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Carbonic anhydrase inhibitors

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ABSTRACT

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread enzymes in all organisms, catalyzing CO_2 hydration to bicarbonate and protons. Their inhibition is exploited clinically for decades for various classes of diuretics and systemically acting antiglaucoma agents. In the last years novel applications of CA inhibitors (CAIs) emerged, such as topically acting antiglaucoma, anticonvulsants, antiobesity, antipain, and antitumor agents/diagnostic tools. Such CAIs target diverse isozymes of the 13 catalytically active α-CA isoforms present in mammals. CAs belonging to the α-, β-, γ-, δ-, and ζ-families are found in many organisms all over the phylogenetic tree, and their inhibition was studied ultimately for some pathogenic protozoa (*Plasmodium falciparum*), fungi (*Cryptococcus neoformans*, *Candida albicans*, *Candida glabrata,* and *Saccharomyces cerevisiae*), and bacteria (*Helicobacter pylori, Mycobacterium tuberculosis,* and *Brucella suis*). Novel interesting chemotypes, in addition to the sulfonamide and sulfamate CAIs, such as coumarins, phenols, and fullerenes, were also reported recently, together with their mechanism of inhibition. This class of enzyme inhibitors shows promise for designing interesting pharmacological agents and understanding in detail protein–drug interactions at molecular level.

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) catalyzes a very simple but essential physiological reaction, carbon dioxide hydration to bicarbonate and protons.[1–3] This reaction also occurs without a catalyst but it is too slow. As CO_2, bicarbonate, and protons are essential molecules/ions in many important physiologic processes in all life kingdoms (*Bacteria*, *Archaea*, and *Eukarya*), throughout the phylogenetic tree, and relatively high amounts of them are present in different tissues/cell compartments of all such organisms, it is no wonder that CAs evolved independently at least five times, with five genetically distinct enzyme families known to date: the α-, β-, γ-, δ-, and ζ-CAs.[1–6] All of them are metalloenzymes, but whereas α-, β-, and δ-CAs use Zn(II) ions at the active site,[1,4] the γ-CAs are probably Fe(II) enzymes (but they are active also with bound Zn(II) or Co(II) ions),[5] whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis.[6] The 3D-fold of the five enzyme classes is very different from each other, as it is their oligomerization state: α-CAs are normally monomers and rarely dimmers; β-CAs are dimers, tetramers, or octamers; γ-CAs are trimers, whereas the δ- and ζ-CAs are probably monomers but in the case of the last family, three slightly different active sites are present on the same protein backbone which is in fact a pseudotrimer.[1–6] Many representatives of all these enzyme classes have been crystallized and characterized in detail, except the δ-CAs.[1–6]

The α-CAs are present in vertebrates, protozoa, algae, and cytoplasm of green plants, and in some *Bacteria*,[1–3] the β-CAs are predominantly found in *Bacteria*, algae, and chloroplasts of both mono- and dicotyledons, but also in many fungi and some *Archaea*.[4,5] The γ-CAs were found in *Archaea* and some *Bacteria*,[1,4,5] whereas the δ- and ζ-CAs seem to be present only in marine diatoms.[6] In many organisms these enzymes are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate, pH, and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes (thoroughly studied in vertebrates),[1–3,7–10] whereas in algae, plants, and some bacteria they play an important role in photosynthesis and other biosynthetic reactions.[1,4,5,11] In diatoms δ- and ζ-CAs play a crucial role in carbon dioxide fixation.[6]

The catalytic mechanism of CAs is understood in detail.[1,2,12] In all enzyme classes a metal hydroxide species (L_3-M^{2+}-OH^-) of the enzyme is the catalytically active species, acting as a strong nucle-

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ophile (at neutral pH) on the CO_2 molecule bound in a hydrophobic pocket nearby.[2d] This metal hydroxide species is generated from water coordinated to the metal ion, which is found at the bottom of the active site cavity. The active center normally comprises M(II) ions in tetrahedral geometry, with three protein ligands (L) in addition to the water molecule/hydroxide ion, but Zn(II) and Co(II) were also observed in trigonal bipyramidal or octahedral coordination geometries, at least in γ-CAs.[5] In many enzymes, generation of the metal hydroxide species from the metal-coordinated water is the rate determining step of the catalytic turnover, which for some α- and ζ-CAs achieve k_{cat}/K_M values $>10^8$ M^{-1} s^{-1}, making CAs among the most effective catalysts known in nature.[1,2] The metal ion ligands are three His residues in α-, γ-, and δ-CAs or one His and two Cys residues in β- and ζ-CAs.[1–6] Some β-class enzymes have four protein zinc ligands, that is, one His, two Cys, and one Asp coordinated to Zn(II).[13] For these enzymes no water coordinated to the metal ion is present at pH values <8, as shown in an excellent crystallographic work from Jones' group on the mycobacterial enzymes Rv3558c and Rv1284.[13] However, at pH values >8, a conserved Arg residue in all β-CAs investigated so far (belonging to a so-called catalytic dyad)[13] makes a salt bridge with the Asp coordinated to Zn(II), liberating the fourth Zn(II) coordination position, which is then occupied by an incoming water molecule/hydroxide ion.[13] The inhibition and activation of CAs are also well understood processes, with most classes of inhibitors binding to the metal center,[1–4,12] and activators binding at the entrance of the active site cavity and participating in the proton shuttling between the metal ion—bound water molecule and the environment.[14] This leads to the enhanced formation of the metal hydroxide, catalytically active species of the enzyme.[14,15]

So far, 16 different α-CA isoforms were isolated and characterized in mammals, where they play important physiological roles, as briefly outlined above. Some of them are cytosolic (CA I, CA II, CA III, CA VII, CA XIII), others are membrane-bound (CA IV, CA IX, CA XII, CA XIV and CA XV), CA VA and CA VB are mitochondrial, and CA VI is secreted in saliva and milk. Three acatalytic forms are also known, the CA-related proteins (CARP), CARP VIII, CARP X, and CARP XI which seem to be cytosolic proteins too.[1–3] The mammalian CAs were the first such enzymes isolated and studied in detail,[1,12] and many of them are established therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders.[1–3,7–10,12–15] However, in the last years, we and others demonstrated that enzymes present in other organisms, such as the protozoan ones from *Plasmodium falciparum*,[3e] the bacterial

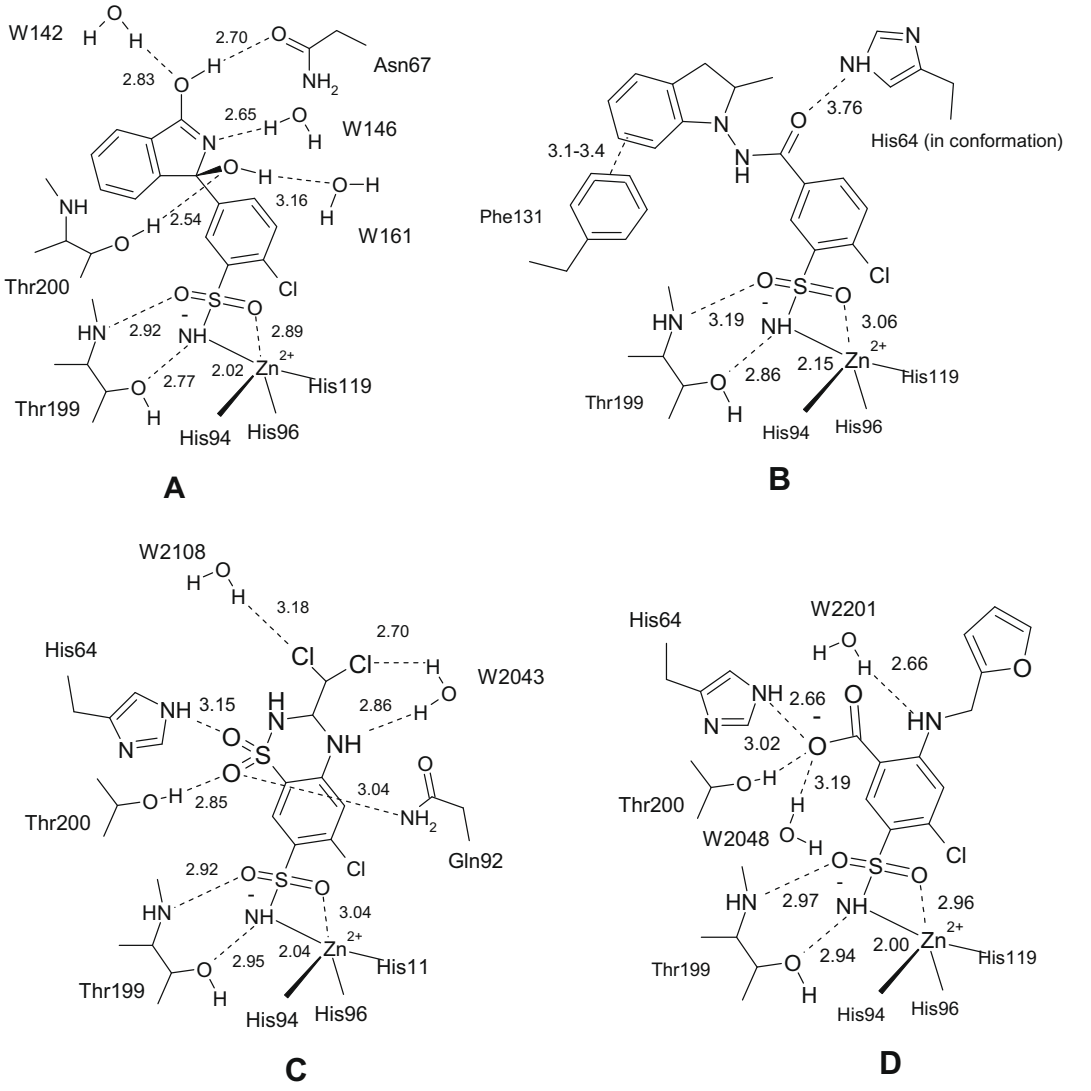
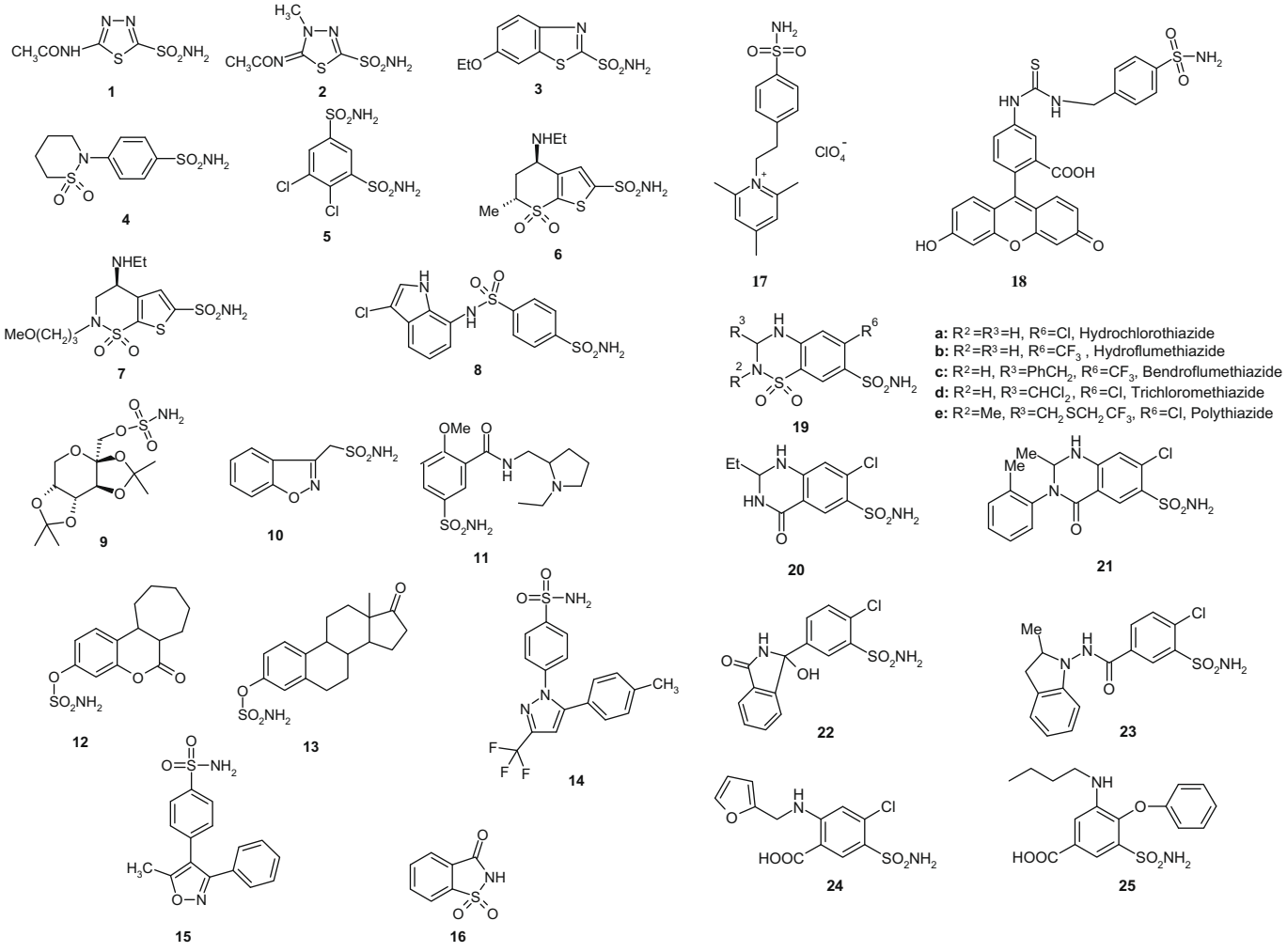


Figure 1. Comparison of interactions in which the thiazide/high-ceiling diuretics chlorthalidone **22** (A), indapamide **23** (B), trichloromethiazide **19d** (C), and furosemide **24** (D) participate when bound to the hCA II active site, as observed in the corresponding X-ray crystal structures (PDB files 3F4X, 3BL1, 1ZGF and 1Z9Y).[19]

ones from *Helicobacter pylori*,[4a] *Mycobacterium tuberculosis*,[4d,13] *Brucella suis*[16], and many other pathogenic bacteria, as well as pathogenic fungi such as *Cryptococcus neoformans*,[17] *Candida albicans*,[17] *Candida glabrata*[18], and *Saccharomyces cerevisiae*[4c] constitute new drug targets with the potential to design anti-infectives and can be used for the structure-based drug design of novel generation, isoform-selective inhibitors (inhibition data of **1**–**25** against all human (h) CA isoforms are provided in Ref. 1). In fact, crucial advances have been made ultimately in this field, some of which will be briefly discussed in this review.



(antimalarials, antibacterial, and antifungal agents) possessing a new mechanism of action.[1] Many of these enzymes belong to the β-CA family, which is not present in humans.[1,4,16–18]

The classical CA inhibitors (CAIs) are the primary sulfonamides, RSO_2NH_2, which are in clinical use for more than 50 years as diuretics and systemically acting antiglaucoma drugs.[1–3,12] In fact there are around 30 clinically used drugs (or agents in clinical development) belonging to the sulfonamide or sulfamate class, of types **1**–**25**, which show significant CA inhibitory activity.[1] In addition to the established role of these CAIs as diuretics and antiglaucoma agents, it has recently emerged that they have potential as anticonvulsant, antiobesity, anticancer, antipain, and antiinfective drugs.[1–3,12] However, critical barriers to the design of CAIs as therapeutic agents are related to the high number of isoforms in humans (i.e., 16 CAs, of which 13 have catalytic activity), their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors of the sulfonamide/sulfamate type.[1–3] In fact, among derivatives **1**–**25** mentioned above, there are no compounds which selectively inhibit some CA isoforms with therapeutic value, although their inhibition profiles against the 13 mammalian isozymes are highly variable

Thiazide and high-ceiling diuretics, such as trichloromethiazide **19d**, chlorthalidone **22**, indapamide **23**, and furosemide **24**, have been discovered in the 60s–70s, when little was known about the various CA isozymes, and these drugs were considered not to interact substantially with the mammalian CAs.[19] Recently we reinvestigated their interactions with the 13 catalytically active mammalian CAs and reported the X-ray crystal structures of their adducts with hCA II.[19] These structurally related sulfonamides showed a very different behavior against the widespread isozyme CA II, with chlorthalidone, trichloromethiazide, and furosemide being efficient inhibitors against CA II (K_Is of 65–138 nM), whereas indapamide was a much weaker one (K_I of 2520 nM). Furthermore, some of these diuretics were quite efficient (low nanomolar) inhibitors of other isoforms, for example, chlorthalidone against hCA VB, VII, IX, and XIII; indapamide against CA VII, IX, XII, and XIII, trichloromethiazide against CA VII and IX, and furosemide against CA I and XIV.[19] Examining the four X-ray crystal structures of their hCA II adducts (Fig. 1), we observed several (2–3) active site water molecules interacting with the chlorthalidone, trichloromethiazide, and furosemide scaffolds which may be responsible for this important difference of activity, in addition to the benzenesulfon-

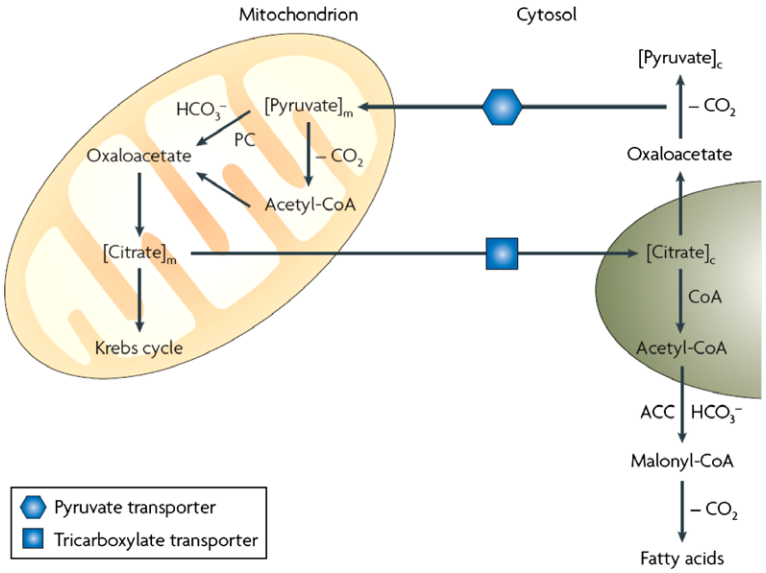
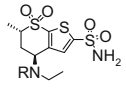


Figure 2. The transfer of acetyl groups from the mitochondrion to the cytosol (as citrate) for the provision of substrate for de novo lipogenesis.[1] All steps involving bicarbonate also need the presence of CA isozymes: CA VA and CA VB in the mitochondrion and CA II in the cytosol.

amide fragment of these molecules which binds to the enzyme active site by coordinating in deprotonated form to the Zn(II) ion.[19] Indeed, indapamide **23** bound to CA II showed no interactions with active site water molecules. Chlorthalidone **22** bound within the CA II active site was in an enolic (lactimic) tautomeric form, with the enolic OH also participating with two strong hydrogen bonds with Asn67 and a water molecule. The newly evidenced binding modes of these diuretics may be thus exploited for designing better CA II inhibitors as well as compounds with selectivity/affinity for various isoforms with medicinal chemistry applications.

Systemic sulfonamide CAIs, such as acetazolamide (**1**; Diamox)[1], are effective antiglaucoma agents but have a limited use due to numerous side effects that arise by inhibition of CAs other than those present in the eye ciliary processes (i.e., CA II, IV, and XII), leading to fatigue, paresthesias, and kidney stones.[1] Topical dosing to the eye of CAIs is possible with water-soluble drugs such as dorzolamide **6**; (Trusopt®)[1] and brinzolamide **7** (Azopt®)[1] together with β-blockers such as timolol, (Timoptol®), and $PGF_{2\alpha}$ analogues[21], latanoprost (Xalatan®), and travoprost (Travatan®). Such compounds are widely used drugs for controlling aqueous humor dynamics and intraocular pressure (IOP) in hypertensive glaucoma patients.[1,3] Recently another approach for obtaining antiglaucoma CAIs has been proposed[20]: hybrid molecules have been obtained which incorporate the dorzolamide **6** scaffold to which nitrate esters have been attached, leading to compounds of type **26**. Some of them showed highly potent and efficacious NO-mediated properties as assessed by their vascular relaxant effect on methoxamine pre-contracted rabbit aortic rings, and exerted potent IOP lowering effects in vivo in normotensive rabbits, thereby anticipating their potential for the treatment of hypertensive glaucoma.[20]



26

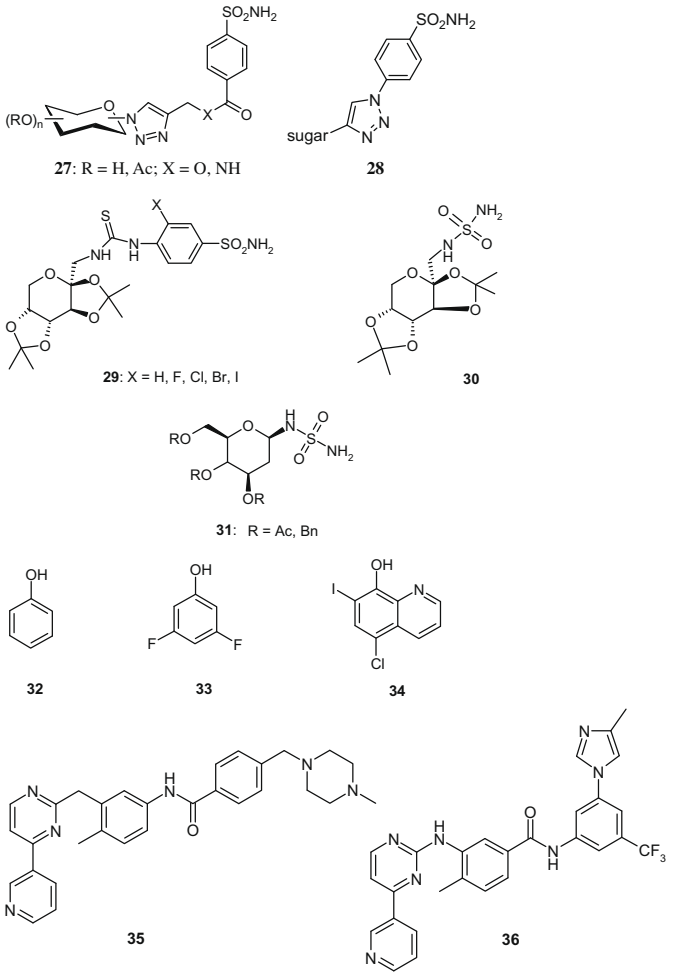
a R = $CO(CH_2)_5ONO_2$ (NCX 274)
b R = CO-p-(C_6H_4)-CH_2ONO_2 (NCX 265)
c R = $COO(CH_2)_3ONO_2$ (NCX 278)
d R = $COO(CH_2)_4ONO_2$ (NCX 245)
e R = $COOCH_2CH(ONO_2)CH_2ONO_2$ (NCX 201)

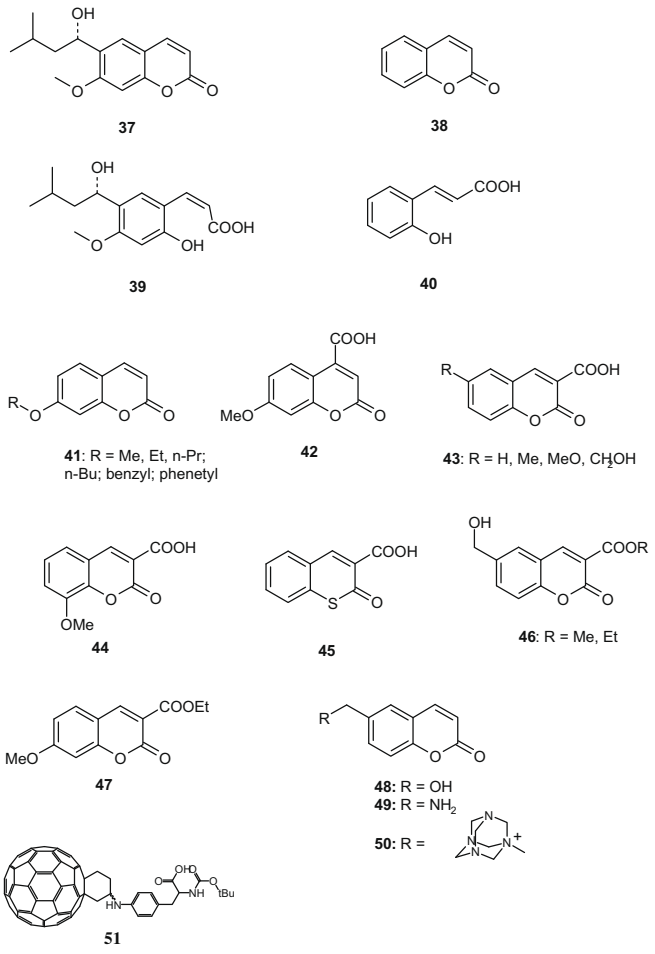
Sulthiame **4**,[21] topiramate **9**,[22] and zonisamide **10**[23] are clinically used antiepileptics also showing potent inhibition of many CA isozymes present in the brain.[24] Their mechanism of anticonvulsant action seems to be rather complex, and the precise isoform(s) target in the CA family is rather difficult to assess at this moment.[24] The X-ray crystal structures of the three drugs in complex with the dominant cytosolic isoform hCA II have been reported by this group, allowing for drug design campaigns based on the sulfamate or sulfonamide zinc-binding groups.[21–24]

Some of the side-effects observed in obese epileptic patients treated with topiramate **9** or zonisamide **10** consisted of a significant weight loss.[1,22–24] This has been rationalized[1] as being due to the lipogenesis inhibition mediated by these two agents, which in turn is mediated by inhibition of some CA isozymes involved in the carboxylation of pyruvate to oxaloacetate (mitochondrial isoforms CA VA and VB) and of acetylcoenzyme A to malonylcoenzyme A (cytosolic isoform CA II), as shown schematically in Figure 2. The overall effect is a potent inhibition of lipogenesis. Presently, a combination of topiramate **9** (sustained release form) with phentermine, Qnexa, is in Phase III clinical trials for the treatment of obesity,[25] making it the first in a class drug, with a novel mechanism of action.[1]

Isozymes CA IX and XII are predominantly found in tumor cells and show a restricted expression in normal tissues.[1,2,7–10] It has been recently proven that by efficiently hydrating carbon dioxide to protons and bicarbonate, these CAs contribute significantly to the extracellular acidification of solid tumors, whereas their inhibition reverts this phenomenon to a certain extent.[7b] CA IX and XII are overexpressed in many such tumors in response to the hypoxia inducible factor (HIF) pathway, and research on the involvement of these isozymes in cancer has progressed significantly in recent years. The report of the X-ray crystal structure of CA IX,[9c] which is a dimeric protein with a quaternary structure not evidenced earlier for this family of enzymes (Fig. 3), allows for structure-based drug design campaigns of inhibitors against this novel antitumor target. Indeed, it has been known for some time that many classes of aromatic/heterocyclic sulfonamides and sulfamates have good affinity for this isoform,[1–3,7–10] but generally they do not show specificity for the inhibition of the tumor-associated isoform versus the remaining CA isozymes (CA I–VII and XII–XV) found in mammals. Several approaches were reported in the last years for obtaining compounds that specifically target the tumor-associated isoforms: (i) fluorescent sulfonamides (such as compound **18**), used for imaging purposes and for determining the role of CA IX in tumor acidification;[1,7] (ii) positively (such as **17**)[26] or negatively

charged compounds, which cannot cross plasma membranes due to their charged character and thus inhibit selectively only extracellular CAs, among which CA IX and XII;[1] (iii) hypoxia-activatable compounds, which exploit the reducing conditions of hypoxic tumors to convert an inactive prodrug into an active CAI;[27] (iv) sugar-containing sulfonamides/sulfamates/sulfamides of types **27**–**31**, which due to their highly hydrophilic character do not easily cross membranes and thus possess an enhanced affinity for extracellular CAs such as CA IX and XII;[28] (v) nanoparticles coated with CAIs;[29] and (vi) diverse chemotypes than the sulfonamides and their bioisosteres, such as the phenols **32**–**34**, protein tyrosine kinase inhibitors **35** and **36**, coumarins **37**–**50**, fullerenes **51**, and other compounds recently investigated as alternative CAIs to the classical types of inhibitors.[30–33]





unsubstituted coumarin **38** are hydrolyzed within the CA active site with the formation of the 2-hydroxy-cinnamic acids **39** and **40**, respectively, which represent the de facto enzyme inhibitors.[30] At least two other interesting facts emerged during these studies: (i) this new class of CAIs, the coumarins/thiocoumarins, binds in hydrolyzed form at the entrance of the CA active site and does not interact with the metal ion, constituting thus an entirely new category of mechanism-based inhibitors; and (ii) for the specific case of compound **37**, the formed substituted-cinnamic acid **39** was observed bound within the CA active site as the *cis*-isomer, although these derivatives are stable in solution as *trans*-isomers. However, for the simpler coumarin **38**, the *trans*-2-hydroxycinnamic acid **40** has been evidenced to be bound within the enzyme active site, by means of X-ray crystallography.[30] The tentative explanation for the unusual geometry of inhibitor **39** within the enzyme active site was that **29** would be too bulky as *trans*-isomer in the restricted space of the CA active site, while the unstable *cis*-isomer in solution would be stabilized when bound within the enzyme cavity. It should also be mentioned that coumarins **37** and **38** were potent inhibitors against some investigated human CA isoforms, which make this entire class of derivatives of paramount interest for designing novel applications for the CAIs. The binding of the hydrolyzed coumarin to hCA II is shown in Figure 4, where the structures of the activators histamine and L-adrenaline are also superposed, evidencing that the coumarin- and activator-binding sites in the CAs are the same.[30]

The protein tyrosine kinase inhibitors (PTKIs) in clinical use as anticancer agents imatinib **35** and nilotinib **36** were recently shown to be nanomolar CA IX/XII inhibitors, although their inhibition mechanism is not yet understood, as no X-ray crystal structures for the adducts of any CA isozymes with the two compounds have been obtained so far. However, this finding which explains the potent antitumor effects of the two compounds in many types of malignancies, which in addition to the PTK inhibition, may be also due to the inhibition of the cancer-associated CA isoforms discussed in this review.[32] Coumarin and thiocoumarins were only recently discovered to act as CAIs, and their inhibition mechanism deciphered in detail by one of our groups.[30] We demonstrated recently that the natural product 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2*H*-chromen-2-one **37** and the simple,

We investigated thereafter a series of derivatives possessing various moieties substituting the (thio)coumarin ring in the 3-,

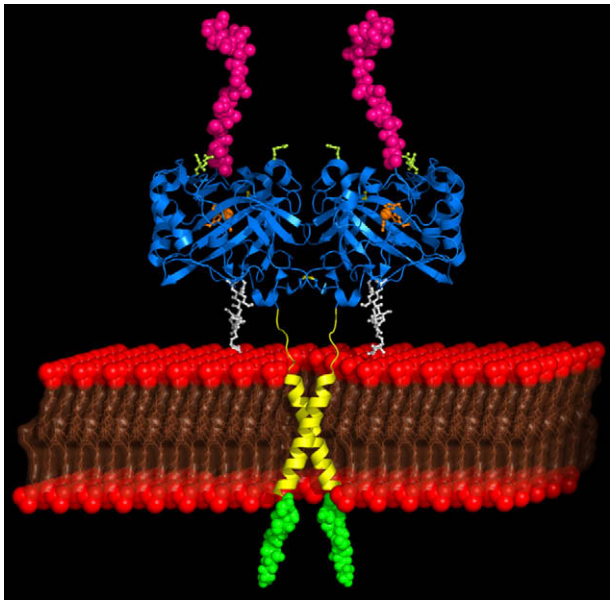


Figure 3. Cartoon representation of the CA IX dimeric protein: in green the short intracytosolic tail, in yellow the transmembrane region, in blue the CA domain as obtained by X-ray crystallography[9c] (PDB file 3IAI) and in magenta the proteoglycan (PG) domain.

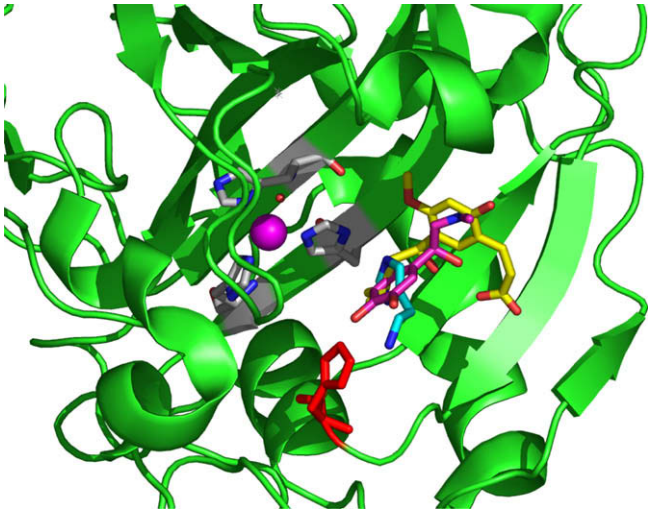


Figure 4. Superposition of hCA II adducts of hydrolyzed coumarin **35** (in yellow, compound **37**) with the CA activators (CAAs) L-adrenaline (magenta) and histamine (blue sky).[30] The coumarin and CAA binding sites are the same, at the entrance of the active site cavity. The catalytic Zn(II) ion is the violet sphere. Its three His ligands are shown in CPK colors, whereas in red is shown His64, an amino acid playing an important role in catalysis as proton shuttle residue.[30]

6-, 7-, 3,6-, 4,7, and 3,8- positions, of types **41**–**50**.[30b] The most significant finding of this second study was that some coumarins are truly isoform-selective CAIs, inhibiting efficiently only one isoform of the 13 catalytically active ones found in humans. For example, thiocoumarin **45** and several coumarins (**43, 44, 46**, and **47**) showed low nanomolar affinity for CA IX, with inhibition constants in the range of 45–98 nM. These coumarins incorporate the 6-hydroxymethyl- and 3-ester moieties (**43** and **46, 47**), with no important differences of activity between the methyl and ethyl esters in this case. The monosubstituted derivatives **48** and **50** on the other hand contained either a compact (CH_2OH) or a rather bulky (hexamethylenetetramine) group in position 6 of the coumarin ring, which render our findings quite important, as it is clear that for effective CA IX inhibition, a large variations of structural motifs are allowed in the 3- and 6-positions of the (thio)coumarin ring. Compound **50** was shown to be a low nanomolar inhibitor of only CA IX (K_I of 48 nM) whereas it inhibited in the micromolar range all other 12 CAs, a feature never evidenced before for a sulfonamide CAI. Thus, this is the first CA IX-selective inhibitor ever reported up to now.[30b]

The in vivo proof-of-concept that potent CA IX inhibitors may indeed show antitumor effects has been only very recently published by Neri's group.[34] By using membrane-impermeant derivatives, based on the acetazolamide scaffold to which either fluorescein-carboxylic acid or albumin-binding moieties were attached, this group demonstrated the strong tumor retardation (in mice with xenografts of a renal clear cell carcinoma line, SK-RC-52) in animals treated for one month with these CA inhibitors. Very recently, Neri's group[35] also published the proof-of-concept study showing that human monoclonal antibodies targeting CA IX can also be used for imaging of hypoxic tumors. The generation of high-affinity human monoclonal antibodies (A3 and CC7) specific to hCA IX, using phage technology, has been reported. These antibodies were able to stain CA IX ex vivo and to target the cognate antigen in vivo. In one animal model of colorectal cancer studied (LS174T), CA IX imaging closely matched pimonidazole staining, with a preferential staining of tumor areas characterized by little vascularity and low perfusion.[35] The same conclusion has been reached by our group by using small molecule CA IX-selective inhibitors of the type **18**.[36] Fluorescent sulfonamides **18** with a high affinity for CA IX have been developed and shown to bind to cells only when CA IX protein was expressed and while cells were hypoxic. NMRI-nu mice subcutaneously transplanted with HT-29 colorectal tumors were treated with 7% oxygen or with nicotinamide and carbogen and were compared with control animals. Accumulation of the CAI **18** was monitored by non-invasive fluorescent imaging. Specific accumulation of **18** could be observed in delineated tumor areas as compared with a structurally similar non-sulfonamide analogue incorporating the same scaffold (i.e., a derivative with the same structure as compound **18** but without the SO_2NH_2 moiety). Administration of nicotinamide and carbogen, decreased acute and chronic hypoxia, respectively, and prevented accumulation of **18** in the tumor. When treated with 7% oxygen breathing, a threefold higher accumulation of **18** was observed. Furthermore, the bound inhibitor fraction was rapidly reduced upon tumor reoxygenation. Such in vivo imaging results confirm previous in vitro data demonstrating that CAI binding and retention require exposure to hypoxia. Fluorescent-labeled sulfonamides may thus provide a powerful tool to visualize hypoxia response in solid tumors. An important step was thus made toward clinical applicability, indicating the potential of patient selection for CA IX-directed therapies.[36]

This group also recently shown[33] that the fullerene derivative incorporating a pendant-protected amino acid moiety, such as the phenylalanine derivative **51**, inhibits CA isozymes (including CA IX) in the submicromolar range by occluding the active site entrance, as shown in Figure 5.

Recently, Kaila's group demonstrated that acetazolamide **1** acts synergistically with midazolam in inhibiting neuropathic pain, an action thought to be due among others to inhibition of the brain CA isoform CA VII.[37] This may open new research directions for CAIs in the management of pain. Indeed, potent, low nanomolar CA VII inhibitors belonging to various classes have been reported.[38]

Returning to sulfonamide CAIs drug design, a brief mention on the two-prong approach which was proposed some years ago.[39] Its rationale is to incorporate in the same molecule an aromatic sulfonamide fragment (that would coordinate to the zinc ion from the active site) and copper(II)-iminodiacetic (IDA) moieties that

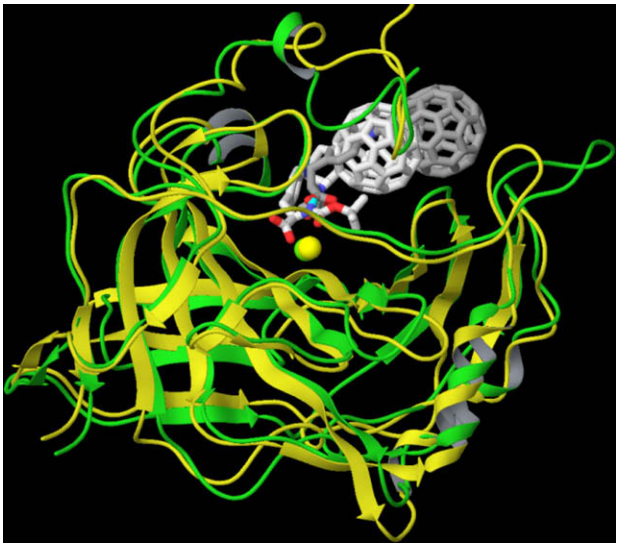


Figure 5. Superimposition of hCA II (shown as yellow-colored ribbon) and hCA IX (shown as green-colored ribbon) docking complexes of fullerene **51**.[33] Dark-colored fullerene shows the binding pose at hCA II and light-colored fullerene shows the binding pose at hCA IX. The Zn(II) ion is shown as yellow (for hCA II) and green (for hCA IX) sphere, respectively.

may bind to different His residues on the rim of the various isoforms active site. It has been claimed that this approach may lead to isozyme-selective CAIs.[39] By using 1,2-dithienylethene-based compounds incorporating benzenesulfonamide and Cu(II)-IDA-, bis-benzenesulfonamide-, bis-Cu(II)-IDA-, and bis-ethyleneglycol-methyl ether moieties, we proved this not to be the case (working with five different CA isoforms, CA I, II, IX, XII, and XIV).[40] This study clearly proved the lack of usefulness of the two-prong approach for designing both tight-binding and isozyme-selective CAIs.[40]

Inhibition studies of the α-CAs from *P. falciparum*[3d] and *H. pylori*[4b] with sulfonamides/sulfamates led to the discovery of low nanomolar in vitro inhibitors, some of which also showed activity in vivo in inhibiting the growth of the pathogen in diverse models. On the other hand the β-CAs from various fungal[4,17,18] (*C. neoformans*,[4c] *C. albicans*,[17] *C. glabrata*[18] or *S. cerevisiae*[4d]) and bacterial[4,13,16] (*H. pylori*,[4b] *M. tuberculosis*,[4e,13], and *B. suis*[16]) pathogens started to be investigated more recently. Potent inhibitors targeting these enzymes were evidenced among sulfonamides, sulfamates, boronic acids, and carboxylates.[4,13–18] However, in vivo the inhibition of the pathogen growth has been observed only for some fungi,[4] and for *H. pylori* and *B. suis*.[4b,16] In the case of *M. tuberculosis* no in vivo inhibition of growth was evidenced so far, probably due to the difficulty of the inhibitor to cross the mycolic acid membrane typical of this bacterium.[4e,13] Thus, future work should address the permeability of CAIs which in many cases are difficult to be delivered at the sites where the enzymes are present in these prokaryotes. On the other hand, more bacterial/fungal genomes are being constantly sequenced and the finding of novel targets belonging to the various CA families is constant, leading to a certain degree of optimism that anti-infectives based on CAIs can be developed.

I shall conclude this review in a rather nonstandard manner, as I work on these enzymes and their inhibitors for more than 20 years and I want to send a message especially to young colleagues. When I started this work, in a Ph.D. program, I was strongly advised by many important scientists to stop it, as my research will be surely unproductive, considering the fact that such 'boring' enzymes were known and thoroughly investigated for so many years (and the same for some of their inhibitors, not very successful as drugs). Throughout these years, and hopefully also in this review, I think we and many others were able to show that this view was distorted. The new applications of this class of enzyme inhibitors range from antiglaucoma agents with topical activity, to anticonvulsants, antipain, antiobesity, and probably soon, antitumor agents/diagnostic tools for cancer. Furthermore, although it is still difficult to make this idea widely acceptable, there is a real potential to develop anti-infectives (antimalarials, antifungal, and antibacterial agents) belonging to the CAIs, targeting enzymes from various pathogens. Last but not least, the recent years saw the discovery of many new chemotypes showing significant CA inhibitory activity and a novel mechanism of action, in addition to the classical sulfonamides and their bioisosteres. All these facts prove how dynamic this research field is.

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