

# Therapeutic applications of the carbonic anhydrase inhibitors

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Inhibitors of the zinc enzyme carbonic anhydrase (CA) targeting different isozymes among the 15 presently reported in humans have various therapeutic applications. These enzymes are efficient catalysts for the hydration of carbon dioxide to bicarbonate and protons, playing crucial physiological/pathological roles related to acid–base homeostasis, secretion of electrolytes, transport of ions, biosynthetic reactions and tumorigenesis. Many CA inhibitors have been reported as antiglaucoma, anticancer and antiobesity agents, or for the management of a variety of neurological disorders, including epilepsy and altitude disease. Furthermore, some CA isozymes appear to be valuable markers of tumor progression in a variety of tissues/organs. Various CAs present in pathogens such as *Plasmodium falciparum*, *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Candida albicans* and *Cryptococcus neoformans*, have started to also be investigated recently as drug targets. Recent progress in all these areas, as well as in the characterization of new such enzymes isolated in many other organisms/tissues, in addition to their detailed catalytic/inhibition mechanisms, are reviewed herein, together with the therapeutic use of these enzyme inhibitors and drug-design studies for obtaining such new-generation derivatives with improved activity.

Among the zinc enzymes extensively studied, the carbonic anhydrases (CAs) occupy a special place for several reasons. These enzymes are ubiquitous in all kingdoms, starting with archaea, bacteria, algae and green plants, and ending with superior animals, including vertebrates [1–8]. Their physiological function is essential for these organisms, as CAs catalyze a very simple physiological reaction, the interconversion between CO<sub>2</sub> and bicarbonate and a proton [1–8]. This reaction is critical for respiration and transport of CO<sub>2</sub> between metabolizing tissues and excretion sites, secretion of electrolytes in a variety of tissues and organs, pH regulation and homeostasis, CO<sub>2</sub> fixation (for algae and green plants), several metabolic biosynthetic pathways (in vertebrates), tumorigenesis and signal transduction [1–8]. Inhibition (and 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) possess a variety of applications in therapy [1]. In mammals, 16 different  $\alpha$ -CA isozymes or CA-related proteins (CARPs) have been described, with very different subcellular localization and tissue distribution [1–8]. There are several cytosolic forms (CA I–III and CA VII), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial

forms (CA VA and CA VB), as well as a secreted CA isozyme (in saliva and milk; CA VI). Among the membrane-bound CAs, isoforms CA IV and XV are anchored to membranes by means of glycosylphosphatidylinositol (GPI) tails, whereas isozymes IX, XII and XIV are transmembrane proteins possessing just one transmembrane domain [1–8]. However, all these five isozymes have their active site outside the cell, and are thus commonly known as extracellular CAs [1–8].

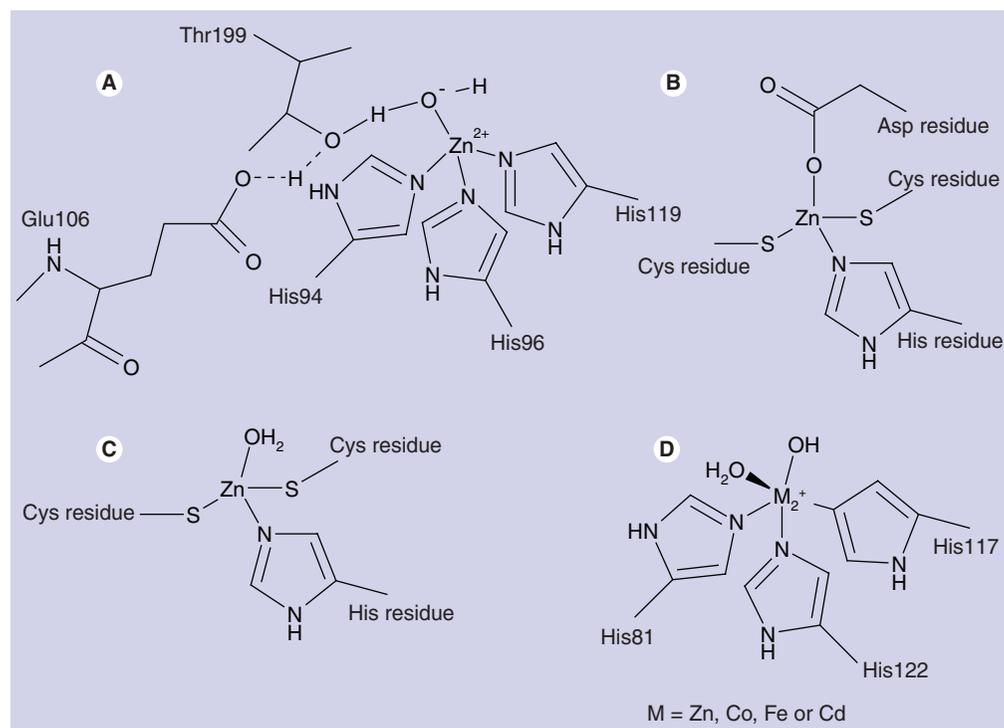
## Catalytic/inhibition mechanism *Isozymes*

Over the phylogenetic tree, CAs are encoded by several (probably four) distinct, evolutionarily unrelated gene families: the  $\alpha$ -CAs (present in vertebrates, bacteria, algae and cytoplasm of green plants); the  $\beta$ -CAs (predominantly in bacteria, algae and chloroplasts of both mono- as well as dicotyledons); the  $\gamma$ -CAs (mainly in archaea and some bacteria); and the  $\delta$ -CAs, evidenced so far in some marine diatoms (Figure 1) [1–8].

CAs are metalloenzymes and most of them have a Zn(II) ion within their active sites, although the  $\delta$ -CAs seem to be Cd(II) enzymes and the  $\gamma$ -CAs use Zn(II), Co(II) or Fe(II) as a metal ion cofactor [1–8]. The metal ion of CAs is essential for catalysis [1–8]. X-ray crystallographic

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**Figure 1. Metal ion coordination sphere in the four classes of carbonic anhydrases.**

**(A)**  $\alpha$ -carbonic anhydrases (CAs; hCA II numbering). **(B)** *Porphyridium purpureum* and *Escherichia coli*  $\beta$ -CAs. **(C)** *Pisum sativum* chloroplast and *Methanobacterium thermoautotrophicum*  $\beta$ -CAs. **(D)**  $\gamma$ -class CA from *Methanosarcina thermophila* (M = Zn(II), Co(II) or Fe(II) – which can be either tetra-, penta- or hexa-coordinated by additional water molecules; Cam numbering of the His residues) and  $\delta$ -class CA from *Thalassiosira weissflogii* (M = Cd(II) or Zn(II) – the exact geometry of the metal ion is not known).

data carried out mainly on the  $\alpha$ -CA family of enzymes showed that the metal ion is situated at the bottom of a 15-Å deep active-site cleft, being coordinated by three histidine residues (His94, 96 and 119) and a H<sub>2</sub>O/OH<sup>-</sup> ion [1–8]. The Zn-bound water is also engaged in H-bond interactions with the hydroxyl moiety of Thr199, which in turn is bridged to the carboxylate moiety of Glu106 (these amino acids are conserved in all  $\alpha$ -CAs currently known [1–5]). These interactions enhance the nucleophilicity of the Zn-bound H<sub>2</sub>O/OH<sup>-</sup> ion, and orient the substrate (CO<sub>2</sub>) in a favorable location for nucleophilic attack (Figure 1A) [1–5].

The first x-ray crystallographic structure of a member of the  $\beta$ -CA family has been determined by Mitsuhashi and colleagues for the enzyme isolated from the red algae *Porphyridium purpureum* (Figure 1B) [9]. This CA monomer is composed of two internally repeating structures, being folded as a pair of fundamentally equivalent motifs of an  $\alpha/\beta$ -domain and three projecting  $\alpha$ -helices. The motif is very distinct from that of either  $\alpha$ - or  $\gamma$ -CAs. This

homodimeric CA appeared like a tetramer with a pseudo 2–2–2 symmetry. The active-site Zn(II) ion was shown to be coordinated by a Cys–Asp–His–Cys tetrad that is strictly conserved among the  $\beta$ -CAs, and no H<sub>2</sub>O molecule was found in the Zn-liganding radius, indicating that the Zn(OH)<sub>2</sub> mechanism in  $\alpha$ - and  $\gamma$ -CAs is not directly applicable to the case in  $\beta$ -CAs [9]. This first report was soon followed by another, in which the structure of the ‘cab’-type  $\beta$ -CA from the archaeon *Methanobacterium thermoautotrophicum* (Cab) has been determined to a resolution of 2.1 Å (Figure 1C) [10]. Cab exists as a dimer with a subunit fold similar to that observed in ‘plant’-type  $\beta$ -CAs. The active-site zinc ion was shown to be coordinated by the amino acid residues Cys32, His87 and Cys90, with the tetrahedral coordination completed by a H<sub>2</sub>O molecule [10]. The major difference between plant- and cab-type  $\beta$ -CAs is in the organization of the hydrophobic pocket (except for the aforementioned Zn coordination). The structure also revealed a HEPES buffer molecule, bound 8 Å away from

the active-site Zn, which suggests a possible proton-transfer pathway from the active site to the solvent [10]. The structure of the  $\beta$ -CA from the dicotyledonous plant *Pisum sativum* at 1.93 Å resolution has also been reported (Figure 1C) [11]. The molecule assembles as an octamer with a novel dimer-of-dimers-of-dimers arrangement. Two distinct patterns of conservation of active-site residues were observed, implying two potentially mechanistically distinct classes of  $\beta$ -CAs. The active site is located at the interface between two monomers, with Cys160, His220 and Cys223 binding the catalytic Zn ion and residues Asp162 (oriented by Arg164), Gly224, Gln151, Val184, Phe179 and Tyr205 interacting with the substrate analogue, acetic acid [11]. The substrate-binding groups have a one-to-one correspondence with the functional groups in the  $\alpha$ -CA active site, with the corresponding residues being closely superposable by a mirror plane. Therefore, despite differing folds,  $\alpha$ - and  $\beta$ -CAs have converged upon a very similar active-site design and are likely to share a common mechanism of action [11].

The prototype of the  $\gamma$ -class of CA has been characterized from the methanogenic archaeon *Methanosarcina thermophila* (Figure 1D) [12]. The crystal structures of Zn-containing and Co-substituted  $\gamma$ -CA from *M. thermophila* were reported in the unbound form and cocrystallized with sulfate or bicarbonate. Relative to the tetrahedral-coordination geometry seen at the active site in the  $\alpha$ -CAs, the active site of  $\gamma$ -CA contains additional metal-bound water ligands, so that the overall coordination geometry is trigonal bipyramidal for the Zn-containing enzyme and octahedral for the Co-substituted enzyme [12]. Ligands bound to the active site make contacts with the side chain of Glu62 in a manner that suggests this side chain to be protonated. In the uncomplexed Zn-containing enzyme, the side chains of Glu62 and Glu84 appear to share a proton; additionally, Glu 84 exhibits multiple conformations. This suggests that Glu84 may act as a proton shuttle, which is an important aspect of the reaction mechanism of  $\alpha$ -CAs, for which a histidine active-site residue generally performs this function (usually His64) [1–8].

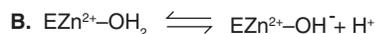
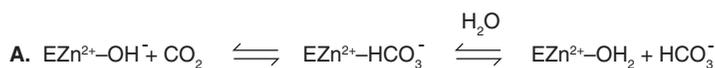
X-ray absorption spectroscopy at the Zn K-edge indicates that the active site of the marine diatom, *Thalassiosira weissflogii* CA, is strikingly similar to that of mammalian  $\alpha$ -CAs. The Zn has three histidine ligands and a single H<sub>2</sub>O molecule, being quite different from the  $\beta$ -CAs of

higher plants in which zinc is coordinated by two cysteine thiolates, one histidine and a H<sub>2</sub>O molecule [13]. The diatom CA shows no significant sequence similarity with other CAs and may represent an example of convergent evolution at the molecular level [13]. In the same diatom, an unusual discovery has been made: the first Cd-containing enzyme, which is a CA-type protein [14]. The marine diatom, *T. weissflogii*, growing under conditions of low Zn, typical of the marine environment, and in the presence of Cd, 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 [14]. <sup>109</sup>Cd labelling comigrates with a protein band that showed this CA activity to be distinct from TWCA1 on native polyacrylamide gel electrophoresis (PAGE) of radiolabeled *T. weissflogii* cell lysates. The levels of the Cd protein were modulated by CO<sub>2</sub> in a manner that is consistent with a role for this enzyme in carbon acquisition. Purification of the CA-active fraction leads to the isolation of a Cd-containing protein of 43 kDa, being clear that *T. weissflogii* expresses a Cd-specific CA, which, particularly under conditions of Zn limitation, can replace the Zn enzyme TWCA1 in its carbon-concentrating mechanism [14]. This enzyme is considered to belong to the  $\delta$ -CA class (Figure 2).

The catalytic mechanism is well understood for the  $\alpha$ -CAs, and will be discussed in detail here. The active form of the enzyme is the basic one, with hydroxide bound to Zn(II) (Figure 1A & 2A) [1–8]. This strong nucleophile attacks the CO<sub>2</sub> molecule bound in a hydrophobic pocket in its neighbourhood (the elusive substrate-binding site comprises residues Val121, Val143 and Leu198 [1–8]) (Figure 2B), leading to formation of bicarbonate coordinated to Zn(II) (Figure 2C). 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 2D), which is catalytically inactive [1–8]. In order to regenerate the basic form A, a proton-transfer reaction from the active site to the environment takes place, which may be assisted either by active-site residues (such as His64 – the proton shuttle in isozyme CA II) or by buffers present in the medium [1–8]. The process may be schematically represented by Equations 1A & B.

The rate-limiting step in catalysis is the second reaction, that is, the proton transfer that regenerates the Zn-OH<sup>-</sup> species of the enzyme (Figure 3) [1–8].

## Equations



Two main classes of CAIs are known: the metal-complexing anions (inorganic inhibitors), and the unsubstituted sulfonamides and their derivatives (e.g., sulfamates or sulfamides) – the organic inhibitors. All inhibitors of interest for their therapeutic applications bind to the Zn(II) ion of the enzyme, either by substituting the nonprotein Zn ligand (Figure 3A) or by adding the metal coordination sphere (Figure 3B), generating trigonal-bipyramidal species [1–8]. Sulfonamides, which are the most important CAIs, bind in a tetrahedral geometry of the Zn(II) ion (Figure 3A), in a deprotonated state, with the nitrogen atom of the sulfonamide group coordinated to Zn(II). Anions may bind either in tetrahedral geometry of the metal ion or as trigonal-bipyramidal adducts, such as the thiocyanate adduct shown in Figure 3B [1–8].

X-ray crystallographic structures are available for many adducts of sulfonamide/sulfamate/sulfamide inhibitors with various isozymes [1–8,15–24]. In all these adducts, the deprotonated sulfonamide is coordinated to the Zn(II) ion of the enzyme, and its NH moiety participates in a H bond with the O<sub>γ</sub> of Thr199, which in turn is engaged in another H bond to the carboxylate

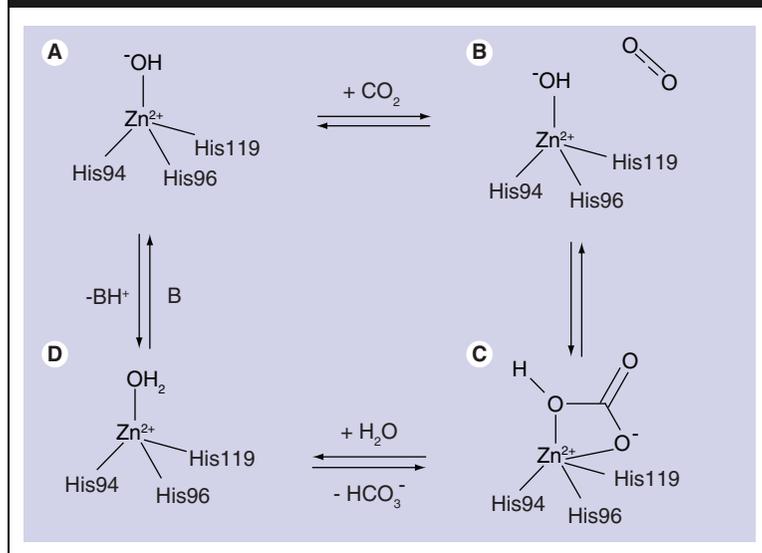
group of Glu106 [15–17]. One of the oxygen atoms of the SO<sub>2</sub>NH moiety also participates in a H bond with the backbone NH moiety of Thr199 [15–24]. In Figure 4, the crystal structures of the human isoform hCA II adducts with the simplest compounds incorporating a sulfamoyl moiety (sulfamide and sulfamic acid, respectively) are shown as examples of the binding of sulfonamide, sulfamate and sulfamide inhibitors [15]. The binegatively charged (NH)SO<sub>3</sub><sup>2-</sup> sulfamate ion and the monoanion of sulfamide NHSO<sub>2</sub>NH<sub>2</sub><sup>-</sup> were shown to bind to the Zn(II) ion within the enzyme active site [15]. These two structures provide some close insights into why this functional group (the sulfonamide) appears to have unique properties for CA inhibition:

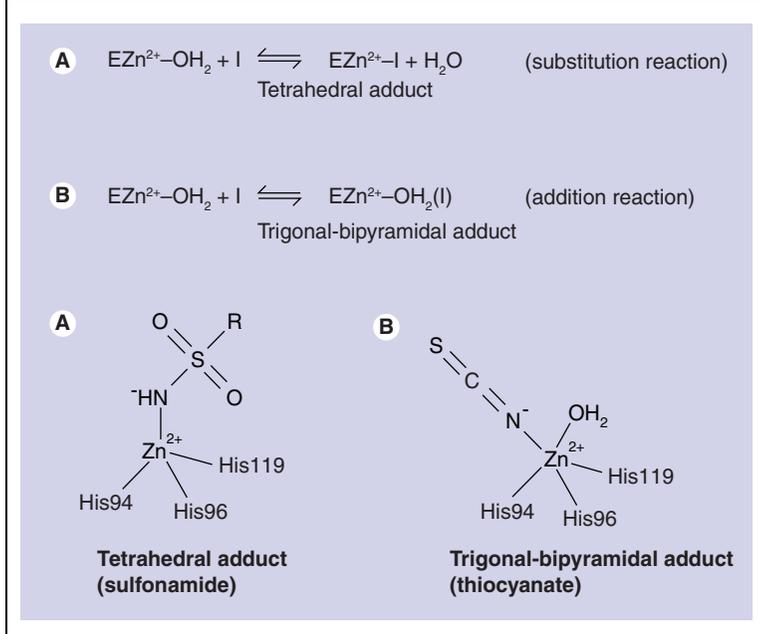
- It exhibits a negatively charged and most likely monoprotonated N coordinated to the Zn(II) ion
- Simultaneously, this group forms a H bond as donor to the oxygen O<sub>γ</sub> of the adjacent Thr199
- A H bond is formed between one of the SO<sub>2</sub> oxygens to the backbone NH of Thr 199

Thus, the basic structural elements explaining the strong affinity of the sulfonamide moiety for the Zn(II) ion of CAs were delineated in all details by using these simple compounds as prototypical CAIs, without the need to analyze the interactions of the organic scaffold, usually present in other inhibitors (generally belonging to the aromatic/heterocyclic sulfonamide class [15]). Despite important similarities of the binding of these two inhibitors to the enzyme with that of aromatic/heterocyclic sulfonamides of the type RSO<sub>2</sub>NH<sub>2</sub> previously investigated, the absence of a C–SO<sub>2</sub>NH<sub>2</sub> bond in sulfamide/sulfamic acid leads to a different H-bond network in the neighborhood of the catalytical Zn(II) ion, which was shown to be useful for the drug design of more potent CAIs, possessing different Zn-binding functions than the classical sulfonamides [15].

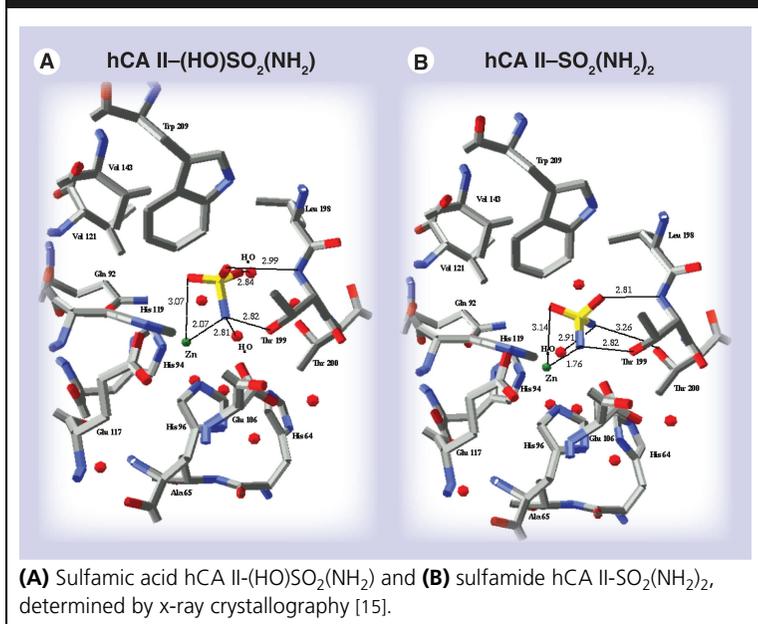
A very important discovery in parasitic CAs has been reported by Krungkrai and colleagues, who discovered the presence of at least two different CAs in *Plasmodium falciparum*, the protozoa causing malaria [25]. Red cells infected with *P. falciparum* contained CA amounts approximately twice as high as those of normal red cells. The three developmental forms of the asexual stages (i.e., ring, trophozoite and schizont) were isolated from their host red cells and found to have stage-dependent CA activity. This enzyme

**Figure 2. Schematic representation of the catalytic mechanism for  $\alpha$ -carbonic anhydrase-catalyzed CO<sub>2</sub> hydration.**



**Figure 3. Carbonic anhydrase inhibition mechanism by organic (sulfonamide) and inorganic (anion) inhibitors.**

was then purified to homogeneity by using multiple steps of fast-liquid chromatographic techniques, showing a Mr of 32, being active in monomeric form (the human red cell enzyme was also purified for comparison with the parasite enzyme in this study) [25]. The parasite–enzyme activity was sensitive to well-known sulfonamide CAIs, such as sulfanilamide and acetazolamide

**Figure 4. Adducts of human carbonic anhydrase II with the simplest sulfonamides.**

(AZ). The kinetic properties and amino-terminal 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 protein (i.e., *P. falciparum* CA). In addition, the aforementioned enzyme inhibitors showed anti-malarial effect against *in vitro* growth of *P. falciparum* [25]. This very important contribution shows that CAIs may represent valuable future drugs for the treatment of malaria.

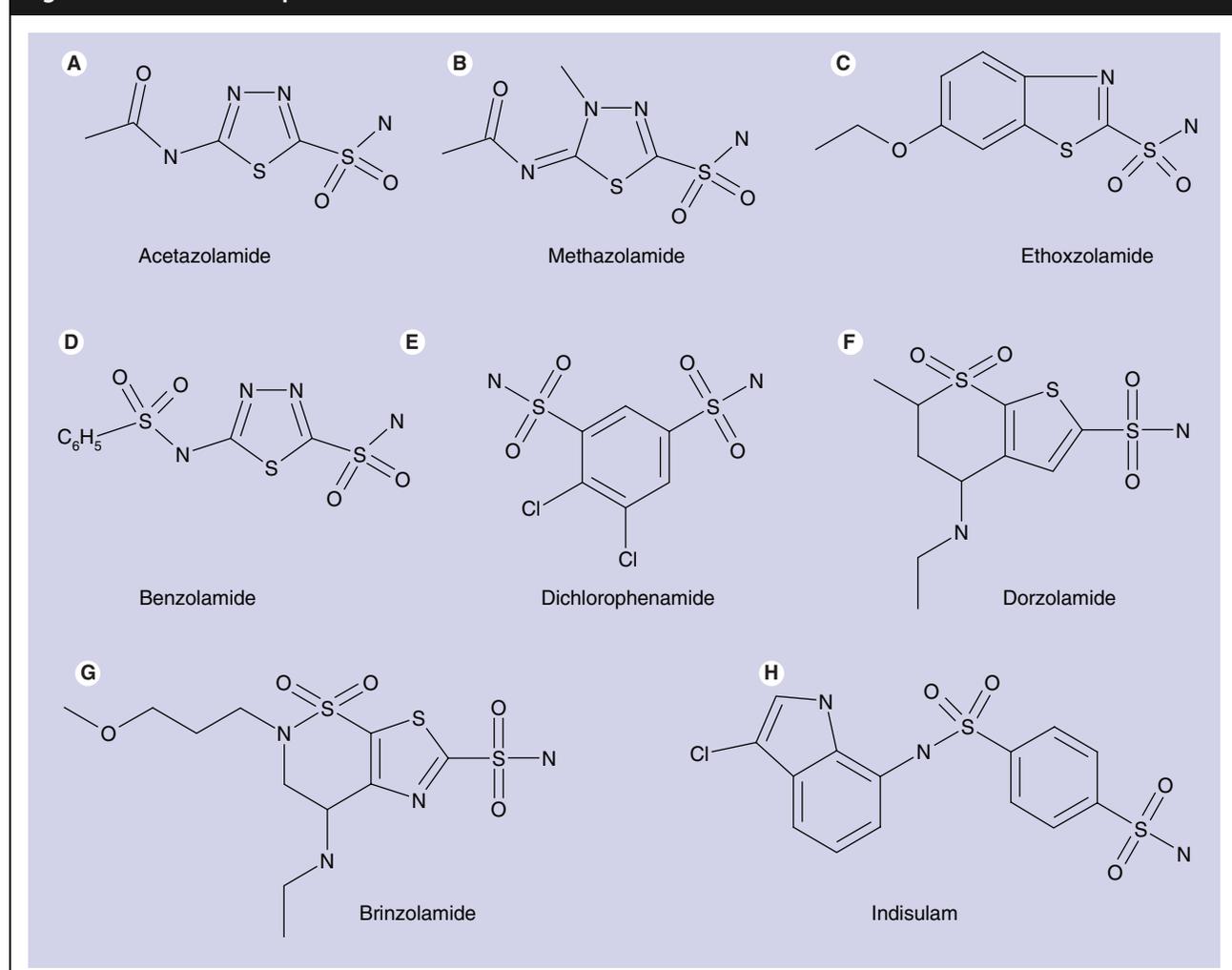
### CAIs & their applications

There are at least 16 clinically used drugs/orphan drugs reported to possess significant CA inhibitory properties (Figures 5 & 6) [1,26–28], and many other such derivatives belonging to the sulfonamide, sulfamate or sulfamide classes are constantly reported, designed and synthesized by means of rational drug-design processes [29–49].

Compounds used clinically, such as AZ (Figure 5A), methazolamide (Figure 5B), ethoxzolamide (Figure 5C), the orphan drug benzolamide (Figure 5D) and dichlorophenamide (DCP; Daranide®) (Figure 5E), have been known for many years and were initially developed in the search for diuretics [26]. Although their diuretic use was not extensive [26–28], it was found that such enzyme inhibitors could be employed for the systemic treatment of glaucoma [26]. Thus, many such drugs (e.g., AZ, methazolamide and DCP) are still presently used in ophthalmology (see discussion later in the text), whereas two novel derivatives, dorzolamide (Figure 5F) and brinzolamide (Figure 5G) have been developed in the 1990s as topically acting antiglaucoma agents [26–28].

The antitumor sulfonamide indisulam (Figure 5H) is in Phase II clinical development for the treatment of solid tumors [50], whereas the antiepileptic sulfamate topiramate (Figure 6A) [51] and sulfonamide zonisamide (Figure 6B) [22] were recently shown to possess significant inhibitory properties against many physiologically relevant CA isozymes. The same is true for the antipsychotic sulpiride (Figure 6C) [52]. The sulfamates possessing steroidal-like scaffolds COUMATE-667 (Figure 6D) [53] and EMATE (Figure 6E) [54] were initially discovered as steroid sulfatase (STS) inhibitors [53] and subsequently shown to be low nanomolar CAIs. These compounds are also in clinical development for the treatment of breast tumors in which both STS and some CA isozymes are overexpressed [1,53,54]. The sulfonamide cyclooxygenase (COX)-2 inhibitors celecoxib (Figure 6F) [55] and valdecoxib (Figure 6G) [23] also act as potent inhibitors of many CA isozymes,

Figure 5. Chemical compounds.



and some of their clinical applications (e.g., the prevention of some gastrointestinal tumors) are correlated with the strong inhibition of some CAs [23,55]. The sulfimine artificial sweetener saccharin (Figure 6H) is also a very potent inhibitor of several physiologically relevant mammalian CA isoforms (Table 1) [56].

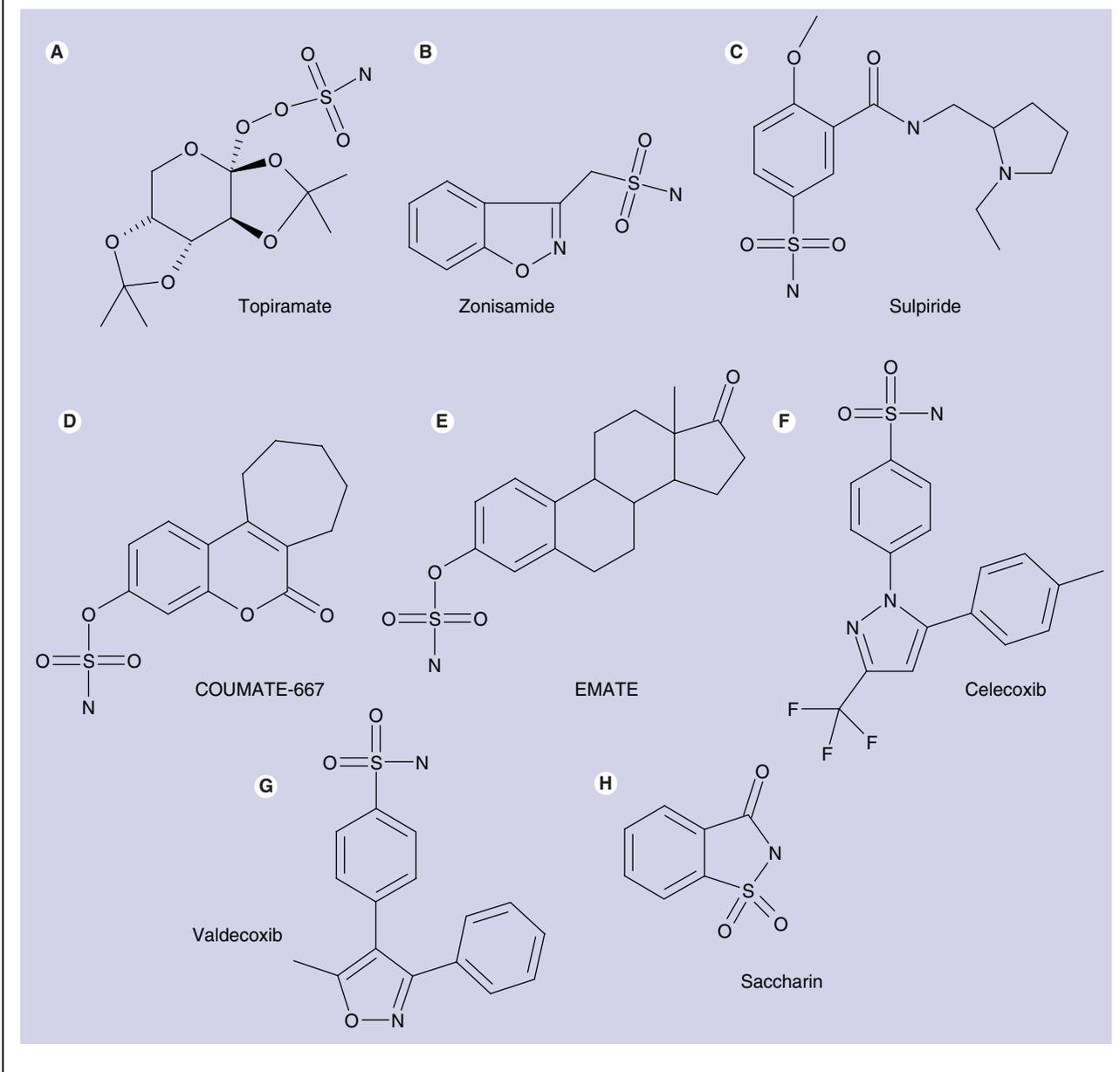
Examples of inhibitory effects of some of these clinically used drugs against the mammalian isoforms CA I–XIV are shown in Table 1. Data in Table 1 show that only isoform III is not susceptible to inhibition by sulfonamides and sulfamates, whereas all other CA isoforms possessing catalytic activity – CA I, II, IV, VA, VB, VI, VII, IX, XII, XIII and XIV (CA XV is not present in humans) – are appreciably inhibited by many of the clinically used drugs, with inhibition constants in many cases in the low nanomolar range. These very diverse inhibition profiles of the various isozymes with derivatives discussed above may explain the very different clinical applications of

the CAIs, ranging from diuretics and antiglaucoma agents, to anticancer, antiobesity and antiepileptic drugs [1–8,26,27].

#### Applications of CAIs in ophthalmology

CAIs constitute an important class of drugs in the armamentarium of antiglaucoma agents [1,2,26,27]. In addition to the classical, systemically used sulfonamides (AZ, methazolamide, DCP and ethoxzolamide [1,2,26,27]), two topically acting agents became available in the last years, dorzolamide and brinzolamide [1,2,26,27]. The two drugs are effective in reducing intraocular pressure (IOP) by 20–25%, and show fewer side effects compared with the systemically applied sulfonamides [1,2]. Still, the observed side effects, including stinging, burning or reddening of the eye, blurred vision, pruritus, and bitter taste, may lead, in many cases, to patient noncompliance and the need to use other drugs, or to develop topically acting sulfonamides possessing less side effects. All but the last

Figure 6. Chemical compounds.



side effects mentioned above are probably due to the fact that dorzolamide and brinzolamide are salts of weak bases (secondary amines) with a very strong acid (HCl), so that the pH of the drug solution is rather acidic (generally around 5.5). Thus, many studies were reported ultimately for the development of topically acting sulfonamide CAIs that can be formulated in solution at near neutral pH [31,32,34,35,37,38,44,45,47]. Conversely, many clinical studies regarding the two drugs from this class available up to now (dorzolamide and brinzolamide), alone or in combination with other agents, were reported in the last few years [57–61]. Thus, in

a randomized, double-blind study the efficacy of dorzolamide 2% and brinzolamide 1% in lowering IOP and in evaluating side effects were recently reported [57]. IOP decreased significantly from baseline for both drugs, with no statistically significant differences between dorzolamide 2% and brinzolamide 1% either before or after eye-drop administration. The most frequent side effect was ocular pain in the case of dorzolamide 2% and blurred vision in brinzolamide 1%. The results suggested that dorzolamide 2% and brinzolamide 1% have similar IOP-lowering efficacies with different side effects [57]. The nonselective  $\beta$ -blocker

**Table 1. Inhibition data with the clinically used sulfonamides, the clinically used sulfamate (topiramate) and saccharin against isozymes I–XIV.**

Isozyme	K <sub>i</sub> (nM)							
	5A*	5B*	5C*	5E*	5F*	5G*	6A*	6H*
hCA I <sup>‡</sup>	250	50	25	1200	50000	45000	250	18540
hCA II <sup>‡</sup>	12	14	8	38	9	3	10	5950
hCA III <sup>‡</sup>	3.10 <sup>5</sup>	1.10 <sup>5</sup>	5000	NT	8000	NT	NT	NT
hCA IV <sup>‡</sup>	74	6200	93	15000	8500	NT	4900	7920
hCA VA <sup>‡</sup>	63	65	25	630	42	50	63	10060
hCA VB <sup>‡</sup>	54	62	19	21	33	30	30	7210
hCA VI <sup>‡</sup>	11	10	43	79	10	0.9	45	935
hCA VII <sup>‡</sup>	2.5	2.1	0.8	26	3.5	2.8	0.9	10
hCA IX <sup>‡</sup>	25	27	34	50	52	37	58 <sup>¶</sup>	103
hCA XII <sup>§</sup>	5.7	3.4	22	50	3.5	3.0	3.8	633
mCA XIII <sup>‡</sup>	17	19	NT	23	18	NT	47	12100
hCA XIV <sup>‡</sup>	41	43	25	345	27	24	1460	773

The isoforms CA VIII, X and XI are devoid of catalytic activity and probably do not bind sulfonamides as they do not contain Zn(II) ions [1].

\*Refers to the figure number from **Figures 5 & 6**.

<sup>‡</sup>Full-length enzyme.

<sup>§</sup>Catalytic domain.

<sup>¶</sup>The data against the full-length enzyme is of 1590 nM.

h: Human; m: Murine isozyme; NT: Not tested (no data available).

timolol and dorzolamide both lower IOP, but the two agents have different mechanisms of action and their effects are additive when administered together [58]. Therefore, these drugs are frequently used concomitantly to treat patients with open-angle glaucoma who have not adequately responded to first-line therapy ( $\beta$ -blockers or sulfonamide CAIs). A barrier to good compliance with concomitant therapy is the need to administer five or six drops of medication on two or four occasions during the day. Timolol 0.5% and dorzolamide 2.0% have therefore been combined in a single formulation, reducing the number of administrations required to two per day [58]. Clinical trials in patients with glaucoma have demonstrated that dorzolamide 2%/timolol 0.5% (dorzolamide/timolol) is superior to monotherapy with the individual components [58]. When dorzolamide/timolol administered twice daily was compared with concomitant treatment with dorzolamide 2% and timolol 0.5%, each administered twice daily for 90 days, both regimens resulted in marked lowering of trough IOP (measured just before the morning dose) compared with baseline (reduction in IOP = 4.2 mmHg). When the combined formulation was compared with a concomitant regimen that included dorzolamide 2% three times a day and timolol 0.5% twice daily, the concomitant regimen was slightly more efficacious

than the combined regimen, after 90 days: IOP was lowered by 3.6 mmHg in the combined group versus 4.1 mmHg in the concomitant group. Dorzolamide/timolol has been compared with concomitant administration of timolol 0.5% and the IOP-lowering miotic drug, pilocarpine 2.0%. This non-blind, patient-preference study found that both regimens reduced IOP. However, the dorzolamide/timolol combination was preferred by patients because of reduced frequency and severity of adverse effects and less frequent administration [58]. Dorzolamide/timolol was well tolerated in clinical trials, the adverse effects reflected those of the individual components and no additional tolerability issues were identified. However, the potential for timolol to cause cardiorespiratory effects must be considered when prescribing this combination, whereas dorzolamide, being a sulfonamide, can cause allergic reactions in those who are hypersensitive to this class of drug [58].

Similarly, the additive effect of latanoprost 0.005% in patients who have uncontrolled IOP using timolol 0.5% and dorzolamide 2% has been investigated [59]. Latanoprost had an additive effect when used as a third drug for patients on timolol and dorzolamide who were in need of further IOP reduction. These results suggested that latanoprost may be very effective in some patients with poorly controlled glaucoma on multiple therapy [59].

The reduction in IOP by topical dorzolamide 2% and oral methazolamide (5 mg/kg) in dogs has also been investigated in order to determine if the combination of both drugs would reduce IOP more than either drug administered alone. Topical dorzolamide 2%, administered twice and three times a day, significantly decreased IOP in glaucomatous dogs on the first day (twice daily 7.6 mmHg and three times a day 16.4 mmHg), which was greater by day 5 (twice daily 10.4 mmHg and three times daily 13.9 mmHg). Oral methazolamide also significantly lowered IOP in both eyes. Oral methazolamide combined with topical dorzolamide 2% (instilled in the eye from day 3 to 5) also significantly lowered IOP of both eyes for all days and, for day 5, the mean IOP was decreased by 7.9 mmHg (methazolamide plus dorzolamide) and 7.5 mmHg (methazolamide only). Topical dorzolamide (instilled in the drug eye for day 1–5) combined with oral methazolamide (administered from day 3 through to day 5) significantly lowered IOP in the eye on day 1 (5 pm, 9.6 mmHg), for day 3 (11 am and 5 pm) and for all of day 5 for both eyes (5 pm: control eye 9.5 mmHg; drug eye 9.0 mmHg). Topical dorzolamide (2%) instilled three times a day produced similar IOP declines compared with the combination of oral methazolamide and dorzolamide 2% administered twice daily. Thus, dorzolamide (2%) instilled twice or three times a day caused significant decreases in IOP in glaucomatous dogs. Twice-daily instillations caused progressive declines in IOP from day 1 to day 5. Dorzolamide (2%) combined with oral methazolamide (5 mg/kg twice daily) produced similar but not additional declines in IOP [60].

A comparison study of topical brinzolamide 1% twice daily with dorzolamide 2% twice daily, each given with timolol 0.5% twice daily, has also been reported [61]. Both treatment regimens significantly reduced IOP at all time points: brinzolamide plus timolol by -3.6 to -5.3 mmHg (-14.2 to -21.9%) and dorzolamide plus timolol by -3.6 to -5.1 mmHg (-14.1 to -21.2%). Clinically relevant IOP reductions (decreases 5 mmHg or greater or absolute IOP values of 21 mmHg or less) were manifested by 50–89.3% of patients under brinzolamide plus timolol and by 43.9–85.4% under dorzolamide plus timolol. The treatments were equivalent in mean IOP lowering. In general, both regimens were well tolerated. However, more patients experienced at least one adverse event with dorzolamide plus timolol (32.8%) compared with

brinzolamide plus timolol (14.7%). In addition, more patients experienced ocular discomfort (stinging and burning) after dorzolamide plus timolol (13.1%) than after brinzolamide plus timolol (1.7%) in this study [61].

Medical treatment of cystoid macular edema (CME) is another major indication for CAIs [1,62]. Initial observations were based on experimental data that suggested that AZ can increase fluid absorption across the retinal pigment epithelium [1,62]. CAIs have also been shown to possess other direct effects, both on retinal and retinal pigment epithelial cell function, by inducing an acidification of the subretinal space, a decrease of the standing potential as well as an increase in retinal adhesiveness [1,62]. It is thought that acidification of the subretinal space is responsible for the increase in fluid resorption from the retina. Several clinical studies have suggested that patients with CME due to retinitis pigmentosa and uveitis may react more favorably to treatment with CAIs than other etiologies, such as diabetic maculopathy or macular edema after retinal vein occlusion [1,62]. A normal clinical starting dose of systemic CAI is 500 mg/day, which should be continued for at least 1 month to see an effect on CME [62].

Recently, the involvement of CA I (an abundant isoform with a rather unknown physiologic function [1]) in eye pathology has emerged [63]. Thus, it is known that excessive retinal vascular occlusion/permeability contributes to the pathogenesis of proliferative diabetic retinopathy and diabetic macular edema, leading causes of vision loss in adults. Using mass spectrometry-based proteomics, Gao and colleagues detected 117 proteins in human vitreous and elevated levels of extracellular CA I in vitreous from individuals with diabetic retinopathy, suggesting that retinal hemorrhage and erythrocyte lysis contribute to the diabetic vitreous proteome [63]. Intravitreal injection of CA I in rats was then shown to increase retinal vessel leakage and to cause intraretinal edema. CA I-induced alkalinization of vitreous increased kallikrein activity and its generation of factor XIIa revealed a new pathway for contact system activation. CA I-induced retinal edema was decreased by complement 1 inhibitor, a neutralizing antibody to prekallikrein- and bradykinin-receptor antagonism. Subdural infusion of CA I in rats induced cerebralvascular permeability, suggesting that extracellular CA I could have broad relevance to neurovascular edema [63]. Inhibition of the enzyme activity of the extracellular CA I by

sulfonamide/sulfamate/sulfamide inhibitors could thus provide new therapeutic opportunities for the treatment of hemorrhage-induced retinal and cerebral edema, a highly relevant medical problem.

### CAIs & cancer

There are many connections between CA and cancer [1,2,64,65]. Some CA isozymes are predominantly found in cancer cells, lacking from their normal counterparts [1,2,64,65]. CA IX was shown to be highly overexpressed in many cancer types and was present in few normal tissues in a quite limited amount [64,65]. CA IX expression is strongly induced by hypoxia present in many tumors, being regulated by the hypoxia-inducible factor (HIF) transcription factor and correlated with a poor response to classical chemo- and radio-therapies [64,65]. CA IX was recently shown to contribute to acidification of the tumor environment by efficiently catalyzing the hydration of  $\text{CO}_2$  to  $\text{HCO}_3^-$  and protons with its extracellularly situated active site [66], leading both to the acquisition of metastatic phenotypes and chemoresistance with weakly basic anticancer drugs [66,67]. Thus, Svastova and colleagues proved that  $\text{CO}_2$  is a significant source of acidity in tumors (in addition to lactic acid) [66], thereby indicating the relevant contribution of CA IX in the tumor acidification processes. This seminal study provided the first evidence for the role of CA IX in the control of the extracellular pH (pHe) in tumors [66]. It was shown that CA IX can acidify the pH of the culture medium in hypoxia but not in normoxia. This acidification could be perturbed by deletion of the enzyme active site or by inhibiting the CA IX catalytic domain with selective sulfonamide CAIs, which were shown to bind only to hypoxic cells expressing CA IX [66]. These findings suggested that hypoxia regulates both expression and activity of CA IX in order to enhance the extracellular acidification processes, which may have important implications for tumor progression [66]. Conversely, inhibition of this enzymatic activity by specific and potent inhibitors was shown to revert these processes, establishing a clear-cut role for CA IX in tumorigenesis and proving this enzyme to be a druggable target. The development of a wide range of potent and selective CA IX inhibitors might thus provide useful tools for highlighting the exact role of CA IX in hypoxic cancers, to control the pH (im)balance of tumor cells and to develop novel diagnostic or therapeutic applications for the management of

such tumors. Indeed, both fluorescent inhibitors [67] or positively charged, membrane-impermeant sulfonamides [68] that were shown to inhibit only these extracellular CAs have recently been developed as CA IX inhibitors and used as proof-of-concept tools for demonstrating that CA IX constitutes a novel and interesting target for anticancer drug development, as well as for imaging purposes of hypoxic tumors [67–69].

The design of fluorescent sulfonamides that preferentially inhibit the activity of CA IX, showing reduced penetration through the plasma membranes and binding to hypoxic cells expressing CA IX, was reported by Cecchi and colleagues [67]. These inhibitors represent promising candidates for developing anticancer therapies based on tumor-associated CA isozyme inhibition and offer very interesting tools for imaging and further investigation of hypoxic tumors, due to the fact that they only accumulate in such tissues [66,67].

A series of positively charged, membrane-impermeant sulfonamides were obtained by reaction of aminobenzolamide [5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide] with tri-/tetra-substituted pyrilium salts possessing alkyl, aryl or combinations of alkyl and aryl groups at the pyridinium ring [68]. The compounds were assayed for the inhibition of four physiologically relevant isozymes: the cytosolic hCA I and II, the membrane-anchored bCA IV and the membrane-bound, tumor-associated isozyme hCA IX. They showed potent inhibitory activity against all investigated isozymes, although with different profiles. For CA I, the derivatives showed inhibition constants in the range of 3–12 nM, for CA II in the range of 0.20–5.96 nM, against CA IV in the range of 2.0–10.3 nM and against CA IX in the range of 3–45 nM. These compounds were demonstrated to be membrane impermeant due to the permanent positive charge present in their molecules. Some of these derivatives were also tested for their inhibitory activity against the  $\text{Cl}^-/\text{HCO}_3^-$  anion exchanger (AE)1. Two derivatives showed inhibitory activity in the low micromolar range, whereas one compound was inactive at these concentrations. The high affinity of these derivatives for the tumor-associated isozyme CA IX and their membrane impermeability make this type of CAI an interesting candidate for the selective inhibition of only the tumor-associated isozyme and not the cytosolic types, for which they also show high potency. Furthermore, it was proved for the first time in

this study that the CA–AE metabolon can be inhibited by the same type of sulfonamide derivatives [68].

In a recent report, the drug design, synthesis, and transepithelial transport of a group of thioureido sulfonamide CAIs, which have been obtained by reaction of isothiocyanate-substituted aromatic sulfonamides with amines, was also investigated [69]. These compounds have potent inhibitory properties against CA IX with  $K_i$  values in the range of 10–37 nM and  $P_{app}$  values greater than  $0.34 \times 10^{-6}$  cm/s for the absorptive transepithelial transport in Caco-2 cells. In these cells, one of these compounds was shown to be a substrate for efflux transporters such as p-glycoprotein (p-gp). p-gp activity is not likely to be rate limiting for intestinal absorption, but might be useful when targeting hypoxic tumors expressing both p-gp and CA IX [69].

Indisulam is a sulfonamide anticancer drug discovered at Eisai Co. and is in Phase II clinical development for the treatment of solid tumors [5–7]. The combination of indisulam with various antitumor drugs, such as carboplatin, oxaliplatin, capecitabine or their salts, for the prevention or treatment of cancer was recently reported [27]. Indisulam is a cell-cycle inhibitor and a potent CAI. The combinations have been found to have strong synergistic activity. The human breast cancer cell line HBC4 was implanted subcutaneously in nude mice and, when tumor volume reached 114 mm<sup>3</sup>, the mice were treated with indisulam at dosages of:

- (I) 30.625 mg/kg/day for 5 days
- (II) 1.3125 mmol/kg/day for 14 days or sequentially with (I) on day 1–5
- (II) on day 6–19

The minimum relative tumor volume (mRTV) between the treatment groups was determined and it was found that (II) alone elicited no reduction in tumor growth, while the mRTV for (I) and the combination groups were 24 and 18% of original tumor size, respectively. Out of 18 mice divided equally among the groups, the only tumor-free mouse was in the combination group [27]. Novel sulfonamide-containing indole compounds structurally related to indisulam, their pharmacologically acceptable salts and their hydrates were claimed by Eisai scientists [27]. In addition, neovascularization inhibitors, anticancer agents, metastasis inhibitors and anti-inflammatory agents for the treatment of rheumatoid arthritis comprising these compounds as the active component were also claimed [27].

Parkkila's group investigated the effect of AZ, a potent CAI (Table 1), on the invasive capacity of four renal carcinoma cell lines (Caki-1, Caki-2, ACHN and A-498) [70]. It was found that AZ 10  $\mu$ M inhibited the relative invasion rate of these cell lines by between 18 and 74%. The Caki-2 and ACHN cell lines displayed the highest responsiveness, and their responses clearly depended on the AZ concentration in the culture medium. Immunocytochemical and western blotting results identified the presence of CA isozyme II in the cytoplasm of all four cell lines and CA XII on the plasma membrane in three of four cell lines. Because AZ alone reduced invasiveness of these cancer cells *in vitro*, it was concluded that the CAs overexpressed in renal cancer cells contribute to invasiveness [70]. This valuable study constituted another demonstration that CAIs may be used in the management of tumors that overexpress one or more CA isozymes.

In a series of important studies [71–78], Parkkila's, Pastorek's and Harris' groups investigated the expression of various CA isozymes (mainly CA IX and XII) and hypoxia as markers of tumor progression in different organs/tissues. CA IX was shown to be expressed in the basolateral plasma membrane of normal biliary epithelial cells, but not in hepatocytes. Pastorek's group recently showed that, in the biliary epithelial tumors, immunostaining for CA IX was mainly localized at the basolateral surface of the epithelial cells, as in normal mucosa [71]. All non-invasive dysplastic lesions and 57% of invasive lesions of the gall bladder expressed this isozyme. In the liver, 78% of cholangiocellular malignant lesions showed a positive reaction for CA IX, whereas only 33% of hepatocellular carcinomas showed a weak immunoreaction [71]. The conclusion was that abnormal expression of CA IX may be linked to malignant transformation of hepatobiliary cells, this enzyme being a promising marker for biliary differentiation in hepatobiliary neoplasms [71]. In another study [72], the same group examined the expression of CA IX in non-small-cell lung cancers. Of 107 cases analyzed, 39 (36.4%) had strong membrane/cytoplasmic expression of CA IX and were grouped as positive. The staining was confined around areas of necrosis, and a significant association of CA IX expression with the extent of necrosis was noted. Nevertheless, 38 out of 74 cases with focal or extensive necrosis did not express this enzyme [72]. A direct association of CA IX expression with epidermal growth factor

receptor, c-erbB-2 and MUC1 expression was also noted. Survival analysis showed that CA IX expression is related to poor prognosis. Multivariate analysis also revealed that CA IX expression was a significant prognostic factor independent of angiogenesis. It was concluded that CA IX is an important molecule in non-small-cell lung cancer, the upregulation of which occurs in highly hypoxic/necrotic regions of the tumors. The expression of CA IX was linked to the expression of a constellation of proteins involved in angiogenesis, apoptosis inhibition, and cell–cell adhesion disruption, which explained the strong association of this enzyme with poor clinical outcome [72].

A similar study was then reported in a cohort of patients with invasive breast cancer [73]. The majority of patients were treated with adjuvant hormonal or chemotherapy. The frequency of CA IX expression, its association with recognized prognostic factors and the relationship with outcome was evaluated by univariate and multivariate statistical analyses. CA IX expression was present in 49 (48%) out of 103 cases. The level of CA IX expression was found to be significantly associated with tumor necrosis ( $p < 0.001$ ), higher grade and negative estrogen receptor status [73]. CA IX expression was associated with worse relapse-free survival and overall survival in an unselected cohort of patients with invasive breast carcinoma. The potential role of CA IX as a marker of hypoxia within breast carcinomas was also indicated by a significant association with necrosis [73].

There is increasing evidence that hypoxia-regulated gene expression influences tumor aggressiveness, contributing to the poorer outcome of patients with hypoxic tumors [74]. The role of the transcriptional complex HIF-1 as an important mediator of hypoxia-regulated gene expression is one of the best documented pathways. Recently, it has emerged that certain tumor-associated CAs can be added to the list of known HIF-responsive genes [72–74]. In such a study, it was proved that the tumor-associated CA IX is correlated with the level of hypoxia in human cervical tumors [74]. There was a significant positive correlation between the level of tumor hypoxia and the extent of CA IX expression. A retrospective study of 130 squamous cell cervical carcinomas demonstrated that a semiquantitative immunohistochemical analysis of CA IX expression in tumor biopsies is a significant and independent prognostic indicator of overall survival and metastasis-free survival after radiation therapy [74]. These

studies provided clinical evidence that CA IX expression is upregulated in hypoxic human cervical tumors and is associated with a poor prognosis. CA IX may act as an intrinsic marker of tumor hypoxia and poor outcome after radiation therapy. The level of CA IX expression may be used to aid in the selection of patients who would benefit most from hypoxia-modification therapies or bioreductive drugs [74]. *CA12*, the gene encoding isozyme XII, has also been identified as a hypoxia-inducible gene [75]. The expression of CA IX and CA XII in relation to necrosis and early breast tumor progression in 68 cases of ductal carcinoma has been recently examined [75]. CA IX expression was rare in normal epithelium and benign lesions, but was present focally in ductal carcinomas (50% of cases) and in associated invasive carcinomas (29%). In comparison, CA XII was frequently expressed in normal breast tissues (89%), in ductal carcinomas (84%) and in invasive breast lesions (71%). Neither CA IX nor CA XII expression was associated with regional or overall proliferation. Assessment of mammographic calcification showed that CA XII expression was associated with the absence of calcification. These results demonstrate that induction of CA IX and CA XII occurs in regions adjacent to necrosis in these tumors [75]. Furthermore, these data suggested that proliferation status does not influence expression of either CA isozyme in breast tissues, that hypoxia may be the dominant factor in the regulation of CA IX and that factors related to differentiation, as determined by tumor grade, dominated the regulation of CA XII. The existence of differential regulation and associations with an aggressive phenotype may be important in the development of selective CAIs useful to prevent/treat tumor invasion [1,2,67]. In another study, the expression and localization of CA IX in head and neck squamous cell carcinoma (HNSCC) was examined and related to the location of tumor microvessels, angiogenesis, necrosis and stage [76]. CA IX was induced by hypoxia in three HNSCC cell lines and overexpressed in HNSCC tumor tissue. Overexpression was localized to the perinecrotic area of the tumor on immunostaining, and the percentage area of the tumor expressing CA IX was significantly higher with more tumor necrosis and advanced stage. CA IX was overexpressed in HNSCC because of hypoxia and may be a potential biomarker for hypoxia in this tumor. Overexpression may help to maintain the intracellular pH, giving tumor cells a survival advantage and

enhancing resistance to radiotherapy and chemotherapy. Thus, CA IX is considered a potential target for future therapy in HNSCC [76]. In a recent study, the localization of isozymes CA I, II, IX and XII in normal large intestine and colorectal tumors has been investigated [77]. While the normal mucosa of the large intestine showed high expression for CA I and II, the intensity of the immunostaining for both isozymes decreased in benign lesions and was very weak in malignant tumors. The reciprocal pattern of expression observed for these cytoplasmic isozymes and transmembrane CA IX and XII in intestinal tissue specimens supported the suggestion that CA IX and XII may be functionally involved in tumor progression to malignancy and/or in invasion [77]. While CA I and II were shown to be prominent in normal colorectal mucosa, playing a role in regulation of pH homeostasis and water and ion transport, loss of expression of these cytoplasmic isozymes was shown to consistently accompany progression to malignant transformation [77].

In another study, the presence of CA XII along the human nephron and collecting duct, together with its cellular and subcellular localization, have been investigated [78]. CA XII has been revealed to be present in the basolateral plasma membrane of the epithelial cells in the thick ascending limb of Henle and distal convoluted tubules, and in the principal cells of the collecting ducts. A weak basolateral signal was also detected in the epithelium of the proximal convoluted tubules. In addition to the normal kidney specimens, this immunohistochemical study included 31 renal tumors [78]. CA XII showed moderate or strong plasma membrane-associated expression in most oncocytomas and clear-cell carcinomas. The segmental, cellular and subcellular distribution of CA XII along the human nephron and collecting duct suggested that it may be one of the key enzymes involved in normal renal physiology, particularly in the regulation of water homeostasis [78]. High expression of CA XII in some renal carcinomas may contribute to its role in von Hippel–Lindau carcinogenesis [78,79].

Breast carcinoma is the most frequent cancer in women and is the second leading cause of death [75]. Choroid metastasis of breast carcinoma can be found either at presentation or in remission, being also frequently encountered in disseminated breast cancer with multiple-organ metastasis. It has recently been proposed that the edema-reducing effect of AZ might be used for

fluid removal from the retina to the choroid. In a 40-year-old female patient on adjuvant chemotherapy for breast cancer with an isolated choroid metastasis, clinical and radiological remission was achieved after orbital radiotherapy, chemotherapy and AZ treatment [75]. Thus, AZ may possess another, only slightly explored up to now, beneficial clinical use in patients with choroid metastasis [75].

### *CAIs in obesity*

Obesity, a multifactorial disorder characterized by an excess of adipose tissue, represents a challenging medical problem in Western countries, with, for example, 65% of the US population affected [8]. Developing countries, such as China, are also not immune to this epidemic, since it was reported that obesity doubled in women and almost tripled in men from 1989 to 1997 in this country [8]. Obesity is caused by an excessively positive energy balance, with the energy intake exceeding the expenditure, but the exact etiology of the disease is largely unknown [8]. Although diet, physical activity and behavioral modifications should theoretically help in controlling this condition, very rarely, patients undergoing these strategies can lose more than 5–10% of their body weight and maintain this loss for a sufficiently long period in order to experience improvements in blood pressure, plasma lipid levels, blood glucose and diabetic control [8]. Thus, pharmacological interventions for the treatment of obesity are essential. Paradoxically, the drugs available for the treatment of obesity are very few, their mechanism of action hardly understood and their side effects generally quite serious [8].

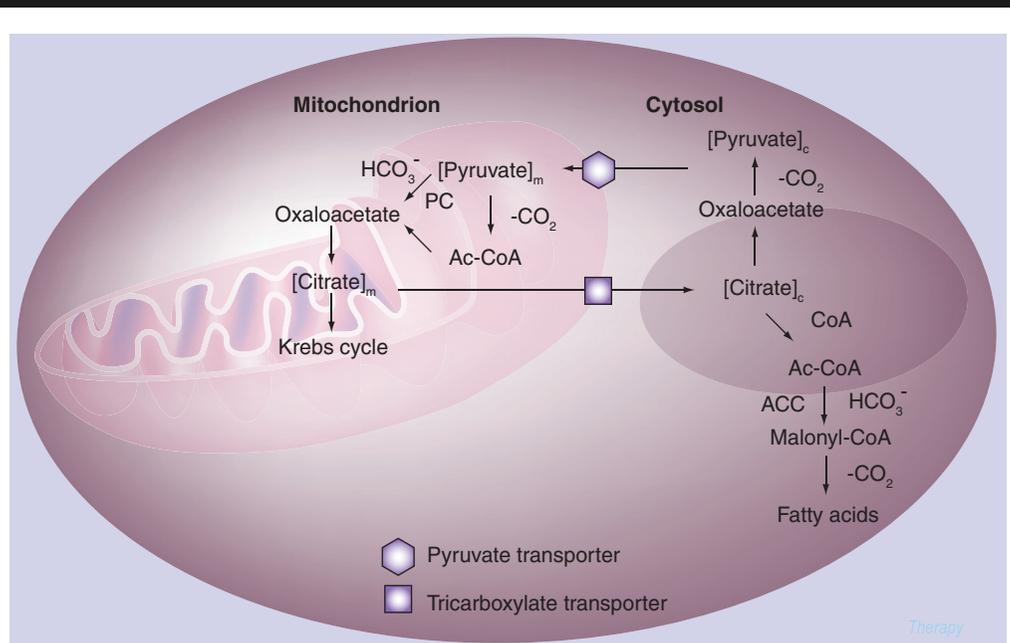
Among the many CA isozymes evidenced so far in diverse organisms all over the phylogenetic tree, CA VA and CA VB are present in mitochondria and are shown to be involved in several biosynthetic processes, such as ureagenesis [1–8], gluconeogenesis [1–8] and lipogenesis (both in vertebrates, such as rodents, as well as invertebrates, such as the locust [8,80–83]). Indeed, in several important biosynthetic processes involving pyruvate carboxylase (PC), acetyl CoA carboxylase (ACC) and carbamoyl phosphate synthetases I and II,  $\text{HCO}_3^-$  and not  $\text{CO}_2$ , is the real substrate of these carboxylating enzyme. The provision of enough  $\text{HCO}_3^-$  is assured mainly by catalysis involving the mitochondrial isozymes CA VA and CA VB (probably assisted by the high-activity cytosolic isozyme CA II) (Figure 7) [8,84–89].

Thus, mitochondrial PC is needed for the efflux of acetyl groups from the mitochondria to the cytosol where the fatty acid biosynthesis takes place [82]. Practically, pyruvate is carboxylated to oxaloacetate in the presence of bicarbonate and PC. The bicarbonate needed for this process is generated under the catalytic influence of the mitochondrial isozymes (CA VA and/or CA VB). The mitochondrial membrane is impermeant to acetyl-CoA, which reacts with oxaloacetate, leading to citrate, which is thereafter translocated to the cytoplasm by means of the tricarboxylic acid transporter. In the cytosol, the citrate is cleaved and regenerates acetyl-CoA and oxaloacetate. As oxaloacetate is unable to cross the mitochondrial membrane, its decarboxylation regenerates pyruvate, which can be then transported into the mitochondria by means of the pyruvate transporter (Figure 7) [8]. The acetyl-CoA generated in the cytosol is in fact used for *de novo* lipogenesis, by carboxylation in the presence of ACC and bicarbonate, with formation of malonyl-CoA. The  $\text{HCO}_3^-$  needed in this process is furnished by the CA II-catalyzed conversion of  $\text{CO}_2$  to  $\text{HCO}_3^-$ . Subsequent steps involving the sequential transfer of acetyl groups lead to longer-chain fatty acids [8]. As a whole,

several CA isozymes are critical to the entire process of fatty acid biosynthesis: CA VA and/or CA VB within the mitochondria (to provide enough substrate to PC) and CA II within the cytosol (for providing sufficient substrate to ACC). It was, in fact, demonstrated that inhibition of CAs by sulfonamides (e.g., trifluoromethanesulfonamide [TFM], a very potent but unstable CAI [8]) can decrease lipogenesis in adipocytes in cell culture [82,83]. In such experiments, an indiscriminate inhibition of all CA isozymes present in these tissues is achieved by the used inhibitor, such as, for example, TFM or AZ [82–84].

Topiramate is an antiepileptic drug possessing potent anticonvulsant effects due to a multifactorial mechanism of action: CA inhibition, blockade of sodium channels and kainate/ $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors,  $\text{CO}_2$  retention secondary to inhibition of the red cell and brain CA isozymes, as well as enhancement of  $\gamma$ -aminobutyric acid (GABA)ergic transmission [51]. A side effect of this drug observed in obese patients was the loss of body weight [90], although no pharmacological explanation of this phenomenon has been provided. Furthermore, topiramate

**Figure 7. The transfer of acetyl groups from the mitochondrion to the cytosol (as citrate) for the provision of substrate for *de novo* lipogenesis.**



All steps involving bicarbonate also need the presence of carbonic anhydrase (CA) isozymes: CA VA and CA VB in the mitochondrion and CA II in the cytosol (see discussion in the text). Adapted from [13].

was shown to reduce energy and fat gain in lean (*Fal?*) and obese (*falfa*) Zucker rats [91]. Conversely, our group recently demonstrated that topiramate is a very potent (low nanomolar) inhibitor of several CA isozymes, such as CA I, II, IV, VA and VAB, and the x-ray crystal structure of its complex with hCA II has also been determined, revealing the basal molecular interactions that explain the high affinity of topiramate for the CA active site [51].

Considering these *in vitro* findings and the reported clinical side effects of the drug that caused weight loss in humans and animals [90,91], we also investigated in detail the inhibition of the mitochondrial isozymes hCA VA [92] and hCA VB [93] with this drug, showing the compound to be a good inhibitor of both ( $K_i$  in the range of 30–63 nM). Thus, we hypothesized that the inhibition of mitochondrial and/or cytosolic CA isozymes leading to impaired lipid biosynthesis may represent a means of controlling weight loss with pharmacological agents such as topiramate or any other sulfamate/sulfonamide CAIs that show effective CA VA/CA VB/CA II inhibition profiles. As a consequence, selective inhibition of mitochondrial CA isozymes (CA VA and CA VB) may lead to diminished lipid biosynthesis and a novel pharmacological approach for the treatment of obesity [8].

Recently, Lynch's group showed that the antipsychotic drug olanzapine (1–8 mg/kg), but not clozapine, increased body weight in female rats only [94]. Weight changes were detectable within 2–3 days and were associated with hyperphagia starting approximately 24 h after the first dose. Chronic administration of the drug (12–29 days) led to adiposity, hyperleptinemia and mild insulin resistance, but no lipid abnormalities or changes in D2-receptor density were observed. Topiramate, which has reversed weight gain from atypical antipsychotics in humans [95], also attenuated weight gain in rats. Olanzapine, but not clozapine, acutely lowered plasma glucose and leptin [94]. Indeed, patients treated with atypical antipsychotic drugs commonly gain excess weight. Because obesity is associated with considerable morbidity and decreased life expectancy, treatment of weight gain in these patients is critical. Topiramate was shown to promote weight loss in healthy obese subjects, patients with bipolar disorder, eating disorder and schizophrenia, without aggravation of their psychotic symptoms [95].

Zonisamide, is another antiepileptic drug used as adjunctive therapy for refractory partial seizures [96]. Owing to its multiple mechanisms of action, it shows a broad spectrum of anti-convulsant activity and has been shown to be effective in several types of seizures, including partial and generalized seizures, tonic-clonic seizures and absence seizures in patients unresponsive to other anticonvulsants [96]. Recent clinical studies have demonstrated additional potential for its therapeutic use in neuropathic pain, bipolar disorder, migraine, obesity, eating disorders and Parkinson's disease, similarly to topiramate [96,97]. Being an aliphatic sulfonamide, we have investigated whether this compound will also interact with the CAs [22].

Unill recently, zonisamide was considered to act as a weak CAI, with a  $K_i$  of 4.3  $\mu\text{M}$  against the cytosolic isozyme hCA II [22]. We proved that this is not true. Indeed, testing zonisamide in classical assay conditions of  $\text{CO}_2$  hydrase activity of hCA II, with incubation times for the enzyme and inhibitor solution of 15 min, a  $K_i$  of 10.3  $\mu\text{M}$  has been obtained. However, when the incubation time between enzyme and inhibitor was increased to 1 h, the obtained  $K_i$  was 35.2 nM, of the same order of magnitude as that of the clinically used sulfonamides/sulfamates AZ, methazolamide, ethoxzolamide and topiramate ( $K_i$  values in the range of 5.4–15.4 nM). Inhibition of the human mitochondrial isozyme hCA VA with these compounds has also been tested by means of a dansylamide competition-binding assay, which showed zonisamide and topiramate to be effective inhibitors, with  $K_i$  values in the range of 20.6–25.4 nM [22]. The x-ray crystallographic structure of the adduct of hCA II with zonisamide has also been examined at a resolution of 1.70 Å, showing that the sulfonamide moiety participates in the classical interactions with the  $\text{Zn}^{2+}$  ion, through coordination to the deprotonated sulfonamide moiety, and also interacting with the Thr199 and Glu106 residues. The benzisoxazole ring of zonisamide has been shown to be oriented toward the hydrophobic area of the active site, establishing a large number of strong van der Waals interactions (<4.5 Å) with residues Gln92, Val121, Phe131, Leu198, Thr200 and Pro202 [22].

Zonisamide, similarly to topiramate, in conjunction with a reduced-calorie diet (deficit of 500 kcal/day), resulted in an additional mean 5-kg weight loss compared with diet alone [98]. This regimen was well-tolerated in obese female patients. In a randomized, controlled trial,

zonisamide therapy was started at 100 mg/day orally, with a gradual increase to 400 mg/day and a further increase to 600 mg/day for patients losing less than 5% of body weight at the end of 12 weeks [99]. The zonisamide group (n = 19) had a mean weight loss of 9.2 kg (1.7 kg; 9.4% loss) at week 32 compared with 1.5 kg (0.7 kg; 1.8% loss) for the placebo group (n = 17). Zonisamide was well tolerated, with few adverse effects [53]. In this preliminary trial, zonisamide and hypocaloric diet resulted in greater weight loss than placebo and hypocaloric diet in the treatment of obesity [99].

#### *Miscellaneous applications of CAIs*

Since CAs are ubiquitous enzymes, their inhibition may have other types of applications in addition to those already mentioned above. Thus, CAIs have been used in the treatment of the primary periodic paralyses (PPs), but their efficacy has not been demonstrated in double-blind, placebo-controlled trials [100]. Therefore, the efficacy of DCP, a potent CAI in the treatment of episodic weakness in primary PPs, has recently been tested [69]. Two multicenter, randomized, double-blind, placebo-controlled crossover trials, one involving 42 subjects with hypokalemic periodic paralysis (HypoPP) and the other involving 31 subjects with potassium-sensitive periodic paralysis (PSPP) have been performed. In each trial, two 8-week treatment periods were separated by an active washout period of at least 9 weeks. The primary outcome variable in the HypoPP trial was the occurrence of an intolerable increase in attack severity or frequency; the primary outcome variable in the PSPP trial was the number of attacks per week. The HypoPP trial had 13 subjects who exhibited a preference for either DCP or placebo, and 11 of these preferred DCP. In the PSPP trial, DCP significantly reduced attack rates relative to placebo [69,100]. DCP also significantly reduced attack rates relative to placebo in the HypoPP subjects. It was concluded that the drug is effective in the prevention of episodic weakness in both HypoPP and PSPP [100].

Essential tremor (ET) is another common movement disorder that often causes functional disability, potentially leading to physical and emotional difficulties [101]. The paucity of data available regarding the underlying pathophysiologic mechanism of ET hinders the development of innovative approaches to pharmacotherapeutic treatments. Options for drug therapy include the use of primidone,

$\beta$ -adrenergic blockers, alcohol, benzodiazepines, gabapentin and CAIs, but few detailed studies in this field are available [101]. In this context, it must be mentioned that, although long suspected from histochemical evidence for CA activity on neurons and observations that CAIs enhance the extracellular alkaline shifts associated with synaptic transmission, an extracellular CA located in the brain had not been identified until recently [102]. A candidate for this CA was suggested by the recent discovery of membrane CA XIV, whose mRNA is expressed in mouse and human brain as well as several other tissues. CA XIV was found by immunostaining on neuronal membranes and axons in both mouse and human brain. The highest expression was seen on large neuronal bodies and axons in the anterolateral part of pons and medulla oblongata. Other CA XIV-positive sites included the hippocampus, corpus callosum, cerebellar white matter and peduncles, pyramidal tract and choroid plexus. These observations made CA XIV a likely candidate for the extracellular CA postulated to have an important role in modulating excitatory synaptic transmission in brain [102].

By inhibition of CA activity, the regional blood flow (rBF) in organs increases as hypercapnia develops [1,103]. However, the effects of AZ-induced vasodilation have not been estimated with respect to vessel size and organs until recently [103]. The aim of a recent study was solely to determine the diameter of the capillaries in various organs that respond to CA inhibition by AZ. While the systolic blood pressure (SBP), pH, hemoglobin concentration and base excess did not change, the partial pressure of arterial O<sub>2</sub> (PaO<sub>2</sub>) increased significantly and the partial pressure of arterial CO<sub>2</sub> (PaCO<sub>2</sub>) decreased significantly with AZ. The rBF was calculated by using three different sizes (15, 25, and 50  $\mu$ M) of colored microspheres (CMs). The rBF measured with 15- $\mu$ M CMs in the brain, kidneys and liver increased in response to AZ, and the rBF in these organs differed between size of CM. However, the rBF, calculated by using the different sizes of CM in the stomach and abdominal muscle, did not change after the administration of AZ. AZ-induced vasodilation occurred in all sizes of vessels in the liver, the small- and medium-sized vessels in kidneys and in the larger capillaries in the brain [103]. Thus, CAIs may be successfully used as diagnostic tools in magnetic resonance imaging or positron-emission tomography [1,103].

The study by Komai and colleagues was designed in order to clarify the effect of Zn deficiency on NaCl preference, the lingual trigeminal and taste nerves transduction and CA activity of the tongue surface and salivary gland [104]. Male Sprague–Dawley rats, 4 weeks old, were divided into four groups and fed Zn-deficient, low-Zn and Zn-sufficient diets with free access and pair-feeding. After taking part in the preference tests for 42 days, the rats took part in the chorda tympani and lingual trigeminal nerve recordings, then finally they were sacrificed and the tongue and submandibular gland were excised to measure CA activity. NaCl preference increased only after 4 days of the feeding of Zn-deficient and low-Zn diets, which means that the taste abnormality appeared abruptly in Zn deficiency, even in marginal deficiency. Reduced CA activities of the taste-related tissues in the Zn-deficient group paralleled well with the decreased taste and lingual trigeminal nerve sensitivities [104].

The secretory isozyme CA VI was previously identified as an essential component of mammalian saliva [1], and recently it has been shown to be an elementary component of human milk [105]. The 42-kDa glycopolypeptide purified from human milk in CAI affinity chromatography shared 100% homology with salivary CA VI in the protein sequence analysis (40% coverage), and its digestion with PNGase F (*N*-glycosidase) resulted in a polypeptide backbone similar in size to salivary CA VI. Quantification of CA VI in milk using a time-resolved immunofluorometric assay revealed an approximately eight-times-higher concentration in human colostrum than in mature milk, the latter corresponding to the levels previously detected in human saliva. The high concentration in the colostrum, in particular its functional and structural stability in an acidic milieu and its growth-supporting role in the taste buds, suggested that milk CA VI is an essential factor in normal growth and development of the infant alimentary tract [105]. This secretory isozyme of human origin, hCA VI, has been cloned, expressed and purified in a bacterial expression system recently [106]. The kinetic parameters for the CO<sub>2</sub> hydration reaction proved that hCA VI possessed a significant catalytic activity for the physiological reaction in the same order of magnitude as the ubiquitous isoform CA I or the transmembrane, tumor-associated isozyme CA IX. A series of sulfonamides and one sulfamate have been tested for their interaction with this isozyme. Simple benzenesulfonamides were rather ineffective hCA VI inhibitors,

with K<sub>i</sub> values in the range of 1090–6680 nM. Better inhibitors were detected among derivatives bearing 2- or 4-amino-, 4-aminomethyl or 4-hydroxymethyl moieties or among halogenated sulfanilamides (K<sub>i</sub> values of 608–955 nM). Some clinically used compounds, such as AZ, methazolamide, ethoxzolamide, DCP, dorzolamide, brinzolamide, topiramate, sulpiride and indisulam, or the orphan drug benzolamide, showed effective hCA VI inhibitory activity, with K<sub>i</sub> values of 0.8–79 nM. The best inhibitors were brinzolamide and sulpiride (K<sub>i</sub> values of 0.8–0.9 nM), the latter compound also being a CA VI-selective inhibitor. The metallic taste reported as a side effect after the treatment with systemic sulfonamides may be due to the inhibition of the salivary CA VI. Some of the compounds investigated in this study might be used as additives in toothpastes for reducing the acidification produced by the relevant CO<sub>2</sub> hydrase activity of enamel CA VI, which leads to the formation of protons and HCO<sub>3</sub><sup>-</sup> and may play a role in carcinogenesis [106].

An immune-mediated reaction to pancreatic structures has been postulated for the pathogenesis of chronic pancreatitis (CP) [107]. Several reports demonstrate the presence of antibodies to the pancreatic ductal epithelium in some patients suffering from CP. Serum antibodies to CA I (anti-CA I) and II (anti-CA II) were shown to be present in patients affected by idiopathic CP. A significant correlation between anti-CA I and anti-CA II serum levels in control subjects and in CP patients has been observed. No correlation was found between serum antibody levels and any of the following variables: length of disease, alcohol consumption, smoking habits, pancreatic surgery, pancreatic calcifications, diabetes and steatorrhea. Serum levels of anti-CA I and anti-CA II are thus quite elevated in patients suffering from CP [107].

Seizures are one of the most common neurological disorders. The triggering mechanisms by which seizures occur remain unclear, but are related to a rapid change in ionic composition, including an increase in intracellular potassium concentration and pH shifts within the brain [108]. pH buffering of extra- and intra-cellular spaces is mainly carried out by the CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> buffer, the equilibration of the two species being assured by the many CA isoforms present in the brain (e.g., CA I, II, IV, VB, VII, XII and XIV) [108–110]. Some CAIs, such as AZ and methazolamide, have been used as anticonvulsants in the treatment of epilepsy, with rather scarce success [108].

Both these sulfonamides are used either in combination therapy with other antiepileptic medications in both children and adults or in refractory epilepsy [108–110]. AZ might be useful in partial, myoclonic, absence and primary generalized tonic–clonic seizures uncontrolled by other classical antiepileptic drugs [110]. However, such sulfonamides did not provide effective long-term therapy for epileptic patients due to reasons currently not well understood [108–110].

Zonisamide is a synthetic 1,2-benzisoxazole-3-methanesulfonamide with potent anticonvulsant properties [111]. The sulfamoyl group on zonisamide was expected to suppress seizures in a similar way to AZ through inhibition of CA, but it was claimed that CA inhibition does not appear to be the primary mechanism of action of this drug [111]. Zonisamide prevents repetitive neuronal firing by blockade of voltage-sensitive Na<sup>+</sup> channels. It also reduces voltage-dependent T-type Ca<sup>2+</sup> channels, facilitating dopaminergic and serotonergic neurotransmission [111]. However, it has recently been reported that zonisamide is also quite an effective CAI against many of the physiologically relevant isoforms [22]. It is unknown at this point to what extent the CA inhibitory properties of the drug have a contribution to its antiepileptic action.

Topiramate is a sulfamate fructopyranose derivative currently available for the treatment of partial-onset epilepsy [112]. As an antiepileptic drug, it is known to be clinically effective against simple or complex partial seizures and also against generalized tonic–clonic seizures [112]. Besides its ability to block the voltage-gated Na<sup>+</sup> channel, to potentiate GABAergic transmission and to block the kainate/AMPA receptor, topiramate occupies a particular place among the new anticonvulsants due to its ability to inhibit CA [113]. In this respect, topiramate-induced changes of GABAergic depolarizations were supposed to be based on a decreased intracellular HCO<sub>3</sub><sup>-</sup> concentration, which may be caused by an inhibition of neuronal CA [112,114]. It has been reported by our group that topiramate strongly inhibits several CA isoforms, among which are the cytosolic CA II and CA VII present in the brain [51,115]. Moreover, its x-ray crystallographic structure in complex with hCA II revealed a tight association, with a network of seven strong H bonds fixing the inhibitor within the active site, in addition to Zn(II) coordination through the ionized sulfamate moiety [51]. The inhibitory effect of topiramate on interstitial and intracellular CAs could modify extra- and intra-cellular

activity-dependent pH changes, as demonstrated for other CAIs [116]. Such pH shifts might have a deep impact on neuronal excitability because many ion channels, gap junctions and neurotransmitter receptors are highly sensitive to change in pH [112]. Local administration of AZ has a similar effect to systemic administration of topiramate or AZ, suggesting that the effect of these drugs on the initial alkalinization is through inhibition of central CA [117]. Both drugs decreased initial alkalinization, which would be consistent with an antiepileptic effect, and may actually contribute to their antiepileptic actions *in vivo* [117]. However, the change in pH regulation is not a fundamental anticonvulsant mechanism since carbamazepine and phenytoin, two antiepileptic drugs, have no effect on the alkalinization [117]. Thus, the effect of CA inhibition on the antiepileptic action of topiramate is not well understood.

CAIs were also shown to be effective in the prevention and treatment of mountain sickness and high-altitude cerebral edema [118–120]. These conditions affect, to varying degrees, all travellers to high altitudes, being characterized by a combination of symptoms such as headache, insomnia, anorexia, nausea, dizziness, vomiting, dyspnea, muscle weakness, oliguria, peripheral edema and retinal hemorrhage [118–120]. In more serious cases, pulmonary or cerebral edema were also observed [118–120]. The primary cause of mountain sickness is related to reduced oxygen content in the air at high altitudes, which leads to hypoxemia and all the symptoms mentioned above. AZ, alone or in combination with dexamethasone, is useful in markedly reducing these symptoms, due to the increased arterial oxygen concentrations after red blood cell/brain enzyme inhibition by the sulfonamide drug [118–120]. It is not clear whether methazolamide or other clinically used CAIs may have a better mode of action compared with AZ in preventing acute mountain sickness, since few comparative studies have been reported [120].

## Conclusion

CAs continue to be surprising enzymes, as many exciting new discoveries are constantly emerging, even though these are quite 'old' enzymes, being first discovered in 1933, and thoroughly investigated since then. In the last few years, a host of interesting such reports have been made, first of all regarding the catalytic/inhibition mechanism as well as isolation/characterization of new isozymes, in addition to CAs of nonvertebrate origin.

The x-ray crystallographic structure adducts of hCA II with the simplest sulfonamides (sulfamide and sulfamate) have been reported. These compounds are considered to be the mother of all sulfonamides, since they incorporate in their structure the essential Zn-binding function present in all potent CAIs currently known, without any other aromatic/heterocyclic scaffold generally present in other inhibitors investigated in detail. The x-ray crystallographic structures of many hCA II and I adducts with sulfonamides and sulfamates currently reported ultimately helped in a better understanding of the molecular interactions between the inhibitor and the enzyme, which may lead to a rational drug design of inhibitors with reduced side effects and selectivity for the target isoform. Among these structures, there are also those with some widely used clinical agents, some of which have originally been designed for other applications, such as the anti-epileptics topiramate and zonisamide, the anti-tumor sulfonamide indisulam, the antipsychotic sulpiride, the steroid sulfatase inhibitors COUMATE and EMATE, the COX-2 inhibitors celecoxib and valdecoxib and the artificial sweetener saccharin. All these compounds show appreciable inhibition (in the low nanomolar range) of some physiologically relevant human CAs.

In the last few years, most of the catalytically active isozymes have been investigated in detail for their inhibition profile against the most important classes of CAIs, the sulfonamides, sulfamates and sulfamides. This is highly important in the search of isozyme-selective compounds and for reducing side effects of the presently available drugs. Indeed, physiologically quite relevant isoforms, such as CA IV, VA, VB, VII, IX, XII, XIII and XIV, have been investigated for the first time in detail for their inhibition with many types of such compounds, and several important drug-design studies have also been reported in which compounds with selectivity for one or other such isozymes were obtained. However, to date, few compounds show a good selectivity for any CA isoform with clinical relevance, although important advances have been reported in the design of compounds with a good selectivity ratio for CA VA over CA II, CA IX over CA II or CA XIII over CA II (CA II is the ubiquitous, catalytically very effective isoform, and this is the reason why many-times its inhibition is regarded as detrimental).

In targeting the tumor-associated isoform CA IX, important advances have been reported in the design of fluorescence labels and of

membrane-impermeant compounds that inhibit only the membrane-associated isoforms (e.g., CA IX) and not cytosolic (e.g., CA I and II). By using fluorescent CAIs, a proof-of-concept study showing the role of CA IX in the tumor acidification processes was reported, and the fact that this phenomenon may be reversed by inhibiting the catalytic activity of the enzyme with CA IX-selective and potent inhibitor. Furthermore, B-containing CA IX inhibitors have been reported, which may be useful in B neutron capture therapy. In another important study, hypoxia-activatable CA IX prodrugs containing disulfide bonds were reported. Such bulky, dimeric sulfonamides are very weak CA I, II and IX inhibitors, but by reduction in hypoxic conditions present in tumors, they lead to thiols that act as low nanomolar inhibitors of these isozymes.

Various ophthalmologic and antiobesity applications of the CAIs were also reported in the patent and scientific literature, together with novel derivatives that act as anticonvulsants.

There are currently very few pharmacological approaches for the treatment of obesity, and most are unsatisfactory. Furthermore, this disease is widespread both in the developed and developing world, and the number of affected individuals is constantly increasing. Thus, new and effective approaches are needed for the development of antiobesity agents possessing different mechanisms of action. A new approach for the treatment and prophylaxis of obesity may be based on the inhibition of CAs, enzymes involved in several steps of *de novo* lipogenesis, both in the mitochondria and the cytosol of cells. Topiramate and zonisamide, clinically used antiepileptic drugs also showing strong CA inhibitory properties, possess a highly desired side effect in obese patients – induction of weight loss. It has been recently proved by our groups that both topiramate and zonisamide act as very potent *in vitro* inhibitors of several CA isozymes (including CA II, CA VA and CA VB), and that this might explain their antiobesity effects. A program of designing such antiobesity sulfonamide CAIs also could lead to the detection of several interesting lead molecules. CAIs targeting isozymes involved in lipogenesis may represent a new approach for the treatment and prevention of obesity.

Several important findings relate to the full structural characterization of CAs belonging to the  $\beta$ - and  $\gamma$ -families (characteristic of bacteria, plants and *Archaea*, respectively). Rather shocking was the finding that, in some  $\beta$ -CAs, there is no H<sub>2</sub>O molecule or OH<sup>-</sup> coordinated to the

catalytically essential Zn(II) ion, making these enzymes quite different to those belonging to the  $\alpha$ - and  $\gamma$ -CA families. Thus, the catalytic mechanism of  $\beta$ -CAs is not fully understood at this time.

Many novel representatives of CAs belonging to the three families mentioned above were isolated and characterized in some other organisms less investigated up to now, such as bacteria (e.g., *Helicobacter pylori*, *Neisseria gonorrhoeae*, *Escherichia coli* and *Mycobacterium tuberculosis*) fungi (*Candida albicans* and *Cryptococcus neoformans*) or in the protozoa causing malaria (*P. falciparum*). However, few inhibition studies of these enzymes are currently available. These discoveries may soon have important therapeutic consequences in the treatment of infections caused by pathogenic agents, in cases where potent and selective inhibitors for the pathogenic over the host enzymes could be detected.

The data presented herein clearly show that CAs and their inhibitors may play an essential role in the development of new therapeutic approaches against a multitude of disorders, in addition to the classical ones for which such agents were and are still in use.

#### Future perspective

As CAs are widespread enzymes in most organisms all over the phylogenetic tree, and as they deal with some of the very simple substrates (such as  $\text{CO}_2$  and  $\text{HCO}_3^-$ ), it is rather probable that the future will see the discovery of many new representatives of these catalysts in many different species. In fact, the recent reports of genomes of many bacterial (pathogenic) species revealed a high number of CAs belonging to the  $\alpha$ -,  $\beta$ -,  $\gamma$ - and/or  $\delta$ -CA gene

families in most of them, although the non-mammalian CAs are highly uninvestigated at the present time. Thus, in my opinion, there will be major breakthroughs in this field with the discovery of many new such enzymes and/or novel gene families encoding for CAs.

It is less probable that novel CAs will be discovered in mammals/humans, since the entire genome of such species has already been analyzed in detail, and 15 isozymes was detected in primates, and 16 in nonprimates. However, the physiological function of many of these isoforms is currently largely not understood, and we estimate that important progress will occur in this field, especially considering the fact that all these isozymes have now been characterized, from the biochemical point of view, and their inhibition profiles have also been investigated with many classes of organic and inorganic inhibitors. As a consequence, it is possible that new CAIs will be developed, some of them with selectivity for various isoforms. Indeed, the presently available drugs belonging to this class indiscriminately inhibit most isozymes. It is probable that the future will see the development of isozyme-selective inhibitors for isozymes I, II, VA, VB, VII, IX, XII, XIII and XIV, which will lead to drugs with less side effects. It is also probable that many new such targets belonging to the bacterial/fungal/protozoan CAs will be validated and lead to novel types of agents fighting diseases provoked by such pathogens. The first results already obtained in this field are very promising.

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#### Executive summary

- Carbonic anhydrases (CAs) are widespread enzymes in bacteria, archaea and eukaryotes that catalyze a critically important physiologic reaction – hydration of  $\text{CO}_2$  to  $\text{HCO}_3^-$  and protons.
- These enzymes are inhibited by several classes of compounds, such as sulfonamides, sulfamates and sulfamides, among others.
- A number of such derivatives are clinically used inhibitors, such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, topiramate, zonisamide, sulpiride, celecoxib and valdecoxib. Several compounds are in clinical development, such as indisulam and COUMATE-667.
- CA inhibitors (CAIs) are used in therapy as antiglaucoma, diuretic, antiobesity and antitumor drugs/diagnostic tools. Some compounds are also used as anticonvulsants or for the treatment of other neurological disorders.
- Most presently available CAIs show undesired side effects due to indiscriminate inhibition of CA isoforms other than the target.
- Many new CAIs are being developed in the search of isozyme-selective compounds as potential drugs with less side effects.
- Bacterial, fungal and protozoan CAs present in many pathogens have recently started to be considered as potential targets for the development of inhibitors with therapeutic applications.

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