CHAPTER 4

Carbonic Anhydrase II as Target for Drug Design

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4.1 INTRODUCTION

CA II is the carbonic anhydrase isoform *par excellence*, since this is the red blood cell isoform first discovered more than 80 years ago with dominant physiological functions (1–5). Subsequently, it has been shown that CA II is a ubiquitous cytosolic isoform, present in a multitude of cells, tissues, and organs in all vertebrates (see Chapter 2 for details) (1,2,6–8), playing a host of crucial, housekeeping, as well as specialized functions (6–15). Among them, the human CA II (hCA II) is involved in the transport of carbon dioxide/bicarbonate from tissues where it is generated to

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the lungs, since it is highly abundant in the blood and shows a very high catalytic activity for the conversion of CO_2 to bicarbonate and protons (k_{cat} of 1.40×10^6 s^{-1} and k_{cat}/K_M of 1.50×10^8 M^{-1} s^{-1} at 20°C and pH 7.5) (1–4). Mammals (including humans) possess two blood isoforms, namely, CA I and CA II, with a total concentration as high as 0.2 mM (16). However, the catalytic activity of hCA I is much lower compared with that of hCA II (see Chapter 2) and in addition hCA I is also inhibited by the chloride and bicarbonate present in the plasma, leaving a lot of questions regarding the physiological function of this isoform (9). Returning to hCA II, this high-activity isoform (also known as the "rapid" blood isoform, in order to distinguish it from the "slow" one, hCA I) is involved not only in the secretion of electrolytes in a multitude of tissues, such as the bicarbonate-rich aqueous humor in the anterior chamber of the eyes (17–19) and the cerebrospinal fluid (20,21), but also in pH and CO_2 homeostasis all over the body (20). Other functions include urine formation and bicarbonate reabsorption in the kidney tubules (20,22), biosynthetic reactions, such as gluconeogenesis, lipogenesis, and ureagenesis (23–25), bone resorption and calcification (26), and probably many other less well-understood physiological/pathological processes. Indeed, a deregulation of the activity of this isoform in one or more tissues has important pathological consequences, such as (i) glaucoma, when excessive aqueous humor is secreted within the eye, with the subsequent increase in the intraocular pressure (IOP) (27–29); (ii) edema, when not enough fluids are secreted/eliminated in the urine, leading to fluid accumulation in the body, processes in which CA II together with several other isoforms such as CA IV, XII, and XIV is involved in the kidneys (30–32); (iii) epilepsy (the involvement of CA II and other brain CA isoforms in this disease is poorly understood but certainly not irrelevant (33–35)); (iv) some forms of cancer, in which CA II was observed to be overexpressed—alone or together with other isoforms such as CA IX and XII (3,36–40). CA II is also involved in other pathologies such as acute mountain sickness (AMS) (41,42) and, apparently, atherosclerosis (43) and osteoporosis (44). The CA II inhibitors are used for treating AMS (41,42), whereas for the last two pathologies there are not only few literature reports on their efficacy but also quite few studies (44,45).

CA II is an established drug target for a multitude of diseases, many of which have been mentioned above. However, CA II is also a target for imaging in various pathological conditions, in organs where the enzyme is present, such as the brain and cerebrospinal fluid (46,47), or the gastrointestinal tract, etc. (48).

In this chapter, we will review the biochemical properties of hCA II related to the drug design of modulators of its activity—inhibitors and activators—considering the various pathologies in which this isoform is involved, and which have been briefly outlined above.

4.2 BIOCHEMICAL PROPERTIES, GENETIC RELATIONSHIP WITH THE OTHER CYTOSOLIC ISOFORMS, AND 3D STRUCTURE OF hCA II

hCA II contains 259 amino acid residues and has a molecular weight of 29.3 kDa. An alignment of its sequence with those of the other human cytosolic isoforms (hCA I, III, VII, and XIII) (49–55) is shown in Figure 4.1, whereas the three-dimensional structure of hCA II as determined by X-ray crystallography (49,50) is shown in Figure 2.2.

All cytosolic CA isoforms have a rather high sequence similarity with each other (hCA II has 59.5% sequence identity with hCA I, 57.7% with hCA III, 56.2% with hCA VII, and 60.2% with hCA XIII, respectively). They contain conserved residues crucial for the catalytic cycle: (i) the Zn(II) ligands, which are three His residues (His94, His96, and His119, hCA I numbering system, which will be used throughout and not mentioned any longer; only if another numbering system will be used, this will be mentioned); (ii) the proton shuttle residue, which is His64 in hCA II, which (see also Chapter 2) is involved in transferring protons from the zinc-coordinated water molecule to the buffer, in the rate-determining step of the catalytic cycle (1–5). Some isoforms, such as hCA III, do not contain a His in position 64 but a Lys, having a much lower catalytic activity compared with hCA II (see Chapter 5). (iii) The "gatekeeping" residues (49,50) that are Thr199 and Glu106 (Figure 4.1) (see discussion later in the text for their role in the catalytic cycle and interaction with inhibitors).

As for all mammalian CAs for which the X-ray crystal structure has been determined, hCA II has an egg-like shape (Figure 2.2) with the approximate dimensions of 50 × 40 × 40 $Å^3$ and a typical fold characterized by a central 10-stranded mainly

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                                                                  64
hCA I      ---ASPDWGYDDKNGPEQWSKLYPIANGNNQSPVDIKTSETKHDTSLKPISVSYNPATAKEIINVGHSFHVNFEDNDNRSVLKGGPFSDSYR
hCA II     ---MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGPLDGTYR
hCA III    ---MAKEWGYASHNGPDHWHELFPNAKGENQSPVELHTKDIRHDPSLQPWSVSYDGGSAKTILNNGKTCRVVFDDTYDRSMLRGGPLPGPYR
hCA VII    -MTGHHGWGYGQDDGPSHWHKLYPIAQGDRQSPINIISSQAVYSPSLQPLELSYEACMSLSITNNGHSVQVDFNDSDDRTVVTGGPLEGPYR
hCA XIII   GSMSRLSWGYREHNGPIHWKEFFPIADGDQQSPIEIKTKEVKYDSSLRPLSIKYDPSSAKIISNSGHSFNVDFDDTENKSVLRGGPLTGSYR

                 94 96          106             119
hCA I      LFQFHFHWGSTNEHGSEHTVDGVKYSAELHVAHWNSAKYSSLAEAASKADGLAVIGVLMKVGEANPKLQKVLDALQAIKTKGKRAPFTNFDP
hCA II     LIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWN-TKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNFDP
hCA III    LRQFHLHWGSSDDHGSEHTVDGVKYAAELHLVHWN-PKYNTFKEALKQRDGIAVIGIFLKIGHENGEFQIFLDALDKIKTKGKEAPFTKFDP
hCA VII    LKQFHFHWGKKHDVGSEHTVDGKSFPSELHLVHWNAKKYSTFGEAASAPDGLAVVGVFLETGDEHPSMNRLTDALYMVRFKGTKAQFSCFDP
hCA XIII   LRQVHLHWGSADDHGSEHIVDGVSYAAELHVVHWNSDKYPSFVEAAHEPDGLAVLGVFLQIGEPNSQLQKITDTLDSIKEKGKQTRFTNFDL

                          199
hCA I      STLLPSSLDFWTYPGSLTHPPLYESVTWIICKESISVSSEQLAQFRSLLSNVEGDNAVPMQHNNRPTQPLKGRTVRASF--
hCA II     RGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEELMVDNWRPAQPLKNRQIKASFK-
hCA III    SCLFPACRDYWTYQGSFTTPPCEECIVWLLLKEPMTVSSDQMAKLRSLLSSAENEPPVPLVSNWRPPQPINNRVVRASFK-
hCA VII    KCLLPASRHYWTYPGSLTTPPLSESVTWIVLREPICISERQMGKFRSLLFTSEDDERIHMVNNFRPPQPLKGRVVKASFRA
hCA XIII   LSLLPPSWDYWTYPGSLTVPPLLESVTWIVLKQPINISSQQLAKFRSLLCTAEGEAAAFLVSNHRPPQPLKGRKVRASFH-
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Figure 4.1 Sequence alignment of the cytosolic isoforms hCAs I, II, III, VII, and XIII. The zinc ion ligands (His94, His96, and His119), the proton shuttle (His64), and the catalytic dyad, involved in orientating the substrate and enhancing the nucleophilicity of the zinc-bound water molecule (Glu106–Thr199), are highlighted in gray.

antiparallel β-sheet surrounded by several helices and additional β-strands (Figure 2.2). The active site is located in a large, cone-shaped cavity that reaches the center of the molecule (Figure 2.4). The catalytic Zn(II) ion is located at the bottom of this cavity, being coordinated by the three conserved His residues and a water molecule/hydroxide ion (2,49–55). The Zn^{2+}-bound solvent molecule is engaged in hydrogen bond interactions with another water molecule (the so-called deep water) and with the hydroxyl moiety of the conserved Thr residue (Thr199), which in turn is bridged to the carboxylate moiety of a conserved Glu residue (Glu106) (2,49–55). These interactions enhance the nucleophilicity of the Zn^{2+}-bound water molecule, and orient the CO_2 substrate in a location favorable for the nucleophilic attack (1–5). This is why the residues Thr199–Glu106, an important catalytic dyad for all α-CAs, are called gatekeeping residues (50).

A striking feature of the hCA II active site (as well as that of all other CAs crystallized so far) (11) is that it contains two very different environments, one lined with hydrophobic residues and the other lined with hydrophilic amino acid residues (Figure 2.4). Residues in positions 91, 121, 131, 135, 141, 143, 198, 202, 204, 207, and 209 delimit the hydrophobic region, whereas those in positions 62, 64, 67, and 92 identify the hydrophilic one (Figure 2.4). Furthermore, the bulky Phe131 residue roughly in the middle of the hydrophobic half subdivides this part of the active site in two subsites in which various classes of inhibitors bind in a specific manner (2,4,9,11). This very particular "bipolar" active site architecture is probably due to the very different chemical nature of the substrate (CO_2) and its hydration reaction products: the hydrophobic part is used to entrap the CO_2 molecule (a hydrophobic gas, as mentioned above). Indeed, the CO_2 binding site was found at the bottom of the hydrophobic part of the cavity, delimited by residues Val121, Val143, Leu198, and Trp209, as shown in the interesting study by McKenna and coworkers for hCA II (56). On the contrary, the hydrophilic half of the active site facilitates the binding of the polar components generated from the CO_2 hydration reaction (bicarbonate and protons) and their release from the cavity toward the environment. At least for the protons, it is, in fact, well demonstrated that a relay of water molecules and several histidines (proton shuttling residues other than His64) are involved in such processes (57,58). His64, on the other hand, is one of the very few amino acid residues having a high flexibility within the hCA II active site (57–59) (the scaffold of the protein is very rigid, and no major conformational changes of the protein or its active site were observed when inhibitors or activators belonging to a variety of classes bind to it) (1,2,4,11). Indeed, two conformations of the imidazole moiety of this important amino acid residue have been evidenced by means of X-ray crystallography: an "in" one, pointing toward the Zn(II) ion, and an "out" one, pointing toward the exit of the cavity (57). Presumably the two conformations are part of the proton transfer mechanism by which this residue shuttles protons between the active site and the reaction medium (57–59). The catalytic mechanism of hCA II has been discussed in detail in Chapter 2 (see also refs. (1,2,4,5)).

4.3 hCA II INHIBITORS

The general aspects of hCA II inhibition have been discussed in Chapter 1. However, one should stress that hCA II was the most investigated isoform for its interaction with inhibitors, and most kinetic, spectroscopic, and crystallographic studies reported so far were done with hCA II (1–11). Furthermore, all new classes of CA inhibitors (CAIs) reported so far were first discovered working with this isoform.

Briefly, we will present an overview on the CAIs targeting hCA II (but one must stress that most of these classes of compounds also inhibit the other hCA isoforms, and in many cases also the other classes of CAs, the β-, γ-, δ-, and ζ-CAs, as well as α-CAs from other organisms) (1–11,60).

As the Zn(II) ion from the active site of hCA II is coordinated by three His residues (neutral ligands) and the fourth ligand is a water molecule/hydroxide ion, the formal positive charge on the metal ion constitutes the initial driving force for the binding of anions, one of the first classes of CAIs investigated in detail (1,2,9,11). Both inorganic (1,2,9,61–64) and organic (e.g., carboxylates, hydroxamates) (1,2,9) anions act as CAIs, whereas the sulfonamides and their isosteres (sulfamates, sulfamides) also bind as anionic species (1,2), but will be treated in the next section of the chapter due to their important pharmacological applications.

The structure of hCA II complexed with bisulfite (with tetrahedral geometry of the Zn(II) ion, Figure 4.2A), bromide (with a distorted tetrahedral geometry of Zn(II) ion, Figure 4.2B), formate (with trygonal bipyramidal geometry of the Zn(II) ion, Figure 4.2C), and nitrate (an inhibitor not coordinated to the zinc ion, Figure 4.2D) is shown in Figure 4.2, in order to notice the versatility of the binding modes even for such simple inhibitors (61–64). It should be mentioned that normally the anions are weak, millimolar hCA II inhibitors, but their investigations are important because it may lead to the discovery of novel classes of potent CAIs. Indeed, trithiocarbonate CS_3^{2-} was reported (65) as a weak hCA II inhibitor recently, but it thereafter led to the discovery of the dithiocarbamates (DTCs) as a class of potent inhibitors (66) (see discussion later in the text).

Other classes of hCA II inhibitors reported so far include the phenols (67), the polyamines (68), the sulfocoumarins (69), and the coumarins and their derivatives (70–72). All these compounds show mechanisms of inhibition distinct from those of the anions discussed above and the sulfonamides that will be discussed shortly, and these particular mechanisms of hCA II inhibition are presented in Chapter 1.

The primary sulfonamides ($R\text{-}SO_2NH_2$) represent the classical, most investigated class of hCA II inhibitors (1,2,15). The key factors responsible for the binding of the sulfonamide moiety to the CA active site were elucidated by means of X-ray crystallography of enzyme–inhibitor adducts (mainly with hCA II) and provide a rationale for explaining the unique properties of this anchoring group for the design of CAIs (1,2,4). In all the studied adducts, the binding of the sulfonamide derivatives is predominantly

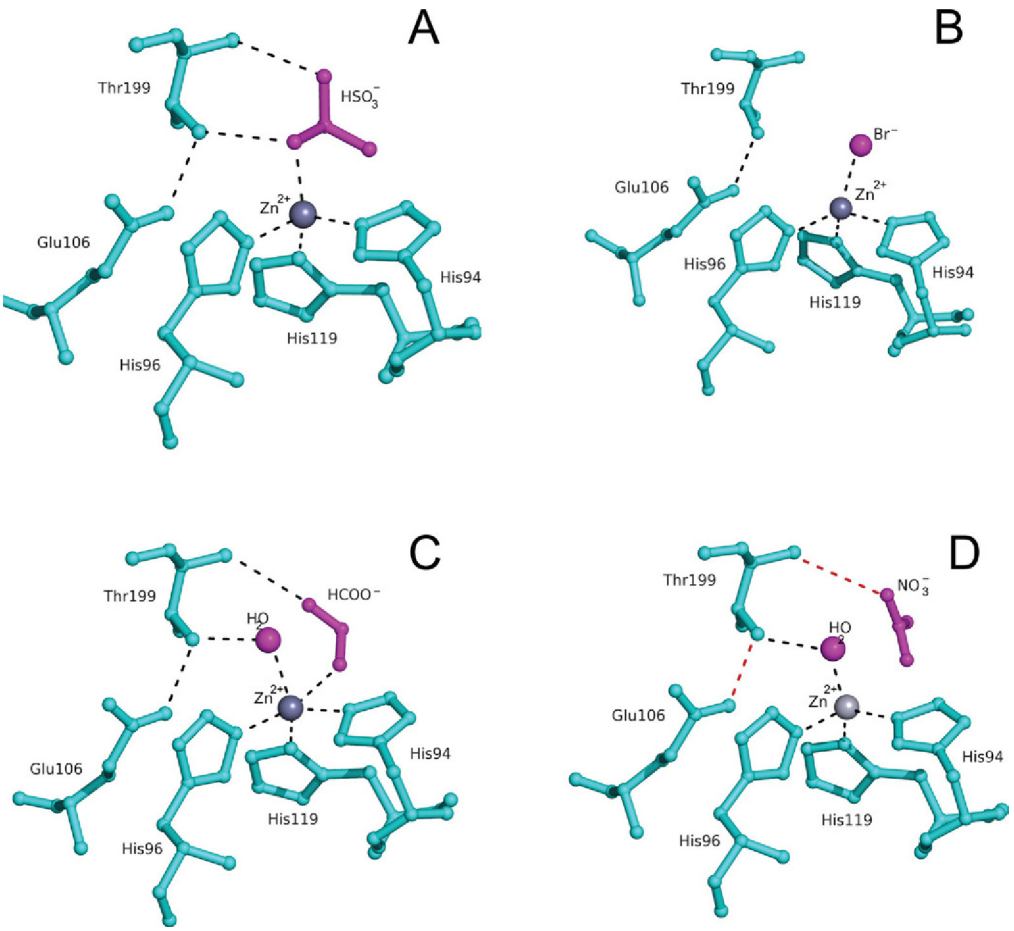


Figure 4.2 Structure of hCA II complexed with (A) bisulfite, (B) bromide, (C) formate, and (D) nitrate (61–64). The three protein zinc ligands (His94, His96, and His119) as well as the other two amino acid residues involved in the catalytic mechanism and binding of inhibitors, Glu106 and Thr199, are evidenced.

driven by the coordination of the deprotonated sulfonamide nitrogen to the Zn^{2+} catalytic ion, with consequent substitution of the zinc-bound water molecule, and by two hydrogen bonds of the sulfonamide moiety with residue Thr199 (Figure 1.4) (1,2,4,49–55).

A large number of studies highlighted that the sulfonamide group is an ideal ligand of the CA active site, since it combines the negative charge of the deprotonated nitrogen with the positively charged zinc ion. The presence of one proton on the coordinated nitrogen atom fits the hydrogen bond acceptor character of Thr199OG1 atom, which forms a strong hydrogen bond with it (1,2,4,49–55). However, the predominant role

played by the sulfonamide moiety itself in the interaction of the sulfonamide derivatives with CA active site has also some unfavorable effects; indeed, any change in the thermodynamics of binding caused by the nature of the R substituent may have a small effect on the enzyme–inhibitor affinity and consequently requires a very careful experimental design. In fact, most primary sulfonamides show potent hCA II inhibitory properties (in the low micromolar to the subnanomolar range) (1–8). However, depending on the nature of the R moiety, additional interactions with the hydrophilic and/or hydrophobic regions of the active site further stabilize (or destabilize) the adduct and lead to compounds with different inhibition patterns, as shown in Figure 4.3 for a very simple aromatic sulfonamide, benzenesulfonamide bound to hCA II (2). Sulfamates and sulfamides incorporate isosteric zinc-binding functions with the sulfamoyl moiety, and bind to hCA II in a very similar manner with the sulfonamides (73).

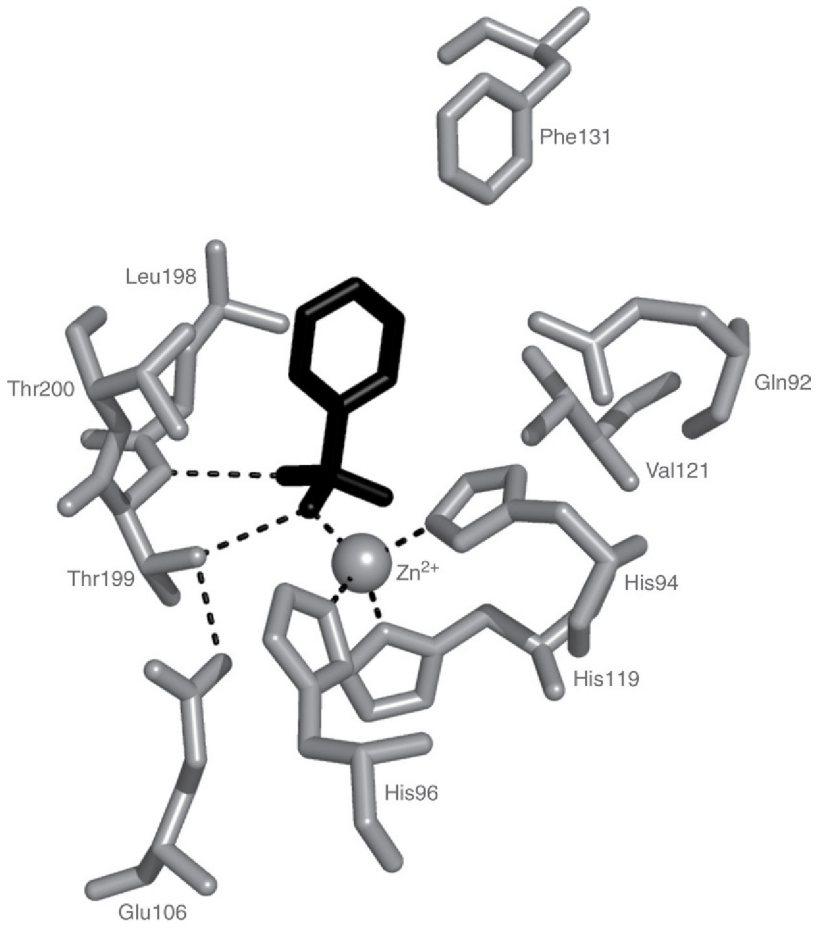


Figure 4.3 Binding of benzenesulfonamide (black) to hCA II active site (gray) (1,2,4,11,49–55).

Sulfonamides are in clinical use as CAIs (targeting principally hCA II) for more than 60 years. The next sections will deal in detail with their pharmacological applications as antiglaucoma agents, diuretics, and agents for the management of altitude sickness, and various other applications for which the role of diverse isoforms is not clearly understood, as well as for the management of tumors overexpressing CA II. The new drug design strategies that led to the discovery of the novel generations of CAIs will be treated at the end of the chapter.

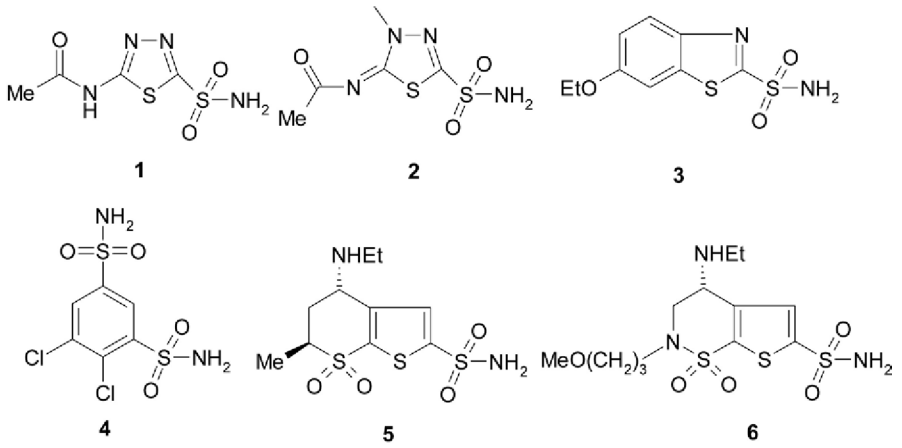
4.4 ANTIGLAUCOMA AGENTS

4.4.1 First-generation inhibitors

Glaucoma is a term covering a group of ophthalmological diseases, a common factor of which is the occurrence of an optic neuropathy believed to be due to elevated IOP (27–29). Glaucoma leads to changes of the optic nerve head and visual field loss, which are commonly linked to the high IOP (27–29). Treatment strategies to prevent glaucoma and the consequent irreversible vision loss are based on the reduction of IOP by using topically acting or systemic hypotensive drugs (27–29).

Heterocyclic sulfonamides such as acetazolamide **1**, methazolamide **2**, and ethoxzolamide **3**, as well as the aromatic compound dichlorophenamide **4**, represent the first generation of clinically used CAIs (74). They are very strong (typically low-nanomolar) inhibitors of most CA isoforms of the 15 presently known in humans (see ref. (1) for details). Except ethoxzolamide that has fewer clinical applications, acetazolamide **1**, methazolamide **2**, and dichlorophenamide **4** are systemically used CAIs, mainly as antiglaucoma drugs, even if they were discovered decades ago, and even if they have a range of side effects (see discussion later in the text) (1,2,27–29,74).

Systemic inhibitors are useful in reducing elevated IOP characteristic of many glaucoma forms, as they represent the most efficient physiological treatment of glaucoma. Indeed by inhibiting the ciliary process enzymes (the sulfonamide susceptible isozymes CA II, CA IV, and CA XII—but the predominant isoform is CA II), a reduced rate of bicarbonate and aqueous humor secretion is achieved, which leads to a 25–30% decrease of IOP (17–20,27–29). However, as mentioned above, these compounds are promiscuous, strong inhibitors of all CA isoforms, and the inhibition of various CA isozymes present in other tissues than the eye leads to an entire range of side effects (27–29). The most prominent ones are numbness and tingling of extremities, metallic taste, depression, fatigue, malaise, weight loss, decreased libido, gastrointestinal irritation, metabolic acidosis, renal calculi, and transient myopia (27–29). As a consequence, there are limitations of their use due to patient compliance. However, acetazolamide and dichlorophenamide are even nowadays components of regimens used to treat refractory glaucoma, which does not respond to adrenergic antagonists, or prostaglandin (PG) analogs (27–29).



4.4.2 Second-generation inhibitors: topically acting sulfonamides

The idea to administer the sulfonamide CAI topically, directly into the eye was already addressed by Becker in the 1950s (75). This and other studies involving the clinically used compounds **1–4** that were administered as suspensions into the eye of experimental animals gave only negative results, and it was concluded that sulfonamide CAIs were effective as antiglaucoma drugs via only the systemic route (75). The lack of efficiency of the first-generation sulfonamide CAIs via the topical route was due to the fact that the drug was unable to arrive at the ciliary processes where CAs are present (76). The inadequate drug penetrability through the cornea was due to the fact that sulfonamides **1–4** possess inappropriate physicochemical properties for such a route of administration.

In 1983, in a seminal paper Maren et al. (76) postulated that a water-soluble sulfonamide, possessing a relatively balanced lipid solubility (in order to be able to penetrate through the cornea) as well as strong enough hCA II inhibitory properties, would be an effective IOP-lowering drug via the topical route, but at that moment no inhibitors possessing such properties existed, as the bioorganic chemistry of this class of compounds was rather unexplored at that time (15). Water-soluble sulfonamide CAIs started to be developed in several laboratories soon thereafter, and by 1995 the first such pharmacological agent, dorzolamide **5**, had been launched for clinical use by Merck, as 2% eye drops (76). A second, structurally related compound, brinzolamide **6** (discovered at Alcon Laboratories), had then been approved for the topical treatment of glaucoma in 1999 (77). These two compounds are still the only topically acting CAIs in clinical use at this moment.

Dorzolamide **5** and brinzolamide **6** are nanomolar hCA II inhibitors (1,2), but they significantly inhibit the other mammalian isoforms too (79). They possess a good water solubility, are sufficiently liposoluble to penetrate through the cornea, and may be administered topically, directly into the eye, as a 2% water solution (of the dorzolamide hydrochloride salt) or as 1% suspension (as the brinzolamide hydrochloride salt) two to

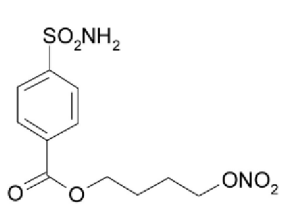
three times a day (76,77). The two drugs are effective in reducing IOP and show fewer side effects as compared with the systemically applied drugs. The observed common side effects include stinging, burning or reddening of the eye, blurred vision, pruritus, and bitter taste (27–29). All but the last are probably due to the fact that dorzolamide (the best studied topical CAI) is administered as the salt of a weak base with a very strong acid, so that the pH of the drug solution is rather acidic (generally around 5.5). The last side effect mentioned above is probably due to drug-laden lachrymal fluid draining into the oropharynx and inhibition of CA present in the saliva (CA VI) and the taste buds (CA II and CA VI), with the consequent accumulation of bicarbonate, and was seen with both systemic and topical CAIs (27–29). Brinzolamide produces less stinging but more blurred vision as compared with dorzolamide (27–29). Unfortunately, dorzolamide already showed some more serious side effects, such as contact allergy, nephrolithiasis, anorexia, depression, and dementia, and irreversible corneal decompensation in patients who already presented corneal problems (27–29). Thus, even if dorzolamide and brinzolamide represent indeed a major progress in the fight against glaucoma with therapies based on CAIs, novel types of topically effective inhibitors belonging to this class of pharmacological agents are still needed.

4.4.3 Third-generation inhibitors

The "tail approach" was reported in 1999 for obtaining novel types of sulfonamide CAIs (targeting hCA II) with good hydrosolubility and IOP-lowering effects (80–83). It consists in attaching water-solubilizing functionalities to the molecules of aromatic/heterocyclic sulfonamides incorporating derivatizable moieties of the amino, imino, or hydroxyl type. Such moieties included, among others, pyridine-carboximido, carboxypyridine-carboxamido, quinoline-sulfonamido, picolinoyl, isonicotinoyl, perfluoroalkyl/arylsulfonyl, as well as amino acyl groups, whereas ring systems that have been derivatized by using the above-mentioned moieties included 2-, 3-, or 4-aminobenzenesulfonamides, 4-(ω-aminoalkyl)-benzenesulfonamides, 3-halogeno-substituted-sulfanilamides, 1,3-benzene-disulfonamides, 1,3,4-thiadiazole-2-sulfonamides, benzothiazole-2-sulfonamides, as well as thienothiopyran-2-sulfonamides (80–83). Compounds prepared by the tail approach showed two to three times more effective topical IOP-lowering effects in rabbits as compared with dorzolamide **5**. They possessed good water solubility (as hydrochlorides, triflates, or trifluoroacetates), inhibition in the low nanomolar range against hCA II and IV, good penetrability through the cornea, and very good IOP-lowering properties in both normotensive and glaucomatous rabbits (widespread animal models of glaucoma) (80–83). What is more important is this effect lasted for a prolonged period of time as compared with the similar effect of dorzolamide. These promising compounds were not developed for clinical use for reasons having nothing to do with science (or their effectiveness as antiglaucoma agents), but because the company that acquired rights on them was incorporated into another one, which had no interest in this type of applications.

The tail approach proved however to be a general and versatile modality to obtain a wide range of CAIs belonging to several classes of compounds, the sulfonamides being just one particular case (reviewed in refs. (1,2,4,8–11)). Its most interesting applications deal with the synthesis of hybrid antiglaucoma drugs, incorporating sulfonamide moieties as well as a second pharmacophore: nitric oxide (NO)–donating moieties or prostaglandin $PGF_{2\alpha}$ analogs.

NO, a radical gas produced by the enzyme nitric oxide synthase (NOS), is also involved in vasodilation, aqueous humor outflow within the eye, local modulation of ocular blood flow, and retinal ganglion cell death by apoptosis (83–86). It appeared thus of interest to combine these two pharmacophores, a sulfonamide CAI, and a moiety able to donate NO, of the nitrate ester type, in the molecule of a hybrid drug. In this way, a large number of sulfonamides with NO-donating properties were reported in the last several years, among which those of types **7**–**9** were the most interesting (83–86). Several aromatic/heterocyclic sulfonamide scaffolds have been used to synthesize compounds incorporating NO-donating moieties of the nitrate ester type, by the tail approach mentioned above. Some of the new compounds showed effective in vitro inhibition of the target isoforms involved in glaucoma (in the low nanomolar range), and the X-ray crystal structure of one of them (compound **9**) revealed factors associated with this marked inhibitory activity (Figure 4.4).



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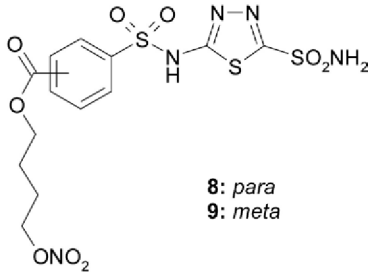


8: *para*
9: *meta*

Compound **9** is a benzolamide derivative possessing a *meta*-COOH moiety on the phenyl ring that has been derivatized as a nitrate ester with an aliphatic, normal C4 chain (85). As for other hCA II–sulfonamide adducts investigated earlier (1,2), the deprotonated sulfonamide moiety of the inhibitor **9** was found coordinated to the Zn(II) ion at a distance of 1.96 Å. The same NH group made a hydrogen bond with the Thr199OG atom. Furthermore, the two endocyclic nitrogens of the 1,3,4-thiadiazole ring participate in two hydrogen bonds with the Thr200OG atom, as reported earlier for a structurally related compound (1,2,85). One oxygen of the secondary SO_2 moiety of inhibitor **9** made a hydrogen bond (of 2.97 Å) with the NH_2 of Gln92 side chain. Due to the *meta*-substituent of the phenyl moiety present in **9**, the conformation of the compound when bound to the hCA II active site is rather particular (Figure 4.4). Indeed, it may

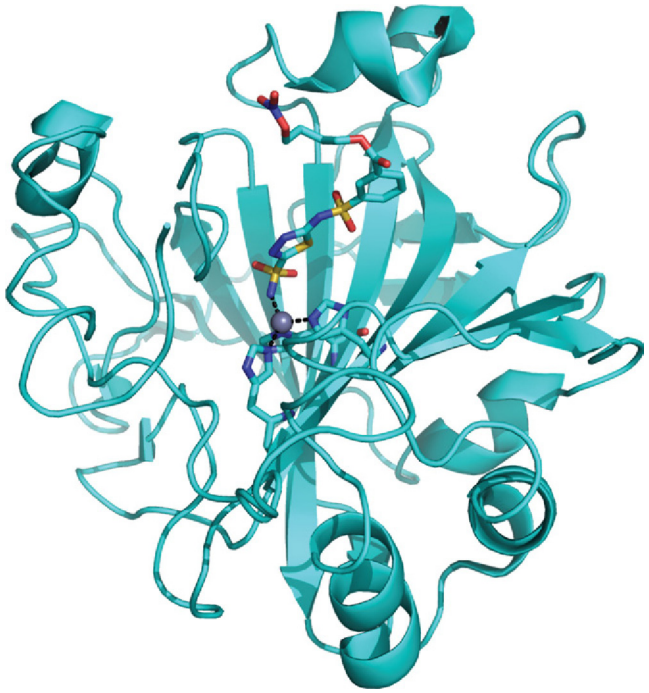


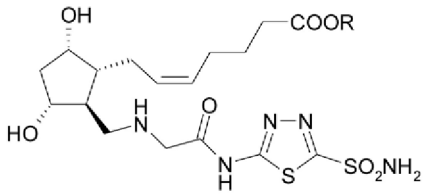
Figure 4.4 Adduct of hCA II with the sulfonamide incorporating an NO-donating moiety ***9***, as obtained by X-ray crystallography (85). The zinc ion and the three coordinating histidines are also shown.

be observed that the amino-1,3,4-thiadiazolyl-2-sulfamoyl moieties of this inhibitor are buried deep within the active site, as for other 1,3,4-thiadizoles for which the structures in adducts with various CAs have been reported (1,2,11). However, the terminal fragment of the inhibitor (the 5-SO_2NH substituent of the thiadiazole ring and 3-substituted phenyl with the nitrate ester moiety incorporated in it) binds in an extended conformation that prolongs toward the external part of the active site, as expected for a molecule with such a long tail. This binding mode explains the potent hCA II inhibitory effects of the compound (K_I of 18 nM) that makes a large number of favorable interactions with various amino acid residues from the enzyme active site (85).

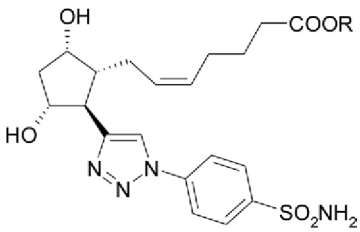
In an animal model of ocular hypertension, one of the new compounds incorporating NO-donating moieties, more precisely **7**, was twice more effective than dorzolamide in reducing the elevated IOP characteristic of this disease, anticipating its potential for the treatment of glaucoma (84–86). A detailed pharmacological study of **7** was thereafter reported (86). Chronic administration of **7** as 2% eye drops to glaucomatous albino rabbits resulted in an important reduction in IOP (of 45–50%) already after the first week of treatment, with a regular decreasing trend during the treatment (86). This reduction was much higher than that observed when dorzolamide at 2% was administered in the

same animal model and with an identical administration schedule (86). Furthermore, in the ophthalmic artery of the treated rabbits, both systolic and diastolic velocities were significantly reduced in eyes treated with the hybrid drug **7** in comparison to dorzolamide **6**, thus suggesting a beneficial effect of this class of CAIs on the blood supply to the optic nerve (in addition to the IOP reduction), which was not observed with dorzolamide or brinzolamide (86). Thus, by using a structure-based drug design approach, hybrid drugs incorporating sulfonamide and NO-donating moieties have been obtained, which showed good in vitro inhibition of the target enzymes. They were observed bound in an interesting way within the active site of the target enzyme (as determined by high-resolution X-ray crystallography), and also showed promising in vivo action in animal models of glaucoma (84–86).

Very recently this hybrid drug approach has been also used for obtaining compounds incorporating sulfonamide and $PGF_{2\alpha}$ moieties in the same molecule (87). The rationale of having two pharmacophores with well-known antiglaucoma effects, that is, the aromatic/heterocyclic sulfonamide one associated with CA inhibition and the PGF one associated with increased outflow of the aqueous humor, has led to molecules of types **10** and **11** (87). Both heterocyclic (1,3,4-thiadiazole-2-sulfonamide, such as **10a,b**) and aromatic (benzenesulfonamides, such as **11a,b**) derivatives have been reported. They incorporate, in addition to the aromatic/heterocyclic sulfonamide moiety, the $PGF_{2\alpha}$ moiety, occurring in eicosanoids such as latanoprost, which are effective antiglaucoma drugs (27,28). The most interesting compound seems to be **11b** that showed an inhibition constant of around 10 nM against hCA II and was also a good PGF receptor (FP) agonist (EC_{50} of 5.7 nM) (87). Although the ocular permeability of this (and related) hybrid drugs was good, no in vivo IOP-lowering data with these derivatives were reported so far.



10a: R = *i*-Pr
10b: R = H

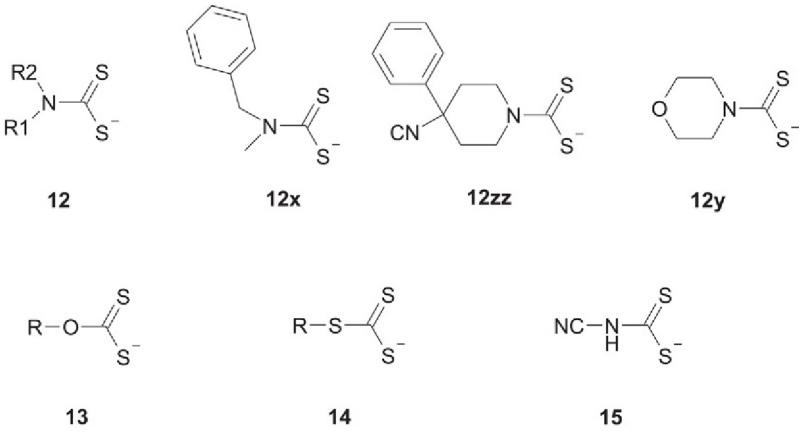


11a: R = Me
11b: R = H

4.4.4 Dithiocarbamates and xanthates as topically acting antiglaucoma CAIs targeting hCA II

Although the sulfonamides dominated the drug design landscape of CAIs for many years, recently, new chemotypes emerged that interact with these enzymes by a similar or different inhibition mechanism as the sulfonamides (66,88,89). Among them, the

DTCs and the xanthates (XTs) are undoubtedly the most interesting ones (66,88,89). These compounds have been rationally discovered as CAIs after our report of trithiocarbonate (CS_3^{2-}) as an interesting (millimolar–micromolar) CAI (65). In the X-ray crystal structure of this inorganic anion bound to CA II, a monodentate coordination of the inhibitor via one sulfur atom to the zinc ion from the enzyme active site has been observed (65). Thus, the CS_2^- was discovered as a new zinc-binding group (ZBG) for generating CAIs of type **12**. As DTCs and XTs are the simplest compounds that incorporate this new ZBG, a rather large series of such compounds was prepared and evaluated for their inhibitory activity against several CAs, including hCA II (66,88,89). Several low-nanomolar and subnanomolar hCA II inhibitors were thus detected, depending on the substitution pattern of the R moiety to which the ZBG is attached (see Tables 4.1 and 4.2) (66,88,89).



X-ray crystal structures were also reported for three DTCs complexed to hCA II, compounds **12x**, **12zz**, and **12y** (66,88). Their binding mode to the enzyme is identical to that of trithiocarbonate, that is, with one sulfur of the CS_2^- moiety coordinated to the metal ion, while the organic scaffold present in these DTCs was observed to make extensive contacts with many amino acid residues from the active site, which explained the wide range of inhibitory power of these derivatives (from the subnanomolar to the micromolar, for the entire series of DTCs reported so far, Table 4.1) (66,88). Interestingly, the highly water-soluble morpholine DTC **12y** was also very effective in vivo as an antiglaucoma agent when administered topically, directly into the eye of hypertensive rabbits (66), a widely used animal model of glaucoma.

A large series of XTs and several thioxanthates (compounds **13** and **14**, as well as a simple, inorganic-like DTC, compound **15**) were also reported as interesting hCA II inhibitors (see Table 4.2) (89). Also in this case, as for the corresponding DTCs, the nature

Table 4.1 Inhibition data of hCA I and II with dithiocarbamates **12** by a stopped-flow, CO_2 hydrase assay (66)

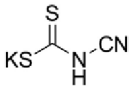
$R^1R^2N\text{-}CSS^-M^+$
12

12	R^1	R^2	K_I (nM)*		M
			hCA I	hCA II	
a	H	Ph	4.8	4.5	Et_3NH
b	H	$O[(CH_2CH_2)]_2N$	4.8	3.6	K
c	H	$MeN[(CH_2CH_2)]_2N$	33.5	33.0	K
d	H	2-Butyl	21.1	29.4	K
e	H	$O[(CH_2CH_2)]_2N(CH_2)_2$	31.8	36.3	K
f†	H	$N[(CH_2CH_2)N]_3$	31.9	13.5	K
g	H	$PhCH_2$	4.1	0.7	Na
h	H	4-PyridylCH_2	3.5	16.6	Et_3NH
i	H	$[(CH_2)_5N]CH_2CH_2$	4.5	20.3	K
j	H	2-Thiazolyl	3.9	4.6	Et_3NH
k	H	$KOOCCH_2$	13.1	325	K
l	H	Imidazol-1-yl-$(CH_2)_3$	8.6	24.7	A
m	Me	Me	699	6910	Na
n	Et	Et	790	3100	Na
o	$(CH_2)_5$	0.96	27.5	Na	
p	*n*-Pr	*n*-Pr	1838	55.5	Na
q	*n*-Bu	*n*-Bu	43.1	50.9	Na
r	*iso*-Bu	*iso*-Bu	0.97	0.95	Na
s	*n*-Hex	*n*-Hex	48.0	51.3	Na
t	Et	*n*-Bu	157	27.8	Na
v	$HOCH_2CH_2$	$HOCH_2CH_2$	9.2	4.0	Na
w	Me	Ph	39.6	21.5	Na
x	Me	$PhCH_2$	69.9	25.4	Na
y	$O[(CH_2CH_2)]_2$		0.88	0.95	Na
z	$NaS_2CN[(CH_2CH_2)]_2$		12.6	0.92	Na
zz	$(NC)(Ph)C(CH_2CH_2)_2$		48.4	40.8	N
zy†	(*S*)-$[CH_2CH_2CH_2CH(COONa)]$		2.5	17.3	Na
1	Acetazolamide		250	12	—

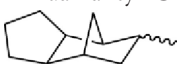
A, imidazol-1-yl-$(CH_2)_3NH_3^+$.
* Errors in the range of ±10% of the reported values, by a CO_2 hydrase assay method (66).
† Tris-dithiocarbamate.
‡ (*S*)-Proline dithiocarbamate.

of the R moiety was the main factor influencing hCA II (but also hCA I) inhibitory properties. The best hCA II inhibitors (**13j**, **13k**, **13n**, **13o**, **13q**, and **13r**, see Table 4.2) incorporated boc-aminoalkyl, arylalkyl, and hetaryl moieties and had affinities for the enzyme in the low nanomolar range. They also showed in vivo IOP-lowering effects. However, no X-ray crystal structures for adducts of XTs with hCA II were obtained so far (89).

Table 4.2 hCA I and hCA II inhibition data with xanthates **13a–r**, thioxanthate **14**, and dithiocarbamate **15** (89).

R-*O*-CSS$^-$K$^+$ R-*S*-CSS$^-$K$^+$ 

13 **14** **15**

No.	R	K_I (nM)*	
		hCA I	hCA II
13a	Me	687 ± 32	346 ± 23
13b	Et	604 ± 18	305 ± 27
13c	*i*-Pr	401 ± 31	307 ± 14
13d	*i*-Amyl	638 ± 50	298 ± 21
13e	*n*-C_5H_{11}	384 ± 17	361 ± 28
13f	*n*-C_8H_{17}	351 ± 21	337 ± 30
13g	Cyclopentyl	532 ± 42	366 ± 22
13h	Cyclohexyl	795 ± 61	293 ± 14
13i	Me_2N-CH_2CH_2	327 ± 15	60.4 ± 5
13j	Me_2N-$CH_2CH_2OCH_2CH_2$	45.1 ± 2	8.1 ± 0.4
13k	Boc-NH-$(CH_2)_4$	5.6 ± 03	9.5 ± 0.8
13l	Boc-NH-$(CH_2)_6$	6.3 ± 0.4	59.0 ± 3
13m	$PhCH_2$	76.1 ± 6	45.1 ± 2
13n	Ph_2CHCH_2	64.1 ± 5	5.4 ± 0.4
13o	2-Pyridyl-CH_2CH_2	74.2 ± 7	13.1 ± 1.2
13p	1-Adamantyl-CH_2	81.3 ± 6	21.5 ± 1.8
13q	1-Adamantyl-CH_2CH_2	59.1 ± 4	6.4 ± 0.5
13r		63.4 ± 3	6.6 ± 0.4
14	$PhCH_2$	73.5 ± 5	75.7 ± 6
15	—	531 ± 26	73.6 ± 7
AAZ	—	250 ± 13	12 ± 0.9

* Errors in the range of ±10% of the reported values, by a CO_2 hydrase assay method (89).

Although few such compounds were investigated in detail up until now, the DTCs/XTs, being readily available, easy to synthesize, and with an excellent water solubility, may have a firm place in the antiglaucoma drug armamentarium (66,88,89).

4.5 DIURETICS WITH CA INHIBITORY PROPERTIES

CAs are highly abundant in the kidneys (a total concentration of about 8–10 μM has been estimated for this organ), and many isoforms have been shown to be present in various tissues of this organ (20,30,90,91). In humans, the CA isoforms present in

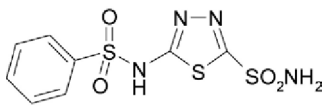
kidneys are CA II, IV,VB, IX, XII, and XIV, and they play crucial functions in at least three physiological processes: (i) the acid–base balance homeostasis (by secreting and excreting protons, due to the carbon dioxide hydration reaction catalyzed by these enzymes); (ii) the bicarbonate reabsorption process; and (iii) the renal NH_4^+ output (20,30,90–92).These important functions are well localized in the different segments of the nephron: bicarbonate reabsorption occurs in the proximal tubule, whereas urinary acidification and NH_4^+ output take place in the distal tubule and collecting ducts (20,30,90–92). In the intercalated cells of the late distal tubule, the collecting tubule, and the collecting ducts, high levels of CA II have been reported (20,30,90–92).The CA II–positive segments along the nephron and collecting ducts include the proximal convoluted (S1 segment) and straight (S2–S3 segments) tubules, the descending thin limb of Henle, the thick ascending limb of Henle, the distal convoluted tubule, and the intercalated and principal cells of the collecting ducts (93–98). Based on the distribution pattern described above, it is obvious that CA II is widely expressed in the kidney where it plays a key role in renal functions. Indeed, such a fundamental role of CA II in renal physiology was reinforced when Sly et al. (99) reported renal tubular acidosis (as well as brain calcification and osteopetrosis) in patients with CA II deficiency syndrome, a rare genetic disease. Thus, a substantial amount of the renal physiology has been understood in detail by inhibiting CAs present in this organ by using sulfonamide CAIs (20,30,92).

Acetazolamide **1** was the first CAI to be used clinically (20). It is a potent but promiscuous inhibitor of most CA isoforms from mammals, but was the first nonmercurial diuretic to be used clinically, starting in 1956 (20). Acetazolamide represents the prototype of a class of pharmacological agents with apparently limited therapeutic usefulness nowadays, but which played a major role in the development of fundamental renal physiology and pharmacology, as well as for the design of many of the presently widely used diuretic agents, such as, among others, the thiazide and high-ceiling diuretics (1–4,30). Following the administration of a CAI, such as acetazolamide, the urine volume promptly increases, and its normally acidic pH (of 6) becomes alkaline (around 8.2) (20). An increased amount of bicarbonate is thus eliminated into the urine (120 times higher than the amount eliminated normally), together with Na^+ and K^+ as accompanying cations, whereas the amount of chloride excreted is diminished (20). The increased alkalinity of the urine is accompanied by a decrease in the excretion of titratable acid and ammonia, and in consequence by metabolic acidosis (20,30). This sequence of events is due to the inhibition of the various CA isozymes in the proximal tubule, which leads to inhibition of H^+ secretion by this segment of the nephron (20). Inhibition of both cytosolic (CA II) and membrane-bound (CA IV, XII, and XIV) enzymes seems to be involved in the diuretic effects of the sulfonamides (1–4, 20,30). Inhibition of such CAs decreases the availability of protons for the Na^+–H^+

antiporter, which maintains a low proton concentration in the cell. The net effect of these processes is the transport of sodium bicarbonate from the tubular lumen to the interstitial space, followed by movement of the isotonically obligated water, and then augmented diuresis (1–4,20,30). CAIs also increase phosphate excretion (by an unknown mechanism) but have little or no effect on the excretion of calcium or magnesium ions (1–4,20,30).

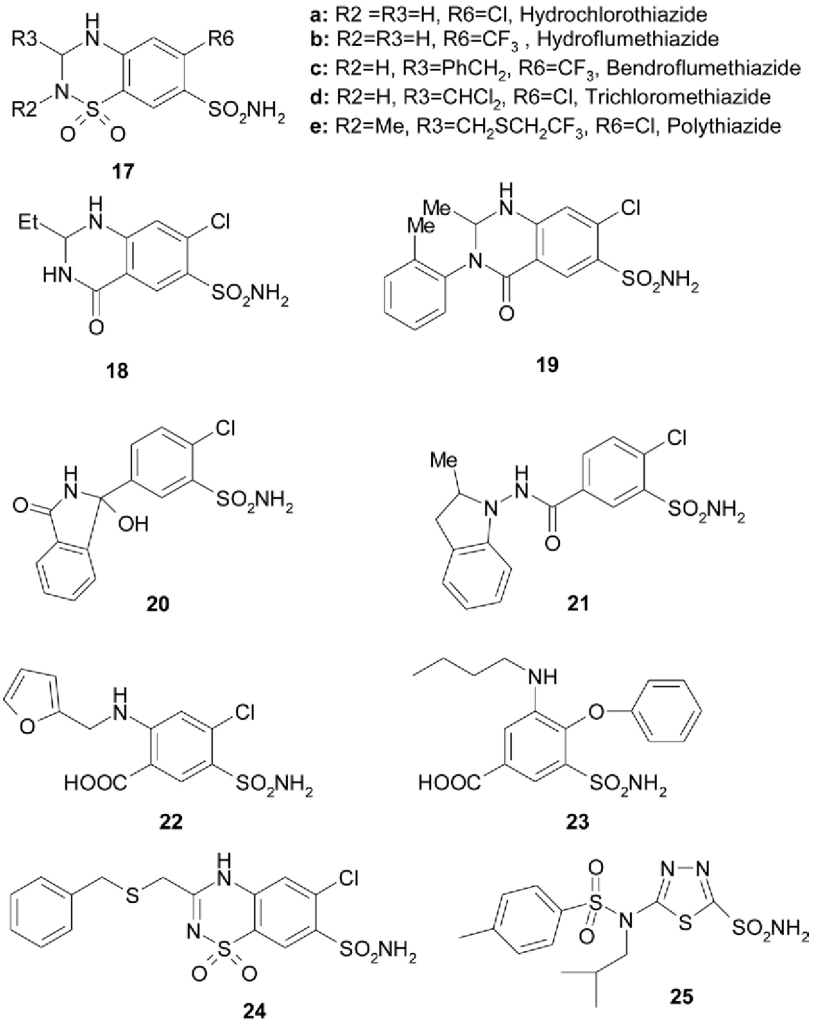
Acetazolamide and structurally related sulfonamides, such as methazolamide **2**, ethoxzolamide **3**, and dichlorophenamide **4**, were and are still used for the treatment of edema due to congestive heart failure, and for drug-induced edema, in addition to their applications as antiglaucoma agents (1–4,20,30). However, these systemic CAIs, due to inhibition of CAs present in organs other than the kidneys, generally produce many undesired side effects, such as metabolic acidosis, nephrolithiasis, CNS symptoms, and allergic reactions. These side effects have limited their exploitation in therapy (1,20,100).

Benzolamide **16** is a compound structurally related to acetazolamide, with a quite acidic pK_a of 3.2 for the secondary sulfonamide group; thus, it is completely ionized at the physiological pH, as sulfonamidate anion (15,20). Its renal effect on bicarbonate excretion is around 10 times as potent as that of acetazolamide, the drug being maximally active at doses of 1 mg/kg, and being actively and rapidly accumulated in the kidney, but its plasma half-life is of only 20 min. All these facts make benzolamide a renal-specific CAI, but the compound remained an orphan drug and has not been developed for wide clinical use, due to its inappropriate pharmacokinetics, although some anecdotical reports indicate that it might be beneficial for patients suffering from chronic obstructive lung disease (15,20).



16

The diuretics are widely employed drugs for controlling hypertension (101–103). They belong to several classes of pharmacological agents, but here we will discuss only the derivatives incorporating a primary sulfonamide moiety in their molecule. Indeed, by using acetazolamide **1** as lead, a large number of other quite successful sulfonamide diuretics were developed in the 1960s and 1970s, such as the benzothiadiazines **17** (hydrochlorothiazide **17a**, hydroflumethiazide **17b**, and the like), quinethazone **18**, metolazone **19**, chlorthalidone **20**, indapamide **21**, furosemide **22**, and bumetanide **23** (104–108). Some of them are among the most widely clinically used diuretics (101–103) alone or in combination with other drugs.



Even if the clinically used drugs **17–23** possess the primary SO_2NH_2 moiety in their molecule, till recently, their CA inhibitory properties were investigated only for one CA isozyme (i.e., CA II), which was presumed to be responsible for all the physiological effects of the sulfonamide drugs in the 1960s and 1970s, when these drugs were initially launched (56–60). Only recently the CA inhibition with these diuretics, against all mammalian CA isoforms, was thoroughly reinvestigated (104–108), offering several interesting findings that can lead to important polypharmacologic applications and drug repositioning of these agents (109).

CA inhibition data with all sulfonamide diuretics (first- and second-generation ones), of types **1–4** and **16–23**, are reported in Table 4.3.

Table 4.3 Inhibition data with sulfonamides **1–4** and **16–23** against isozymes CAs I–XV (56–60)

Isozyme	K_I (nM)*												
	1	***2***	***3***	***4***	***16***	***17a***	***17b***	***18***	***19***	***20***	***21***	***22***	***23***
hCA I†	250	50	25	1,200	15	328	2,840	35,000	54,000	348	51,900	62	4,930
hCA II†	12	14	8	38	9	290	435	1,260	2,000	138	2,520	65	6,980
hCA III†	2×10^5	7×10^5	1×10^6	6.8×10^5	1.4×10^5	7.9×10^5	8.7×10^5	nt	6.1×10^5	1.1×10^4	2.3×10^5	3.2×10^6	3.4×10^6
hCA IV†	74	6,200	93	15,000	nt	427	4,780	nt	216	196	213	564	303
hCA VA†	63	65	25	630	37	4,225	10,200	nt	750	917	890	499	700
hCA VB†	54	62	19	21	34	603	429	nt	312	9	274	322	nt
hCA VI†	11	10	43	79	93	3,655	8,250	nt	1,714	1,347	1,606	245	nt
hCA VII†	2.5	2.1	0.8	26	0.45	5,010	433	nt	2.1	2.8	0.23	513	nt
hCA IX†	25	27	34	50	49	367	412	nt	320	23	36	420	25.8
hCA XII†	5.7	3.4	22	50	3.5	355	305	nt	5.4	4.5	10	261	21.1
mCA XIII†	17	19	50	23	nt	3,885	15,400	nt	15	15	13	550	2,570
hCA XIV†	41	43	25	345	33	4,105	360	nt	5,432	4,130	4,950	52	250
mCA XV†	72	65	58	95	70	135‡	141‡	nt	79‡	143‡	234‡	176‡	431‡

* h, human; m, murine isozyme; nt, not tested, data not available.
† From refs. (56–60).
‡ Unpublished data from our laboratory.

Data of Table 4.3 show that similarly to the clinically used/orphan drug classical sulfonamide CAIs, that is, compounds **1–4**, the clinically used sulfonamide diuretics **17–23** act as inhibitors of all 13 investigated CA isozymes, but with an inhibition profile quite different from that of inhibitors investigated earlier, and particularly different from that of the first-generation CAIs **1–4**.

Based on data of Table 4.3, there are several aspects that need to be considered here regarding the polypharmacology of these diuretic sulfonamides. Many of these compounds do inhibit substantially several CA isoforms involved in crucial physiological and pathological processes. In contrast to the classical CAIs of types **1–4**, generally low-nanomolar CA II inhibitors, compounds **17–23** (except furosemide **22**) are much weaker inhibitors of this isozyme, usually in the micromolar range. Only furosemide **22** is a good CA II inhibitor among these diuretics, with a K_I of 65 nM, whereas all others show K_I in the range of 138–6980 nM (Table 4.3). Again with the exception of furosemide **22**, the diuretics **17–23** have a low affinity for CA I, the other isoform known when these drugs had been discovered (20). Data of Table 4.3 also show that many of the drugs **17–23** appreciably inhibit CAs discovered after their introduction in clinical use, with some low-nanomolar (or even subnanomolar) inhibitors against many of them. Examples of such situations are, among others, metolazone **19** against CA VII, XII, and XIII; chlorthalidone **20** against CA VB, VII, IX, XII, and XIII; indapamide **21** against CA VII, IX, XII, and XIII; furosemide **22** against CA I, II, and XIV; and bumetanide **23** against CA IX and XII (Table 4.3). Bumetanide **23** is a tumor-specific (targeting CA IX and XII) CAI, of equal potency to acetazolamide **1**, but without the promiscuity of acetazolamide. Indeed, bumetanide, differently from acetazolamide, is a weak inhibitor of all isoforms except CA IX and XII, which are overexpressed in tumors (3). Indapamide **21** and chlorthalidone **20** are also strong inhibitors of the tumor-associated CAs, but they are also effective in inhibiting CA VII and XIII (Table 4.3). It is thus clear that these old drugs may indeed have newer applications in therapy or as experimental agents, in situations in which the selective inhibition of some CA isozymes is needed, and which cannot be obtained with the clinically used compounds of types **1–4**. As far as we know, for the moment, no clinical trials are being conducted to understand in detail the polypharmacologic aspects related to CA inhibition of these diuretics. This would be desirable, as it may help the discovery of new agents (mention should be made that no diuretic possessing sulfonamide moieties has been discovered during the last 40 years).

A mention should be made regarding benzthiazide **24**, another diuretic in clinical use for many years (30). Recently it has been shown that this is a very potent hCA II inhibitor (K_I of 8.8 nM), but the compound also inhibits significantly hCA I and IX (K_I of 10 and 8.0 nM, respectively) (110). In tumor cell cultures (lines overexpressing hCA IX such as squamous lung cancer cells HCC-1588), the compound was highly cytotoxic, killing specifically only the cancer cells. It has thus been proposed that benzthiazide should be repositioned as an antitumor drug for treating hypoxic tumors (110).

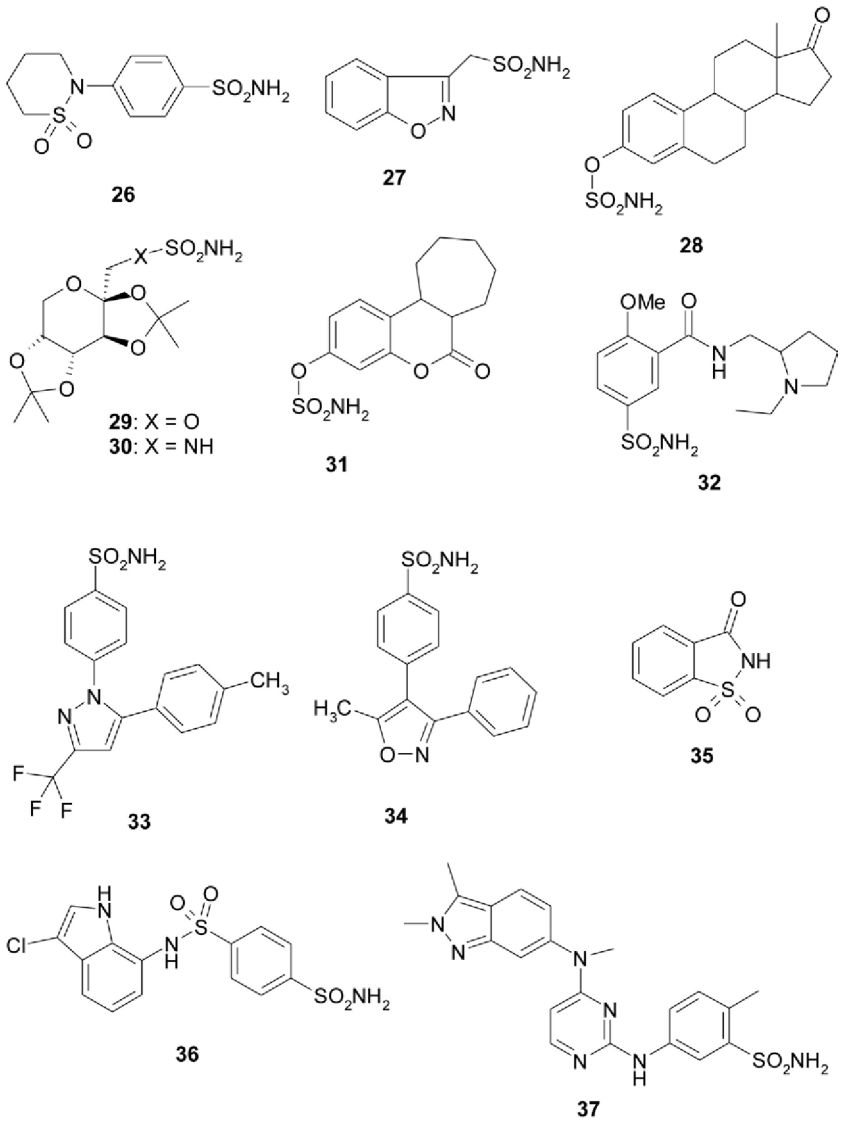
4.6 AGENTS FOR THE MANAGEMENT OF ALTITUDE SICKNESS

It is known for a long time that low-dose acetazolamide **1** (125 mg twice daily) or other sulfonamide CAIs (of types **2–4**) may be useful for the prevention and treatment of AMS (20,41,42). Indeed, CAIs are effective in the management of AMS due to the inhibition CAs present in the blood and/or brain (mainly hCA II as the predominant isoform), thus increasing the arterial oxygen concentration (41,42). The main problem with this treatment is that even after so many years of clinical use of such sulfonamides in the management of AMS, the isoform (or isoforms) responsible for these effects is not precisely known (CA I and II are abundant in the blood as mentioned above; CA II, VII, XII, and XIV, among others, are present in the brain). As shown above, acetazolamide (and also the other classical sulfonamide inhibitors **2–4**) is a promiscuous, potent inhibitor of all these CAs present in the brain and blood (Table 4.3), and, probably, such clinical studies as the ones mentioned above (41,42) should be conducted with more isoform-selective CAIs in order to understand in more detail the isoform(s) responsible for the efficacy of sulfonamide CAIs in the management of AMS.

Recently, Wang et al. (111) reported a benzolamide-like CAI, tolsultazolamide **25**, designed for the prophylaxis and treatment of AMS. This compound has been reported in an earlier study (112), but the CA inhibition data provided there were very imprecise and difficult to evaluate. It seems to be a potent CA II inhibitor, and due to the presence of the *iso*-butyl moiety substituting the secondary sulfonamide (lacking thus the acidic character of benzolamide discussed above) it is also liposoluble and easily penetrates the blood–brain barrier. If this compound will be approved for clinical use, it will be the only CAI approved in the last 15 years (after brinzolamide, approved in 1999).

4.7 VARIOUS PHARMACOLOGICAL ACTIONS CONNECTED WITH CA II INHIBITION: SERENDIPITY OR OFF-TARGETING WITH IMPRESSIVE EFFICACY?

A range of clinically used drugs or agents in clinical development that incorporate sulfonamide or sulfamate moieties were shown to act as potent CAIs, targeting hCA II in the low nanomolar range (1–4,109). For many of them, the X-ray crystal structure at high resolution in complex with hCA II was also reported, allowing for the understanding of their inhibition mechanism at the molecular level (1–4,11). They include the antiepileptic drugs sulthiame **26** (113) and zonisamide **27** (114) (Figure 4.5), and sulfamates such as EMATE **28** (115) and topiramate **29** (an antiepileptic and antiobesity drug) (116), whereas the topiramate sufamide analog **30** (117) is also mentioned here due to its very low inhibitory properties against hCA II, compared with other sulfonamides, sulfamates, and sulfamides (1–4).



Other examples of such drugs/investigational agents that inhibit hCA II include irosustat **31**, a steroid sulfatase inhibitor in clinical development (118), the antipsychotic sulpiride **32** (119), the cyclooxygenase COX-2 inhibitors celecoxib **33** (120) and valdecoxib **34** (121), as well as the sweetener saccharin **35** (122). Most of these drugs are low-nanomolar hCA II inhibitors (see Table 4.4). The X-ray crystallography of the hCA II adducts with these compounds helped to understand this behavior in detail (2,113–122). As seen from Figures 4.5A–C and 4.6A–C, where some of these adducts are reported,

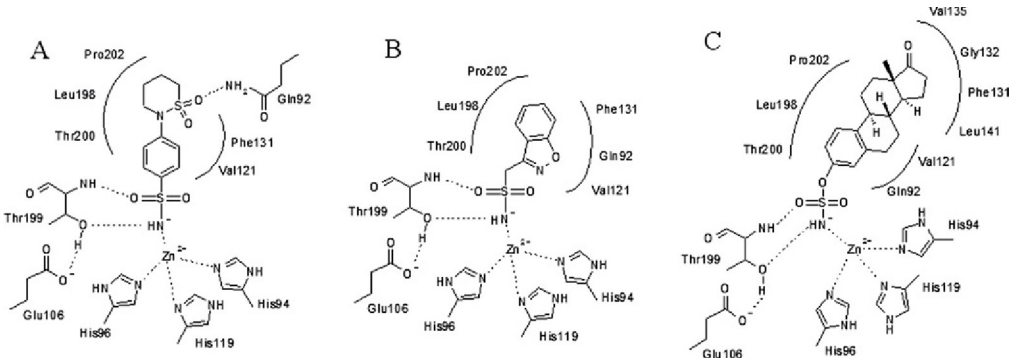


Figure 4.5 Schematic representation of the binding to hCA II of the anticonvulsant sulfonamides sulthiame ***26*** (A) and zonisamide ***27*** (B) and of the sulfamate EMATE ***28*** (C), as obtained by means of X-ray crystallography (25–30).

these sulfonamides/sulfamates effectively bind to hCA II (as well as other isoforms, see Figure 4.6A and C) similar to other sulfonamides investigated earlier (1,2), with the nitrogen of the deprotonated sulfamoyl moiety coordinated to the Zn(II) ion and participating in interactions with Thr199. Furthermore, a host of other favorable interactions were observed between the scaffold of the inhibitor and amino acid residues and/or water

Table 4.4 hCA I and hCA II inhibition data with compounds **26–39** and coumarins **41**, **42**, **45** (R = Me), and **46** (1–4,70,71).

Compound	Name	K_I (nM)	
		hCA I	hCA II
26	Sulthiame	25	8
27	Zonisamide	56	35
28	EMATE	37	10
29	Topiramate	250	10
30	Topiramate sulfamide	3,450	2,135
31	Irosustat	3,450	21
32	Sulpiride	12,000	40
33	Celecoxib	50,000	21
34	Valdecoxib	54,000	43
35	Saccharin	18,540	5,950
36	Indisulam	31	15
37	Pazopanib	12.1	32.4
38	Imatinib	31.9	30.2
39	Nilotinib	29.3	4.1
41	—	78	59
42	—	3,100	9,200
45	—	5,900	66
46	—	3,720	99

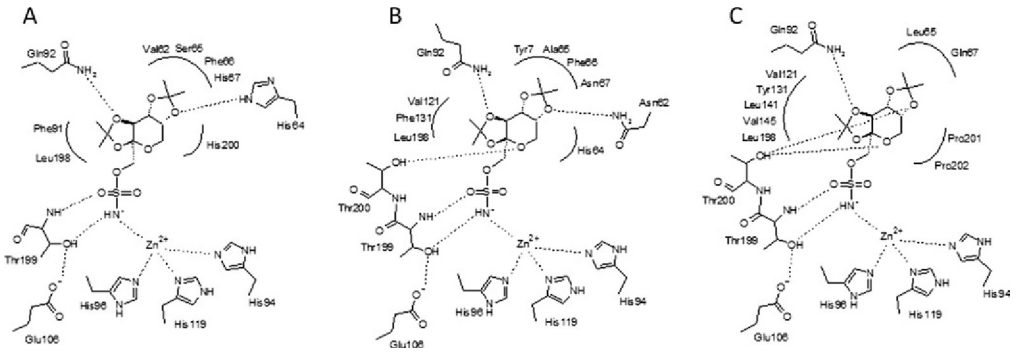


Figure 4.6 Schematic representations for the binding of topiramate to ***29*** hCA I (A), hCA II (B), and hCA VA (C) active site. "A" and "B" were obtained by X-ray crystallography, whereas "C" was obtained by homology modeling (2,116,123).

molecules from the hCA II active site (113–122). For example, in the case of topiramate **29**, the inhibitor participates in five direct hydrogen bonds with amino acid residues of the hCA II active site (116), and in four such interactions in the case of the hCA I adduct (123) (Figure 4.6). This explains its behavior as a highly potent hCA II inhibitor (K_I of 10 nM).

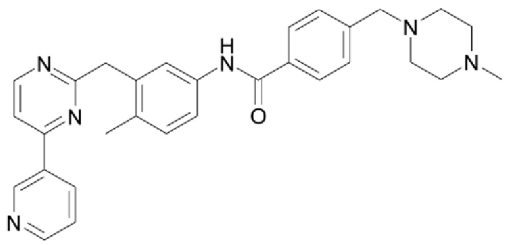
Sulpiride **32**, celecoxib **33**, and valdecoxib **34** all contain primary sulfamoyl moieties in their molecules, and act as potent inhibitors of several CA isozymes, including hCA II (Table 4.4) with affinity for some of them of the same order of magnitude as those of clinically used CAIs, that is, in the nanomolar range (Table 4.4) (119–121). Furthermore, the X-ray crystal structure of adducts of these drugs bound to the physiologically dominant isoform hCA II has also been reported, confirming the solution inhibition data (119–121). The potent inhibition of physiologically relevant CA isozymes by the COX-2 inhibitors **33** and **34** also may lead to novel clinical applications of these agents. We have, in fact, showed that both celecoxib and valdecoxib are effective systemic antiglaucoma agents in hypertensive rabbits, possessing an activity similar to the clinically used agent acetazolamide (120). However, the two compounds also strongly inhibit the tumor-associated isoforms CA IX and XII, in the low nanomolar range (120), and there are many reports in the literature regarding the beneficial effects of mainly celecoxib in diverse cancer types (124). Such effects may be explained by a dual mechanism of action: in addition to COX-2 inhibition, these compounds also interfere with the activity of CA isozymes critical for the development and invasion of cancer cells, such as CA II, IX, and XII. This additional mechanism is, in fact, observed only with the sulfonamide and not with the methylsulfone COX-2 inhibitors (120,124). Thus, these two compounds may be used in clinical trials as antitumor agents and may constitute leads for developing more effective antitumor sulfonamides possessing diverse mechanisms of antitumor action.

Saccharin **35**, an acylated sulfonamide, is the only compound examined here with a secondary sulfonamide moiety in its molecule (122). It also acts as a weak hCA II inhibitor (K_I of 5.95 μM), but its X-ray crystal structure in adduct with hCA II afforded the identification of a new inhibition mechanism. In fact, the compound binds in deprotonated form

with the nitrogen coordinated to the zinc ion and participating in other polar interactions with Thr199 (122). Based on these crystallographic data, saccharin has been used as a lead molecule for obtaining much stronger CAIs with this particular binding mode (1,2).

4.8 CA II INHIBITORS IN THE MANAGEMENT OF TUMORS?

The primary sulfonamide indisulam **36** was shown to possess significant antitumor activity, and this agent was in clinical trials for the treatment of solid tumors till recently when its development was stopped (125,126). It has been shown by our group that indisulam is also a potent CAI, and its X-ray crystal structure in adduct with hCA II (K_I of 15 nM) was also reported (125). More recently, our group also showed that the protein tyrosine kinase inhibitor (PTKI) pazopanib **37** (also incorporating a primary sulfonamide moiety in its molecule) significantly inhibits all mammalian isoforms, with a K_I of 32.4 nM against hCA II and of 9 nM against the tumor-associated hCA IX (127). Furthermore, pazopanib is a subnanomolar hCA XII inhibitor (K_I of 0.88 nM) (127). It is interesting to note that pazopanib was originally approved for the treatment of renal clear cell carcinomas, which are known to have huge amounts of CA IX overexpressed due to hypoxia or the von Hippel-Lindau phenotype (3,127). Pazopanib is also used nowadays for the treatment of lung cancers, breast cancers, ovarian cancers, gliomas, and soft tissue sarcomas, all of which express a high amount of CA IX (less is known in the literature on the CA XII expression of many of these cancers) (3). Based on these data, it has been proposed that a significant therapeutic effect of pazopanib on hypoxic tumors is due to its strong CA inhibitory properties (127).



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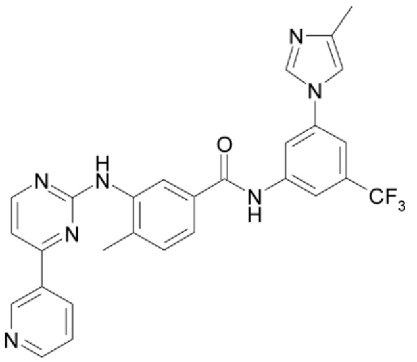

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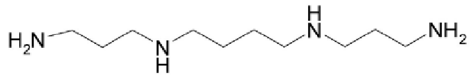

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Serendipitously, we have recently discovered (40) the potential of two other PTKIs, imatinib **38** and nilotinib **39**, to act as inhibitors of all the catalytically active mammalian CA isoforms. Imatinib and nilotinib were observed to act as very potent inhibitors of two CA isozymes, that is, hCA I and II, with inhibition constants in the range of 4.1–31.9 nM (Table 4.4) (40). The isoform with the highest affinity for these drugs was the ubiquitous, physiologically dominant hCA II. Effective inhibition with imatinib and nilotinib was also observed against the cytosolic isoform, hCA VII, the tumor-associated, transmembrane hCA IX, and the membrane-anchored enzyme mCA XV (40). These isoforms were inhibited by the two compounds with K_I in the range of 41.9–109 nM. We were thus tempted to hypothesize that part of the excellent anticancer effects of these drugs may also be due to their interaction with the CA isoforms involved in carcinogenesis (40). Given the systemic exposures achieved at the standard recommended doses of both imatinib (steady-state C_{max}/C_{min} 5.2 and 2.5 μM at 400 mg q.d.) and nilotinib (steady-state C_{max}/C_{min} 4.0 and 1.8 μM at 400 mg b.i.d.), it seems likely that the inhibition of at least some of the CAs by these drugs might be physiologically relevant (40). The recently reported long-term effects of imatinib therapy in promoting bone formation in some leukemia patients mimic quite well the action of acetazolamide observed in the same clinical settings, and might be related to CA II inhibition in osteoclasts and osteoblasts, in addition to the inhibition of kinases (45). It is, in fact, well known that several CA isozymes (mainly CA II, but also CA XII and XIV) are involved in the acidification processes in osteoclasts, leading to inorganic matrix dissolution that precedes enzymatic removal of the organic bone matrix (45). By inhibiting these CA isozymes with sulfonamides, the osteoclast acidification and bone dissolution processes are also inhibited.

As shown earlier in this chapter, there are some types of tumors, such as gastrointestinal stromal tumors (GISTs), that overexpress high amounts of hCA II (38). Other cancers that were shown to express (or overexpress) CA II are some uterine tumors (36), medulloblastomas (37), and some supratentorial primitive neuroectodermal tumors (37). In all of them, CA IX and XII overexpression was also observed (36,37) but not for the GISTs (38). Thus, CAIs targeting hCA II may have a place in the treatment of some tumors.

4.9 NEW STRATEGIES TO DESIGN CA II–SELECTIVE INHIBITORS

Phenols were shown to act as CAIs quite some time ago (67,128), but no real drug design studies for detecting hCA II–selective (or other isoform-selective) compounds have been performed for this class of CAIs, and they will not be discussed in detail here. However, the phenol binding mode is characteristic, as this was the first compound observed to be anchored (by means of hydrogen bonds) to the zinc-coordinated water molecule/hydroxide ion (128). Afterwards, such a binding has been observed for polyamines (68) such as spermine (Figure 4.7) as well as the sulfonic acids formed by hydrolysis of sulfocoumarins (69).

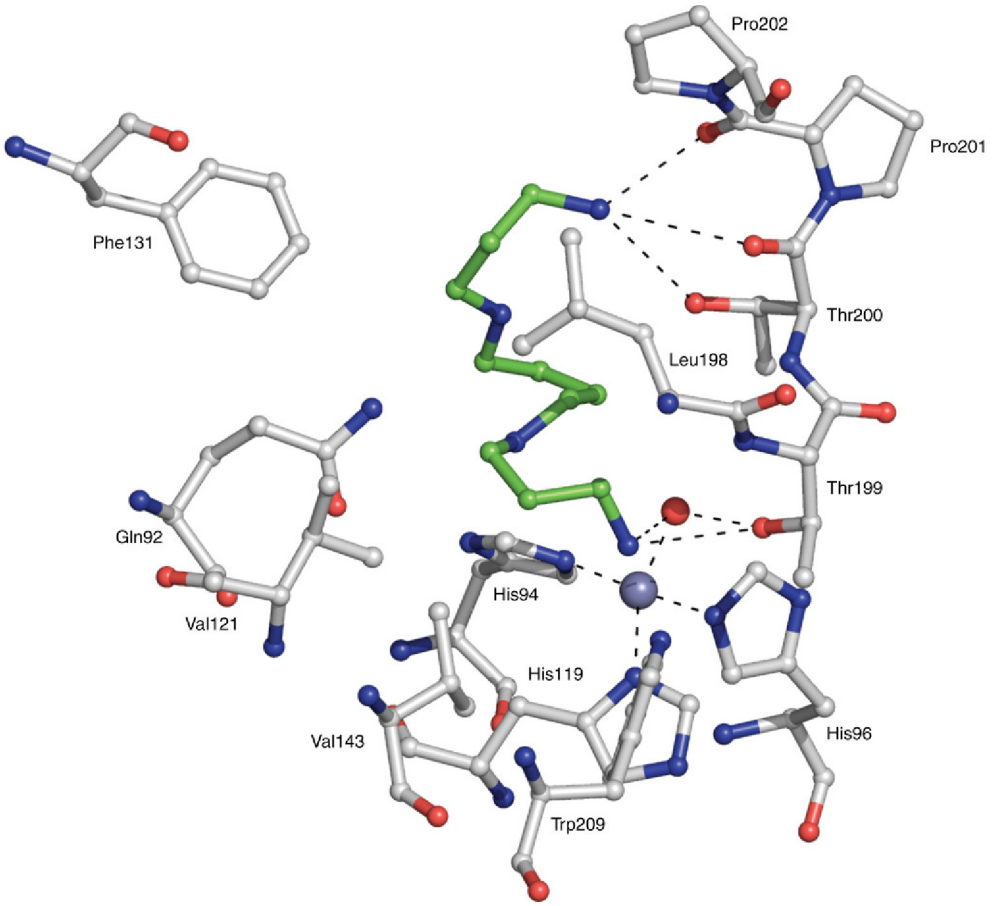
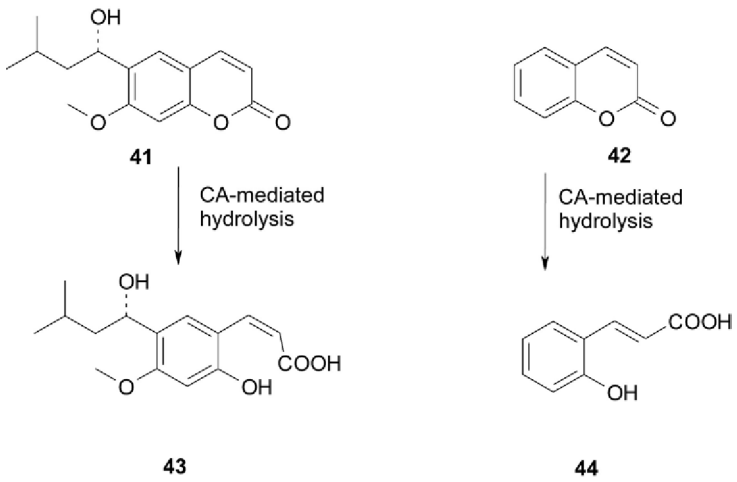


Figure 4.7 Active site of the hCA II/40 adduct showing interactions in which spermine ***40*** (as tetracation) participates when bound to the enzyme (68).

The X-ray crystal structure of the CA II–spermine adduct was solved, and allowed to understand the inhibition mechanism of this molecule (Figure 4.7) (68). Spermine, which in the experimental conditions used is probably a tetracation, adopts a coiled conformation when bound to the enzyme active site. It was found bound deep within the hCA II active site, but interestingly it was not directly coordinated to the metal ion. In particular, the polyamine was anchored to the Zn^{2+}-bound hydroxide ion by means of a strong hydrogen bond involving one of its terminal ammonium groups, in a way reminiscent of the binding of phenol. The same moiety participates in a second hydrogen bond with the Thr199OG1 atom. Additional hydrogen bonds with residues Thr200 and Pro201 and several van

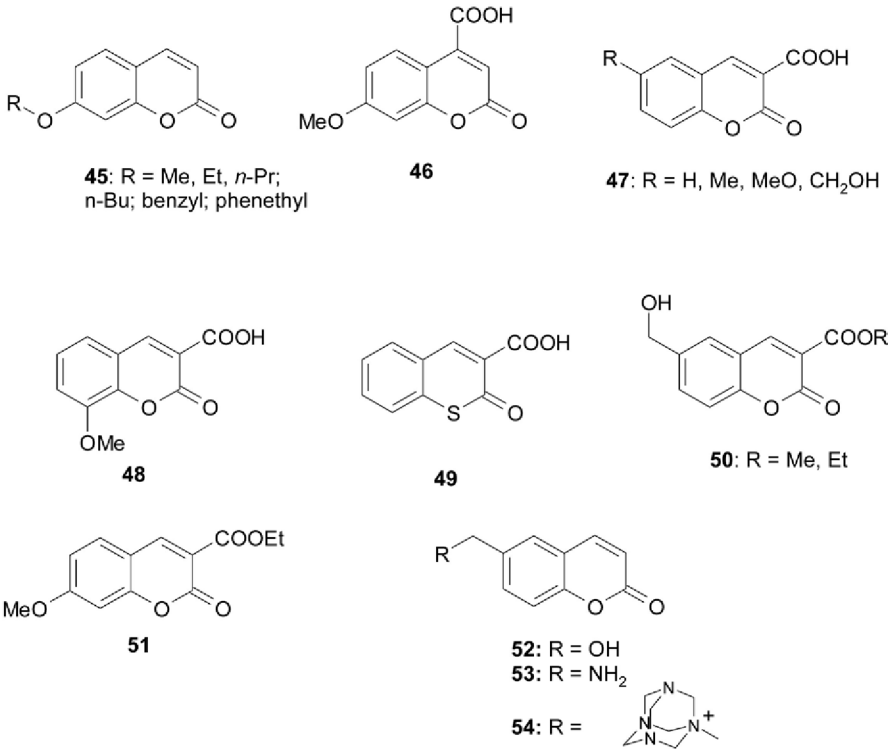
der Waals contacts with enzyme residues Gln92, Val121, Phe131, Val143, Leu198, Thr199, Thr200, Pro201, and Pro202 further stabilize the adduct. Spermine thus binds differently compared with sulfonamides, the other main classes of CAIs, rendering it possible to develop CAIs with a diverse inhibition mechanism, profile, and selectivity for various isoforms (68). Indeed, spermine was a low-nanomolar inhibitor of only CA IV (K_I of 10 nM (68)), whereas its inhibition of other isoforms was in the micromolar range. Some of its derivatives possessing bulky moieties at one of the terminal primary amine groups were shown, on the other hand, to act as low-nanomolar hCA II inhibitors (68).

There are few sulfocoumarins acting as hCA II–selective inhibitors (69,129), but the coumarins, on the other hand, represent a much more interesting case for the design of isoform-selective CAIs (70–73).



Coumarins (and some of their derivatives such as the thiocoumarins) were only recently discovered to act as CAIs (70–73), and their inhibition mechanism was deciphered in detail by Maresca et al. (70). We demonstrated that the natural product 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one **41** (isolated from the Australian plant *Leionema ellipticum* (70)) as well as the simple, unsubstituted coumarin **42** were hydrolyzed within the CA active site with formation of the 2-hydroxy-cinnamic acids **43** and **44**, respectively, which represent the de facto enzyme inhibitors (70,71). The formed 2-hydroxy-cinnamic acids occlude the entrance of the enzyme active site, a mechanism never evidenced before for CA inhibition, as shown in Figure 4.8 (70,71).

It should also be mentioned that coumarins **41** and **42** were potent inhibitors against some investigated hCA isoforms, which makes this entire class of derivatives of paramount interest for designing novel applications for the CAIs.



We investigated thereafter a series of derivatives possessing various moieties substituting the (thio)coumarin ring in the 3-, 6-, 7-, 3,6-, 4,7-, and 3,8- positions, of types **45–54** (71). The most significant finding of this second study was that some coumarins are truly isoform-selective CAIs, inhibiting efficiently only 1 isoform of the 13 catalytically active ones found in humans (71). For example, **45** and **46** were nanomolar hCA II inhibitors (K_I of 66 and 99 nM, respectively, Table 4.4), whereas they inhibited the other mammalian isoforms in the micromolar range (71). The thiocoumarin **49** and several coumarins (**47**, **48**, **50**, and **51**) showed low-nanomolar affinity for CA IX, with inhibition constants in the range of 45–98 nM. Compound **54** was shown to be a low-nanomolar inhibitor of only CA IX (K_I of 48 nM), whereas it inhibited all other 12 CAs in the micromolar range, a feature never evidenced before for sulfonamides or other types of CAIs. Thus, this was the first case of isoform-selective inhibitors for many (almost all) CA isozymes ever reported up to now, but many other such compounds were detected in further studies on coumarins, their derivatives, and sulfocoumarins (130–133).

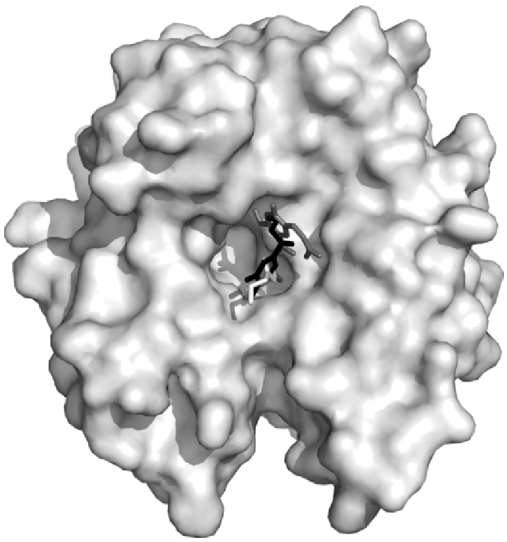


Figure 4.8 Binding of the hydrolyzed coumarin (compound ***43***, in gray) to the CA II active site (70), superposed with the CAAs L-adrenaline (black) (140) and histamine (57) (white): the coumarin and CAA binding sites are the same. The hCA II solvent accessible surface is shown.

4.10 CA II ACTIVATION

The CA activators (CAAs) still remain an orphan field. Initially all activator studies have been performed on hCA II (or the corresponding bovine isoform, bCA II) (134). Nowadays, all CA isoforms from mammals have been investigated for their activation with several classes of activators, most of which are amines, amino acids, and oligopeptides, and the CA activation mechanism is also thoroughly understood due to extensive X-ray crystallographic and kinetic work from several groups (135–140). The rate-determining step for the CO_2 hydration reaction catalyzed by CAs is the proton transfer reaction from the water bound to the Zn(II) ion to the reaction medium, with generation of the zinc hydroxide species of the enzyme (1–10). This step is achieved in many α-CA isoforms by the active site residue His64, which being flexible acts as a proton shuttle between the active site and the reaction medium (59). Compounds able to participate in such proton transfer reactions, of the amine, amino acid, and oligopeptide type, have been shown to act as efficient CAAs (57,58,135–140). It should be mentioned that the first X-ray crystal structure of an activator bound to CA was the adduct of hCA II with histamine (57). In that work, it has been shown that the activator binding site is at the entrance of the enzyme active site, far away from the catalytic metal ion. In this position, the activators are able to participate in supplementary proton release pathways with enhanced generation of the nucleophilic species of the enzyme, and thus more efficient catalysis (57). Since this first work, many other adducts of activators complexed to various CA isoforms have been reported (57,58,135–140).

In Figure 4.8, the superposition of the hCA II adducts of the activators histamine (57) and L-adrenaline (140) with the hydrolyzed coumarin natural product (compound **43**) is shown, which demonstrates that the coumarin and the activator binding sites are, in fact, the same. Thus, it took more than 20 years to understand the deep interrelations between the binding of inhibitors and activators to the active site of this enzyme (57,70,71). In fact, the activator binding site can be exploited by inhibitors too, as recently exemplified for the binding of coumarin inhibitors and of amine/amino acid activators (70,71).

Some CAAs, such as phenylalanine and imidazole, when administered to experimental animals produced an important pharmacological enhancement of synaptic efficacy, spatial learning, and memory, proving that this class of relatively unexplored enzyme modulators may have pharmacological applications in conditions in which learning and memory are impaired, such as Alzheimer's disease or aging (141,142). One must mention that it was also reported that the levels of CA II are significantly diminished in the brain of patients affected by Alzheimer's disease, and these facts strongly support the involvement of different CA isozymes in cognitive functions (141,142).

At this moment, the most significant finding in this field during the last years was that the activator binding site is the same as the coumarin (inhibitor) binding site (Figure 4.8), as stressed above, which facilitates interesting drug design campaigns. Further work in the field of the CAAs is warranted in order to understand the significance of this phenomenon in vivo. A lot of CA activation drug design studies have also been reported, with CA II being one of the most targeted isoforms (143–150).

4.11 CONCLUSIONS

A wealth of X-ray structural data has been accumulated in the last 15 years for CAI complexes, including not only the main classes of inhibitors—the pharmacologically relevant sulfonamides and their isosteres (sulfamates, sulfamides, ureates, and hydroxamates), and the simple inorganic anions—but also the less investigated ones, such as, among others, the carboxylates and the newly identified DTCs, XTs, polyamines, sulfocoumarins, and coumarins. Most of these X-ray crystal structures reported so far were complexes with inhibitors/activators of isozyme CA II. These data are important for the drug design of isozyme-selective CAIs, and important advances have been made in the last years. In fact, the main problem with the classical, clinically used sulfonamides (including also the second-generation agents dorzolamide and brinzolamide) was related to the fact that they are promiscuous inhibitors of all (or most of the) CA isozymes found in mammals. Some low levels of isozyme selectivity were shown by dorzolamide and brinzolamide, which have been designed in such a way to act as much weaker CA I than CA II inhibitors, but similarly to acetazolamide, methazolamide, and ethoxzolamide, these 2 second-generation inhibitors strongly inhibit the remaining 10 CA isozymes. Thus, considering only the zinc-binding functionality and the organic scaffold, it is

quite difficult to design isoform-selective CAIs, as the interactions around the metal ion and the organic scaffold (normally positioned at the bottom and in the middle of the active site cavity, respectively) are almost the same between the inhibitors and most CA isozymes with medicinal chemistry applications. This also explains why the first- and second-generation CAIs are normally devoid of any isozyme selectivity. They are indeed rather small, compact molecules that bind deeply within the enzyme active site. However, around 10 years ago the "tail-approach" was reported, which afforded the rather facile synthesis of a large number of CAIs starting from aromatic/heterocyclic scaffold also containing derivatizable amino, imino, or hydroxy groups, to which various moieties (tails) were introduced by normal chemical modification reactions (acylation, alkylation, arylsulfonylation, condensation, etc.). In this way, it was possible to modulate both the physicochemical properties of the synthesized inhibitors (e.g., by introducing tails that induce high water solubility, enhanced lipophilicity, positive/negative charges that lead to membrane impermeability, or fluorescence or spin-labeled groups) and their affinity to the various isozymes, as the tail(s) usually interact with amino acid residues toward the exit of the active site or on its edge. In fact, those are the amino acids that are less conserved among the various mammalian CAs, and this explains why most of these novel-generation inhibitors showed much more interesting inhibition profiles as compared with the classical ones. X-ray crystal structures are available for many of these compounds, which proved that both favorable interactions and clashes with particular amino acids present only in some isozymes are critical for the inhibition profile and isozyme selectivity issues. Several interesting examples of inhibitors designed by the tail approach that showed efficient antiglaucoma and anticancer activities in vivo have been presented in this chapter, which also validate this highly versatile method of generating interesting CAIs, with valuable pharmacological properties.

X-ray crystal structures of DTCs complexed to hCA II allowed a deep understanding of the interactions between enzyme and this new class of CAIs at atomic level, showing that the CS_2^- ZBG, found in DTCs (and XTs), is an excellent alternative to the sulfonamide ZBG present in the classical CAIs. Furthermore, such compounds are water-soluble and easy to prepare, and afford exploration of a wide chemical space, which for the sulfonamides is not always possible.

The other new classes of CAIs discovered recently, such as the polyamines and the coumarins, were for the moment less investigated for their pharmacological applications in vivo, but certainly they are also very promising.

CA II is thus one of the most intricate isoforms among the 15 human ones, although it was and is the most studied one. On one hand, its strong inhibition may lead to antiglaucoma drugs, efficient diuretics, agents against AMS, and probably anticonvulsants and antitumor agents with a new mechanism of action. On the other hand, CA II is many times considered as an off-target isoform, when selective inhibition of mitochondrial or transmembrane CA isoforms must be achieved. This was recently shown to be possible

both with the classical compounds (sulfonamides) and with the newly discovered classes of CAIs (coumarins and sulfocoumarins, principally). Whether this will also lead to new therapeutic agents is for the moment difficult to predict, but there is a stringent need of new such drugs considering that the last approved one is already 15 years old. Still, CA II is probably the "star" CA isoform, with most of our understanding of this enzyme superfamily being due to its detailed investigations.

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