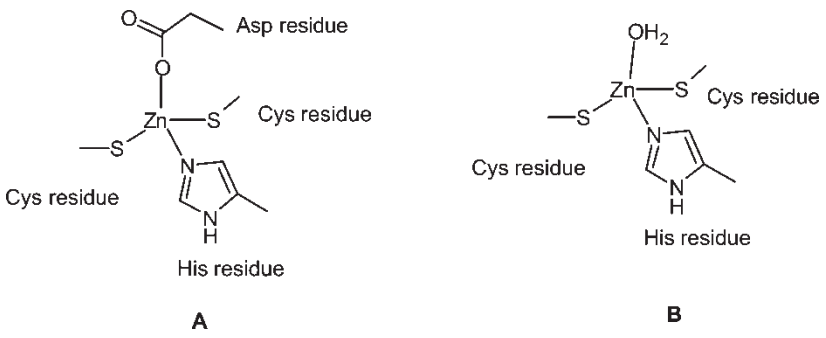

FIGURE 7 Schematic representation of the Zn(II) coordination sphere in β-CAs: **A**—*Porphyridium purpureum* and *Escherichia coli* enzymes;[9,23] **B**—*Pisum sativum* chloroplast and *Methanobacterium thermoautotrophicum* enzyme,[22,24] as determined by X-ray crystallography.

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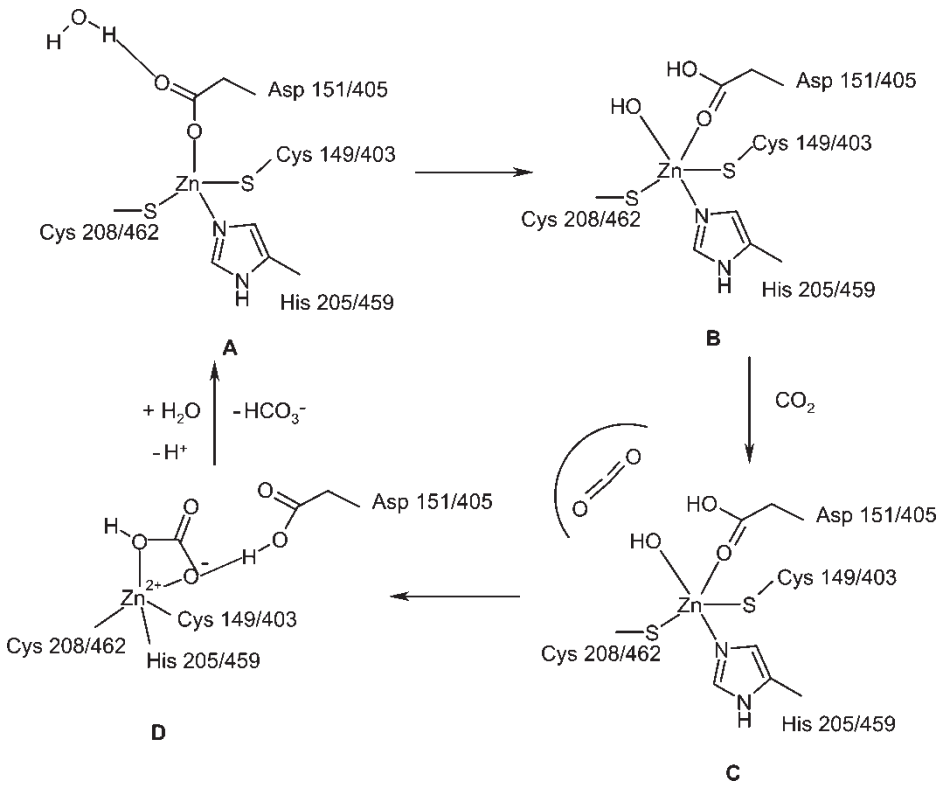

FIGURE 8 Proposed catalytic mechanism for prokaryotic β-CAs(*Porphyridium purpureum* enzyme numbering).[9]

2-fold axis, there are two Zn(II) ions coordinated by the four amino acids mentioned above. In this case these pairs are: Cys 149/Cys 403, His 205/His 459, Cys 208/Cys 462, and Asp 151/Asp 405.[10] A water molecule is also present in the neighborhood of each metal ion, but it is not directly coordinated to it, forming a hydrogen bond with an oxygen belonging to the zinc ligand Asp 151/Asp 405 (Figure 8A). 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 that may then be coordinated to Zn(II) which acquires a trigonal bipyramidal geometry (Figure 8B). Thus, the strong nucleophile which may attack CO_2 bound within a hydrophobic pocket of the enzyme is formed (Figure 8C), with generation of bicarbonate bound to Zn(II) (Figure 8D). This intermediate is rather similar to the reaction intermediate proposed for the α-CA catalytic cycle (Figure 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 (Figure 8D). 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 generated aspartate 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 *Pisum sativum* at 1.93 Å resolution, has also been reported.[23] The molecule assembles as an octamer with a novel dimer of dimers of dimers arrangement. The active site is located at the interface between two monomers, with Cys 160, His 220 and Cys 223 binding the catalytic zinc ion and residues Asp 162 (oriented by Arg 164), Gly 224, Gln 151, Val 184, Phe 179 and Tyr 205 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.[23]

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 Cys 32, His 87, and Cys 90, with the tetrahedral coordination completed by a water molecule.[25] 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.[25]

No structural data are available at this moment regarding the binding of inhibitors to this type

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of CAs, except for the fact that acetate coordinates to the Zn(II) ion of the *Pisum sativum* enzyme.[23]

The prototype of the γ-class CAs, "Cam" has been isolated from the methanogenic archaeon *Methanosarcina thermophila*.[26] The crystal structures of zinc-containing and cobalt-substituted Cam were reported in the unbound form and co-crystallized with sulfate or bicarbonate.[26] 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.[26,27] 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.[26,27] The catalytic mechanism of γ-CAs was proposed to be similar with the one presented for the α-class enzymes. Nevertheless, the finding that Zn(II) is not tetracoordinated as originally reported[27] but pentacoordinated,[26] 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 the equilibrium exists between the trigonal bipyramidal and the tetrahedral species of the metal ion from the active site of the enzyme. Ligands bound to the active site were shown to make contacts with the side chain of Glu 62 in a manner that suggests this side chain to be probably protonated. In the uncomplexed zinc-containing Cam, the side chains of Glu 62 and Glu 84 appear to share a proton; additionally, Glu 84 exhibits multiple conformations. This implies that Glu 84 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 His 64. Anions, such as bicarbonate or sulfate were shown to bind to Cam, but no information is available regarding its inhibition by sulfonamides.[26,27]

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.[28] 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. We propose here that this enzyme is the first member of the δ-CA family. In the same diatom a rather perplexing discovery was then made, that of the first cadmium-containing enzyme, which is a CA-type protein.[29] The marine diatom *Thalassiosira 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.[29,109] Cd labelling comigrates with a protein band that showed this CA activity to be distinct from TWCA1 on native PAGE of radiolabeled *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 lead to the isolation of a Cd-containing protein of 43 kDa, it 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.[29]

NEW REPRESENTATIVES AND THEIR CHARACTERIZATION

As mentioned above, CAs were recently shown to be present in a multitude of prokaryotes, where these enzymes play important functions such as respiration, transport of carbon dioxide and photosynthesis.[21,22] The possibility to develop CA inhibitor-based antibiotics, by inhibiting bacterial CAs present in pathogenic species, raised much interest some years ago, with promising results in the use of ethoxzolamide for the treatment of meningitis.[30,31] This type of inhibition has also been exploited for developing selective culture media for other pathogenic bacteria, such as *Branhamella catarrhalis*,[31] in the presence of different *Neisseria* species. Some strains of *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Serratia* and *Proteus*, were reported to strongly express a gene product immunologically related to CA.[31] On the other hand, α-, β- and γ-CAs have been purified in many species of bacteria, such as *Neisseria spp.*, *E. coli*, *Synechocystis spp.*, *Acetobacterium woodi*, *Anabaena variabilis* and *Rhodospirillum rubrum*, but it is established that these enzymes are nearly ubiquitous in prokaryotes.[22,31–34] Lindskog's group reported the isolation, purification and characterization of some α-CAs from pathogenic bacteria, such as

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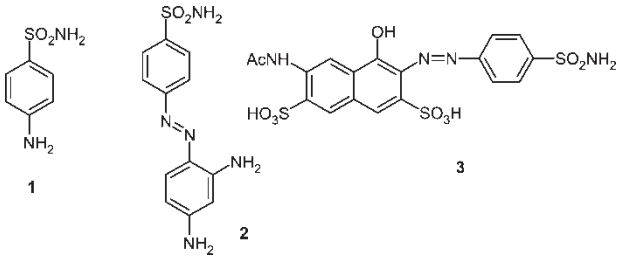
Helicobacter pylori or *Neisseria gonorrhoeae*.[31–34] Because the CA from *Helicobacter pylori* strain 26695 was toxic to *Escherichia coli*, a modified form of the gene lacking a part that presumably encodes a cleavable signal peptide was used for the expression. This truncated gene could be expressed in *E. coli* yielding an active enzyme comprising 229 amino acid residues, with the amino acid sequence showing 36% identity with that of the enzyme from *Neisseria gonorrhoeae* and 28% with that of hCA II.[34] The *H. pylori* CA was purified by sulfonamide affinity chromatography and its kinetic parameters for CO_2 hydration were determined. Thiocyanate showed an uncompetitive inhibition pattern at pH 9 indicating that the maximal rate of CO_2 hydration is limited by proton transfer reactions between a zinc-bound water molecule and the reaction medium in analogy to higher vertebrate α-CAs. The 4-nitrophenyl acetate hydrolase activity of the *H. pylori* enzyme was shown to be quite low, whereas the esterase activity against 2-nitrophenyl acetate as substrate was much better.[34] The kinetic properties of the CA isolated from *Neisseria gonorrhoeae* (NGCA) as well as for some mutant of such enzymes, have also been investigated by the same group.[33] Qualitatively, the enzyme shows the same kinetic behaviour as the well studied hCA II, suggesting a ping-pong mechanism with buffer as the second substrate. The ratio k_{cat}/K_m is dependent on two ionizations with pK_a values of 6.4 and 8.2, suggesting that His 66 in NGCA has the same function as a proton shuttle, as His 64 in hCA II. The kinetic defect in some of the NGCA mutants lacking His 66 can partially be overcome by some buffers, such as imidazole and 1,2-dimethylimidazole, acting as endogenous activators. The bacterial enzyme shows similar K_i values for the inhibitors cyanate, thiocyanate and azide as hCA II, while cyanide and the sulfonamide ethoxzolamide are considerably weaker inhibitors of the bacterial enzyme than of hCA II.[33] An excellent review on prokaryotic CAs has recently been published by Smith and Ferry.[22]

The recent report of parasitic CAs by Krungkrai *et al.*,[35,36] who discovered the presence of at least two different α-CAs in *Plasmodium falciparum*, the malaria provoking protozoa, opens new vistas to the development of pharmacological agents based on CA inhibitors. Red cells infected by *Plasmodium falciparum* contained CA amounts approximately 2-fold higher than those of normal red cells. The three developmental forms of the asexual stages of the parasite (i.e., ring, trophozoite and schizont) were isolated from their host red cells and found to have stage-dependent CA activity. The enzyme was then purified to homogeneity, showing a M_r of 32 kDa, being active in monomeric form (the human red cell enzyme was also purified for comparison with the parasite enzyme in this study).[35,36] The parasite enzyme activity was sensitive to well-known sulfonamide-CA inhibitors such as sulfanilamide and acetazolamide. The kinetic properties and the aminoterminal sequences of the purified enzymes from the parasite and host red cell were found to be different, indicating that the purified protein was a distinct enzyme, i.e., *P. falciparum* CA. In addition, the above-mentioned enzyme inhibitors showed antimalarial effect against *in vitro* growth of *P. falciparum*. This very important contribution shows that CA inhibitors may represent valuable future drugs for the treatment of malaria.[35,36]

In higher plants, algae and cyanobacteria, all members of the three CA families were found to be present.[37] For example, analysis of the *Arabidopsis* database 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.[37] In algae, CAs were found in mitochondria, the chloroplast thylakoid, cytoplasm and periplasmic space.[37–39] 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.[39] 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.[39] The plant CAs are exhaustively reviewed by Badger and Price.[39] These enzymes are highly abundant in terrestrial vegetation and seem to be correlated with the content of atmospheric CO_2, and thus with the global warming processes.[40]

DRUG DESIGN OF CA INHIBITORS

CA inhibition with sulfanilamide (**1**) discovered by Mann and Keilin[41] was the beginning of a great scientific adventure that led to important drugs, such as the antihypertensives of benzothiadiazine and high-ceiling diuretics type,[42] the sulfonamides with CA inhibitory properties mainly used as antiglaucoma agents,[2,4,42,43] some anti-thyroid drugs,[42] the hypoglycemic sulfonamides[44] and, ultimately, some novel types of anticancer agents.[45]



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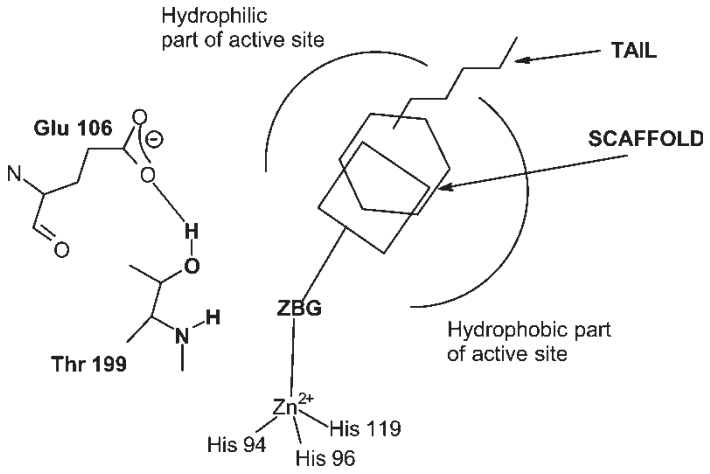

FIGURE 9 The general structure of a CAI complexed to the enzyme active site: ZBG = zinc binding group; the organic scaffold may be present or absent; the tail too. These structural elements interact both with the hydrophobic as well as the hydrophylic halves of the active site, whereas ZBG interacts with Thr 199 and Glu 106.

Up to recently, only sulfonamides were known to possess high affinity for CAs. Some recent data indicate that CA inhibitors (CAIs) may be designed from many other types of compounds. Figure 9 shows the most general structure of a CAI complexed to the enzyme active site. Thus, such a compound must possess: (i) a zinc binding group (ZBG) by which it interacts with the metal ion of the enzyme and the residues Thr 199 and Glu 106 in its neighborhood; (ii) the organic scaffold-usually an aromatic or heterocyclic moiety, which may be present or absent in new generation CAIs; (iii) a tail attached to the scaffold, which usually was absent in the first and second generation of sulfonamide CAIs, but which is extremely important (and generally present) for the last generation of such derivatives.[4] All these structural elements interact both with the hydrophobic as well as the hydrophilic halves of the active site, whereas ZBG interacts with Thr 199 and Glu 106 as shown in the Figure.[4] Thus, sulfonamides constitute just a particular case for this type of general interaction.

Indeed, in recent years, an entire range of new ZBGs were reported, mostly by our group,[4] as shown in Figure 10. These new ZBGs include, in addition to the classical sulfonamides sulfamates, sulfamides, substituted sulfonamide, Schiff's bases, urea and hydroxyurea, as well as hydroxamates.

The binding of some non-classical CAIs to the most investigated isozyme, hCA II, is shown in Figure 11. Thus, urea binds as ureate to the Zn(II) ion and participates in a network of eight hydrogen bonds which make this complex the most potent CAI ever reported (Figure 11A).[5] Hydroxamates bind to hCA II[46] in a very different manner (Figure 11B) as compared to their binding to matrix metalloproteinases (MMP-8)[47]—(Figure 11C)—coordinating to the Zn(II) ion monodentately through the nitrogen atom of the hydroxamate moiety.[48]

Returning to the classical CAIs, the report of Krebs[49] that mainly the unsubstituted aromatic sulfonamides of type $ArSO_2NH_2$ act as strong CAIs, and that the potency of such compounds is

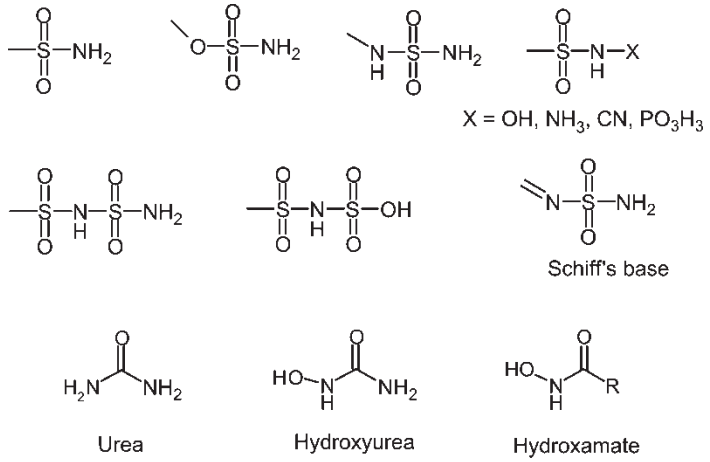

FIGURE 10 Zinc binding groups: sulfonamides, sulfamates, sulfamides, substituted sulfonamides, Schiff's bases, urea, hydroxyurea and hydroxamates.

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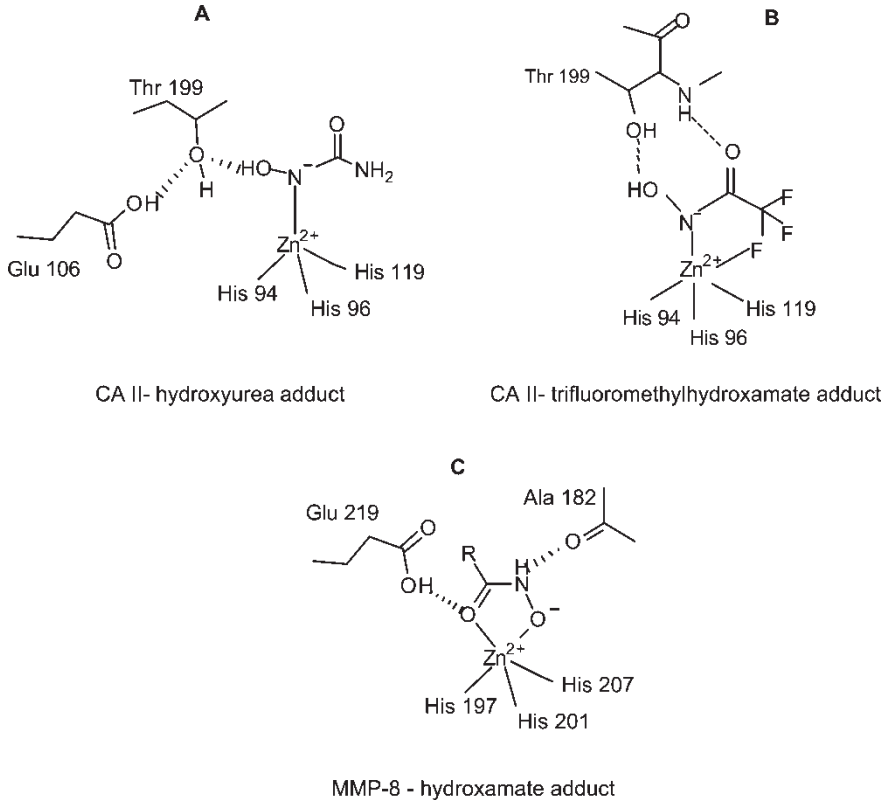
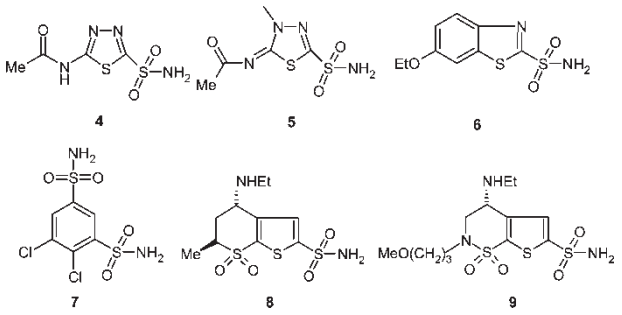


FIGURE 11 The binding of some non-classical CAIs to hCA II: **A**. Urea binds as ureate the Zn(II) ion and participates in a network of eight hydrogen bonds;[5] **B**. Hydroxamates bind to hCA II[45] in a very different manner as compared to their binding to matrix metalloproteinases (MMP-8)[46]—**C**—coordinating to the Zn(II) ion monodentately through the nitrogen atom of the hydroxamate moiety.[47]

drastically reduced by *N*-substitution of the sulfonamide moiety, constituted the beginning of extensive structure–activity correlations, which led to some valuable drugs during a short period of time. Among the active structures found by Krebs were also the azodyes **2** (prontosil red) and **3**, derived from sulfanilamide.

Four systemic sulfonamide drugs have been used clinically mainly as antiglaucoma agents, for a long time: acetazolamide (**4**), methazolamide (**5**), ethoxzolamide (**6**), and dichlorophenamide (**7**)[2,42,50]. Systemic inhibitors are useful in reducing elevated intraocular pressure (IOP) characteristic of this disease. They represent the most efficient physiological treatment of glaucoma, since by inhibiting the ciliary process enzyme (the sulfonamide susceptible isozymes CA II and CA IV), a reduced rate of bicarbonate and aqueous humor secretion is achieved, which leads to a 25–30% decrease of IOP, but the inhibition of various CA isozymes present in tissues other than the eye leads to an entire range of side effects.[2,4,42] The most prominent ones are: numbness and tingling of extremities, metallic taste, depression, fatigue, malaise, weight loss, decreased libido, gastrointestinal irritation, metabolic acidosis, renal calculi and transient myopia.[2,4,42] Indeed, compounds **4**–**7** indiscriminately inhibit several CA isozymes (such as CA I, CA II, CA IV, CA V, CA VII, CA IX and CA XIII) abundant in organs other than the eye, such as blood, kidneys, lungs, gastrointestinal tract, CNS, etc.[4,42]



Therefore, it appeared of interest to try to administer such sulfonamide CAIs topically, directly into the eye.[2,4,42] However, not one of the clinically used inhibitors **4**–**7** (or other structurally-related investigational agents) proved to be effective when administered topically in reducing elevated IOP, and thus, for a long period, it was considered that sulfonamide CAIs cannot be administered *via* the topical route. In a classical study, Maren's[43] group then showed that this is principally due to the innappropriate physico-chemical properties of the existing CAIs available at that time, and provided the basis for the discovery of the topically

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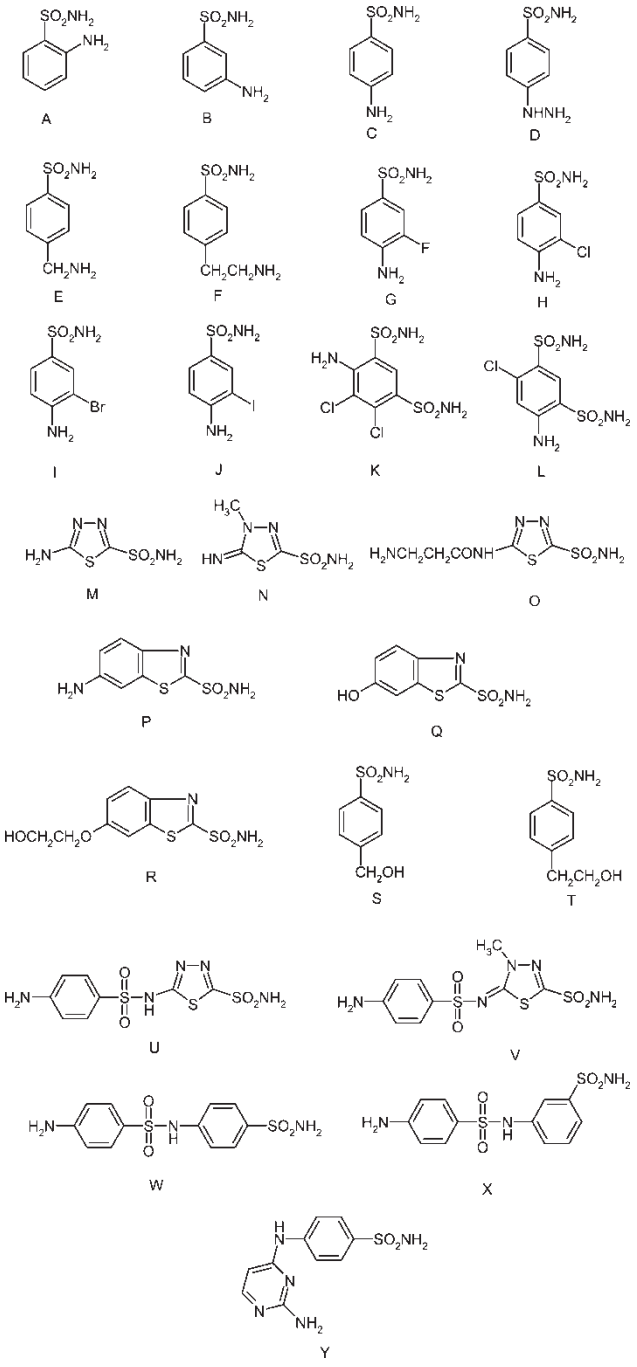
acting sulfonamides: the first such drug, dorzolamide (**8**), entered clinics in 1995, and the second one (structurally related to dorzolamide)-brinzolamide (**9**) entered clinics in 1999.[4,50,51] Thus, in addition to the systemically acting inhibitors **4**–**7**, the clinical antiglaucoma armamentarium comprises these two new drugs, which show much less side effects as compared to the first drugs mentioned above, but which also basically inhibit all the physiologically relevant CA isozymes.[4,50,51]

A large number of novel types of CAIs have been reported in recent years. Most of them were developed as candidate topically acting antiglaucoma drugs, but other applications were also envisaged, such as the design of CAIs with anticancer properties, anti-osteoporosis agents, etc. Two main approaches were used for the drug design of effective CAIs of the sulfonamide type:[4,50,51] (i) The "ring" approach, used for the discovery of dorzolamide and brinzolamide, consisted in exploring a great variety of ring systems on which the sulfonamide group (and eventually other moieties) were attached. This approach was really beneficial for the chemistry of this class of derivatives as a very large number of new ring systems was explored in this way, but generally led to few effective *in vivo* IOP lowering agents (except for the two drugs mentioned above), and the largest majority of the obtained derivatives were potent allergens; (ii) The second approach, the "tail" one, consisted in attaching water-solubilizing tails to different scaffolds of well-known aromatic/heterocyclic sulfonamides possessing affinity for the CA active site, assuring in this way the possibility of modulating in greater detail the physico-chemical properties of these pharmacological agents. This approach did not lead at the time to a clinically used drug (it was reported relatively recently), but led to the development of a very large number of much more effective antiglaucoma sulfonamides as compared to **8** and **9** (at least in animal models of the disease) in comparison with the ring approach. It must also be mentioned that these two aproaches are both valuable, and complementary, since it was proved for example that dorzolamide may also be further derivatized by the tail strategy, leading to even more effective IOP lowering compounds.[4,51]

Many brinzolamide-like derivatives of type **10** were reported by Alcon scientists.[52] These non-chiral 2H-thieno[3,2-e]- and [3,2-e]-1,2-thiazine-6-sulfonamide 1,1-dioxides were synthesized for evaluation as potential candidates for the treatment of glaucoma, and showed high affinity towards hCA II ($K_i < 0.5$ nM). Some of them were evaluated for their ability to lower intraocular pressure (IOP) in naturally hypertensive Dutch-belted rabbits, and showed significant efficacy (>20% decrease) in this model following topical ocular administration.[52]

The "tail" approach consisted in using well-known aromatic/heterocyclic sulfonamide scaffolds (of type A–Y) to which tails that will induce water solubility (or other desired pharmacological/physico-chemical properties) are attached at the amino, hydroxy, imino or hydrazino moieties contained in the precursor sulfonamides A–Y.[4,51]



The starting sulfonamides derivatized by this simple approach included 2-; 3- or 4-aminobenzenesulfonamides; 4-(ω-aminoalkyl)-benzenesulfonamides/thiadiazole-sulfonamides; 3-halogeno-substituted-sulfanilamides;

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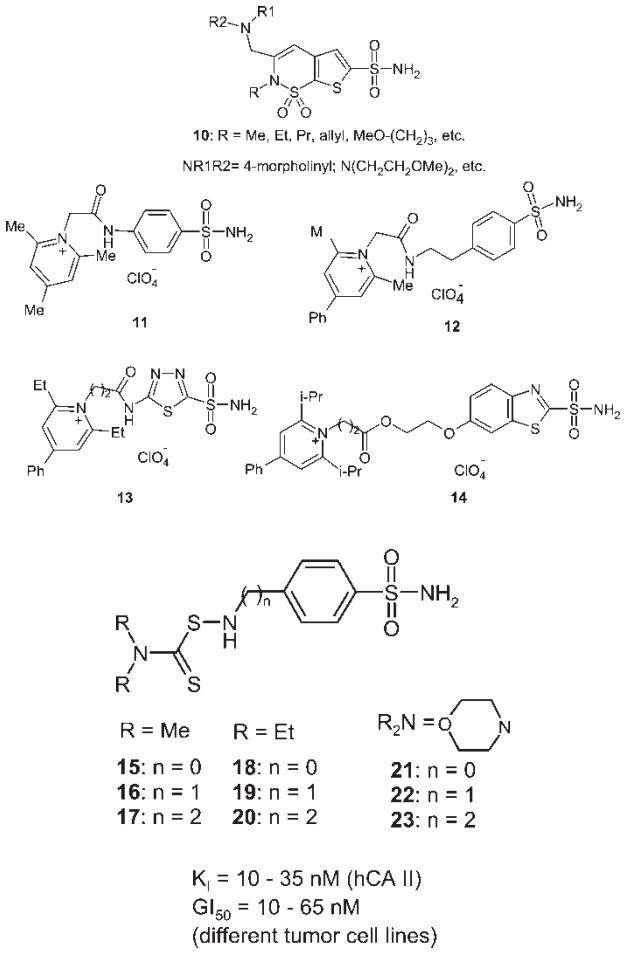
1,3-benzene-disulfonamides; 1,3,4-thiadiazole-2-sulfonamides; benzothiazole-2-sulfonamides as well as sulfanilyl-substituted aromatic/heterocyclic sulfonamides (structures U–X) among others, and were chosen in such a way as to prove that this is a general approach.[4,51] Tails that were introduced in the molecules of such new CAIs contained either moieties protonatable at endocyclic nitrogen atoms (such as pyridine- or quinoline rings), or at amino groups belonging to amino acids and some of their derivatives (such as glycine, β-alanine, GABA, sarcosine, creatine, diethylenetriaminopentaacetic acid (dtpa), or glycyl-glycine moieties), as well as perfluoroalkyl/aryl moieties. The water solubility of the first type of such derivatives is assured by formation of salts with hydrochloric, trifluoroacetic or triflic acid, or by formation of sodium salts, for the derivatives possessing carboxylic acid moieties. The tail was generally attached to the sulfonamide scaffold either as an amide (CONH), ester (COO), sulfonamide (SO_2NH) or imide moiety.[4,51] Thus, a sulfonamide CAI obtained by the tail approach is generally described by a figure (corresponding to the tail moiety) and a letter (corresponding to the sulfonamide scaffold to which the tail has been attached, through a carboxamide, ester or secondary sulfonamide bond).

By applying this approach,[4,51] a large number of novel sulfonamide CAIs belonging to a variety of aromatic/heterocyclic ring systems and incorporating many types of new tails have been generated.[53–72] Furthermore, the first nanomolar, non-sulfonamide CAIs were reported by the same group, as belonging to the monosubstituted sulfonylated amino acid hydroxamates.[72]

A new approach for the selective *in vivo* inhibition of membrane-bound versus cytosolic CA isozymes with a new class of positively charged, membrane-impermeant sulfonamides has been also reported.[53,57] This approach is based on the attachment of trisubstituted-pyridinium-methyl/ethylcarboxy moieties (obtained from 2,4,6-trisubstituted-pyrylium salts and glycine/β-alanine) to the molecules of classical aromatic/heterocyclic sulfonamides possessing free amino and hydroxyl groups in their molecules, leading thus to compounds of types **11**–**14**. Efficient inhibition (in the nanomolar range) was observed with many of these new derivatives against three isolated CA isozymes: i.e., hCA I, hCA II (cytosolic forms), and bCA IV (membrane-bound isozyme).[53,57] Due to their salt-like character, this new type of inhibitor, unlike the classical, clinically used compounds (such as acetazolamide, methazolamide, ethoxzolamide, etc.), are unable to penetrate through biological membranes, inhibiting in this way only membrane-associated CAs and not the cytosolic isozymes.[53,57]



Novel sulfonamide inhibitors of types **15**–**23** were prepared by reaction of aromatic or heterocyclic sulfonamides containing amino moieties with *N*, *N*-dialkyldithiocarbamates in the presence of oxidizing agents (sodium hypochlorite or iodine).[54,67] The N,N-dialkylthiocarbamylsulfenamido-sulfonamides **15**–**23** synthesized in this way behaved as strong inhibitors of human CA I and CA II (hCA I and hCA II) and bovine CA IV (bCA IV). For the most active compounds, inhibition constants ranged from 10^{-8} to 10^{-9} M (for isozymes II and IV). Some of the derivatives belonging to this new class of CA inhibitors were also tested as inhibitors of tumor cell growth *in vitro*, showing potent inhibition of growth against several leukemia, non small-cell lung, ovarian, melanoma, colon, CNS, renal, prostate and breast cancer cell lines. With several cell lines, GI_{50} (molarity of inhibitor producing a 50% inhibition of tumour cell growth) values of 10–75 nM were observed.[54,67]

The same potent antitumor activity has been discovered for some 1,3,4-thiadiazole-sulfonamides of type **24**–**30**[66] or several aromatic derivatives of types **31**–**35**[65]. These derivatives showed GI_{50} values typically in the range of 0.1–30 μM against several leukaemia, non-small cell lung cancer, ovarian, melanoma, colon, CNS, renal, prostate and breast

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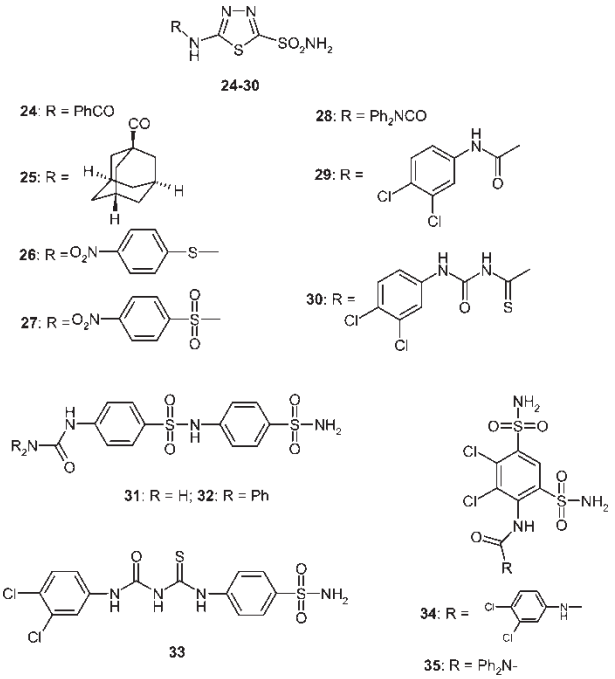
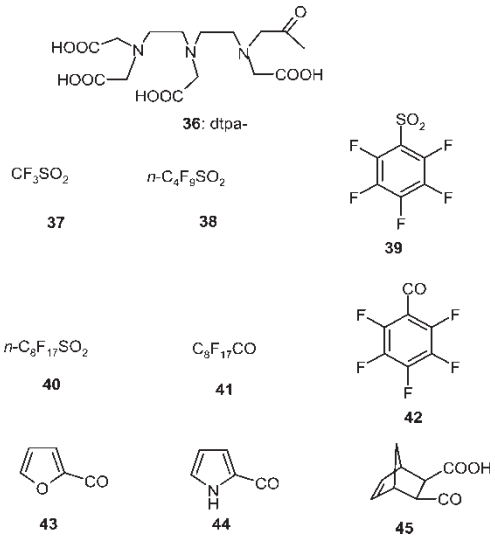


cancer cell lines. The mechanism of antitumour action with such sulfonamides is unknown, but it might involve either inhibition of CA isozymes (such as CA IX and CA XII) predominantly present in tumour cell membranes, acidification of the intracellular environment as a consequence of CA inhibition, uncoupling of mitochondria and/or strong CA V inhibition, or a combination of several such mechanisms. Such derivatives might lead to the development of effective novel types of anticancer agents/therapies.[65,66]



A large number of derivatives possessing powerful topical antiglaucoma activity, were also reported[55–57,61,62,68,69,71] whereas in two QSAR studies attemps have been made to correlate this activity with physico-chemical and quantum properties of such derivatives.[59,72] Aromatic/heterocyclic sulfonamides **A**–**Y** possessing a free amino/imino/hydrazino/hydroxy group were reacted with a variety of acyl/alkyl/arylsulfonyl halides or carboxylic acid anhydrides present in tails **36**–**45** shown above. The tails were chosen in such a way as to assure desired physico-chemical properties, appropriate for topical activity as antiglaucoma drugs for many such compounds.[55–57,61,62,68,69,71] Indeed, both in normotensive as well as glaucomatous albino rabbits, such derivatives showed potent and prolonged IOP lowering properties, as compared to the clinically used drugs dorzolamide and brinzolamide. Some thioureas of types **46**–**48** were obtained by reaction of 4-isothiocyanatobenzenesulfonamide with amines, amino acids, and oligopeptides.[62] The new thiourea derivatives showed strong affinities toward isozymes I, II, and IV. *In vitro* inhibitory power was good (in the low-nanomolar range) for the derivatives of β-phenylserine and α-phenylglycine, for those incorporating hydroxy and mercapto amino acids (Ser, Thr, Cys, Met), hydrophobic amino acids (Val, Leu, Ile), aromatic/heterocyclic amino acids (Phe, His, Trp, Tyr, DOPA), and dicarboxylic amino acids as well as di/tri/tetrapeptides among others. Such CA inhibitors displayed very good water solubility (in the range of 2–3%) mainly as sodium (carboxylate) salts, with the pH value of the obtained solutions being 6.5–7.0. Some of these preparations (such as the derivatives of Ser, β-Ph-Ser, Leu, Asn, etc.) strongly lowered intraocular pressure (IOP) when applied topically, directly into the normotensive/glaucomatous rabbit eye, as 2% water solutions. It is interesting

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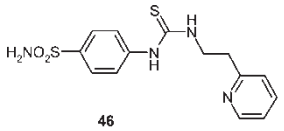
to note that not all the powerful CA inhibitors designed in that study showed topical IOP-lowering effects (such as, for instance, the Cys and Lys derivatives, devoid of such properties) whereas the Pro, Arg, and oligopeptidyl thiourea derivatives showed reduced efficacy when administered topically.[62] This has been explained as being be due to the very hydrophilic nature of some of these compounds, whereas only inhibitors with balanced hydro- and liposolubility showed optimal *in vivo* activity. The interesting pharmacological properties of these new type of CA inhibitors, correlated with the neutral pH of their solutions used in ophthalmologic applications, making them attractive candidates for developing novel antiglaucoma drugs devoid of major ocular side effects.



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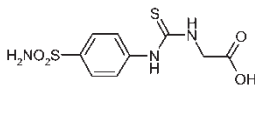

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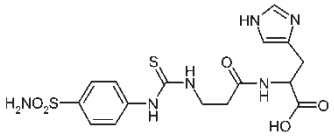

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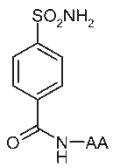
Reaction of 4-carboxy-benzenesulfonamide or 4-chloro-3-sulfamoyl benzoic acid with carboxy-protected amino acids/dipeptides (or aromatic/heterocyclic sulfonamides/mercaptans, data not shown) afforded the corresponding benzene-carboxamide derivatives of types **49** and **50**.[71] These were tested as inhibitors of three isozymes, CA I, II and IV and showed affinity in the low nanomolar range for isozymes CA II and IV, involved in aqueous humor secretion within the eye. Some of them were tested as topically acting anti-glaucoma agents, in normotensive and glaucomatoous rabbits. Good *in vivo* activity and prolonged duration of action was observed for some of these derivatives, as compared to the clinically used drugs dorzolamide and brinzolamide. Some of the 4-chloro-3-sulfamoyl benzene-carboxamides showed higher affinity for CA I than for the sulfonamide avid isozyme CA II.[71]



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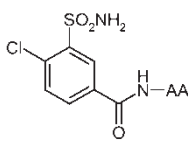
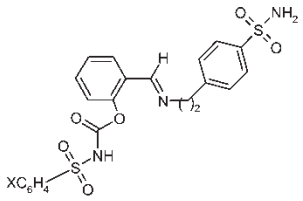


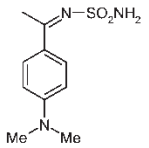
50

AA = amino acid/dipeptide residue

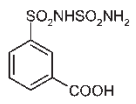
Metal complexes of a sulfonamide possessing strong CA inhibitory properties, 5-(2-chlorophenyl)-1,3,4-thiadiazole-2-sulfonamide (chlorazolamide) have been obtained from the sodium salt of the sulfonamide and the following metal ions: Mg(II), Zn(II), Mn(II), Cu(II), Co(II), Ni(II), Be(II), Cd(II), Pb(II), Al(III), Fe(III) and La(III)[66]. The original sulfonamide and its complexes were assayed for the in vitro inhibition of three CA isozymes, CA I, II, and IV, some of which play a critical role in ocular fluid secretion. All these compounds (the sulfonamide and its metal complexes) behaved as powerful inhibitors against the three investigated isozymes. The parent sulfonamide possessed an extremely weak topical pressure lowering effect when administered as a 1–2% suspension into the rabbit eye, but some of its metal complexes, such as the Mg(II), Zn(II), Mn(II) and Cu(II) derivatives, lower intraocular pressure (IOP) in experimental animals very well. *Ex vivo* data showed a 99.5–99.9% CA II inhibition in ocular fluids and tissues of rabbits treated with these agents, proving that the observed IOP lowering is due to CA inhibition.[66]



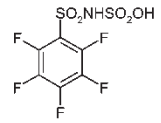
51: X = H, p-Me; o-Me; p-F; p-Cl



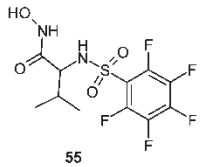
52: K_I = 20 nM (hCA II)



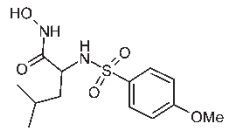
53: K_I = 10 nM (hCA II)



54: K_I = 1200 nM (hCA II)
K_I = 50 nM (hCA I)



55
(K_I hCA I= 8 nM; hCA II = 11 nM; bCA IV = 13 nM)



56

(K_I hCA I= 50 nM; hCA II = 5 nM; bCA IV = 39 nM)

Some Schiff's bases of aromatic sulfonamides or sulfamide, of types **51** and **52** have also been reported, together with some structurally related sulfamide/sulfamic acid derivatives **53** and **54**[60,64]. Reaction of *o*- or *p*-hydroxybenzaldehydes with sulfanilamide, homosulfanilamide and *p*-(2-aminoethyl)-benzene-sulfonamide afforded several new Schiff bases which

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were subsequently derivatized at the phenolic hydroxy moiety by reaction with arylsulfonylisocyanates leading to derivatives of type **51**.[64] The new arylsulfonylcarbamates obtained in this way possessed interesting inhibitory properties against three isozymes, hCA I, hCA II and bCA IV. Generally, the *p*-hydroxybenzaldehyde derivatives were more active than the corresponding *ortho* isomers. An interesting behavior was seen for some of the *ortho*-substituted arylsulfonylcarbamato-sulfonamides, which showed higher affinities for the isozyme hCA I, as compared to hCA II and bCA IV (generally hCA I is 10–1000 less sensitive to "normal" sulfonamide inhibitors, such as acetazolamide, methazolamide or dorzolamide, as compared to hCA II). This made the new derivatives attractive leads for designing isozyme I-specific inhibitors.[64]

Since sulfamide and sulfamic acid are the simplest compounds containing the SO_2NH_2 moiety, responsible for binding to the Zn(II) ion within the CA active site, the novel classes of CA inhibitors obtained by their derivatizations, possessing the general formula $RSO_2NH–SO_2X$ ($X = OH, NH_2$), contain new zinc-binding functions inducing strong CA inhibitory properties.[60] A smaller series of derivatives has been obtained by reaction of aromatic aldehydes with sulfamide, leading to Schiff bases of the type $ArCH=NSO_2NH_2$. All these compounds acted as strong inhibitors of isozymes I, II and IV, and their mechanism of CA inhibition was evaluated based on electronic spectroscopic measurements on adducts with the Co(II)-substituted CA II. These experiments led to the conclusion that the new inhibitors are directly coordinated (in a monodentate manner) to the metal ion within the enzyme active site, similarly to the classical inhibitors, the aromatic/heterocyclic sulfonamides.[60]

In the search for new zinc binding functions for CAIs, we tested whether sulfonylated amino acid hydroxamates of the type $RSO_2NX–AA–CONHOH$ (X = H, benzyl, substituted benzyl; AA = amino acid moiety, such as Gly, Ala, Val, Leu) with well-known inhibitory properties against matrix metalloproteinases[74] (MMPs) and *Clostridium histolyticum* collagenase (ChC, another zinc enzyme related to the MMPs) might also act as CA inhibitors.[73] We also investigated whether N-hydroxysulfonamides of the type RSO_2NHOH (which are effective CA inhibitors[4]) inhibit MMPs and ChC. We have thus discovered several potent sulfonylated amino acid hydroxamate such as **55** and **56** which act as CA inhibitors (with inhibition constants in the range of 5–40 nM, against the human isozymes hCA I and hCA II, and 10–50 nM, against the bovine isozyme bCA IV).[73] Some *N*-hydroxysulfonamides were also shown to possess inhibitory properties (in the micromolar range) against MMP-1, MMP-2, MMP-8, MMP-9, and ChC. Thus, the SO_2NHOH group is a new zinc-binding function for the design of MMP inhibitors. Both CA as well as MMPs are involved, among others, in carcinogenesis and tumor invasion processes.[73] On the basis of these findings, we suggested that the mechanism of antitumor action with some hydroxamate inhibitors might also involve inhibition of some CA isozymes (such as CA IX, and CA XII) present only in tumor cell membranes, in addition to collagenases/gelatinases of the MMP type. Our data also suggested that it should be possible to develop dual enzyme inhibitors that would strongly inhibit both these metalloenzymes, CAs and MMPs, based on the nature of the R, AA, and X moieties in the above formula. Compact X (such as H) and AA (such as Gly) moieties favored CA over MMP inhibition, whereas bulkier X (benzyl, substituted benzyl, etc.) and AA (such as Val, Leu) moieties and substituted-aryl R groups are advantageous for obtaining potent MMP and ChC inhibitors, which showed lower affinity for CAs.[73]

In the same context, the interaction of hCA I and II with boron derivatives was investigated by kinetic and spectroscopic studies.[70] Such derivatives, tested as new inhibitors, were sulfonamide and non-sulfonamide boron derivatives and some of them proved to be moderately efficient inhibitors of the isozymes mentioned above, their activities being comparable to those of the unsubstituted sulfonamides, the classical inhibitors of these zinc enzymes. Ph_2BOH, one of the compounds with the highest affinity for hCA II in this study (K_I of 3.4 μM), has been docked within the active site of hCA II. After minimisation it was found situated at 7.9 Å from zinc, within the hydrophobic half of the active site, in Van der Waals contacts with the amino acid residues: Val 121, Phe 130, Val 135, Leu 141, Val 143, Val 207 and Pro 201. This was the first time that a CA inhibitor has been found to bind at the edge of the active site cavity, similarly to the CA activator histamine,[75] which binds on the hydrophilic half. This finding may be of importance also for the design of novel types of inhibitors with increased affinity for the different CA isozymes.[70]

The tail approach has also been further developed, and applied for the preparation of other CAIs with interesting biological activity. Thus, sulfonamides of type **57** incorporating 2-carboxy-benzenecarboxamido (phthaloyl) tails were prepared by reaction of phthalic anhydride with aromatic/heterocyclic sulfonamides **A**–**Y** under mild conditions.[76] Another closely related series of derivatives was prepared by reaction of the same reagents under more forcing conditions, when the corresponding phthalimides 58 were obtained.[76] Some of these compounds showed very good *in vitro* inhibitory properties against CA isozymes I, II and IV, with affinities for the enzymes in the low nanomolar range for the best inhibitors, and some of them were formulated as sodium salts, in aqueous solutions for topical administration as

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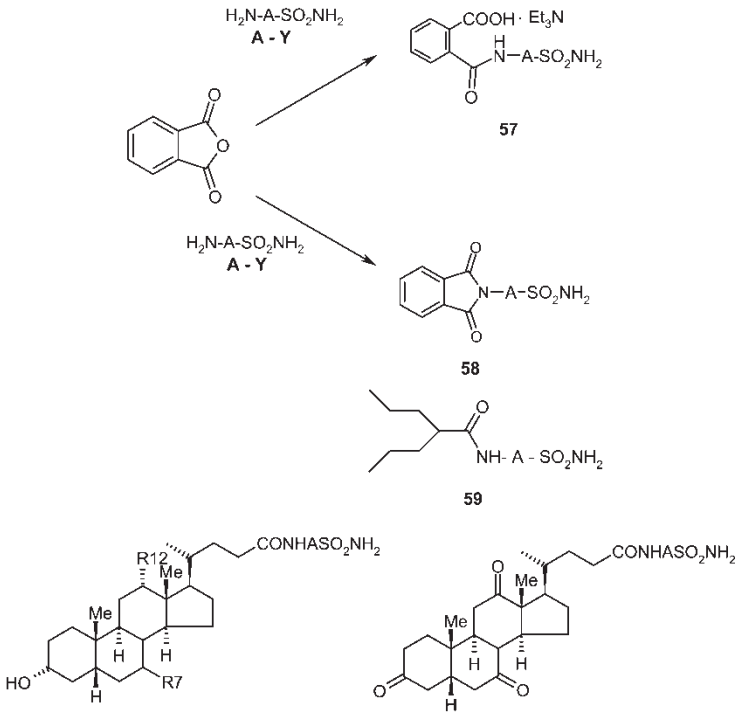
antiglaucoma agents, in normotensive/glaucomatous rabbits. Only the derivatives possessing good water solubility showed effective and longer lasting topical intraocular pressure (IOP) lowering than dorzolamide and brinzolamide. All the *in vivo* effective new compounds belonged to the first series of derivatives (of type **57**), whereas the corresponding phthalimides **58**, although stronger *in vitro* CAIs were devoid of topical IOP lowering properties, probably due to their unsuitable physico-chemical properties.[76] Another series of aromatic/heterocyclic sulfonamides incorporating valproyl moieties of type **59** were reported, in order to design anti-epileptic compounds possessing in their structure two moieties known to induce such a pharmacological activity: valproic acid, one of the most widely used anti-epileptic drugs, and the sulfonamide residue included in acetazolamide and topiramate, two CAIs with anti-epileptic properties.[77] Some of the derivatives **59** showed very high inhibitory potency against three CA isozymes involved in important physiological processes, such as CA I, CA II and CA IV, and also showed promising *in vivo* anticonvulsant properties in mice. Such compounds may be considered as interesting leads for developing anticonvulsant or selective cerebrovasodilator drugs.[77]

Reaction of *tert*-butyl-dimethylsilyl (TBDMS)-protected bile acids (such as cholic, chenodeoxycholic, deoxycholic, lithocholic, ursodeoxycholic acids) or dehydrocholic acid with aromatic/heterocyclic sulfonamides possessing free amino/hydroxy moieties, of type **A**–**Y**, in the presence of carbodiimides, afforded after deprotection of the OTBDMS ethers, a series of sulfonamides incorporating bile acid moieties in their molecules, of types **60**–**65**.[78] Many such derivatives showed strong inhibitory properties against three isozymes CA I, II and IV. Some of the most active derivatives, incorporating 1,3,4-thiadiazole-2-sulfonamide or benzothiazole-2-sulfonamide functionalities in their molecules, showed low nanomolar affinity for CA II and CA IV. Furthermore, the bioavailability of these derivatives in rabbits was comparable to that of acetazolamide, being in the range of 85–90%, showing them as promising candidates for systemically acting CAIs.[55] Some of the derivatives reported above[78] were also investigated thereafter by another group, which prepared them by a slightly different method.[79]

Reaction of (poly)amino-polycarboxylic acids or their dianhydrides with aromatic/heterocyclic sulfonamides **A**–**Y**, possessing a free amino/imino/hydrazino/hydroxy group afforded mono- and



60: R7 = R12 = α-OH (cholic acid derivatives)

61: R7 = α-OH; R12 = H (chenodeoxycholic acid derivatives)

62: R7 = H; R12 = α-OH (deoxycholic acid derivatives)

63: R7 = R12 = H (lithocholic acid derivatives)

64: R7 = β-OH; R12 = H (ursodeoxycholic acid derivatives)

65: dehydrocholic acid derivatives

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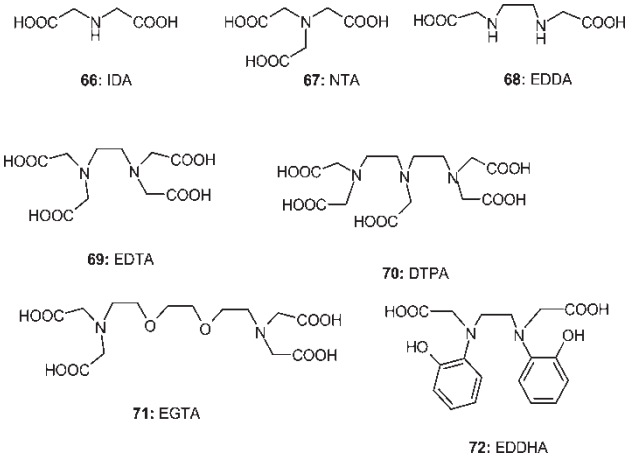
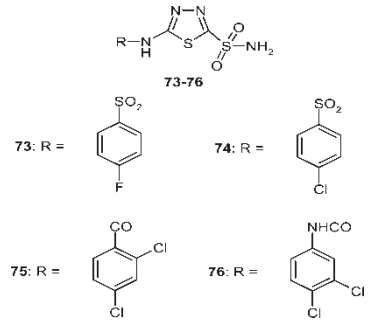
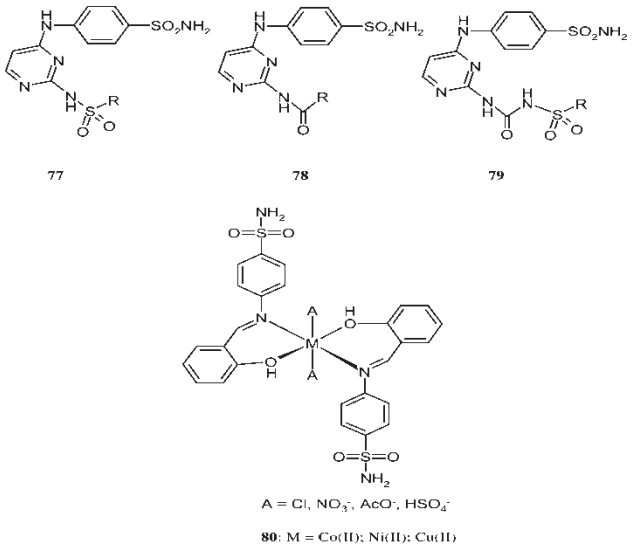
bis-sulfonamides containing (poly)amino-polycarboxylic tails in their molecule.[80] The acids/anhydrides used in synthesis included: IDA **66**, NTA **67**, EDDA **68**, EDTA **69** and EDTA dianhydride, DTPA **70** and DTPA dianhydride, EGTA **71** and EGTA dianhydride, and EDDHA **72** among others. All the newly prepared derivatives showed strong affinity towards isozymes I, II and IV. Metal complexes of the new compounds have also been prepared. Metal ions used in such preparations included di- and trivalent main-group and transition cations, such as Zn(II); Cu(II); Al(III); etc. Several new sulfonamides/disulfonamides obtained in this way, as well as their metal complexes behaved as nanomolar CAIs against isozymes II and IV, being slightly less effective in inhibiting isozyme I. Some of these sulfonamides as well as their metal complexes strongly lowered IOP when applied topically, directly into the normotensive/glaucomatous rabbit eye, as 1–2% water solutions/suspensions. The good water solubility of these sulfonamide CA inhibitors, correlated with the neutral pH of their water solutions used in the ophthalmologic applications and the long duration of action of the IOP lowering effect, makes them interesting candidates for developing novel types of antiglaucoma drugs devoid of serious topical side effects.[80]



Another approach for obtaining compounds for ophthalmologic applications as antiglaucoma drugs consisted in obtaining complexes of several 1,3,4-thiadiazole-2-sulfonamide derivatives of types **73–76** with β-cyclodextrin and hydroxypropyl-β-cyclodextrin.[81] Although the investigated CAIs possessed very powerful inhibitory properties against the two CA isozymes involved in aqueous humor production within the eye, i.e., CA II and CA IV, these compounds were topically ineffective as IOP lowering agents in normotensive/hypertensive rabbits, due to their very low water solubility. On the contrary, the cyclodextrin complexes of sulfonamides **73–76** proved to be effective and long lasting IOP lowering agents in the two animal models of glaucoma mentioned above.[81]



Reaction of 4-(2-amino-pyrimidin-4-yl-amino)-benzenesulfonamide with alkyl/aryl-sulfonyl halides, acyl halides or arysulfonyl isocyanates afforded a series of derivatives of type **77–79** which were tested for inhibition of three CA isozymes.[82] These compounds were designed in such a way as to (i) strongly inhibit the isozymes involved in aqueous humor secretion within the eye (CA II and CA IV), and (ii) to possess a pharmacokinetic profile that allows an easy penetration through the cornea, when administered as eye drops, in solution or suspension, constituting thus a valuable therapeutic approach for glaucoma. Several of the obtained inhibitors **77–79** showed low nanomolar affinities for the two isozymes involved in aqueous humor secretion, CA II and CA IV, and in normotensive and hypertensive rabbits, some of them showed an effective and prolonged IOP lowering when administered topically, as 2% suspensions/solutions.[82] Complexes of type **80**, a Schiff base CAI incorporating different metal ions (Co(II), Ni(II), Cu(II)) were also reported and tested as CAIs.[60] Some of these derivatives are potent inhibitors of the physiologically relevant isozymes I, II and IV.[83]



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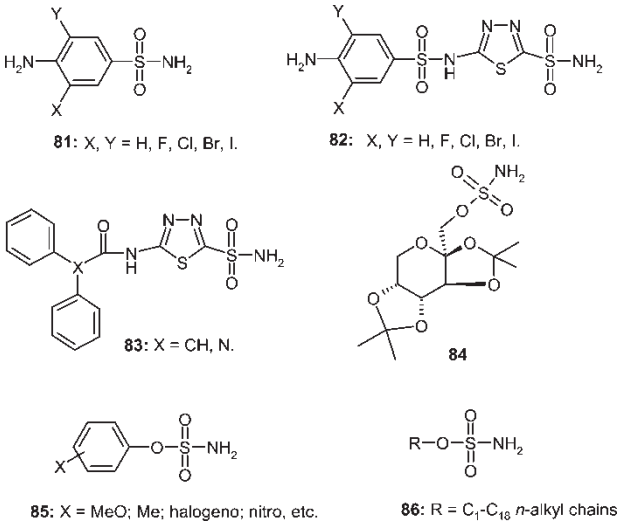
Two CA isozymes, CA IX and CA XII, are found in cancer tissues, while being absent from or decreased in their normal counterparts.[84] The first tumor-associated CA isozyme discovered was CA IX, a transmembrane protein with a suggested function in maintaining the acid–base balance and intercellular communication, consisting of a signal peptide, a proteoglycan-like region, a CA catalytic domain, a transmembrane region, and a short intracytoplasmic tail.[85,86] It has subsequently been shown that this protein is present in only a few normal tissues, predominantly in normal gastric, intestinal and billiary mucosa,[87,88] but is ectopically expressed in a multitude of tumors, such as cervical carcinomas, renal cell carcinomas, lung carcinomas, breast cancers, carcinomas of the esophagus etc.[89–95] On the other hand, CA XII (with a domain composition similar to CA IX, but lacking the proteoglycan region) was initially identified as an antigen overexpressed in 10% of renal cell carcinomas and in breast and colon carcinomas.[96–99] Nevertheless, it is also present in many corresponding normal tissues, including kidney, breast, colon, lung etc.[97–100]

Both of these tumor-associated isoenzymes have been recently connected with hypoxia and tumor progression.[101,95,98] Expression of CA IX in various tumor types is linked to poor prognosis and the clinical and experimental data indicate that hypoxia is a critical factor in its regulation.[98,102] In contrast, the factors related to differentiation appear to dominate the regulation of CA XII and its expression may predict good prognosis in breast cancer.[98]

In fact, acetazolamide, one of the best-studied, classical CAIs used clinically, was shown to function as a modulator in anticancer therapies, in combination with different cytotoxic agents, to suppress tumor metastasis and to reduce the invasive capacity of several renal carcinoma cell lines (Caki-1, Caki-2, ACHN, and A-498).[103–105] Such valuable studies constituted a proof-of-concept demonstration that CAIs may be used in the management of tumors that overexpress one or more CA isozymes. Thus, recently, the first CA IX inhibition study has been reported by our group.[106–111] The inhibition of the tumor-associated, membrane bound isozyme CA IX with a series of aromatic and heterocyclic sulfonamides, among which were also the six clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide, has been investigated. A very interesting and unusual inhibition profile against CA IX with these sulfonamides was observed, which is a promising discovery for the potential design of CA IX-specific inhibitors with applications as antitumor agents. Several nanomolar (K_I in the range of 14–50 nM) CA IX inhibitors were detected, both among the aromatic (such as orthanilamide, homosulfanilamide, 4-carboxy-benzenesulfonamide, 1-naphthalenesulfonamide and 1,3-benzenedisulfonamide derivatives) as well as the heterocyclic (such as 1,3,4-thiadiazole-2-sulfonamide, benzothiazole-2-sulfonamide, etc.) sulfonamides investigated.[106]

CA IX inhibition has also been investigated with a series of halogenosulfanilamides **81** and halogeno-aminobenzolamides **82**,[107] with lipophilic sulfonamides **83**,[108] as well as with sulfamates/sulfamides.[109–112] Indeed, the antiepileptic drug topiramate **84** was recently shown to act as a low nanomolar inhibitor of several CA isozymes, including CA IX, and its X-ray crystal structure in complex with hCA II has also been reported.[117] Winum *et al.*[110,111] showed then that aromatic sulfamates of type **85** as well as aliphatic derivatives **86**, not only act as very potent inhibitors of several isozymes (CA I, II, IV and IX), but some of them also show clear differences in affinity between these isozymes, it becoming thus possible to detect compounds with a certain selectivity for the target isozyme, the tumor-associated one CA IX.



Casini *et al.*[112] also investigated CA I and II inhibition with sulfamides of type **87**, detecting low nanomolar inhibitors, whereas carboxy-benzolamide derivatives of type **88** were shown to act as very potent CA I, II and IV inhibitors, and to constitute effective IOP lowering agents in a model of glaucoma.[113] The same type of behavior was then reported for glycosylated sulfanilamides of type **89**.[114] Finally, the high resolution X-ray crystal structures were reported for the hCA II complexes with sulfonamides/sulfamates **90–96**, some of which are clinically used or are in advanced clinical trials (E7070 **92** and EMATE **93** as anticancer drugs, sulpiride **94** as an antipsychotic drug, and celecoxib **96**—a COX-2 selective inhibitor—as an anti-inflammatory agent).[115–121]

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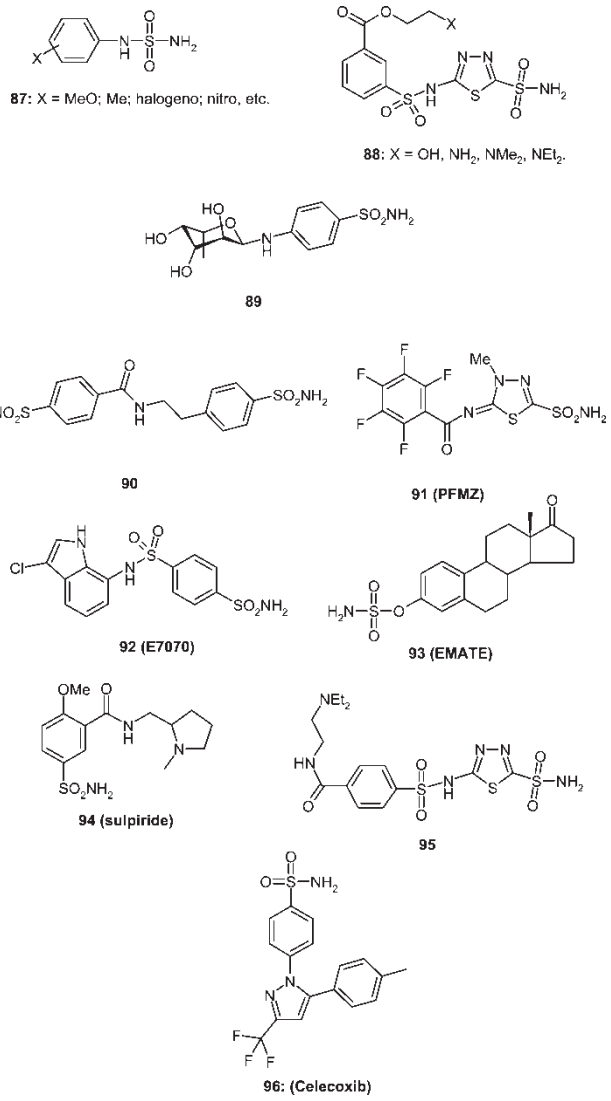

It has also been shown that CA XIII is an isozyme with nanomolar affinity for sulfonamide inhibitors (such as acetazolamide) and also highly sensitive to inhibition by anions.[122]

CA ACTIVATORS

CA inhibition by sulfanilamide, discovered in England by Mann and Keilin[41] and its activation by different classes of compounds, reported in Germany by Leiner,[123] although simultaneous, had completely different consequences for CA research. Whereas CAIs were extensively studied in the next decades, leading to a detailed understanding of the catalytic and inhibition mechanisms, but also to several valuable pharmacological agents,[1–4] CA activators (CAAs) constituted a controversial issue immediately after they were first described.[124–129] Thus, activation of crude human red cell enzyme by diverse tissue extracts or by selected pure compounds, such as histamine, amino acids and some purine derivatives has been reported and retracted several times by the above-mentioned and other authors (reviewed in ref.[129]), without arriving at a clear-cut answer regarding the mere existence of such a class of CA activity modulators. This topic then received little attention from the scientific community in the period from 1950 for at least two reasons: (i) the statement by Clark and Perrin[130] that activators of CA do not exist, and (ii) the idea[131,132] that the reported activation is not a phenomenon *per se*, but an artefact generally due to restoration of CA activity possibly lost in the presence of adventitious metal ions or other impurities, or due to enzyme adsorption at interfaces, or even due to enzyme denaturation followed by renaturation in the presence of activators. Regarding the above two factors, one should note that Clark and Perrin[130] did their experiments in the presence of high concentrations of peptone, which like many peptides/oligopeptides or simple amino acids, has an important CA activating effect, and this might explain why these authors did not observe activation with the other investigated compounds, such as glutathione, histamine, glycine, adrenaline, etc. On the other hand, the generally irreproducible results published in the first decade of research into CA activators[129] were due not only to experimental difficulties related to the use of the manometric measurements, but probably also to the low purity of enzyme preparations as well as of the activators used in the experiments. Thus Leiner,[123] the researcher whose role in discovering this important class of modulators of CA activity should be completely re-evaluated, observed that the activation is more readily detected when working with highly purified enzyme preparations.

Research into CA activators progressed little in the period 1960–1980, except for the report of Ho and Sturtevant[133] that EDTA and other polyaminopolycarboxylic acids act as strong activators. As in the case of the previous researchers,[123,129] this fact has been erroneously attributed to conformational changes induced in the enzyme by the activator molecule, or to the stabilization of the enzyme in the presence of the activator.

Although important advances were reported in the late 70's in understanding the CA catalytic/inhibition mechanism, these discoveries had no consequences for the study of activators, except for the controversial work of Silverman's group on the activation of red cell CAs by histidine or hemoglobin.[134] This study also took into consideration the possibility that the CA activation mechanism might involve an enhanced proton transfer facilitated by the activator molecule. Still, the term "activator" was avoided in the above study, as it was in the only other important work on this topic,

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by Parkes and Coleman,[135] who studied the activity "enhancement" of isozymes I and II in the presence of erythrocyte membranes.

A totally different atmosphere in the area of CAAs research was inaugurated in the early 90's with the work from our laboratory on activators of isozymes I and II, together with a hypothesis for explaining the mechanism of such processes.[136–139] Finally, one had to wait as late as 1997, for the first X-ray crystallographic structures of adducts of CA II with different activators, which were reported by this group,[11] proving undoubtedly the existence of this class of compounds as well as elucidating their mechanism of action.

Actually, the phenomenon of CA activation is explained by considering the catalytic mechanism of this enzyme.[1–4,75] The generally accepted catalytic mechanism for the physiological reaction involves the nucleophilic attack of zinc-bound hydroxide on CO_2, optimally activated and oriented in the hydrophobic pocket of the CA active site. Bicarbonate formed in this way is then replaced by a water molecule, with generation of the catalytically inactive form of the enzyme $EZn^{2+}–OH_2$ (Equation *14*). In order to regenerate the catalytically active form, a proton transfer reaction must occur, from the water bound to Zn(II) within the enzyme active site, to the external medium. In isozyme CA II, this step (Equation *15*) was considered to be assisted by the active site residue His 64, placed at the entrance of the active site, as well as by external buffer molecules.[1–4,75] This step is also rate-determining for the whole catalytic cycle[140] and the shuttling effects of His 64 would explain the very high efficiency of CA II as catalyst, with a maximal turnover number of $1.6 \times 10^6\ s^{-1}$.[141] Indeed, in several crystal structures, the His 64 side chain has been observed disordered over two orientations, one towards the inside and the other one towards the outside of the active site cavity, indicating its flexibility and hence supporting its involvement in the proton shuttling.[142,143]

$$EZn^{2+}–OH^- + CO_2 \Leftrightarrow EZn^{2+}–HCO_3^- \overset{H_2O}{\Leftrightarrow} EZn^{2+}–OH_2 + HCO_3^- \quad (14)$$

$$EZn^{2+}–OH_2 \Leftrightarrow EZn^{2+}–HO^- + H^+ \quad (15)$$

In the presence of activators, again for isozymes II and III, it was proposed that an enzyme–activator complex is formed, in which the activator participates in proton transfer processes.[136–139] The enhanced catalytic rate is due to the fact that intramolecular reactions are more rapid than intermolecular ones.[136–139] Thus, in the presence of activators (symbolized as "A"), Equation (*15*) becomes (*16*):

$$EZn^{2+}–OH_2 + A \Leftrightarrow [EZn^{2+}–OH_2–A] \Leftrightarrow [EZn^{2+}–HO^–AH^+] \Leftrightarrow EZn^{2+}–HO^- + AH^+ \quad (16)$$

enzyme–activator complexes.

Although originally proposed for isozyme II, the above mechanism is probably valid also for other isozymes.[75] Nonetheless, the kinetic, spectroscopic and crystallographic evidence supporting this mechanism was generally obtained working with human CA II.

The first adduct of a CAA studied by means of X-ray crystallography was the histamine–hCA II complex, characterized at a resolution of 1.95 Å.[11] The histamine molecule is bound at the entrance of the active site cavity, where it is anchored by hydrogen bonds to amino acid side-chains and to water molecules. It is noteworthy that such hydrogen bonds involve only the nitrogen atoms of the imidazole moiety. The terminal aliphatic amino group does not have any contact with the enzyme, but extends from the cavity into the solvent.[11] The Nδ1 and Nε2 atoms of the histidine imidazole ring were shown to be engaged in hydrogen bonds with the side-chains of Asn 62 and of Gln 92 as well as to Wat 152. Comparison of the refined model of the complex with that of the native enzyme refined at 1.54 Å produced evidence of some relevant differences. In native hCA II, as well as in most of its small molecule adducts, the zinc coordination polyhedron has always been a quite regular tetrahedron with three histidine nitrogens and a water molecule/hydroxide ion as ligands. On the other hand, in the hCA II–histamine complex, the electron density corresponding to the metal coordinated water/hydroxide molecule had an elongated shape. The binding of histamine to hCA II displaced at least three water molecules from the active site cavity and this has been accompanied by a substantial rearrangement of the water structure in the cavity. A further difference with respect to the native structure was found regarding the orientation of the His 64 side chain.[11] While this residue has almost always been found to be disordered both in the native and in many hCA II complexes, in the histamine adduct structure the side chain of His 64 appeared well defined and oriented towards the inside of the cavity, pointing towards the metal site.[11] The His 64 imidazole ring was shown to be involved in a hydrogen bond with a nearby water molecule and to make short contacts with the histamine imidazole moiety. Inspection of the refined model of the hCA II–histamine complex also revealed the presence of a hydrogen bond pathway linking the zinc bound water

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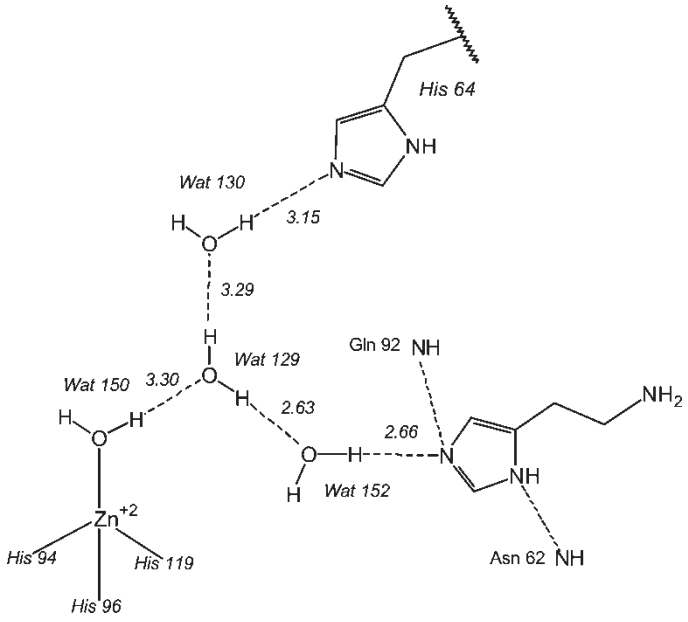

FIGURE 12 Schematic binding of histamine to hCA II active site. The catalytic metal ion, the hydrogen bond network for the proton transfer reactions, as well as the proton shuttle residue His 64 are evidenced, in addition to the histamine molecule, which participates in three hydrogen bonds with a water molecule (Wat 152) and the backbone amide moieties of Asn 62 and Gln 92 [10].

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(numbered as Wat 150) molecule to histamine through two water molecules present within the active site, Wat 129 and Wat 152 as shown in Figure 12. A second alternative pathway exists through Wat 129 and Wat 130, reaching then His 64 (Figure 12). This is similar to the pathway existing in the native enzyme between the zinc-bound water and His 64 which has been considered as the normal proton release pathway.[1,2,4,75]

The hydrogen bond pathway linking the zinc-bound water (Wat 150) to the histamine molecule reported in Figure 12 provides the alternative route for proton release besides the His 64 shuttling. The mere availability for the proton of more than one pathway to leave the active site appears to be a reliable explanation for the activation effect of histamine and related compounds towards hCA II. The entropic contribution to the histamine free energy of binding to hCA II provided by the release of water molecules appears to dominate the complex formation, although the ability of histamine to make two hydrogen bonds simultaneously clearly provides further stabilisation to the complex. The magnitude of the interaction seems to be such as to favour the activating effect. Indeed the ability of histamine to leave the active site cavity easily and act as a second proton shuttle seems to fit the activation mechanism perfectly as has emerged from the structural findings.

The X-ray structure of hCA II complexed with histamine provided important insights for the design of novel CAAs using this simple compound as lead. Indeed, the aliphatic amino group of the activator does not participate in any interaction with the CA active site. Thus, the most logical rational was to derivatize it, in order to obtain better interactions with amino acid residues at the rim of CA active site, assuring in this way a higher affinity for the enzyme. Indeed, many such derivatives were obtained by a reaction strategy which involved treatment of histamine with tetrabromophthalic anhydride and protection of its imidazole moiety with tritylsulfenyl chloride, followed by hydrazinolysis.[144–147] The obtained N-1-tritylsulfenyl histamine, the key intermediate, was further derivatized at its aminoethyl moiety by a variety of procedures. Carboxamido derivatives **97–100** were obtained by reaction of the key intermediate with carboxylic acid anhydrides, acyl chlorides or carboxylic acids in the presence of carbodiimides.[145] Reaction of the same key intermediate with isocyanates, isothiocyanates, cyanamide or dicyandiamide afforded another series of compounds. Deprotection of the above-mentioned intermediates with hydrochloric acid in dioxane afforded two series of compounds, histamine derivatives possessing carboxamido, ureido, thioureido or guanidino moieties in their molecule. These derivatives were assayed as activators of three isozymes, hCA I, hCA II and bCA IV. Efficient activation was observed against all three isozymes, but especially against hCA I and bCA IV, with affinities in the nanomolar range for the best compounds. hCA II was, on the other hand, activatable with affinities around 10–25 nM.[145]

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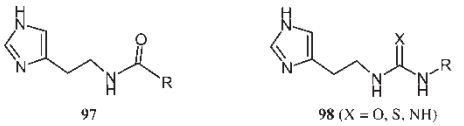
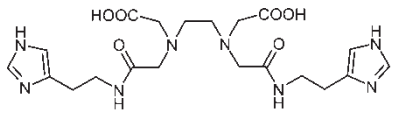


97 **98** (X = O, S, NH)

R = alkyl, aryl, etc.



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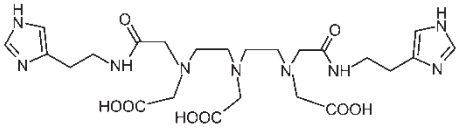

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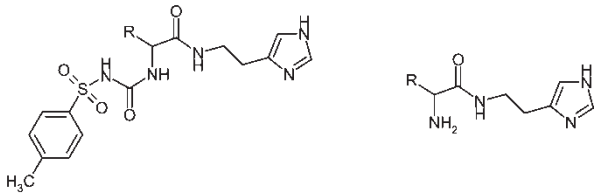
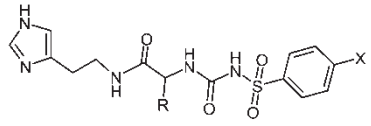
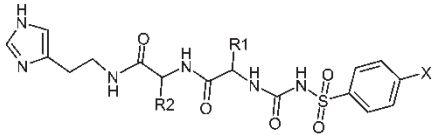


101 (R belongs to natural and non-natural amino acid) **102**



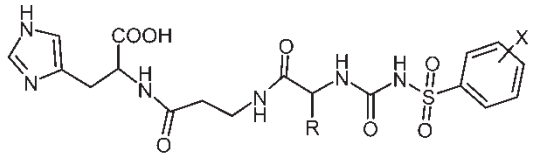
103



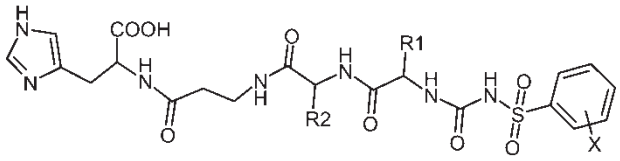
104

X = F, Cl, Me; R1, R2 belong to natural and non -natural amino acid

Reaction of the key intermediate mentioned above with arylsulfonylureido amino acids in the presence of carbodiimides, afforded after deprotection, several series of compounds with the general formula arylsulfonylureido-AA-Hst (arylsulfonylureido = 4-$FC_6H_4SO_2NHCO$; 4-$ClC_6H_4SO_2NHCO$; 2- or 4-$MeC_6H_4SO_2NHCO$; Hst = $NHCH_2CH_2$-imidazole-4-yl). Some structurally related dipeptides with the general formula arylsulfonylureido-AA1-AA2-Hst (AA, AA1 and AA2 represent amino acyl moieties) were also prepared, by a strategy similar to that used for the simple amino acyl compounds above. These derivatives of types **101**–**104** proved to be efficient *in vitro* activators of three CA isozymes. Best activity was shown against hCA I and bCA IV, for which some of the new compounds (such as the Lys, Arg, His or the dipeptide derivatives) showed affinities in the 2–12 nm range. hCA II was on the other hand somehow less prone to activation by the new derivatives, which possessed affinities around 30–60 nM for this isozyme. *Ex vivo* experiments showed some of the new activators to strongly enhance red cell CA activity (180–230%) after incubation with human erythrocytes.[145–147]



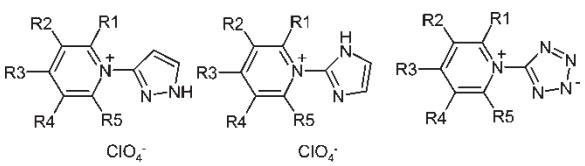
105: X = 4-F
106: X = 2-Me



107: X = 4-F
108: X = 2-Me

R1, R2 belong to natural and non-natural amino acid

A novel class of tight binding CAAs was recently designed by using histamine and histidine as lead molecules.[148] Carnosine (β-Ala-His) derivatives of types **105** and **106** were synthesized by reaction of appropriately derivatized β-alanines with imidazole/carboxy-protected histidine in the presence of carbodiimides, followed by removal of the various protecting groups. The derivatized β-alanines mentioned above were in turn obtained by coupling of 4-fluorophenylsulfonylureido amino acids (fpu-AA) or 2-toluene-sulfonylureido amino acids (ots-AA) with β-Ala. Some structurally related dipeptides with the general formula fpu/ots-AA1–AA2 (AA, AA1 and AA2 represent amino acyl moieties) of types **107** and **108** were also prepared by a similar strategy, and used thereafter for obtaining CAAs incorporating a modified tetrapeptide scaffold. Many of the new tri-/tetrapeptide derivatives proved to be efficient *in vitro* activators of three CA isozymes. Very good activity was detected against hCA I and bCA IV, for which some of the new compounds showed affinities in the 1–20 nanomolar range, whereas against hCA II, their affinities were in the range of 10–40 nM. *Ex vivo* experiments showed some of the new activators to strongly enhance cytosolic red cell CA activity after incubation with human erythrocytes.[148]



109 **110** **111**

(R1-R5 = alkyl, aryl or combination of both)

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A series of CAAs was obtained then by reaction of amino-azoles (3-amino-pyrazole, 2-amino-imidazole, and 5-amino-tetrazole) with tri- or tetra-substituted pyrylium salts.[149] Many of the new pyridinium salts incorporating azole moieties of types **109–111** proved to be efficient *in vitro* activators of the three CA isozymes mentioned above, i.e., CA I, II and IV. Very good activity was detected against hCA I and bCA IV, for which some of the compounds **109–111** showed affinities in the low nanomolar range, whereas against hCA II, their affinities were in the range of 95–150 nM. Substitution patterns of the pyridinium ring leading to best activity included 4-phenyl-2,6-dialkyl moieties or 2,4,6-tri- and 2,3,4,6-tetraalkyl groups. *Ex vivo* experiments showed some of the new activators to strongly enhance CA activity after incubation with human erythrocytes. Furthermore, due to their cationic nature, some of these compounds (the imidazole and pyrazole derivatives) were shown to be membrane-impermeant, discriminating thus between cytosolic and membrane-bound CA isozymes. These compounds represent thus the first membrane-impermeant CAAs. The pyridinium tetrazole derivatives on the other hand were shown to penetrate through biological membranes.[149]

A very important discovery[150] deals with the CA activating properties of phenylalanine (Phe), a compound reported by us to possess such biological activity.[151] In the presence of CAAs, such as Phe or imidazole, co-microstimulation of cholinergic inputs from stratum oriens and GABA-ergic inputs from stratum pyramidale at low intensities switched the hyperpolarizing GABA-mediated inhibitory postsynaptic potentials to depolarizing responses. In the absence of the CAAs, however, the same stimuli were insufficient to trigger the synaptic switch.[150] This synaptic switch changed the function of the GABA-ergic synapses from excitation filter to amplifier and was prevented by CAIs, indicating a dependence on bicarbonate availability. Intralateral ventricular administration of these same CAAs caused the rats to exhibit superior learning of the Morris water maze task, suggesting that the GABA-ergic synaptic switch is critical for gating the synaptic plasticity that underlies spatial memory formation. Increased CA activity might, therefore, also enhance perception, processing, and storing of temporally associated relevant signals and represents an important therapeutic target in learning and memory pharmacology.[150] Thus, CA activators might lead to the development of drugs/diagnostic tools for the pharmacological enhancement of synaptic efficacy, spatial learning and memory. This may constitute a new approach for the treatment of Alzheimer disease and other conditions in need of achieving memory therapy.[150,152]

In the context of the physiological function of CAAs an important discovery,[153–156] must be mentioned regarding the human chloride/bicarbonate anion exchanger (AE1) which possesses a binding site within its 33 residue carboxyl-terminal region (Ct) for CA II. The amino acid sequence comprising this CA II binding site was determined by peptide competition and by testing the ability of truncation and point mutants of the Ct sequence to bind CA II with a sensitive microtiter plate binding assay. A synthetic peptide consisting of the entire 33 residues of the Ct (residues 879–911) could compete with a GST fusion protein of the Ct (GST-Ct) for binding to immobilized CA II, while a peptide consisting of the last 16 residues (896–911) could not. A series of truncation mutants of the GST-Ct showed that the terminal 21 residues of AE1 were not required for binding CA II. Removal of four additional residues (887–890) from the Ct resulted in loss of CA II binding. Acidic residues in this region (D887ADD) were critical for binding since mutating this sequence in the GST-Ct to DAAA, AAAA, or NANN caused loss of CA II binding. A GST-Ct construct mutated to D887ANE, the homologous sequence in AE2, could bind CA II. AE2 is a widely expressed anion exchanger and has a homologous Ct region with 60% sequence identity to AE1. Tethering of CA II to an acidic motif within the Ct of anion exchangers was hypothesized to be a general mechanism for promoting bicarbonate transport across cell membranes.[153–156] This may be the case indeed, but we have also recently shown that the tetrapeptide DADD as well as some related oligopeptides (such as for example DA, DD, DAD, DDD, or DDDD) act as very potent activators just of isozyme CA II involved in the interaction with the anion exchanger AE1 mentioned above.[157] It is thus possible that in the complex between hCA II–AE1, the last protein acts as a natural activator of this isozyme, facilitating the formation and transport of bicarbonate in the erythrocytes.

BIOMEDICAL APPLICATIONS OF INHIBITORS/ACTIVATORS OF CAs

Principal involvement of CAs in various physiological processes implies that their deregulated expression and/or abnormal performance may have important pathological consequences. In fact, there are several human diseases whose pathophysiological characteristics include imbalance in conversion between carbon dioxide and bicarbonate resulting in perturbed ion transport, shift in pH, abnormal fluid secretion etc.[1,2] Therefore, it seems plausible that modulation of CA activity to normal levels either by inhibition or activation offers a feasible therapeutic option.

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Clinical testing and/or use of the first CAIs dates several decades back to a period before the recognition of a diversity of isoforms within the CA family and their differential distribution in various human tissues and organs.[42,43] Traditionally, application of inhibitors was based on histochemical, biochemical and functional evidences for the presence of active CA potentially contributing to disease and consequences of treatment were evaluated mainly symptomatically. Because of generally favourable outcomes, sulfonamides became widely accepted drugs in treatment of several CA-based diseases, especially as anti-glaucoma agents and diuretics[1,2]. However, systemic and even topically administered CA inhibitors regularly showed serious side effects.[2,4,42,43] We now understand that these undesired effects are due to existence of at least twelve different active CA isoforms, that are indiscriminately inhibited irrespective of whether they play a real role in a disease or are just co-expressed in the same tissue and elsewhere in the body. In addition, some CAIs can also block other biologically active molecules, such as metalloproteinases, what can further expand their action.[74] Moreover, certain drugs directed primarily against different CA-unrelated molecules may also inhibit the activity of CAs, which could be utilised to achieve more efficient therapeutic effects. This may be exemplified by the antiinflammatory cyclooxygenase-2-selective drug celecoxib that shows nanomolar affinity to several CA isoforms; it is well tolerated and gives clinical responses in several disorders.[121]

This symptomatic attitude to the study of therapeutically used CA inhibitors persists to some extent to the present time, although significant efforts have been undertaken towards design, synthesis and evaluation of the new generations of inhibitors with the selective targeting of particular isoforms as described in detail above. Of course, progress in biomedical applications of CAIs clearly requires not only improvements in the physicochemical properties of sulfonamides, but also more precise knowledge on the distribution and role of individual enzymatically active CA isoforms. The first task has been systematically followed by the production of ever more efficient inhibitors with preferential selectivity to surface *versus* cytosolic CA isoenzymes and specificity for individual isoforms. Evaluation of these inhibitors relies primarily on their inhibitory activity against single purified or recombinant CA isoforms which complicates the extrapolation of results to *in vivo* situations, where several CAs work in parallel within one cell and cooperate with other types of molecules as components of metabolons or complex networks underlying various metabolic and signal transduction pathways. Some progress in obtaining more relevant data can be achieved by the use of cultured cells genetically modified to ectopically express certain CA isoenzyme and their mutants, but the real therapeutic efficacy can still be only assessed when using *in vivo* models or clinical testing.

The amount of data related to the expression of isozymes is also rapidly increasing as the result of isozyme-specific antibodies and sensitive molecular detection techniques that can discriminate one isoform among the others. These tools allow an insight into distribution of the isoenzymes especially in normal human tissues, while the information linking abnormal expression of isoforms to diseases is rare (Table II). In fact, our knowledge that overexpression of CAs may underlie development of disease is principally limited to CA IX and CA XII in cancer and to a recent finding of increased CA XII level in the glaucomatous eye.[89–102,158] Specific biological roles of the isozymes are also largely unknown, although some functions can be derived from a few, so far, generated models of CA deficiency—i.e. chemically induced loss of CA II (also naturally present as genetic defect in human population)[159], and targeted null mutations of CA IX[160], CA III (by Levine and Kim, so far unpublished), and CA VA/VB (Sly's group, so far unpublished). On the other hand, we completely lack information on the functional consequences of manipulated *in vivo* overexpression of isozymes. We could possibly learn more from transgenic models with ectopic tissue-specific expression of separate isozymes, but no such models have yet been generated. Despite these weak points, there is substantial empirical and experimental evidence approving the clinical use of CAIs in several pathologic states and raising hopes for their application in additional diseases (Table III).

Classical example of a disease whose treatment involves sulfonamides is glaucoma. This is based on the fact that carbonic anhydrases present in ciliary bodies contribute to the development of the disorder by excessive production of bicarbonate ions thus stimulating secretion of aqueous humor. This results in increased intraocular pressure, which severely affects the eye lens, reduces the blood flow in retina, damages light-sensitive rods and cones and destroys optic nerve, leading eventually to blindness unless the condition is detected and treated before the damage becomes permanent. CA II was long considered as a major player in this process, but CA III, CA IV and CA XII have emerged as additional candidates. Especially interesting is detection of the five-fold increased CA XII mRNA level and higher CA XII protein expression in non-pigmented ciliary epithelial cells from a glaucoma patient when compared to a normal donor.[158] In addition, CA XII was among the five most abundantly expressed genes in human corneal endothelium as determined

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TABLE II Tissue distribution and proposed function of catalytically active mammalian CA isozymes

Isozyme	Tissue of expression	Proposed role(s)
CA I	Esophageal and laryngeal epithelium[181]	Antireflux defense
	Erythrocytes	Gas exchange
	GI mucosa	Ion transport
CA II	Various tissues/organs including: Nasal mucosa[182]	Nasal chemosensitivity to CO_2
	Esophageal and laryngeal epithelium[181]	Antireflux defense
	Bone osteoclasts	Bone resorption
	Eye	Production of aqueous humor
	Testis[183]	Sperm motility
	Kidney (renal tubular epithelium)	Urine acidification
	Brain (glia)	CSF secretion
	Lung	Gas exchange
	Erythrocytes	Gas exchange
	Gastrointestinal epithelium	H^+ secretion, HCO_3^- secretion
CA III	Esophageal and laryngeal epithelium[181]	Antireflux defense
	Skeletal muscle, Adipocytes	Fatty acid metabolism
	Eye (ciliary processes and lens)[184]	Ocular fluid transport and homeostasis
CA IV	Nasal mucosa[182]	Nasal chemosensitivity to CO_2
	Esophageal epithelium[181]	Antireflux defense
	Kidney	Bicarbonate reabsorption, NH_4^+ output
	Pancreas, salivary glands[185]	pH regulation
	Heart muscle (endothelial and muscle cells)[186]	pH regulation
	Eye	Production of ocular fluid
	Lung	Gas exchange
	Brain capillaries	Cerebral blood flow
	Colon[190]	pH regulation
CA VA	Liver[187]	Biosynthetic reactions
CA VB	Heart and skeletal muscle, pancreas, kidney, salivary glands, spinal cord[187]	Biosynthetic reactions
	Gastrointestinal mucosa[188]	Detoxification of ammonia
CA VI	Salivary glands, mammary glands[191]	pH regulation, antireflux defense, protection from cariogenesis, taste function
CA VII	CNS	CSF production
CA IX	Various tumors[89–95,98,101,102]	pH regulation, cell adhesion
	Gastrointestinal mucosa (basolateral membrane)[87,88]	Cell proliferation and differentiation, ion transport
	Male excurrent ducts[189]	Concentration and acidification of testicular fluid
CA XII	Nasal mucosa[182]	Nasal chemosensitivity to CO_2
	Renal, intestinal and reproductive epithelia (basolateral membrane)[96–100]	Renal HCO_3^- absorption, H^+ secretion
	Eye (overexpressed in glaucoma)[158]	Aqueous humor production
	Male excurrent ducts[189]	Concentration and acidification of testicular fluid
	Tumors of kidney, breast, colon and lung[95–99]	pH regulation
CA XIII	Salivary glands, kidney, brain, lung, gut, uterus, testis[122]	pH regulation
CA XIV	Kidney (luminal membrane)[167]	Bicarbonate reabsorption
	Brain neurons and axons[164]	Modulation of neuronal transmission
	Liver[192]	

by serial analysis of gene expression.[161] These results suggest that an abnormal level of CA XII may be an important factor in the progression of glaucoma. Different sulfonamide derivatives described above can reduce the intraocular pressure by inhibiting CA-catalyzed production of bicarbonate ions and hence secretion of the ocular fluid.

An additional ophthalmologic disorder whose management involves application of CAIs is cystoid macular oedema that may be linked with different conditions including diabetic retinopathy, ischemic retinopathies and retinitis pigmentosa. It is characterized by a swelling process due to decreased fluid absorption across the retinal epithelium with accumulation of metabolic waste products. It may also be associated with decreased retinal adhesiveness and damage of the blood vessels in the retina. Experimental data indicates that CAIs can induce acidification of the subretinal space and thereby increase fluid absorption and adhesiveness of retina. They also act as local vasodilators that improve blood flow in the eye and clearing of metabolic waste products.

CAIs and/or drugs with CA inhibitory properties (e.g. topiramate) are also used in acute pharmacotherapy of a number of neurological and neuromuscular syndromes, such as epilepsy, hemiplegic migraine, ataxia, hyperkalemic periodical paralysis,

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TABLE III Diseases conventionally or potentially treated by CA inhibitors and their proposed isozyme targets

Disease	Patho-physiological characteristics	Potential isozyme targets	CAI-based drugs in use/in testing
Glaucoma	Increased production of aqueous humor → increased intraocular pressure → optic nerve damage	CA II, CA IV, CA XII	systemic CAIs (e.g. acetazolamide) topical CAIs (e.g. dorzolamide, brinzolamide) aromatic/heterocyclic CAI derivatives
Cystoid macular oedema, diabetic retinopathy	Decreased fluid absorption across the retinal epithelium, Decreased retinal adhesiveness	CA II, CA IV, CA XIV	Acetazolamide
Epilepsy	Increased excitatory function of neurons, Channelopathy	CA IV, CA XIV	Acetazolamide methazolamide topiramate
Neurological/neuromuscular disorders (hemiplegic migraine, ataxia, periodic paralysis, neuropathic pain essential tremor)	Channelopathies with various features (e.g. abnormal electrical excitability, increased serum potassium levels, metabolic acidosis)	CA II, CA IV, CA V, CA VII, CA XIV	Acetazolamide topiramate
Edema (due to congestive heart failure, renal/hepatic disease, diabetes, drugs)	Decreased diuresis	CA II, CA IV, CA V, CA XII, CA XIV	Acetazolamide furosemide
Obstructive pulmonary disease	Increase in arterial CO_2	CA II, CA IV	acetazolamide
Sleep apnea	Hypercapnic ventilatory failure		
Osteoporosis	Increased acid formation and depletion of bone calcium	CA II	acetazolamide
Cancer	Extracellular acidification, abnormal metabolism, increased growth and survival	CA IX (ectopic) CA XII (overexpressed), CA II, CA V (sustained)	Indisulam aromatic/heterocyclic CAIs

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essential tremor etc. Most of these syndromes have a complex genetic background and are classified as channelopathies, because of their apparent relationship to mutations in genes coding for different ion channels.[162] These mutations lead to channel defects that severely affect ion transport homeostasis, disturb the physiology of body fluids and impair the function of blood vessels that supply the brain. Resulting symptoms comprise brain hyperexcitability with febrile seizures, acute headache with visual disturbances, impaired coordination, and muscle weakness (loss of electrical excitability in muscle fibers).[162] What could be the role of CAs in these diseases? It is now well accepted that CAs (both extracellular and intracellular isoforms) can directly cooperate with certain types of ion transporters (anion exchangers, Na/bicarbonate transporters and Na/K pumps) to improve their efficiency.[153–156] In this way, they influence flux of ions through the cell membranes and together with ion channels participate in the generation of physiologically relevant ion gradients that are important for proper synaptic transmission, muscular contraction and other functions. These gradients are created via a subtle balance in import-versus-export continuum and therefore, deregulated activity of one component may perturb the whole network and result in shifted activities of other components. Being aware of the complexity of these processes and of the functional interconnection among their molecular players, it is conceivable that defects in ion channels may induce changes that reflect on the enzymatic activities of the CAs. Indeed, several CAs are expressed in the brain and muscle. Some of them (CA II, CA IV, and CA VII) are believed to influence production of CSF (whose important component is bicarbonate), intracranial pressure and cerebral blood flow.[163] The other, i.e. CA XIV, was found to be present in axons and neuronal membranes in specific areas of brain where it can affect neuronal signalling.[164] CA III is expressed in the cytoplasm in skeletal muscle cells and is proposed to play a role in fatty acid metabolism vital for muscle performance and in scavenging oxygen radicals.[165] Despite the fact that the precise roles of CAs in the neurological and neuromuscular pathologies are incompletely understood, their inhibitors consistently show anticonvulsant and vasodilatory properties and have beneficiary effects providing at least partial relief from the disabling symptoms. Recently, new series of sulfonamides have been prepared by using acetazolamide, topiramate and valproate as lead molecules. Some of them act as very strong anticonvulsants in mouse models and are promising for the development of future medications.[77]

The diuretic activities of CAIs are utilized in various pathological situations that result in development of oedema, a localized or general swelling caused by retention of fluid within body tissues.

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Excess fluid may be a result of poor blood circulation, heart and kidney failure, reduction in the amount of protein in the blood due to cirrhosis or nephritis etc. Diuretics, that stimulate the kidneys to eliminate excess salt and water, often produce an immediate improvement. This occurs through inhibition of several CA isoforms present in kidney—intracellular CA II and membrane-bound CA IV, CA XII and CA XIV. They all seem to play an important role in kidney function facilitating proton secretion, bicarbonate reabsorption and ammonium output.[166] Until now, luminal CA IV acting in the metabolon with AE and CA II was thought to mediate most bicarbonate absorption. CA XIV, which is also localized in the apical membranes of renal epithelial cells, appears to have a similar role, while the basolateral CA XII was proposed to enhance the efficiency of basolateral bicarbonate transporters.[99,167] Interestingly, a number of commonly used diuretic drugs (such as benzothiadiazines, chlorthalidone and furosemid) inhibit CAs which further supports their crucial involvement in renal physiology.

Targeting CAs to block their activity with therapeutic intent seems promising in additional pathological conditions including obstructive pulmonary disease, osteoporosis, obesity and cancer. The rationale for the application of CAIs in some of these diseases is explained elsewhere.[1,2,168] However, the possibility of using sulfonamides to treat cancer deserves particular attention. It is supported by two lines of data coming on one hand from investigations on the capacity of CAIs to reduce tumor cell proliferation/invasion *in vitro* and on the other hand from expression studies of CA isoforms in cancer tissues. The first series of experiments has shown that many sulfonamides inhibit proliferation of cancer cells in a monolayer culture. The classical CAIs that are clinically used as anti-glaucoma drugs can achieve this at micromolar concentrations,[169] while the new generation of sulfonamide derivatives created by ring or tail modifications are efficient at nanomolar levels or even below.[65–67] The former compounds were proposed to interfere with the essential biosynthetic pathways that utilize bicarbonate as a substrate, but accurate molecular targets have been defined neither for them, nor for the newer classes of inhibitors.[84] Also the observations that acetazolamide is able to block *in vitro* invasion of renal carcinoma cells and alone or combined with conventional chemotherapy diminish tumor growth *in vivo* have not been satisfactorily explained.[103,104]

Some ideas about these biological effects of CAIs came from the studies of tumor-associated transmembrane CA isoforms CA IX and CA XII and their potential roles in tumor development. CA IX is normally expressed in epithelia of the gastrointestinal tract but is ectopically induced in a broad variety of tumors including carcinomas of kidney, uterine cervix, lung, breast, colon, head and neck.[87–95,98,101,102] CA XII is present in many normal tissues, but its expression is increased in some renal, lung, breast and colon carcinomas.[95–102] The genes coding for these isoforms are both negatively controlled by the wild type von Hippel Lindau tumor suppressor protein (pVHL) in the transfection experiments,[97] although the link between pVHL and CA XII is less consistent when inspected in its natural context in renal cancer cell lines.[170] Both CA IX and CA XII are also induced by hypoxia, a condition linked to insufficient oxygen supply by imperfect intratumoral vasculature.[101] CA IX is a direct transcriptional target of HIF-1 transcription factor, the master regulator of hypoxic responses whose degradation under normoxia is dependent on interaction with pVHL (which clarifies the relationship between CA IX and pVHL).[171] HIF-1 activates a number of genes functionally involved in tumor expansion, metabolism, invasion, metastasis and resistance to treatment and thus, hypoxia is an important trigger of tumor progression.[172] A phenomenon that is invariably associated with these processes is acidification of the extracellular pH in the tumor microenvironment.[173] It was believed to result from increased production of lactate by anaerobic glycolysis, but recent investigations support a significant contribution of the catalytic products of carbonic anhydrases.[174] Highly active extracellular isoforms CA IX/CA XII were proposed to provide bicarbonate ions for intracellular pH buffering and protons for extracellular pH acidification. Acidic extracellular milieu enhances the aggressive behaviour of tumor cells and facilitates their invasion and metastasis.[173–175] Therefore, inhibition of CA activity by sulfonamides is expected to bring benefit by blocking extracellular acidification and retarding tumor progression. This anticipation is supported by very new experimental data demonstrating the capacity of CA IX to acidify extracellular pH under hypoxia and inhibition of this phenomenon by the membrane-impermeable sulfonamides (unpublished data). Inhibition of CA activity may also diminish neutralization of intracellular pH and trigger apoptosis. In addition the pH gradient across the plasma membrane of tumor cells plays an important role in uptake and efficiency of chemotherapeutic drugs that are weak electrolytes (e.g. the anti-cancer drug doxorubicin is less cytotoxic when the extracellular pH is acidic).[176] Thus, the modulation of extracellular pH by inhibition of CA activity may represent another meaningful application of CAIs. Although some observations conform to this suggestion, more thorough investigations are needed for its approval.[103,105]

To achieve better understanding of the consequences of CA inhibition in cancer, it is also

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necessary to characterize the inhibition profiles of the cancer-associated isoforms. A concentrated effort towards this aim brought a number of data showing that the catalytic domain of CA IX behaves as a sulfonamide-avid enzyme with differential responses to different groups of sulfonamide derivatives when compared to some other CA isoforms. Several compounds that exhibit a certain degree of selectivity towards CA IX represent promising leads for the development of CA IX-specific drugs potentially useful for cancer treatment.[106–111]

Similarly to other pathological conditions, some drugs, which were originally generated to block other targets in cancer cells, also possess inhibitory activity against CA. For example, indisulam that was identified via elaborate pre-clinical screening as a cell cycle inhibitor with multiple effects on different types of regulatory molecules blocks CA II activity.[117,177–179] Steroid sulfatase inhibitors that were developed to prevent the production of steroids with estrogenic properties in breast tissues can dock into the active sites of CA II and CA XII and inhibit CA II activity.[118,180] Thus, it is possible that the anti-tumor effects of these drugs may be partially mediated by inhibition of CAs.

Abnormally high activity of different CA isoenzymes may certainly have deleterious consequences, but its decrease or absence is also very harmful. This is documented by the well-characterized CA II deficiency that leads to defined clinical signs comprising osteopetrosis, renal tubular acidosis and mental retardation. Furthermore, CA dysfunction (potentially related to brain isoforms II, VII and XIV) can impair cognitive capacity and memory storage, which are associated with mental retardation, Alzheimer's disease and aging. Cognitive decline is connected with significant decreases in brain CA activity at least partially due to beta amyloid-induced intracellular zinc depletion. Therefore, these conditions appear appropriate for therapeutic application of CA activators which is recently under intense investigation.[150–152]

Altogether, numerous facts and thoughts presented in this overview as well as much other data that were not cited here, illustrate the enormous progress which has been made in the field of carbonic anhydrases during the last decade. A very complex picture of knowledge about these important enzymes and their physiological and pathological roles is slowly acquiring momentum. It is more and more apparent, that modulation of carbonic anhydrase activities offers new pharmacological opportunities and that development of CA-based drugs with improved therapeutic potencies is not straightforward but feasible.

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References

[1] Supuran, C.T. and Scozzafava, A. (2002) *Exp. Opin. Ther. Patents* **12**, 217–242.
[2] Supuran, C.T. and Scozzafava, A. (2000) *Exp. Opin. Ther. Patents* **10**, 575–600.
[3] Hewett-Emmett, D. (2000) In: Chegwidden, W.R., Edwards, Y. and Carter, N., eds, *The Carbonic Anhydrases—New Horizons* (Birkhäuser Verlag, Basel), pp 29–78.
[4] Supuran, C.T., Scozzafava, A. and Casini, A. (2003) *Med. Res. Rev.* **23**, 146–189.
[5] Briganti, F., Mangani, S., Scozzafava, A., Vernaglione, G. and Supuran, C.T. (1999) *J. Biol. Inorg. Chem.* **4**, 528–536.
[6] Guerri, A., Briganti, F., Scozzafava, A., Supuran, C.T. and Mangani, S. (2000) *Biochemistry* **39**, 12391–12397.
[7] Kim, G., Selengut, J. and Levine, R.L. (2000) *Arch. Biochem. Biophys.* **377**, 334–340.
[8] Stams, T. and Christianson, D.W. (2000) In: Chegwidden, W.R., Edwards, Y. and Carter, N., eds, *The Carbonic Anhydrases—New Horizons* (Birkhäuser Verlag, Basel), pp 159–174.
[9] Whittington, D.A., Waheed, A., Ulmasov, B., Shah, G.N., Grubb, J.H., Sly, W.S. and Christianson, D.W. (2001) *Proc. Natl Acad. Sci. USA* **98**, 9545–9550.
[10] Mitsuhashi, S., Mizushima, T., Yamashita, E., Yamamoto, M., Kumasaka, T., Moriyama, H., Ueki, T., Miyachi, S. and Tsukihara, T. (2000) *J. Biol. Chem.* **275**, 5521–5526.
[11] Briganti, F., Mangani, S., Orioli, P., Scozzafava, A., Vernaglione, G. and Supuran, C.T. (1997) *Biochemistry* **36**, 10384–10392.
[12] Christianson, D.W. and Fierke, C.A. (1996) *Acc. Chem. Res.* **29**, 331–339.
[13] Lindskog, S. and Silverman, D.W. (2000) In: Chegwidden, W.R., Edwards, Y. and Carter, N., eds, *The Carbonic Anhydrases—New Horizons* (Birkhäuser Verlag, Basel), pp 175–196.
[14] Bertini, I., Luchinat, C. and Scozzafava, A. (1982) *Struct. Bonding* **48**, 45–92.
[15] Briganti, F., Pierattelli, A., Scozzafava, A. and Supuran, C.T. (1996) *Eur. J. Med. Chem.* **31**, 1001–1010.
[16] Lindahl, M., Vidgren, J., Eriksson, E., Habash, J., Harrop, S., Helliwell, J., Liljas, A., Lindeskog, M. and Walker, N. (1991) In: Botrè, F., Gros, G. and Storey, B.T., eds, *Carbonic Anhydrase* (VCH, Weinheim), pp 111–118.
[17] Abbate, F., Supuran, C.T., Scozzafava, A., Orioli, P., Stubbs, M.T. and Klebe, G. (2002) *J. Med. Chem.* **45**, 3583–3587.
[18] Stams, T., Nair, S.K., Okuyama, T., Waheed, A., Sly, W.S. and Christianson, D.W. (1996) *Proc. Natl Acad. Sci. USA* **93**, 13589–13594.
[19] Ferraroni, M., Briganti, F., Chegwidden, W.R., Supuran, C.T., Wiebauer, K.E., Tashian, R.E. and Scozzafava, A. (2002) *Biochemistry* **41**, 6237–6244.
[20] Ferraroni, M., Briganti, F., Chegwidden, W.R., Supuran, C.T. and Scozzafava, A. (2002) *Inorg. Chim. Acta* **339**, 135–144.
[21] Smith, K.S. and Ferry, J.G. (1999) *J. Bacteriol.* **181**, 6247–6253.
[22] Smith, K.S. and Ferry, J.G. (2000) *FEMS Microbiol. Rev.* **24**, 335–366.
[23] Kimber, M.S. and Pai, E.F. (2000) *EMBO J.* **15**, 2323–2330.
[24] Cronk, J.D., Endrizzi, J.A., Cronk, M.R., O'Neill, J.W. and Zhang, K.Y.J. (2001) *Protein Sci.* **10**, 911–922.
[25] Strop, P., Smith, K.S., Iverson, T.M., Ferry, J.G. and Rees, D.C. (2001) *J. Biol. Chem.* **276**, 10299–10305.
[26] Iverson, T.M., Alber, B.E., Kisker, C., Ferry, J.G. and Rees, D.C. (2000) *Biochemistry* **39**, 9222–9231.

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[27] Kisker, C., Schindelin, H., Alber, B.E., Ferry, J.G. and Rees, D.C. (1996) *EMBO J.* **15**, 2323–2330.
[28] Cox, E.H., McLendon, G.L., Morel, F.M., Lane, T.W., Prince, R.C., Pickering, I.J. and George, G.N. (2000) *Biochemistry* **39**, 12128–12130.
[29] Lane, T.W. and Morel, F.M. (2000) *Proc. Natl Acad. Sci. USA* **97**, 4627–4631.
[30] Eickhoff, T.C. and Nelson, M.S. (1966) *Antimicrob. Agents Chemother.* **6**, 389–392.
[31] Nafi, B.M., Miles, R.J., Butler, L.O., Carter, N.D., Kelly, C. and Jeffery, S. (1990) *J. Med. Microbiol.* **32**, 1–7.
[32] Chirica, L.C., Elleby, B., Jonsson, B.H. and Lindskog, S. (1997) *Eur. J. Biochem.* **244**, 755–760.
[33] Chirica, L.C., Elleby, B. and Lindskog, S. (2001) *Biochim. Biophys. Acta* **1544**, 55–63.
[34] Elleby, B., Chirica, L.C., Tu, C., Zeppezauer, M. and Lindskog, S. (2001) *Eur. J. Biochem.* **268**, 1613–1619.
[35] Krungkrai, S.R., Suraveratum, N., Rochanakij, S. and Krungkrai, J. (2001) *Int. J. Parasitol.* **31**, 661–668.
[36] Reungprapavut, S., Krungkrai, S.R. and Krungkrai, J. (2004) *J. Enz. Inhib. Med. Chem.* **19**, 249–256.
[37] Moroney, J.V., Bartlett, S.G. and Samuelsson, G. (2001) *Plant Cell Environ.* **24**, 141–153.
[38] Park, Y., Karlsson, J., Rojdestvenski, I., Pronina, N., Klimov, V., Oquist, G. and Samuelsson, G. (1999) *FEBS Lett.* **444**, 102–105.
[39] Badger, M.R. and Price, G.D. (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45**, 369–392.
[40] Gillon, J. and Yakir, D. (2001) *Science* **291**, 2584–2587.
[41] Mann, T. and Keilin, D. (1940) *Nature* **146**, 164–165.
[42] Maren, T.H. (1967) *Physiol. Rev.* **47**, 595–781.
[43] Maren, T.H. (1976) *Annu. Rev. Pharmacol. Toxicol.* **16**, 309–327.
[44] Drew, J. (2000) *Science* **287**, 1960–1964.
[45] Supuran, C.T. (2002) *Investig. Drugs* **5**, 1075–1079.
[46] Scolnick, L.R., Clements, A.M., Liao, J., Crenshaw, L., Hellberg, M., May, J., Dean, T.R. and Christianson, D.W. (1997) *J. Am. Chem. Soc.* **119**, 850–851.
[47] Supuran, C.T. and Scozzafava, A. (2002) In: Smith, H.J. and Simons, C., eds, *Proteinase and Peptidase Inhibition: Recent Potential Targets for Drug Development* (Taylor and Francis, London), pp 35–61.
[48] Scozzafava, A. and Supuran, C.T. (2003) *Bioorg. Med. Chem.* **11**, 2241–2246.
[49] Krebs, H.A. (1948) *Biochem. J.* **43**, 525–528.
[50] Supuran, C.T. (1994) In: Puscas, I., eds, *Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism* (Helicon, Timisoara, Romania), pp 29–111.
[51] Supuran, C.T. and Scozzafava, A. (2001) *Curr. Med. Chem.-Imm. Endoc. Metab. Agents* **1**, 61–97.
[52] Chen, H.H., Gross, S., Liao, J., McLaughlin, M., Dean, T., Sly, W.S. and May, J.A. (2000) *Bioorg. Med. Chem.* **8**, 957–975.
[53] Scozzafava, A., Briganti, F., Ilies, M.A. and Supuran, C.T. (2000) *J. Med. Chem.* **43**, 292–300.
[54] Scozzafava, A. and Supuran, C.T. (2000) *Bioorg. Med. Chem. Lett.* **10**, 1117–1120.
[55] Barboiu, M., Supuran, C.T., Menabuoni, L., Scozzafava, A., Mincione, F., Briganti, F. and Mincione, G. (2000) *J. Enz. Inhib.* **15**, 23–46.
[56] Briganti, F., Tilli, S., Mincione, G., Mincione, F., Menabuoni, L. and Supuran, C.T. (2000) *J. Enz. Inhib.* **15**, 185–200.
[57] Supuran, C.T., Scozzafava, A., Ilies, M.A. and Briganti, F. (2000) *J. Enz. Inhib.* **15**, 381–401.
[58] Ilies, M., Supuran, C.T., Scozzafava, A., Casini, A., Mincione, F., Menabuoni, L., Caproiu, M.T., Maganu, M. and Banciu, M.D. (2000) *Bioorg. Med. Chem.* **8**, 2145–2455.
[59] Clare, B.W. and Supuran, C.T. (2000) *Eur. J. Med. Chem.* **35**, 859–865.
[60] Scozzafava, A., Banciu, M.D., Popescu, A. and Supuran, C.T. (2000) *J. Enz. Inhib.* **15**, 443–453.
[61] Scozzafava, A., Menabuoni, L., Mincione, F., Briganti, F., Mincione, G. and Supuran, C.T. (2000) *J. Med. Chem.* **43**, 4542–4551.
[62] Casini, A., Scozzafava, A., Mincione, F., Menabuoni, L., Ilies, M.A. and Supuran, C.T. (2000) *J. Med. Chem.* **43**, 4884–4892.
[63] Mastrolorenzo, A., Scozzafava, A. and Supuran, C.T. (2000) *J. Enz. Inhib.* **15**, 517–531.
[64] Scozzafava, A., Banciu, M.D., Popescu, A. and Supuran, C.T. (2000) *J. Enz. Inhib.* **15**, 533–546.
[65] Supuran, C.T. and Scozzafava, A. (2000) *J. Enz. Inhib.* **15**, 597–610.
[66] Supuran, C.T. and Scozzafava, A. (2000) *Eur. J. Med. Chem.* **35**, 867–874.
[67] Supuran, C.T., Briganti, F., Tilli, S., Chegwidden, W.R. and Scozzafava, A. (2001) *Bioorg. Med. Chem.* **9**, 703–714.
[68] Scozzafava, A., Menabuoni, L., Mincione, F., Mincione, G. and Supuran, C.T. (2001) *Bioorg. Med. Chem. Lett.* **11**, 575–582.
[69] Casini, A., Mincione, F., Ilies, M.A., Menabuoni, L., Scozzafava, A. and Supuran, C.T. (2001) *J. Enz. Inhib.* **16**, 113–123.
[70] Chazalette, C., Riviere-Baudet, M., Scozzafava, A., Abbate, F., Ben Maarouf, Z. and Supuran, C.T. (2001) *J. Enz. Inhib.* **16**, 125–133.
[71] Mincione, F., Starnotti, M., Menabuoni, L., Scozzafava, A., Casini, A. and Supuran, C.T. (2001) *Bioorg. Med. Chem. Lett.* **11**, 1787–1791.
[72] Supuran, C.T. and Clare, B.W. (2001) *SAR QSAR.Environ. Res.* **12**, 17–29.
[73] Scozzafava, A. and Supuran, C.T. (2000) *J. Med. Chem.* **43**, 3677–3687.
[74] Smith, H.J. and Simons, C., eds (2002) *Proteinase and Peptidase Inhibition: Recent Potential Targets for Drug Development* (Taylor & Francis, London).
[75] Supuran, C.T. and Scozzafava, A. (2000) In: Chegwidden, W.R., Carter, N. and Edwards, Y., eds, *The Carbonic Anhydrases—New Horizons* (Birkhauser Verlag, Basel, Switzerland), pp 197–219.
[76] Scozzafava, A., Mincione, F., Menabuoni, L. and Supuran, C.T. (2001) *Drug Des. Dev.* **17**, 337–348.
[77] Masereel, B., Rolin, S., Abbate, F., Scozzafava, A. and Supuran, C.T. (2002) *J. Med. Chem.* **45**, 312–320.
[78] Scozzafava, A. and Supuran, C.T. (2002) *Bioorg. Med. Chem. Lett.* **12**, 1551–1557.
[79] Bulbul, M., Saracoglu, N., Kufrevioglu, O.I. and Ciftci, M. (2002) *Bioorg. Med. Chem.* **10**, 2561–2567.
[80] Scozzafava, A., Menabuoni, L., Mincione, F. and Supuran, C.T. (2002) *J. Med. Chem.* **45**, 1466–1476.
[81] Maestrelli, F., Mura, P., Casini, A., Mincione, F., Scozzafava, A. and Supuran, C.T. (2002) *J. Pharm. Sci.* **91**, 2211–2219.
[82] Casini, A., Mincione, F., Vullo, D., Menabuoni, L., Scozzafava, A. and Supuran, C.T. (2002) *J. Enz. Inhib. Med. Chem.* **17**, 9–18.
[83] Ul-Hassan, M., Scozzafava, A., Chohan, Z.H. and Supuran, C.T. (2001) *J. Enz. Inhib.* **16**, 499–505.
[84] Chegwidden, W.R., Spencer, I.M. and Supuran, C.T. (2001) "The roles of carbonic anhydrase isozymes in cancer", In: Xue, G., Xue, Y., Xu, Z., Holmes, R., Hammond, G.L. and Lim, H.A., eds, *Gene Families: Studies of DNA, RNA, Enzymes and Proteins* (World Scientific, Singapore), pp 157–170.
[85] Pastorek, J., Pastorekova, S., Callebaut, I., Mornon, J.P., Zelnik, V., Opavsky, R., Zatovicova, M., Liao, S., Portetelle, D., Stanbridge, E.J., Zavada, J., Burny, A. and Kettmann, R. (1994) *Oncogene* **9**, 2877–2888.
[86] Opavsky, R., Pastorekova, S., Zelnik, V., Gibadulinova, A., Stanbridge, E.J., Zavada, J., Kettmann, R. and Pastorek, J. (1996) *Genomics* **33**, 480–487.
[87] Pastorekova, S., Parkkila, S., Parkkila, A.K., Opavsky, R., Zelnik, V., Saarnio, J. and Pastorek, J. (1997) *Gastroenterology* **112**, 398–408.
[88] Saarnio, J., Parkkila, S., Parkkila, A.K., Waheed, A., Casey, M.C., Zhou, X.Y., Pastorekova, S., Pastorek, J., Karttunen, T., Haukipuro, K., Kairaluoma, M.I. and Sly, W.S. (1998) *J. Histochem. Cytochem.* **46**, 497–504.
[89] Zavada, J., Zavadova, Z., Pastorekova, S., Ciampor, F., Pastorek, J. and Zelnik, V. (1993) *Int. J. Cancer* **54**, 268–274.
[90] Liao, S.Y., Brewer, C., Zavada, J., Pastorek, J., Pastorekova, S., Manetta, A., Berman, M.L., DiSaia, P.J. and Stanbridge, E.J. (1994) *Am. J. Pathol.* **145**, 598–609.
[91] Liao, S.Y., Aurelio, O.N., Jan, K., Zavada, J. and Stanbridge, E.J. (1997) *Cancer Res.* **57**, 2827–2831.

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[92] Turner, J.R., Odze, R.D., Crum, C.P. and Resnick, M.B. (1997) *Hum. Pathol.* **28**, 740–744.
[93] Vermylen, P., Roufosse, C., Burny, A., Verhest, A., Bossehaerts, T., Pastorekova, S., Ninane, V. and Sculier, J.P. (1999) *Eur. Respir. J.* **14**, 806–811.
[94] Bartosova, M., Parkkila, S., Pohlodek, K., Karttunen, T.J., Galbavy, S., Mucha, V., Harris, A.L., Pastorek, J. and Pastorekova, S. (2002) *J. Pathol.* **197**, 1–8.
[95] Ivanov, S., Liao, S.Y., Ivanova, A., Danilkovich-Miagkova, A., Tarasova, N., Weirich, G., Merrill, M.J., Proescholdt, M.A., Oldfield, E.H., Lee, J., Zavada, J., Waheed, A., Sly, W., Lerman, M.I. and Stanbridge, E.J. (2001) *Am. J. Pathol.* **158**, 905–919.
[96] Türeci, O., Sahin, U., Vollmar, E., Siemer, S., Göttert, E., Seitz, G., Parkkila, A.K., Shah, G.N., Grubb, J.H., Pfreundschuh, M. and Sly, W.S. (1998) *Proc. Natl Acad. Sci. USA* **95**, 7608–7613.
[97] Ivanov, S.V., Kuzmin, I., Wei, M.H., Pack, S., Geil, L., Johnson, B.E., Stanbridge, E.J. and Lerman, M.I. (1998) *Proc. Natl Acad. Sci. USA* **95**, 12596–12601.
[98] Wykoff, C.C., Beasley, N., Watson, P.H., Campo, L., Chia, S.K., English, R., Pastorek, J., Sly, W.S., Ratcliffe, P. and Harris, A.L. (2001) *Am. J. Pathol.* **158**, 1011–1019.
[99] Parkkila, S., Parkkila, A.K., Saarnio, J., Kivela, J., Karttunen, T., Kaunisto, K., Waheed, A., Sly, W.S., Tureci, O., Virtanen, I. and Rajaniemi, H. (2000) *J. Histochem. Cytochem.* **48**, 1601–1608.
[100] Kivela, A., Parkkila, S., Saarnio, J., Karttunen, T.J., Kivela, J., Parkkila, A.K., Waheed, A., Sly, W.S., Grubb, J.H., Shah, G., Tureci, O. and Rajaniemi, H. (2000) *Am. J. Pathol.* **156**, 577–584.
[101] Wykoff, C., Beasley, N., Watson, P., Turner, L., Pastorek, J., Wilson, G., Turley, H., Maxwell, P., Pugh, C., Ratcliffe, P. and Harris, A. (2000) *Cancer Res.* **60**, 7075–7083.
[102] Potter, C.P.S. and Harris, A.L. (2003) *Br. J. Cancer* **89**, 2–7.
[103] Teicher, B.A., Liu, S.D., Liu, J.T., Holden, S.A. and Herman, T.S. (1993) *Anticancer Res.* **13**, 1549–1556.
[104] Parkkila, S., Rajaniemi, H., Parkkila, A.K., Kivela, J., Waheed, A., Pastorekova, S., Pastorek, J. and Sly, W.S. (2000) *Proc. Natl Acad. Sci. USA* **97**, 2220–2224.
[105] Xiang, Y., Ma, B., Li, T., Yu, H.M. and Li, X.J. (2002) *Acta Pharmacol. Sin.* **23**, 745–751.
[106] Vullo, D., Franchi, M., Gallori, E., Pastorek, J., Scozzafava, A., Pastorekova, S. and Supuran, C.T. (2003) *Bioorg. Med. Chem. Lett.* **13**, 1005–1009.
[107] Ilies, M.A., Vullo, D., Pastorek, J., Scozzafava, A., Ilies, M., Caproiu, M.T., Pastorekova, S. and Supuran, C.T. (2003) *J. Med. Chem.* **46**, 2187–2196.
[108] Franchi, M., Vullo, D., Gallori, E., Pastorek, J., Russo, A., Scozzafava, A.S., Pastorekova, S. and Supuran, C.T. (2003) *J. Enz. Inhib. Med. Chem.* **18**, 333–338.
[109] Casini, A., Antel, J., Abbate, F., Scozzafava, A., David, S., Waldeck, H., Schäfer, S. and Supuran, C.T. (2003) *Bioorg. Med. Chem. Lett.* **13**, 841–845.
[110] Winum, J.Y., Vullo, D., Casini, A., Montero, J.L., Scozzafava, A. and Supuran, C.T. (2003) *J. Med. Chem.* **46**, 2197–2204.
[111] Winum, J.Y., Vullo, D., Casini, A., Montero, J.L., Scozzafava, A. and Supuran, C.T. (2003) *J. Med. Chem.* **46**, 5471–5477.
[112] Casini, A., Winum, J.Y., Montero, J.L., Scozzafava, A. and Supuran, C.T. (2003) *Bioorg. Med. Chem. Lett.* **13**, 837–840.
[113] Casini, A., Scozzafava, A., Mincione, F., Menabuoni, L., Starnotti, M. and Supuran, C.T. (2003) *Bioorg. Med. Chem. Lett.* **13**, 2867–2873.
[114] Winum, J.Y., Casini, A., Mincione, F., Starnotti, M., Montero, J.L., Scozzafava, A. and Supuran, C.T. (2004) *Bioorg. Med. Chem. Lett.* **14**, 225–229.
[115] Casini, A., Abbate, F., Scozzafava, A. and Supuran, C.T. (2003) *Bioorg. Med. Chem. Lett.* **13**, 2759–2763.
[116] Abbate, F., Casini, A., Scozzafava, A. and Supuran, C.T. (2003) *J. Enz. Inhib. Med. Chem.* **18**, 303–308.
[117] Abbate, F., Casini, A., Owa, T., Scozzafava, A. and Supuran, C.T. (2004) *Bioorg. Med. Chem. Lett.* **14**, 217–223.
[118] Abbate, F., Winum, J.Y., Potter, B.V.L., Casini, A., Montero, J.L., Scozzafava, A. and Supuran, C.T. (2004) *Bioorg. Med. Chem. Lett.* **14**, 231–234.
[119] Abbate, F., Coetzee, A., Casini, A., Ciattini, S., Scozzafava, A. and Supuran, C.T. (2004) *Bioorg. Med. Chem. Lett.* **14**, 337–341.
[120] Abbate, F., Casini, A., Scozzafava, A. and Supuran, C.T. (2004) *Bioorg. Med. Chem. Lett.* **14**, in press.
[121] Weber, A., Casini, A., Heine, A., Kuhn, D., Supuran, C.T., Scozzafava, A. and Klebe, G. (2004) *J. Med. Chem.* **47**, 550–557.
[122] Lehtonen, J., Shen, B., Vihinen, M., Casini, A., Scozzafava, A., Supuran, C.T., Parkkila, A.K., Saarnio, J., Kivelä, A., Waheed, A., Sly, W.S. and Parkkila, S. (2004) *J. Biol. Chem.* **278**, 2719–2727.
[123] Leiner, M. (1940) *Naturwissenschaft* **28**, 316–317.
[124] Kiese, M. (1941) *Naturwissenschaft* **29**, 116–117.
[125] Leiner, M. and Leiner, G. (1941) *Naturwissenschaft* **29**, 195–197.
[126] Leiner, M. and Leiner, G. (1941) *Biochem. Z.* **311**, 119–145.
[127] Bakker, A. (1943) *Enzymologia* **11**, 70–80.
[128] Main, R.E. and Locke, A. (1941) *J. Biol. Chem.* **110**, LXXXI.
[129] van Goor, H. (1948) *Enzymologia* **13**, 73–164.
[130] Clark, A.M. and Perrin, D.D. (1951) *Biochem. J.* **48**, 495–503.
[131] Roughton, F.J.W. (1943) *Harvey Lect.* **39**, 96–110.
[132] Roughton, F.J.W. and Booth, H. (1946) *Biochem. J.* **40**, 319–329.
[133] Ho, C. and Sturtevant, J.M. (1960) *Biochem. Biophys. Res. Commun.* **3**, 20–23.
[134] Silverman, D.N., Backman, L. and Tu, C. (1979) *J. Biol. Chem.* **254**, 2588–2591.
[135] Parkes, J.L. and Coleman, P. (1989) *Arch. Biochem. Biophys.* **275**, 459–468.
[136] Supuran, C.T. (1992) *Rev. Roum. Chim.* **37**, 411–421.
[137] Puscas, I., Supuran, C. and Manole, G. (1990) *Rev. Roum. Chim.* **35**, 683–689.
[138] Supuran, C.T., Dinculescu, A., Manole, G., Savan, F., Puscas, I. and Balaban, A.T. (1991) *Rev. Roum. Chim.* **36**, 937–946.
[139] Supuran, C.T. and Puscas, I. (1994) "Carbonic anhydrase activators", In: Puscas, I., ed., *Carbonic anhydrase and modulation of physiologic and pathologic processes in the organism* (Helicon Press, Timisoara), pp 113–145.
[140] Steiner, H., Jonsson, B.H. and Lindskog, S. (1975) *Eur. J. Biochem.* **59**, 253–259.
[141] Silverman, D.N. and Lindskog, S. (1988) *Acc. Chem. Res.* **21**, 30–36.
[142] Nair, S.K. and Christianson, D.W. (1991) *J. Am. Chem. Soc.* **113**, 9455–9458.
[143] Smith, G.M., Alexander, R.S., Christianson, D.W., McKeever, B.M., Ponticello, G.S., Springer, J.M., Randall, W.C., Baldwin, J.J. and Habecker, C.N. (1994) *Protein Sci.* **3**(3), 118–125.
[144] Scozzafava, A. and Supuran, C.T. (2000) *Eur. J. Pharm. Sci.* **10**, 29–41.
[145] Scozzafava, A. and Supuran, C.T. (2000) *Eur. J. Med. Chem.* **35**, 31–39.
[146] Scozzafava, A., Iorga, B. and Supuran, C.T. (2000) *J. Enz. Inhib.* **15**, 139–161.
[147] Supuran, C.T. and Scozzafava, A. (2000) *J. Enz. Inhib.* **15**, 471–486.
[148] Scozzafava, A. and Supuran, C.T. (2002) *J. Med. Chem.* **45**, 284–291.
[149] Ilies, M., Banciu, M.D., Ilies, M.A., Scozzafava, A., Caproiu, M.T. and Supuran, C.T. (2002) *J. Med. Chem.* **45**, 504–510.
[150] Sun, M.K. and Alkon, D.L. (2001) *J. Pharmacol. Exp. Ther.* **297**, 961–967.
[151] Clare, B.W. and Supuran, C.T. (1994) *J. Pharm. Sci.* **83**, 768–773.
[152] Sun, M.K. and Alkon, D.L. (2002) *Trends Pharmacol. Sci.* **23**, 83–89.
[153] Vince, J.W. and Reithmeier, R.A. (2000) *Biochemistry* **39**, 5527–5533.
[154] Sterling, D., Reithmeier, R.A. and Casey, J.R. (2001) *J. Biol. Chem.* **276**, 47886–47894.
[155] Sterling, D., Alvarez, B.V. and Casey, J.R. (2002) *J. Biol. Chem.* **277**, 25239–25246.
[156] Alvarez, B., Loiselle, F.B., Supuran, C.T., Schwartz, G.J. and Casey, J.R. (2003) *Biochemistry* **42**, 12321–12329.
[157] Scozzafava, A. and Supuran, C.T. (2002) *Bioorg. Med. Chem. Lett.* **12**, 1177–1180.

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[158] Liao, S.Y., Ivanov, S., Ivanova, A., Ghosh, S., Cote, M.A., Keefe, K., Coca-Prados, M., Stanbridge, E.J. and Lerman, M.I. (2003) *J. Med. Genet.* **40**, 257–261.
[159] Sly, W.S. and Hu, P.Y. (1995) *Ann. Rev. Biochem.* **64**, 375–401.
[160] Gut, M.O., Parkkila, S., Vernerova, Z., Rohde, E., Zavada, J., Hocker, M., Pastorek, J., Karttunen, T., Gibadilunova, A., Zavadova, Z., Knobeloch, K.P., Wiedenmann, B., Svoboda, J., Horak, I. and Pastorekova, S. (2002) *Gastroenterology* **123**, 1889–1903.
[161] Gottsch, J.D., Seitzman, G.D., Margulies, E.H., Bowers, A.L., Michels, A.J., Saha, S., Jun, A.S., Stark, W.J. and Liu, S.H. (2003) *Arch. Ophthalmol.* **121**, 252–258.
[162] Lehman-Horn, F. and Jurkat-Rott, K. (1999) *Physiol. Rev.* **79**, 1317–1372.
[163] Cammer, W.B. and Brion, L.P. (2000) In: Chegwidden, W.R., Edwards, Y. and Carter, N., eds, *The Carbonic Anhydrases—New Horizons* (Birkhäuser Verlag, Basel), pp 475–489.
[164] Parkkila, S., Parkkila, A.K., Rajaniemi, H., Shah, N.G., Grubb, J.H., Waheed, A. and Sly, W.S. (2001) *Proc. Natl Acad. Sci. USA* **98**, 1918–1923.
[165] Carbiscol, E. and Levine, R.L. (1995) *J. Biol. Chem.* **270**, 14742–14747.
[166] Breton, S. (2001) *J. Pancreas* **2**, 159–164.
[167] Kaunisto, K., Parkkila, S., Rajaniemi, H., Waheed, A., Grubb, J. and Sly, W.S. (2002) *Kidney Int.* **61**, 2111–2118.
[168] Casini, A., Scozzafava, A., Mastrolorenzo, A. and Supuran, C.T. (2002) *Curr. Cancer Drug Targets* **2**, 55–75.
[169] Chegwidden, W.R., Dodgson, S.J. and Spencer, I.M. (2000) In: Chegwidden, W.R., Edwards, Y. and Carter, N., eds, *The Carbonic Anhydrases—New Horizons* (Birkhäuser Verlag, Basel), pp 343–363.
[170] Ashida, S., Nishimori, I., Tanimura, M., Onishi, S. and Shuin, T. (2002) *J. Cancer Res. Clin. Oncol.* **128**, 561–568.
[171] Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykof, C.C., Pugh, C.W., Maher, E.R. and Ratcliffe, P.J. (1999) *Nature* **399**, 271–275.
[172] Hockel, M. and Vaupel, P. (2001) *J. Natl Cancer Inst.* **93**, 266–276.
[173] Stubbs, M., McSheehy, P.M.J., Griffiths, J.R. and Beshford, C.L. (2000) *Mol. Med. Today* **6**, 15–19.
[174] Helmlinger, G., Sckell, A., Dellian, M., Forbes, N.S. and Jain, R.K. (2002) *Clin. Cancer Res.* **8**, 1284–1291.
[175] Martinez-Zaguilan, R., Seftor, E.A., Seftor, R.E., Chu, Y.W., Gillies, R.J. and Hendrix, M.J. (1992) *Clin. Exp. Metastasis* **14**, 176–186.
[176] Raghunand, N. and Gillies, R.J. (2001) *Novartis Found. Symp.* **240**, 199–211.
[177] Owa, T. and Nagasu, T. (2000) *Exp. Opin. Ther. Patents* **10**, 1725–1740.
[178] Fukuoka, K., Usuda, J., Iwamoto, Y., Fukumoto, H., Nakamura, T., Yoneda, T., Narita, N., Saijo, N. and Nishio, K. (2001) *Investig. New Drugs* **19**, 219–227.
[179] Yokoi, A., Kuromitsu, J., Kawai, T., Nagasu, T., Sugi, N.H., Yoshimatsu, K., Yoshino, H. and Owa, T. (2002) *Mol. Cancer Ther.* **1**, 275–286.
[180] Ho, Y.T., Purohit, A., Vicker, N., Newman, S.P., Robinson, J.J., Leese, M.P., Ganeshapillai, D., Woo, L.W.L., Potter, B.V.L. and Reed, M.J. (2003) *Biochem. Biophys. Res. Commun.* **305**, 909–914.
[181] Axford, S.E., Sharp, N., Ross, P.E., Pearson, J.P., Dettmar, P.W., Panetti, M. and Koufman, J.A. (2001) *Ann. Otol. Rhinol. Laryngol.* **110**, 1099–1108.
[182] Tarun, A.S., Bryant, B., Zhai, W., Solomon, C. and Shusterman, D. (2003) *Chem. Senses* **28**, 621–629.
[183] Mezquita, P., Mezquita, C. and Mezquita, J. (1999) *Mol. Hum. Reprod.* **5**, 199–205.
[184] Jampel, H.D., Chen, X., Chue, C. and Zack, D.J. (1997) *Investig. Ophthalmol. Vis. Sci.* **38**, 539–543.
[185] Fujikawa-Adachi, K., Nishimori, I., Sakamoto, S., Morita, M., Onishi, S., Yonezawa, S. and Hollingsworth, M.A. (1999) *Pancreas* **18**, 329–335.
[186] Sender, S., Decker, B., Fenske, C.D., Sly, W.S., Carter, N.D. and Gros, G. (1998) *J. Histochem. Cytochem.* **46**, 855–861.
[187] Fujikawa-Adachi, K., Nishimori, I., Taguchi, T. and Onishi, S. (1999) *J. Biol. Chem.* **274**, 21228–21233.
[188] Saarnio, J., Parkkila, S., Parkkila, A.K., Waheed, A., Karttunen, T. and Sly, W.S. (1999) *J. Histochem. Cytochem.* **47**, 517–524.
[189] Karhumaa, P., Kaunisto, K., Parkkila, S., Waheed, A., Pastorekova, S., Pastorek, J., Sly, W.S. and Rajaniemi, H. (2001) *Mol. Hum. Reprod.* **7**, 611–616.
[190] Fleming, R.E., Parkkila, S., Parkkila, A.K., Rajaniemi, H., Waheed, A. and Sly, W.S. (1995) *J. Clin. Investig.* **96**, 2907–2913.
[191] Karhumaa, P., Leinonen, J., Parkkila, S., Kaunisto, K., Tapanainen, J. and Rajaniemi, H. (2001) *Proc. Natl Acad. Sci. USA* **98**, 11604–11608.
[192] Parkkila, S., Kivelä, A.J., Kaunisto, K., Parkkila, A.K., Hakkola, J., Waheed, A., Sly, W.S. and Rajaniemi, H. (2002) *BMC Gastroenterol.* **2**, 13–20.

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