Chapter 15
Carbonic Anhydrase Inhibitors Drug Design

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Abstract Inhibition of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) has pharmacologic applications in the field of antiglaucoma, anticonvulsant, antiobesity, and anticancer agents but is also emerging for designing anti-infectives (antifungal and antibacterial agents) with a novel mechanism of action. As a consequence, the drug design of CA inhibitors (CAIs) is a very dynamic field. Sulfonamides and their isosteres (sulfamates/sulfamides) constitute the main class of CAIs which bind to the metal ion in the enzyme active site. Recently the dithiocarbamates, possessing a similar mechanism of action, were reported as a new class of inhibitors. Other families of CAIs possess a distinct mechanism of action: phenols, polyamines, some carboxylates, and sulfocoumarins anchor to the zinc-coordinated water molecule. Coumarins and five/six-membered lactones are prodrug inhibitors, binding in hydrolyzed form at the entrance of the active site cavity. Novel drug design strategies have been reported principally based on the tail approach for obtaining all these types of CAIs, which exploit more external binding regions within the enzyme active site (in addition to coordination to the metal ion), leading thus to isoform-selective compounds. Sugar-based tails as well as click chemistry were the most fruitful developments of the tail approach. Promising compounds that inhibit CAs from bacterial and fungal pathogens, of the dithiocarbamate, phenol and carboxylate types have also been reported.

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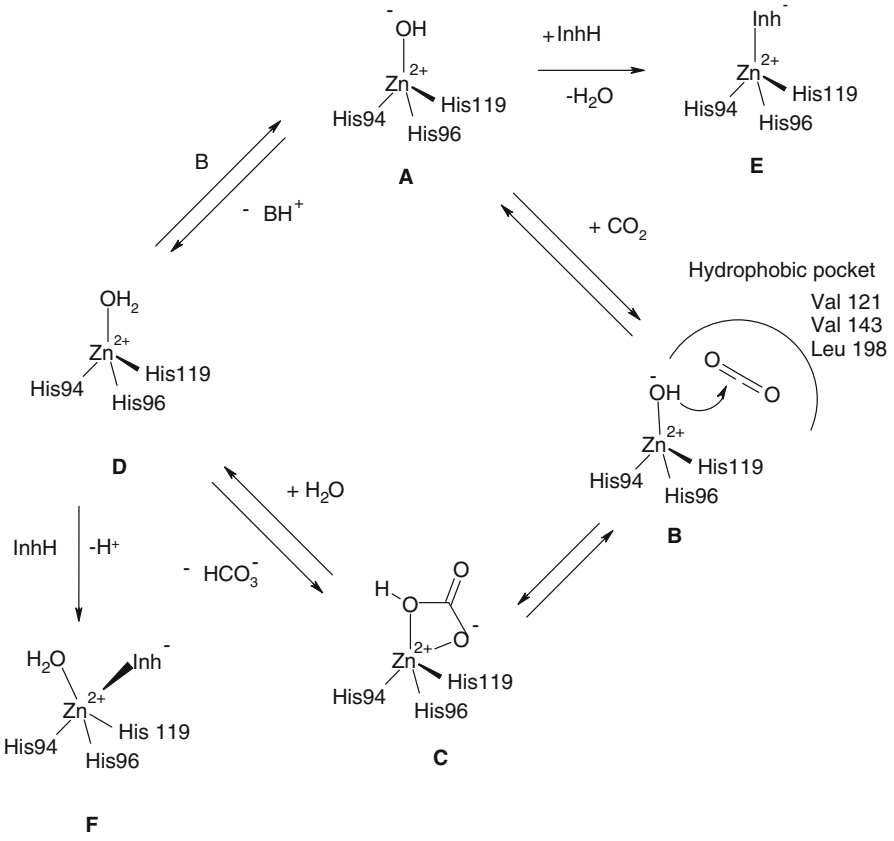
1 Introduction

CO_2, bicarbonate and protons are essential molecules/ions in many important physiologic processes in all life kingdoms (Bacteria, Archaea, and Eukarya); hence, relatively high amounts of carbonic anhydrases (CAs, EC 4.2.1.1) are present in different tissues/cell compartments of most investigated organisms [1–9]. The α-CAs are present in vertebrates, protozoa, algae and cytoplasm of green plants and in some bacteria [1–9], the β-CAs are predominantly found in bacteria, algae and chloroplasts of both mono- as well as dicotyledons, but also in many fungi and some archaea [1–9]. The γ-CAs were found in archaea and some bacteria [3], whereas the δ- and ζ-CAs seem to be present only in marine diatoms [2]. 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 (e.g., gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes (thoroughly studied in vertebrates) [1, 4–7, 9]; whereas in algae, plants and some bacteria they play an important role in photosynthesis and other biosynthetic reactions [2, 3]. In diatoms δ- and ζ-CAs play a crucial role in carbon dioxide fixation [2]. Many such enzymes from vertebrates, fungi and bacteria are well-known drug targets [1, 4].

The CAs are a superfamily of metalloenzymes which catalyze the interconversion between CO_2 and bicarbonate by using a metal hydroxide nucleophilic mechanism [1–9]. Five distinct genetic CA families are known to date: the α-, β-, γ-, δ- and ζ-CAs, which differ in their preference for metal ions used within the active site for performing the catalysis. Zn(II) ions may be used by all five classes mentioned above but the γ-CAs are probably Fe(II) enzymes (being active also with Zn(II) or Co(II) ions) [3], whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis [2]. In all cases, the apo-enzymes are devoid of catalytic activity, the presence of the metal ion being essential both for catalysis as well as binding of inhibitors [1–9]. The catalytic/inhibition mechanisms are shown in Scheme 15.1.

In all CA classes a metal hydroxide species $\left(L_3\text{-}M^{2+}\text{-}OH^{-}\right)$ of the enzyme is the catalytically active nucleophile (working at neutral pH) which attacks the CO_2 molecule bound in a hydrophobic pocket nearby [10]. This metal hydroxide species is generated from a water coordinated to the metal ion, which is placed at the bottom of the active site cavity (Scheme 15.1, A). The active center normally comprises M(II) ions in a tetrahedral geometry, with three protein ligands (L) in addition to the water molecule/hydroxide ion coordinating the metal, but Zn(II) or Co(II) were also observed in trigonal bipyramidal or octahedral coordination geometries, at least in γ-CAs [11]. In many CAs, generation of the metal hydroxide species from the metal-coordinated water one, is the rate determining step of the catalytic turnover, which for some α- and ζ-CAs achieve k_{cat}/K_M values $> 10^8$ $M^{-1} \times s^{-1}$, making CAs among the most effective catalysts known in nature [1, 2]. The metal



Scheme 15.1 Catalytic and inhibition mechanisms (with zinc ion binders) of α-CAs (hCA I amino acid numbering of the zinc ligands). A similar catalytic/inhibition mechanism is valid also for CAs from other classes (β-, γ- and ζ-CAs) but either the metal ion is coordinated by other amino acid residues or a Cd(II) ion is present instead of zinc at the active site

ion ligands are three His residues in α-, γ-, and δ-CAs or one His and two Cys residues in β- and ζ-CAs [1–9, 12]. Some β-class enzymes have four protein zinc ligands, i.e., 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 < 8, as shown in an excellent crystallographic work from Jones' group on the mycobacterial enzymes Rv3558c and Rv1284 [13]. However, at pH > 8 a conserved Arg residue in all β-CAs investigated so far (belonging to a so-called catalytic dyad) 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 substrate CO_2 was found bound (for the α-class enzymes) in a hydrophobic pocket near the Zn(II) ion, defined among others by residues Val121, Val143 and Leu198 [in the human (h) isoform hCA II, for which the X-ray crystal structure in

complex with CO_2 was reported in a seminal paper by McKenna's group [10]], as represented in Scheme 15.1, B. Orientated in such a favorable position for the nucleophilic attack, CO_2 is transformed into bicarbonate which is coordinated bidentately to the Zn(II) ion, as shown in Scheme 15.1, C. There are X-ray crystal structures of adducts of hCA II with bicarbonate [10] (shown in Scheme 15.1, C) or thrithiocarbonate (CS_3^{2-}) [14] which is an interesting mimic of bicarbonate, having higher binding affinity for the metal ion compared to bicarbonate itself [14]. As the binding of bicarbonate to zinc is rather labile, intermediate C is readily transformed to D by reaction with water, which liberates the bicarbonate into solution. In this way, the acidic form of the enzyme is generated, with water coordinated to the metal ion (Scheme 15.1, D), which is catalytically ineffective. Generation of the nucleophilically active species of the enzyme A is achieved though a proton transfer reaction from the zinc-coordinated water (species D) to the buffer, which is also the rate-determining step of the entire process [15–17]. In many CA isoforms and probably in many enzyme classes (not only the α ones which are the most investigated CAs), this process is assisted by an active site residue able to participate in proton transfer processes, which for the specific case examined in Scheme 15.1 (i.e., hCA II) is a histidine residue placed in the middle of the active site [15–17]. For hCA II and several other human isoforms this residue is His64, which may be further assisted in the proton transfer process by a cluster of histidines prolonging from he middle of the cavity to its entrance and till the surface of the enzyme around the edge of the cavity [15]. In hCA II this cluster includes residues His4, His3, His10 and His15 [15].

The inhibition and activation of CAs are well understood processes, with most classes of inhibitors binding to the metal center [1–9], whereas activators bind at the entrance of the active site cavity and participate in proton shuttling processes between the metal ion – bound water molecule and the environment [15]. This leads to the enhanced formation of the metal hydroxide, catalytically active species of the enzyme [1, 15, 16]. Inhibitors generally bind to the metal ion from the enzyme active site in deprotonated state (as anions), as shown schematically in steps E and F of Scheme 15.1, for a tetrahedrally bound inhibitor (E) and for one in which the Zn(II) ion is in a trigonal bipyramidal geometry (F), case in which a water molecule in addition to the inhibitor is also coordinated to zinc [1–9]. Although the mechanisms of Scheme 15.1 were depicted for an α-CA, they are valid even if another metal ion is present within the active site cavity, i.e., Cd(II) or Fe(II), since the corresponding hydroxides have similar nucleophilicity with zinc hydroxide. The same is true for a different coordination pattern of the metal ion (i.e., two Cys and one His residues), as for the β- and ζ-class CAs [1–9].

It should be mentioned that recently other inhibition mechanisms than the binding to the metal center were reported for α-CAs, which do not directly involve the metal ion from the enzyme active site. For example polyamines bind to the enzyme by anchoring to the zinc-coordinated water/hydroxide ion [18], whereas coumarins act as prodrugs and bind at the entrance of the active site cavity, rather far away from the metal ion [19–21].

2 Drug Design Studies of Sulfonamides, Their Bioisosteres and Dithiocarbamates as CAIs

Sulfonamides are the most important class of CAIs [1, 4–9], with several compounds such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, sulthiame **4**, dichlorophenamide **5**, dorzolamide **6**, brinzolamide **7**, sulpiride **8** and zonisamide **9** in clinical use for many years, as diuretics, antiglaucoma agents, as well as antiepileptics [1] (Fig. 15.1). Sulfamates such as topiramate **10**, EMATE **12**, and irosustat **11** although developed independently of their potential CA inhibitory properties [1], are also potent CAIs and are clinically used as antiepileptics/antiobesity agents (topiramate [1, 4–9]) or are in advanced clinical trials as dual, steroid sulfatase inhibitors/CAIs with anticancer effects [22] (Fig. 15.1). Irosustat **12** is in fact the first-in-class steroid sulfatase inhibitor to be used clinically in patients with advanced hormone-dependent cancers [22] but is also a potent CAI [1, 22].

Sulfonamides and sulfamates strongly inhibit CAs belonging to most families, not only the α-class enzymes [23–28]. Other compounds possessing potent CA inhibitory activity and developed for other uses are celecoxib **13** and valdecoxib **14**, originally described as cyclooxygenase 2 (COX-2) inhibitors [29, 30]. Saccharin **15**, widely used sweetener, is also a CAI, as recently reported by Klebe's groups [31]. Compounds **1**–**15** may be considered as first/second generation CAIs. Their main problem is that they indiscriminately inhibit most of the human isoforms known to date [1]. Indeed, 16 such isozymes were described in non-primates, CAs I-XV with two V-type isoforms, CA VA and CA VB, and 15 isoforms are known in primates, as CA XV is not expressed in these mammals [32].

Both sulfonamides and sulfamates bind in deprotonated form, as anions, to the Zn(II) ion from the enzyme active site, which is in a tetrahedral geometry as shown in Scheme 15.1, step E. CA inhibition data of all mammalian CAs with compounds **1**–**15** (and other sulfonamides in clinica use such as among other the thiazide and high-ceiling diuretics) were published in ref. [1] and will be not discussed here in detail.

The side effects of many of the compounds belonging to the first/second generation CAIs of types **1**–**15** (among which metabolic acidosis, kidney stones, bone loss, etc. [1, 4]) are due to the potent inhibition of all mammalian CA isoforms, and not only of the target one [1, 4]. Indeed, among the many mammalian isoforms investigated to date it is generally considered that CAs II, IV and XII are the targets for antiglaucoma agents [1]; CAs VA and VB for antiobesity agents [1], CAs IX and XII for antitumor agents/diagnostic tools for imaging of hypoxic tumors [1, 4]. Whereas it is not at all clear which isoforms are targeted by the antiepileptic sulfonamides/sulfamates, but CAs VII and XIV seem to be among them [1]. There are several isoforms for which the physiologic role/targeting with inhibitors are not clearly established and among them are CAs I, III, VI and XIII [1]. It should be also noted that CAs VIII, X and XI are acatalytic isoforms, i.e., they are devoid of CO_2 hydrase activity [1].

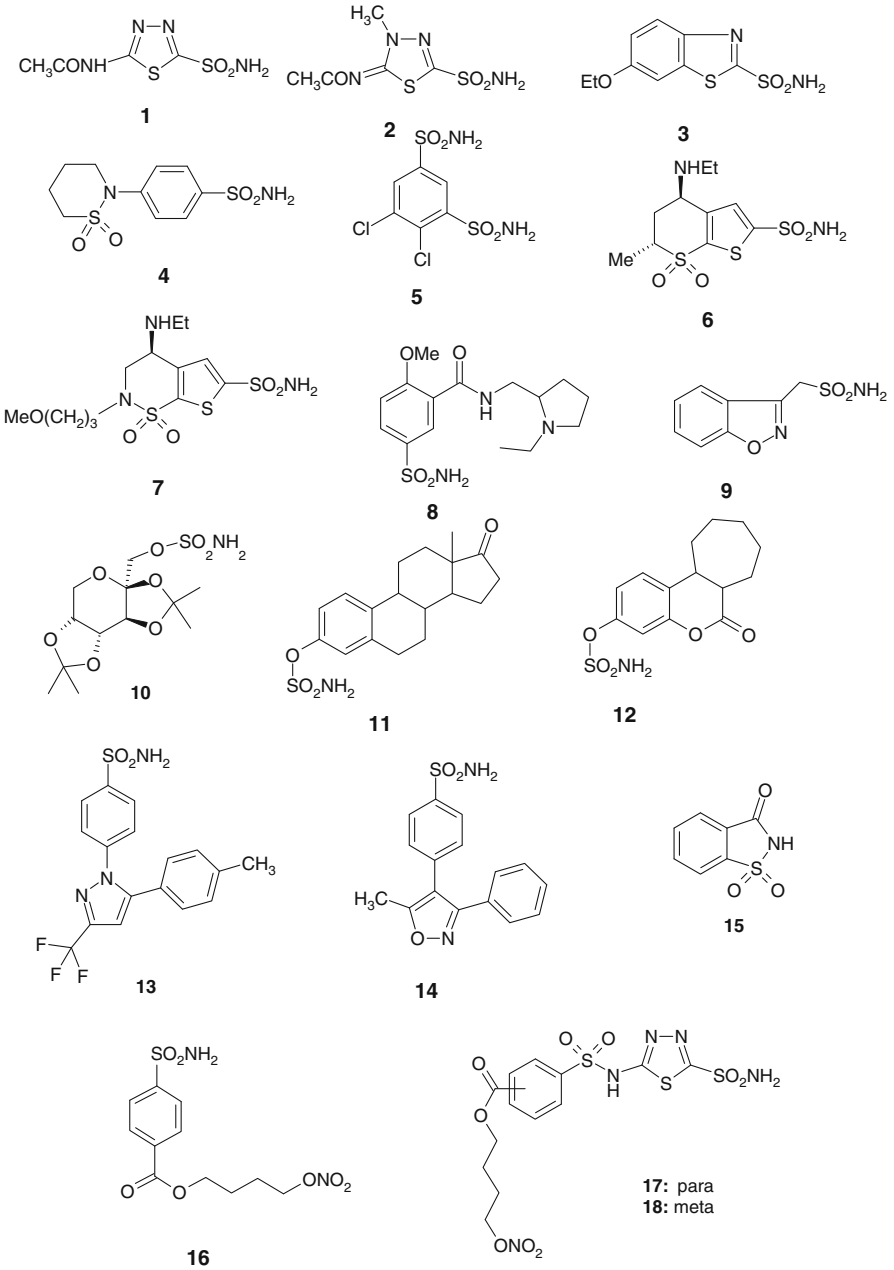


Fig. 15.1 Clinically used/preclinical sulfonamide and sulfamate CAIs **1**–**1**5 and compounds developed in the last period by the tail approach **(16–27)**

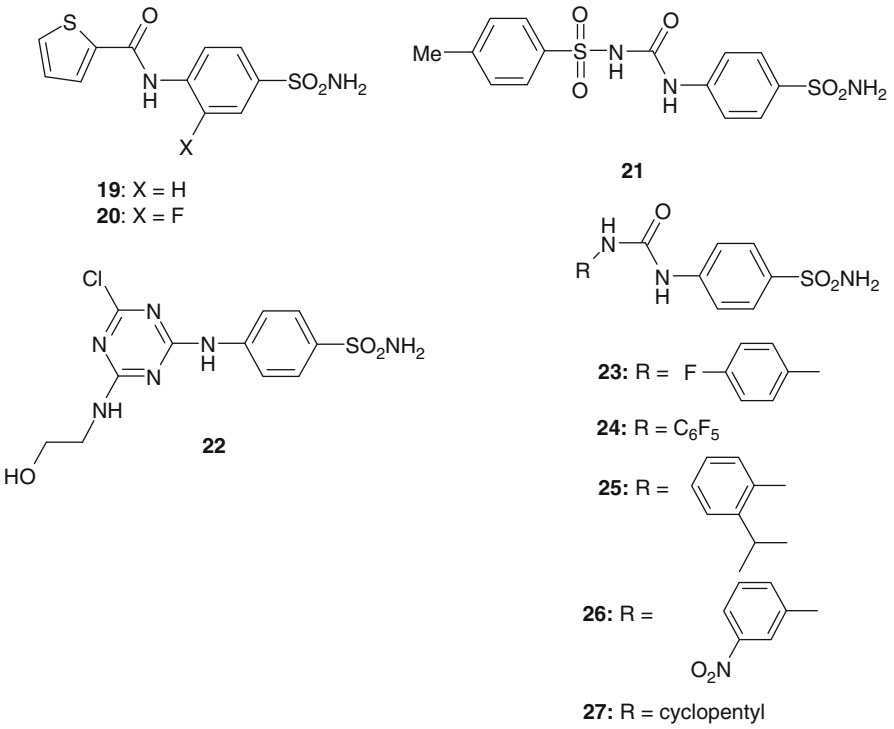


Fig. 15.1 (continued)

The main scope of the drug design campaigns in the last years has been to obtain isoform-selective CAIs for the various isoforms involved specifically in different pathologies, as detailed above. This is however not an easy task, considering that the 13 catalytically active isoforms have an active site architecture quite similar to each other [28, 33–36]. In fact, all human isoforms have the three conserved His residues (His94, 96 and 119) as zinc ligands, two other conserved residues acting as "gate keepers", i.e., Thr199 (hydrogen bonded through its -OH group with the water molecule/hydroxide ion coordinated to the zinc) and Glu106 (hydrogen bonded to Thr199, as well as half the active site mainly lined with hydrophobic residues and the opposite one with hydrophilic residues) [28, 33–36]. However, there are also important differences in amino acid residues mainly in the middle and towards the exit of the active site cavity [1, 4, 28, 33–36]. Most of the inhibitors mentioned above, of types **1**–**15**, do not make extensive contacts with them as their are rather compact molecules binding deep within the active site cavity which is rather similar in all mammalian isoforms [28, 33–36]. Thus, it appeared of considerable interest to devise alternative approaches for obtaining isoform-selective CAIs. One of the most successful one was termed "the tail approach" [37, 38]. It consists of appending "tails" to the scaffolds of aromatic/heterocyclic sulfonamides possessing derivable

moieties of the amino, imino or hydroxy type, in such a way that an elongated molecule is obtained with its tail being able to interact with amino acid residues from the middle and the edge of the active site cavity [1, 37, 38]. By choosing tails with a diverse chemical nature, it was also possible to modulate the physico-chemical properties of such CAIs, which are crucial for their biological activity [1, 37, 38]. For example, antiglaucoma agents should possess an appropriate hydrophilicity and water solubility (in order to be formulated as eye drops) but also a balanced lipophilicity (for being able to penetrate through the plasma membranes and arrive at the ciliary processes where the enzymes responsible for aqueous humor secretion are found, such as CAs II, IV and XII) [37–39].

For targeting CAs IX and XII, which are transmembrane isoforms with an extracellular active site, and are predominantly found in tumors [40], charged, membrane-impermeable derivatives are preferred, which selectively inhibit only these and not the cytosolic/mitochondrial CAs [41–45]. These and other types of compounds could be obtained easily by this simple approach. Several examples will be provided in the following part of the chapter for the design of antiglaucoma hybrid drugs and antitumor sulfonamides targeting hypoxia. It should be noted that as this is a very dynamic field, in which many classes of compounds are being synthesized constantly and their structure in complex with various isoforms are reported every now and then. Due to space limitations, many interesting cases will be not discussed here. We concentrate the remaining part of this review on several relevant examples for which the structure-based drug design was also completed by in vivo data which may validate these derivatives as candidates for clinical trials.

Glaucoma is a disorder encompassing a group of ophthalmological diseases a common factor of which, is the occurrence of an optic neuropathy believed to be due to elevated intraocular pressure (IOP) [46, 47]. 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 [46, 47]. Sulfonamide CAIs, among which acetazolamide **1**, dichlorophenamide **5** (systemically-acting agents) as well as dorzolamide **6** and brinzolamide **7** (topically-acting drugs), are commonly used antiglaucoma agents [1, 47]. They reduce bicarbonate secretion in the aqueous humor by inhibiting CA isoforms present in the ciliary processes (CA II, IV and XII) with a consequennt reduction of IOP [46, 47]. Nitric oxide (NO), a radical gas produced by the enzyme nitric oxide synthase (NOS) is also involved in vasodilation, aqueous humour outflow within the eye, local modulation of ocular blood flow and retinal ganglion cells death by apoptosis [39, 48, 49]. 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 by using the tail approach mentioned above [39, 48, 49]. In this way, a large number of sulfonamides with -NO donating properties were reported in the last several years, among which those of types **16–18** were the most interesting ones [39, 48, 49]. Several aromatic/heterocyclic sulfonamide scaffolds have been used to synthesize compounds incorporating NO-donating moieties of the nitrate ester type [48, 49]. Some of the new compounds showed effective in vitro inhibition of the

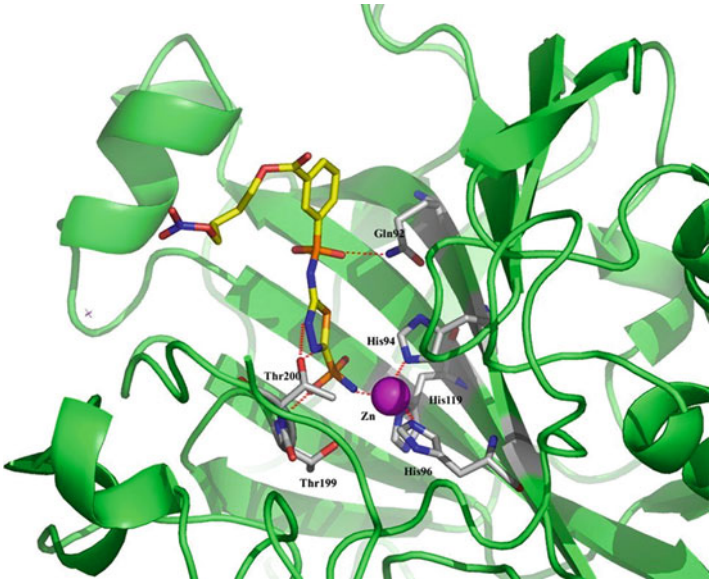


Fig. 15.2 hCA II in adduct with the sulfonamide incorporating an NO-donating moiety **18**, as obtained by X-ray crystallography [49]

target isoforms involved in glaucoma (in the low nanomolar range), and the X-ray crystal structure of one of them (compound **18**) revealed factors associated with this marked inhibitory activity (Fig. 15.2).

Compound **18** is a benzolamide derivative possessing a *meta*-COOH moiety on the phenyl ring which has been derivatized as a nitrate ester with an aliphatic, normal C_4 chain [49]. As for other hCA II-sulfonamide adducts investigated earlier [50–59], the deprotonated sulfonamide moiety of **18** was found coordinated to the Zn(II) ion at a distance of 1.96 Å [49]. The same NH group made a hydrogen bond with the OH of Thr199 (of 2.9 Å). Furthermore, the two endocyclic nitrogen of the 1,3,4-thidiazole ring participate in two hydrogen bonds (of 2.5–2.8 Å) with the -OH of Thr200, as reported earlier for a structurally related compounds [50–59]. One oxygen of the secondary SO_2 moiety of inhibitor **18** made a hydrogen bond (of 3.0 Å) with the NH_2 of Gln92. Due to the *meta*-substituent of the phenyl moiety present in **18** the conformation of the compound when bound to the hCA II active site is rather particular (Fig. 15.2). Indeed, it may be observed that the amino-1,3,4-thiadiazolyl-2-sulfamoyl moieties of this inhibitor is buried deep within the active site, as for other 1,3,4-thiadizoles for which the structure in adducts with various CAs have been reported [33, 34, 50]. 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) bind in an extended conformation which prolongs towards 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) which makes a large number of favorable interactions with various amino acid residues from the enzyme active site [49]. In an animal model of ocular hypertension, one of these new compounds incorporating NO-donating moieties, more precisely **16**, was twice more effective than dorzolamide in reducing elevated IOP characteristic of this disease, anticipating their potential for the treatment of glaucoma [49]. A detailed pharmacologic study of **16** was also reported thereafter [39]. 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, as they were observed bound in an interesting way within the active site of the enzyme. These compounds also showed promising in vivo action in animal models of glaucoma [39, 48, 49].

In the previous paragraphs the role of the tail present in a sulfonamide CAI for the binding of the inhibitor within the active site, and its influence on the inhibition profile against the many mammalian isoforms was discussed. However, small structural changes in the ring on which the sulfonamide zinc-binding group (ZBG) is appended, may also markedly influence the binding of the sulfonamide to the enzyme. This has been demonstrated in a recent work [54] in which the thienyl-carboxamido benzenesulfonamides **19** and **20** were crystallized bound to hCA II.

These two sulfonamides differ only by the presence of a fluorine atom in *meta* to the sulfonamide moiety in compound **20**. By means of X-ray crystallography it has been shown that the benzenesulfonamide fragments of the two compounds bind in a superposable manner to the enzyme [and in a similar fashion to most benzenesulfonamides crystallized up until now in complex with CAs [50–53]] but the orientation of the two thienylcarboxamide fragments was variable, as it also was the inhibition profile of these compounds against various CAs. For example, **20** was a weak hCA II inhibitor (K_I of 390 nM) whereas **19** was much more effective one (K_I of 50 nM) [60, 61]. Indeed, these compounds and some of their congeners showed isoforms selectivity for inhibiting CA VA/VB and CA VII [60, 61]. For example, **20** was a CA VII-selective inhibitor (over CA II), with a K_I for CA VII of 7 nM and a selectivity ratio for the inhibition of the two isoforms of 55.7 [60]. None of the clinically used sulfonamides shows such a selectivity ratio for inhibiting the brain enzyme CA VII [1].

Other interesting structure-based examples for obtaining isoform selective CAIs of the sulfonamide type are **21**–**27** which will be discussed here as tumor-associated, CA IX/XII-selective derivatives. Compound **21** incorporates a tosylureido tail [62]. This class of derivatives has been reported earlier [62] but only recently it was observed an interesting selectivity profile for inhibiting the transmembrane, tumor-associated isoforms (CAs IX and XII) over the cytosolic off-target CA II [56]. Indeed, **21** has K_Is of 12 nM against hCA II, 1.3 nM against hCA IX and 1.5 nM against hCA XII; whence, a selectivity ratio of 9.2 (hCA IX versus hCA II) and of 8.0 (hCA XII versus hCA II) [56]. However, the most CA IX/XII-selective compounds reported up until now are those incorporating triazinyl tails, of which **22** is an interesting example [56, 63, 64]. Compound **22** had inhibition constants of 1098 nM against hCA I, 37 nM against hCA II, 0.7 nM against hCA IX and 1.6 nM

against hCA XII; whence, a selectivity ratios of 49.3 (CA IX versus CA II) and 23.1 (CA XII versus CA II) [56]. Some congeners of **22** showed even higher selectivity ratios (up to 700) for inhibiting the tumor-associated isoform hCA IX over hCA II [63, 64]. By comparing these inhibition data with the X-ray crystallographic structure of **21** and **22** in complex with hCA II [56], it was observed that the benzenesulfonamide fragment present in the two inhibitors binds in the expected manner (coordinating to the Zn(II) ion), being superposable for the two compounds. However, the tosylureido and substituted-triazinyl tails of the two compounds adopted extended conformation orientated towards opposing parts of the active site cavity [56]. This surely was reflected in the different inhibition/selectivity profiles of the two compounds, which were mentioned above.

Undoubtedly, the most interesting case of CA IX-selective compounds is furnished by derivatives **23**–**27**, which incorporate again the benzenesulfonamide head, but this time 4-aryl/alkylureido tails [45, 55]. A large series of such compounds has been reported [45] and for many of them good selectivity ratios (in the range of 16–53) for inhibiting CA IX over CA II were detected [45]. Another interesting feature for this series of compounds was that some of its members were also excellent hCA II inhibitors [in addition to strongly inhibiting the tumor-associated isoforms hCA IX and XII, in the low nanomolar range [45]]. For example, the inhibition constants of compounds **23**–**27** against hCA II were 96 nM, 50 nM, 3.3 nM, 15 nM and 226 nM, respectively [55]. The K_Is for the inhibition of hCA IX were 45 nM, 5.4 nM, 0.5 nM, 0.9 nM and 7.3 nM, respectively [45, 55]. By resolving the X-ray crystal structures of five such sulfonamides bound to hCA II, it was observed that the benzenesulfonamide fragments of the inhibitors were again quite superposable; whereas, the tails adopted a variety of conformations and orientations within the enzyme active site. This variability of binding was probably made possible by the flexible ureido linker between the benzenesulfonamide part and second moiety of the 1,3-disubstituted ureas **23**–**27**. Indeed, some of the groups from the terminal part of these molecules were observed in the hydrophobic pocket, others in the hydrophilic one and some of them between these two binding sites. Such different orientations may explain the range of inhibitory activity against hCA II (3.3–226 nM) and obviously hCA IX, as well as the selectivity ratios for inhibiting the two isoforms. However, a very interesting feature of some of these compounds (e.g., **26**) was their potent inhibition of growth of primary tumors and metastases in an animal model of breast cancer which strongly over-expresses CA IX [44, 45, 65]. In similar tumor cell lines without CA IX, no inhibition of the tumor growth has been observed, which demonstrated that the drug target of these compounds is indeed CA IX [44, 45, 65].

It may be observed from all data presented above that the sulfonamides dominated the drug design landscape of CAIs for many years. However, recently, new important chemotypes have emerged. Among them, the dithiocarbamates (DTCs) are undoubtedly very interesting ones [59, 66–68]. These compounds have been rationally discovered as CAIs after the report of trithiocarbonate (CS_3^{2-}) as a weak (milli – micromolar) CAI [14]. In the X-ray crystal structure of this inorganic anion bound to CA II, a monodentate coordination of the inhibitor is observed via one

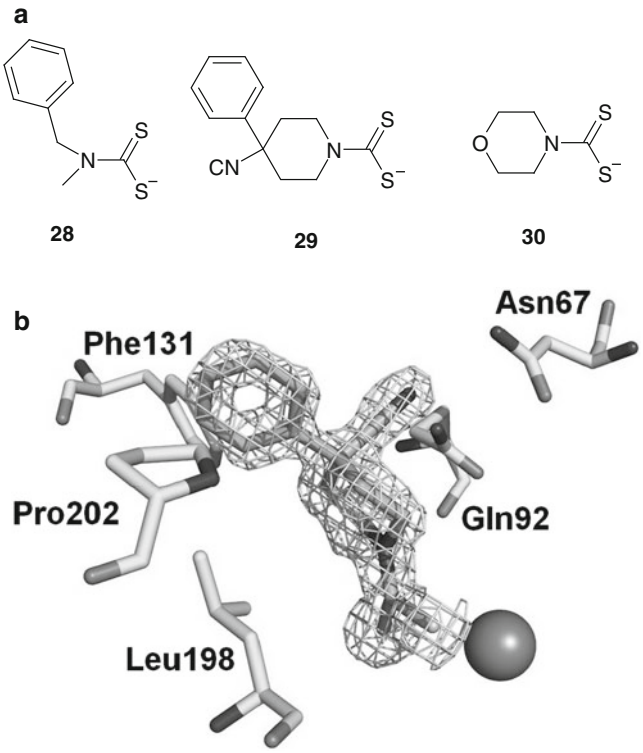


Fig. 15.3 **Panel A.** Structure of DTCs **28–30**. **Panel B.** Electronic density for the adduct of dithiocarbamate **29** bound within the active site of hCA II [59]. The zinc ion is shown as the central sphere and the amino acid residues involved in the binding are evidenced and numbered (hCA I numbering system) [59]

sulfur atom to the zinc ion from the enzyme active site, and a hydrogen bond in which another sulfur and the -OH of Thr199 were involved. Thus, the CS_2^- was discovered as a new ZBG. As DTCs incorporate this new ZBG, a rather large series of such compounds was then prepared and evaluated for their inhibitory activity against several mammalian, fungal and bacterial CAs [59, 66–68]. Several low nanomolar and subnanomolar CAIs were thus detected against all these isoforms. The X-ray crystal structures were also reported for three DTCs complexed to hCA II, compounds **28–30** (Fig. 15.3). DTCs **28–30** inhibited hCA II with K_Is of 25 nM, 41 nM and 0.95 nM, respectively, and hCA IX with K_Is of 53 nM, 757 nM and 6.2 nM, respectively [66]. As seen in Fig. 15.3, the binding mode of the ZBG present in **29** is identical to that of trithiocarbonate (with one sulfur coordinated to the metal ion), but the organic scaffold present in the investigated DTCs was observed to make extensive contacts with many amino acid residues from the active site, which may explain the wide range of inhibitory power of these derivatives [from the subnanomolar to the micromolar, for the entire series of around 30

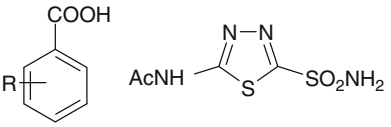
DTCs reported so far [59, 66]]. Interestingly, the highly water soluble DTC **30** was also effective in vivo as an antiglaucoma agent when administered topically directly into the eye of hypertensive rabbits [66], a widely used animal model of glaucoma [39].

3 Drug Design Studies Targeting Fungal CAs with Sulfonamides, DTCs and Carboxylates

β-CAs were recently characterized in a high number of human pathogens, such as *Candida albicans*, *Candida glabrata, Cryptococcus neoformans*, *Saccharomyces cerevisiae* etc. [26, 69, 70]. Inhibition of such enzymes has been investigated with sulfonamides/sulfamates of types **1**–**14** among others [26, 27] and more recently with DTCs [67] and carboxylates [71]. Several ureidosulfonamides similar to those discussed above (compounds **23**–**27**) [27] or DTCs from the same series as compounds **28**–**30** [67] showed interesting inhibitory activity (low nanomolar inhibition) against many of the β-CAs from these fungal pathogens, but they will be not discussed here in detail. Instead, we will discuss some carboxylates as fungal β-CA inhibitors since the COO^- ZBG was less investigated in the drug design of CAIs until recently [11, 71]. Both aliphatic and aromatic carboxylates were investigated as inhibitors of α- and β-CAs in the recent period [11, 71]. The most interesting data have been obtained for the aromatic derivatives **31**–**46** shown in Table 15.1 [71].

It may be observed that these aromatic carboxylates (benzoic acid derivatives with various substitution patterns at the aromatic ring) were micromolar inhibitors of hCA I, hCA II (human off-target isoforms), Can2, Nce103, and β-CAs from the fungal pathogens. However, some of them showed low nanomolar activity against the fungal pathogenic enzymes. For example, 4-hydroxy- and 4-methoxy-benzoate inhibited Can2 with K_Is of 9.5–9.9 nM and Nce103 with inhibition constants of 0.1–0.2 μM. The methyl esters, hydroxamates, hydrazides and carboxamides of some of these derivatives were also effective inhibitors of these α- and β-CAs. Unfortunately, no X-ray crystal structures for the binding of these inhibitors to the fungal enzymes are available but Can2 has been co-crystallized in complex with acetate, a weak inhibitor [26]. Acetate was found directly bound to zinc in a monodentate manner, substituting the zinc-bound water molecule, with the metal ion in a slightly distorted tetrahedral geometry [26, 70]. Thus, the structure-based drug design of CAIs targeting the fungal enzymes is still in its infancy, due to the very limited number of X-ray structures of enzyme-inhibitor adducts available at this moment [26, 70]. It is anyhow rather probable that the benzoates/DTCs investigated later [67, 71] bind in a similar manner to the enzyme but the detailed interactions in which the organic scaffold participate are unknown for the moment.

Table 15.1 Inhibition constants of aromatic carboxylates **31**–**46** against isozymes hCA I and II (α-CA class), and β-CAs Can2 (from *Cryptococcus neoformans*) and Nce103 (from *Candida albicans*), for the CO_2 hydration reaction, at 20°C. The standard sulfonamide inhibitor acetazolamide (**1**) was also included as standard [71]



31-46 1

No	R[a]	K_I (μM)[b] hCA I[c]	hCA II[c]	Can2[d]	Nce103[d]
31	H	730	30	1,267[c]	24.4[c]
32	2,3,5,6-F_4-	74	6	49.1[c]	61.6[c]
33	4-F-	7.39	3.34	0.13	1.09
34	4-Cl-	7.61	3.81	3.40	2.65
35	4-Br-	8.00	4.70	4.24	4.18
36	4-I-	8.18	5.85	5.46	5.17
37	4-O_2N-	8.80	8.56	0.12	4.85
38	4-HO-	7.61	7.28	0.0095	0.11
39	4-MeO-	9.06	8.03	0.0099	0.15
40	4-H_2N-	8.40	8.13	0.12	2.95
41	3-H_2N-	8.79	8.63	3.06	3.40
42	4-NC-	6.92	6.79	0.14	4.47
43	4-AcHN-	4.04	7.28	0.32	0.34
44	4-$ClCH_2$-	3.88	4.36	3.65	4.71
45	4-Me_2N-	3.94	3.33	0.13	5.20
46	4-Cl_2NSO_2-	3.88	1.96	0.12	4.97
1		0.25	0.012	0.010	0.13

[a]As sodium salt
[b]Errors were in the range of 3–5 % of the reported values, from three different assays
[c]Human cloned isoforms, assay at pH 7.5
[d]Fungal enzymes, at pH 8.3

4 Targeting Bacterial CAs

Inhibition of bacterial CAs was recently reviewed [8]. Many pathogenic bacteria encode such enzymes belonging to the α-, β-, and/or γ-CA families [8]. In the last years, the α-CAs from *Neisseria spp*. and *Helicobacter pylori* as well as the β-class enzymes from *Escherichia coli, H. pylori, Mycobacterium tuberculosis*, *Brucella spp., Streptococcus pneumoniae*, *Salmonella enterica Haemophilus influenzae* and *Vibrio cholerae* have been cloned and characterized in detail [72–86]. For some of these enzymes the X-ray crystal structures were determined, and in vitro and in vivo inhibition studies with various classes of inhibitors, such as anions, sulfonamides and sulfamates reported, but no X-ray structures in complex with inhibitors are available so far. Bacterial CAs represent promising targets for obtaining antibacterials devoid of the resistance problems of the clinically used such agents but further studies are needed to validate these and other less investigated enzymes as novel drug targets [72–83].

Table 15.2 CAs from pathogenic bacteria cloned and characterized so far, and their inhibition studies [8]. The genome of many other bacteria contain CAs which have not yet been cloned and characterized

			Inhibition study	
Bacterium	Family	Name	In vitro	In vivo
Neisseria gonorrhoeae	α	–	Sulfonamides, anions	Sulfonamides
Neisseria sicca	α	–	Sulfonamides	Sulfonamides
Helicobacter pylori	α	hpαCA	Sulfonamides, anions	Sulfonamides
	β	hpβCA	Sulfonamides, anions	Sulfonamides
Escherichia coli	β	–	NI	NI
Haemophilus influenzae	β	HICA	Bicarbonate	NI
Mycobacterium tuberculosis	β	mtCA 1	Sulfonamides	Phenols
	β	mtCA 2	Sulfonamides	NA
	β	mtCA 3	Sulfonamides	NA
Brucella suis	β	bsCA 1	Sulfonamides	Sulfonamides
	β	bsCA2	Sulfonamides	Sulfonamides
Streptococcus pneumoniae	β	PCA	Sulfonamides, anions	NI
Salmonella enterica	β	stCA 1	Sulfonamides, anions	NI
	β	stCA 2	Sulfonamides, anions	NI
Vibrio cholerae	α	VchCA	Sulfonamides, anions	NI

- means not named, *NA* no activity in vivo (presumably due to penetration problems), *NI* not investigated

The enzymes which have been so far characterized in detail are shown in Table 15.2. It can be observed that there are detailed data regarding the CAs present in their genome only for nine bacterial pathogens and some of them cause serious infections worldwide, with important antibiotic resistance problems too [8]. Many of them contain more than one such enzyme (e.g., *M. tuberculosis* has 3 β-CAs; *B. suis* two such enzymes, *H. pylori* one α- and one β-CA, etc.), and in many cases there are members both from the α- and β-class in the same pathogen (e.g., *H. pylori*). However, most bacteria for which at least part of the genome was sequenced contain such enzymes and the near future will probably see important developments. As for fungi, it seems that the β-CAs are the most abundant such enzymes among bacteria.

As shown in Table 15.2, the inhibition of these enzymes has been studied in vitro with the main classes of inhibitors, i.e., inorganic anions and sulfonamides/sulfamates [the clinically used derivatives **1**–**14** as well as compounds which were designed on purpose to target the bacterial enzymes [72–81]]. The *M. tuberculosis* CAs are the most investigated ones due to the extensive drug resistance problems of this pathogen to the clinically used antibiotics. Thus, the three CAs from this bacterium were investigated also for their inhibition with DTCs [68] and carboxylates [83].

Many sulfonamide/sulfamate CAIs showed low nanomolar in vitro inhibition against many bacterial CAs [72–81], but in vivo, only for *Nessseria spp., H. pylori, B. suis*, and *S. pneumoniae* enzymes it has been possible to evidence inhibition of

bacterial growth in vivo [8, 84, 85]. Probably the lack of correlation between the in vitro CA inhibition and in vivo inhibition of growth of the bacterial pathogen is due to penetration problems of the sulfonamides, which are highly polar molecules [8]. However, very recently, in vivo inhibition of growth of *M. tuberculosis* has been evidenced for several phenol type CAIs [87].

Indeed, in the last period, interesting chemotypes different of the sulfonamides were investigated as bacterial CAIs [68, 83]. Thus, a series of *N*-mono- and *N,N*-disubstituted DTCs of types **47**–**73**, recently discovered CAIs as shown earlier [59], were investigated as inhibitors of two β-CAs from the bacterial pathogen *M. tuberculosis*, mtCA 1 (Rv1284) and mtCA 3 (Rv3273) [68] – Table 15.3. Both enzymes were inhibited with efficacies between the subnanomolar to the micromolar one, depending on the substitution pattern at the nitrogen atom from the DTC ZBG. Aryl, arylalkyl-, heterocyclic as well as aliphatic and amino acyl such moieties led to potent mtCA 1 and 3 inhibitors in both the *N*-mono- and *N,N*-disubstituted dithiocarbamate series. For example, DTC **70** was a subnanomolar CAI of both mycobacterial enzymes [68]. This new class of β-CA inhibitors may have the potential for developing antimycobacterial agents with a diverse mechanism of action compared to the clinically used drugs for which many strains exhibit multi-drug/extensive multi-drug resistance [68]. No X-ray structures are available so far for adducts of these β-CAs with DTCs, but already the first drug design study identified extremely potent CAIs from this series. Work is in progress to evaluate the efficacy of such compounds in vivo.

The growth of *M. tuberculosis* is known to be strongly inhibited by weak acids although the mechanism by which these compounds act is not well understood [83]. A series of substituted benzoic acids, nipecotic acid, *ortho*- and *para*-coumaric acid, caffeic acid and ferulic acid, of types **74**–**83** were recently investigated as inhibitors of three β-CAs from this pathogen, mtCA 1 (Rv1284), mtCA 2 (Rv3588c) and mtCA 3 (Rv3273) [83] – Table 15.4. All three enzymes were inhibited with efficacies between the submicromolar to the micromolar one, depending on the scaffold present in the carboxylic acid. mtCA 3 was the isoform mostly inhibited by these compounds (K_Is in the range of 0.1–1.0. μM); followed by mtCA 2 (K_Is in the range of 0.6–8.1 μM) whereas against mtCA 1 these carboxylic acids showed inhibition constants in the range of 2.2–7.1 μM (Table 15.4). This class of relatively under explored β-CAIs warrants further, in vivo studies, as they may have the potential for developing antimycobacterial agents with a diverse mechanism of action compared to the clinically used drugs for which many strains exhibit multi-drug or extensive multi-drug resistance [83].

Table 15.3 Inhibition data of the human (h) α-CA isoforms hCA I and II, and mycobacterial β-CA isoforms mtCA 1 and 3 with dithiocarbamates **47–73** by a stopped-flow, CO_2 hydrase assay [68]

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

47-73

			K_I (nM)[a]				
Cmpnd	R^1	R^2	hCA I	hCA II	mtCA 1	mtCA 3	M
47	H	Ph	4.8	4.5	5.6	2.5	Et_3NH
48	H	$O((CH_2CH_2))_2N$	4.8	3.6	6.1	2.4	K
49	H	$MeN((CH_2CH_2))_2N$	33.5	33.0	4.7	2.6	K
50	H	2-butyl	21.1	29.4	6.0	3.6	K
51	H	$O((CH_2CH_2))_2N(CH_2)_2$	31.8	36.3	7.1	2.8	K
52[b]	H	$N((CH_2CH_2)N)_3$	31.9	13.5	4.2	4.0	K
53	H	$PhCH_2$	4.1	0.7	7.1	87.3	Na
54	H	4-PyridylCH_2	3.5	16.6	5.4	5.7	Et_3NH
55	H	$((CH_2)_5N)CH_2CH_2$	4.5	20.3	9.1	8.8	K
56	H	2-thiazolyl	3.9	4.6	89.4	9.5	Et_3NH
57	H	$KOOCCH_2$	13.1	325	7.8	8.3	K
58	H	imidazol-1-yl-$(CH_2)_3$	8.6	24.7	5.3	8.7	A
59	Me	Me	699	6,910	893	659	Na
60	Et	Et	790	3,100	615	431	Na
61		$(CH_2)_5$	0.96	27.5	90.5	4.1	Na
62	n-Pr	n-Pr	1,838	55.5	74.8	80.0	Na
63	n-Bu	n-Bu	43.1	50.9	81.7	72.8	Na
64	iso-Bu	iso-Bu	0.97	0.95	86.2	43.0	Na
65	n-Hex	n-Hex	48.0	51.3	95.4	51.7	Na
66	Et	n-Bu	157	27.8	91.6	63.5	Na
67	$HOCH_2CH_2$	$HOCH_2CH_2$	9.2	4.0	7.5	6.0	Na
68	Me	Ph	39.6	21.5	25.2	46.8	Na
69	Me	$PhCH_2$	69.9	25.4	72.0	62.5	Na
70		$O((CH_2CH_2))_2$	0.88	0.95	0.94	0.91	Na
71		$NaS_2CN((CH_2CH_2))_2$	12.6	0.92	7.7	8.0	Na
72		$(NC)(Ph)C(CH_2CH_2)_2$	48.4	40.8	93.0	61.2	N
73[c]	(*S*)-$(CH_2CH_2CH$ $CH(COONa))$		2.5	17.3	7.1	6.4	Na
1	acetazolamide		250	12	481	104	–

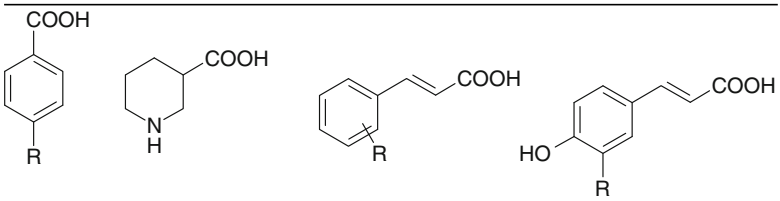
A = imidazol-1-yl-$(CH_2)_3NH_3^+$

[a]Errors in the range of ± 10 % of the reported values, by a CO_2 hydrase assay method [68]

[b]Tris-dithiocarbamate

[c](*S*)-proline dithiocarbamate

Table 15.4 Inhibition data of mycobacterial β-CA isoforms mtCA 1–3 with carboxylic acids **74–83** and sulfanilamide **84** by a stopped-flow, CO_2 hydrase assay [83]



74-78 79 80,81 82,83

		K_I (μM)[a]		
Cmpnd	R	mtCA 1	mtCA 2	mtCA 3
74	Br	3.91	2.66	0.97
75	I	4.94	2.14	0.14
76	CN	6.20	2.12	0.19
77	AcNH	4.76	3.67	0.48
78	NO_2	5.87	8.10	0.52
79	–	4.89	5.27	0.11
80	2-OH	2.25	4.17	0.12
81	4-OH	3.03	1.56	0.60
82	OH	5.92	0.59	0.57
83	OMe	7.13	3.50	0.36
84	sulfanilamide	9.84	29.6	7.11

[a]Errors in the range of ± 10 % of the reported values, by a CO_2 hydrase assay method (from three different measurements) [30]

5 Alternative CA Inhibition Mechanisms: Phenols, Polyamines, Coumarins/Lactones, Sulfocoumarins and Proteinaceous Inhibitors

Phenol **85** was investigated as a CAI several decades ago, but its X-ray crystal structure in adducts with hCA II has been reported only in 1994 by Christianson's group [88]. As shown from the schematic representation of Fig. 15.4A, phenol has a totally new inhibition mechanism compared to the sulfonamides or inorganic anions (the only classes of CAIs known at that time), as this molecule is not coordinated to the Zn(II) ion but anchored to the zinc coordinated water molecule/hydroxide ion (it is most probable that the last species is the one present in the adduct, at the pH of 7.5 at which the experiments were performed [88]). Afterwards, a rather wide range of synthetic and natural product phenols were investigated for the inhibition of most mammalian CAs [89–93]. Many of them were medium potency CAIs, with K_Is typically in the millimolar range, although some more effective compounds (among the natural products with a more complex scaffold [92] were also detected). Recently, Martin and Cohen [94] reported the X-ray structure of several other such compounds (some of which also incorporate carboxylate moieties) which bind in a

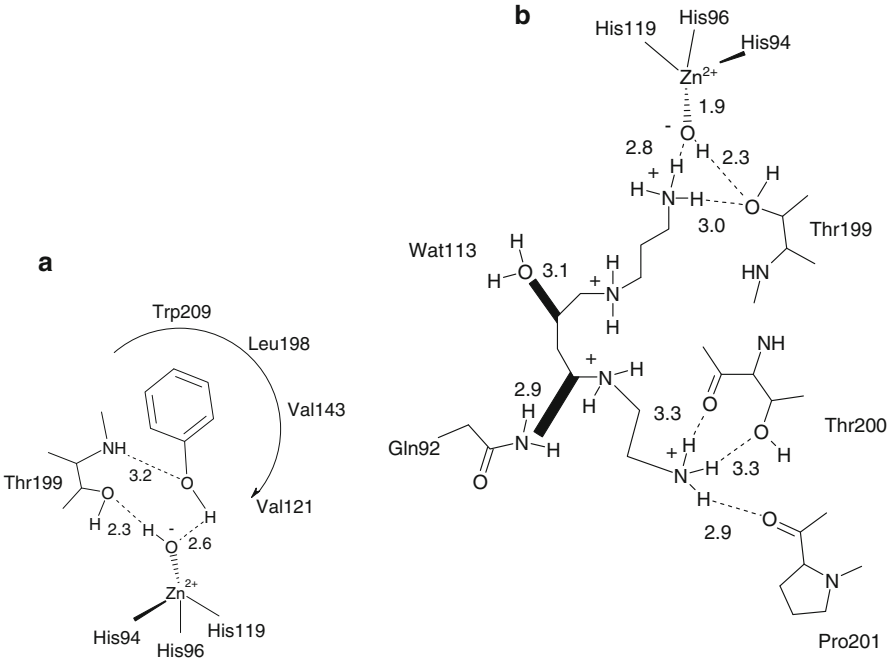


Fig. 15.4 **Panel A.** Schematic representation of the binding of phenol **85** to hCA II as determined by X-ray crystallography [88]. **Panel B.** Schematic representation of interactions in which spermine **86** (as tetracation) participates when bound to the hCA II active site. Figures represent distances (in Å). Hydrogen bonds are represented as dashed lines. In bold are shown two clashes involving some carbon atoms (C5 and C7) of the spermine scaffold with a water molecule (Wat113) and Gln92. The non-protein zinc ligand is represented as a hydroxide ion, which should be the preponderant species at the pH at which the experiments were done (7.5) [18]

fashion similar to the simple phenol **85**, i.e., anchoring to the non-protein zinc ligand by means of a hydrogen bond. Afterwards, the polyamine spermine **86** was also shown to act as a weak CAI and its crystal structure resolved in adduct with hCA II [18]. Unexpectedly, as seen from Fig. 15.4A, spermine has a similar mechanism of CA inhibition with phenol, as one of its ammonium terminal moieties is anchored to the zinc-coordinated water molecule/hydroxide ion by means of a hydrogen bond.

Figure 15.5 shows a superposition of three CAIs bound to hCA II, as determined by X-ray crystallography, the phenol **85**, spermine **86** and the hydrolysed coumarin (*trans*-2-hydroxycinnamic acid) **87** [18, 20, 88]. The first two compounds share the same inhibition mechanism but due to their chemical nature they occupy different parts of the active site. As will be discussed shortly, coumarins constitute a completely different class of CAIs.

Coumarins such as the simple derivative **87a** or the natural product **88a**, isolated from the Australian plant *Leionema ellipticum*, P.G. Wilson (Rutaceae) [19], were recently discovered to act as effective CAIs [19, 20]. Coumarins **87a** and **88a**

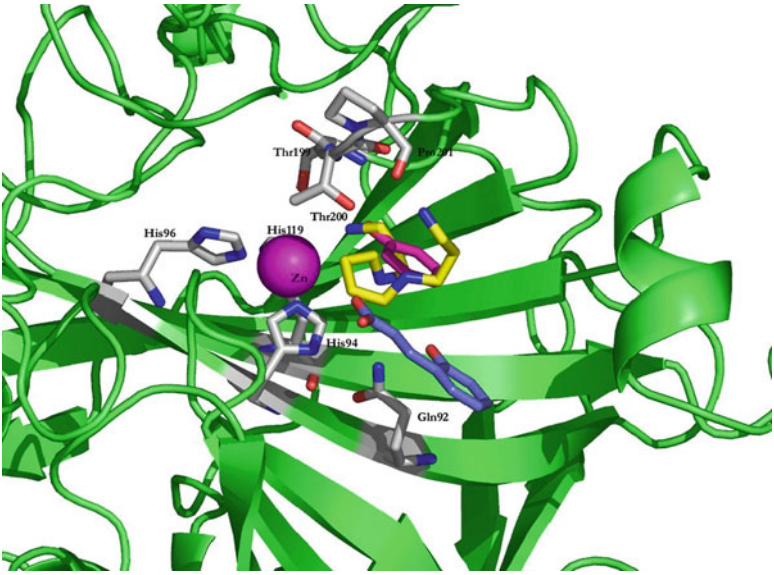
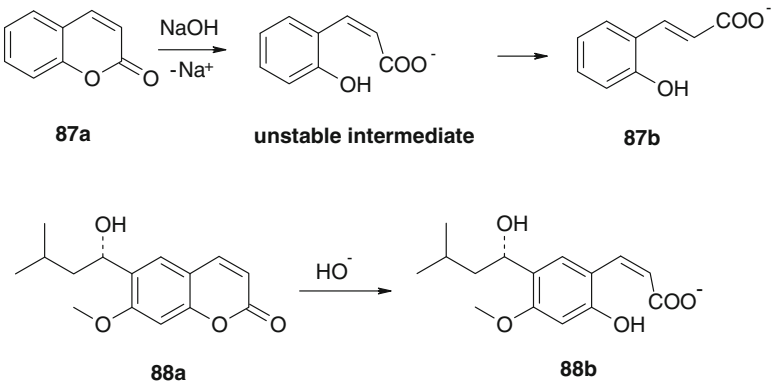


Fig. 15.5 Superimposition of the spermine **86** (*yellow*) [18], phenol **85** (*magenta*) [88] and *trans*-2-hydroxycinnamic acid **87b** [20] (*violet*) adducts with hCA II. The Zn(II) ion is the violet sphere. The Zn(II) histidine ligands as well as residues 92 and 199–201 involved in the binding of inhibitors are also evidenced. One terminal amino moiety of spermine (which hydrogen bonds the non-protein zinc ligand) is fully superimposed on the phenol OH moiety which is interacting with the same group of the enzyme. The phenyl moiety of phenol and the organic scaffold of spermine are not superimposable. 2-Hydroxycinnamic acid **87b** (in *violet*), the hydrolysis product of coumarin **87a** binds towards the exit of the active site and is not superposable with phenol **85** and spermine **86**

were shown to act as "prodrug" inhibitors, being hydrolyzed by the esterase CA activity to the corresponding 2-hydroxy-cinammic acids **87b** and **88b**, which are the de facto CAIs, as shown by kinetic, X-ray crystallographic and MS methods (Scheme 15.2) [19, 20]. Interestingly, these compounds were found bound at the entrance of the CA active site cavity in a region where only CA activators were observed earlier in the many CA – modulators of activity adducts reported in the literature [15, 16]. In this region of the enzyme active site, the classical CAIs such as the sulfonamides, sulfamates, sulfamides, DTCs or other (in)organic anions were never observed [1, 2, 9, 11, 12]. A large number of diversely substituted-coumarins was subsequently screened for their inhibitory activity against all the 13 catalytically active mammalian CA isoforms [95–99]. Thus, coumarins are a relevant class of CAIs because: (i) they are mechanism-based, "prodrug" inhibitors; (ii) they bind in a totally different active site region compared to the classical inhibitors (sulfonamides and congeners); and (iii) they led to highly isoform-selective compounds for many mammalian CA isoforms, such as CAs IX, XII, XIII and XIV among others. In addition, coumarins or they derivatives are easy to synthesize, being possible to incorporate in their molecule a large variety of substitution patterns, which

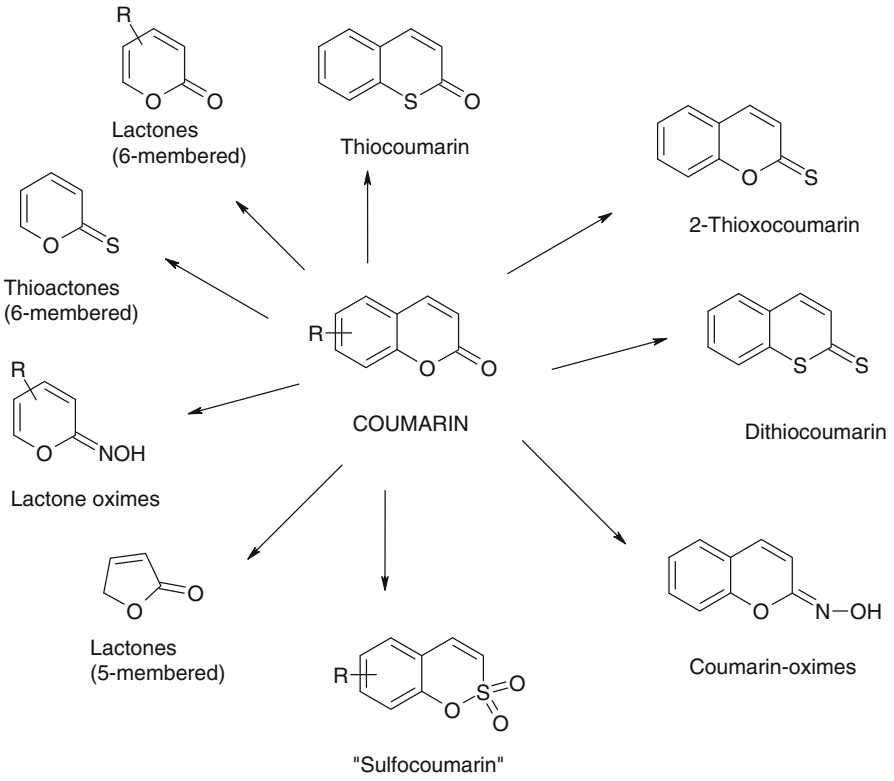


Scheme 15.2 Active site, CA-mediated hydrolysis of coumarins **87a** and **88a** affording the hydroxycinnamic acids **87b** or **88b,** as evidenced by X-ray crystallography or enzyme-inhibitor adducts [19, 20]

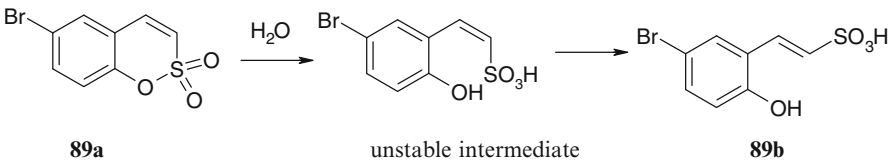
lead to the possibility of exploring a vast chemical space, difficultly accessible for other classes of CAIs. For example, thiocoumarins, 2-thioxocoumarins, dithiocoumarins, coumarin-N-oximes, 5-/6-membered lactones and thiolactones, or lactone oximes were recently investigated for their CA inhibitory properties [95–100] (Scheme 15.3). "Sulfocoumarins", i.e., 1,2-benzoxathiin 2,2-dioxides were also very recently explored as CAIs [101].

As for the coumarins, the sulfocoumarins are mechanism based inhibitors [101]. By means of X-ray crystallography it has been observed (in a mutant hCA II complexed with compound **89a**) that the hydrolysis product of **89a**, i.e., 2-dihydroxy-5-bromophenyl-vinyl sulfonic acid **89b** (Scheme 15.4) perfectly fitted within this electron density (Fig. 15.6).

As seen in Fig. 15.6, the electron density of **89b** is perfectly defined for all its atoms. It is probable that the sulfocoumarin **89a** is initially hydrolyzed (by the zinc hydroxide, nucleophilic species of the enzyme) to the *cis* vinyl sulfonic acid unstable intermediate which subsequently isomerizes spontaneously to the *trans* derivative **89b** (Scheme 15.4) which is observed in the electron density of the CA II/IX – **89a** adduct (Fig. 15.6). Interestingly, the sulfonic acid moiety (presumably as a sulfonate group, at the pH of 7.8 at which the crystallization was done) was not coordinated to the Zn(II) ion but was anchored to the zinc-bound water molecule/hydroxide ion (Fig. 15.6), a binding mode initially observed for phenol and spermine (and discussed above). With the X-ray structure of the sulfocoumarin adduct, it is obvious that anchoring to the zinc-coordinated water molecule may be considered as a quite general CA inhibition mechanism [we prefer to use the term "anchoring to the zinc-coordinated water" for the inhibition mechanism of these compounds, as originally proposed by Christianson [88] over the more recent term "nucleophile recognition" proposed by Martin and Cohen [94]]. The distance between the zinc-coordinated water/hydroxide ion and an oxygen atom of the sulfonate moiety of **89b** was of



Scheme 15.3 CAIs obtained from coumarins as lead molecules [95–101]



Scheme 15.4 Active site CA-mediated hydrolysis of **89a**

2.5 Å. Another oxygen of this moiety was at 2.8 Å from the metal ion, being "half-coordinated". The organic scaffold of **89b** did not participate in other interactions with residues from the enzyme active site, except for the -OH moiety in *ortho* to the ethenylsufonate group. This moiety participated in a bifurcated hydrogen bond with the hydroxyl of Thr200 (of 3.3 Å) and through a bridging water molecule, with the carbonyl oxygen of Pro201 (of 2.2 Å). The relatively few interactions between the inhibitor scaffold and the active site may in fact explain the not so good inhibitory power of this compound against the CA II/IX mimic [K_I of 0.9 μM [101]].

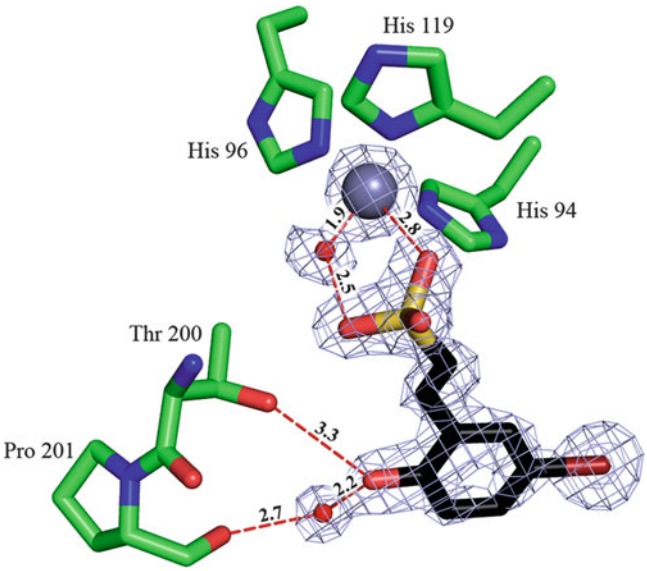
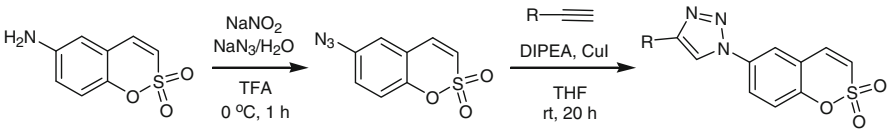


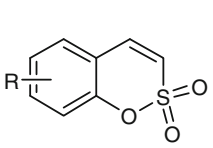
Fig. 15.6 Binding of compound **89b** within the active site of the CA II/IX mimic [101]. The $2F_o$-F_c map was calculated in the absence of ligand and contoured at 1σ. The hydroxyphenylvinylsulfonic acid (in *black*), the zinc ion (*grey sphere*), its ligands (His94, 96 and 119) and water molecules (*red spheres*) are shown. Polar interactions (with Thr200 and Pro201) are shown with dashed lines and the distances are indicated in Å



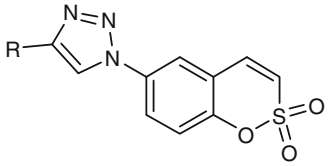
Scheme 15.5 Preparation of derivatives **90–99** by reaction of azide **89j** with alkynes (click chemistry) [101]

A series of mono- or disubstituted sulfocoumarins possessing various functionalities on the benzene ring, of types **89a-i** were reported recently and investigated as CAIs [101]. They were synthesized by an intramolecular aldol cyclization reaction of mesylsalicyl aldehydes in the presence of strong bases, such as 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU). The amine **89 h** was diazotized and subsequently converted to the azide **89j**, which by reaction with alkynes was transformed to the 1,2,3-triazoles **90–99** by the classical click chemistry cycloaddition reaction (Scheme 15.5). Compounds **90–99** incorporated several groups at the triazole ring (such as substituted aryl, carboxyalkyl, substituted silyl, alkylaminomethyl, etc.), being structurally diverse when compared to compounds **89a-i**. These structural variations are essential in order to investigate the structure-activity relationship of this new class of CAIs. Indeed, as seen from Table 15.5, these compounds possessed

Table 15.5 Inhibition of isozymes hCA I, II, IX, XII and a CA II/IX active site mimic with coumarins **87a, 88a**, and sulfocoumarins **89a-89i** and **90–99** [101]



89a-i



90-99

		K_I (μM)[a, b]				
Compound	R	hCA I[c]	hCA II[c]	hCA IX[d]	hCA XII[d]	CA II/IX mimic
87a	–	3.10	9.20	>100	>100	–
88a	–	0.080	0.062	54.5	48.6	–
89a	6-Br	>100	>100	6.83	4.51	0.93
89c	6-OH	91	>100	0.300	0.234	0.80
89d	6-$MeSO_3$	99	>100	0.324	0.254	2.03
89e	6-BnO	93	>100	0.275	0.219	2.37
89f	5,6-Benzo	>100	>100	0.375	0.717	–
89 g	6-O_2N	92	>100	3.77	3.16	–
89 h	6-H_2N	6.78	8.89	0.046	0.023	–
89i	6,8-Cl_2	>100	>100	3.26	2.93	–
90	Ph	6.86	7.76	0.029	0.032	–
91	COOMe	8.05	6.33	0.095	0.012	–
92	COOEt	8.88	9.21	0.086	0.013	–
93	Me_3Si	6.00	7.20	0.060	0.009	–
94	$HOCH_2$	7.20	9.29	0.058	0.016	–
95	Et_2NCH_2	8.11	9.37	0.025	0.007	–
96	4-F_3CO-C_6H_4	8.43	9.64	0.074	0.014	–
97	4-MeO-C_6H_4	8.93	9.35	0.018	0.039	–
98	3-F_3C-C_6H_4	6.71	7.72	0.048	0.013	–
99	3-MeO-C_6H_4	7.47	8.61	0.049	0.021	–
Acetazolamide	–	0.25	0.012	0.025	0.005	–

[a]Errors in the range of ± 10 % of the reported data, from three different assays
[b]Pre-incubation of 6 h between enzyme and inhibitor
[c]Cytosolic full length, recombinant enzyme
[d]Catalytic domain, transmembrane, recombinant isoform

interesting CA inhibitory activity against four isoforms, hCAs I, II, IX and XII. As the coumarins [95–99], many sulfocoumarins were also relatively weak inhibitors of hCAs I and II, whereas they act as much more powerful hCA IX and XII inhibitors (Table 15.5): It may be observed that sulfocoumarins **90–99** were low nanomolar inhibitors of these tumor-associated isoforms, whereas their affinity for hCAs I and II was in the micromolar range. The simpler compounds **89a-g** and **89i** were submicromolar hCA IX/XII inhibitors and did not significantly inhibit hCAs I and II (Table 15.5). Thus, the sulfocoumarins, like the coumarins, lead to isoform-selective CAIs targeting the tumor-associated, transmembrane isoforms hCAs IX and XII [101].

6 Conclusion

By catalyzing the simple but highly important hydration of CO_2 to bicarbonate and protons, CAs are involved in critical steps of the life cycle of many organisms, including eukaryotes, bacteria and archaea. A wealth of X-ray structural data have been accumulated in the last 15 years for CA-inhibitor complexes, including 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 the carboxylates, phenols, polyamines, coumarins/sulfocoumarins and the newly identified DTCs. Although X-ray crystal structures are already available for the majority of the 12 catalytically active hCAs (i.e., isozymes I-VA, IX, XII, XIII and XIV), most of the reported complexes are for isozyme II (and to a less extent isozyme I). These data are relevant for the drug design of isozyme-selective CAIs, and important such advances have been made in the last years, mainly by rationalizing the various subpockets for the binding of sulfonamide/sulfamide inhibitors done by McKenna's group [102, 103]. In fact, the main problem with the classical, clinically used sulfonamides (including also the second generation agents dorzolamide and brinzolamide) is 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 are shown by dorzolamide and brinzolamide, which have been designed in such a way as to act as much weaker CA I than CA II inhibitors, but similar to acetazolamide, methazolamide and ethoxzolamide, these two second generation inhibitors strongly inhibit the remaining ten CA isozymes [1, 3]. Thus, considering only the ZBGs and the organic scaffold, it is practically impossible 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 basically the same between the inhibitors and most CA isozymes with medicinal chemistry applications [1, 50]. This also explains why the first and second generation sulfonamide/sulfamate CAIs were devoid of any isozyme-selectivity. They are indeed rather small, compact molecules which bind deeply within the enzyme active site. However, 13 years ago the "tail-approach" was reported, which afforded a 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, cycloaddition, glycosylation, etc.) [1, 50]. In this way, it was possible both to modulate the physico-chemical properties of the synthesized inhibitors (for example by introducing tails which induce high water solubility, or enhanced lipophilicity, or positive/negative charges which lead to membrane impermeability, or fluorescence or spin-labeled groups, etc.), and also their affinity to the various isozymes, as the tail(s) usually interact with amino acid residues towards the exit of the active site or on its edge [50]. In fact, those are the amino acids which 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 to the classical ones. X-Ray crystal structures and homology modeling are available for some of these compounds, which proved that both favorable interactions as well as clashes with particular amino acids present only in some isozymes, are critical for the inhibition profile and isozyme selectivity issues [50, 102, 103]. In fact, several interesting examples of inhibitors designed by the tail approach, which show efficient antiglaucoma and anticancer/antimetastatic activity in vivo have been discussed in this chapter. The tail approach has been in fact successfully applied also to generate other classes of CAIs, such as DTCs, coumarins/sulfocoumarins and even phenols. By using the diverse inhibition mechanisms of these compounds, these diverse scaffolds may offer advantages over the sulfonamides and their bioisosteres as CAIs targeting a variety of enzymes.

The carboxylate as an alternative ZBG to the sulfonamide one, was intensely studied in the last period, with several interesting developments, especially for the inhibition of fungal β-CAs from pathogenic fungi/yeasts [71]. Such enzymes were also highly inhibited by DTCs, a new class of CAIs targeting both α- and β-class CAs, recently discovered by our group [59]. X-ray crystal structures of DTCs complexed to hCA II allowed a deep understanding of the interactions between enzyme and inhibitor at atomic level. In fact the CS_2^- ZBG found in DTC is an excellent alternative to the sulfonamide ZBG present in the classical CAIs. Furthermore, such compounds are water soluble, easy to prepare and afford the exploration of a wide chemical space, which for the sulfonamides is not always possible (due to synthetic limitations).

In bacteria, many CAs were shown to be important for survival, invasion and pathogenicity. Bacteria encode CAs belonging to the α-, β-, and/or γ-families, but up to now only the first two classes have been investigated in some detail in different species. Indeed, the α-CAs from *Neisseria spp*. and *H. pylori* as well as the β-class enzymes from *E. coli, H. pylori, M. tuberculosis*, *Brucella spp., S. pneumoniae*, *S. enterica, V. cholerae* and *H. influenzae* have been cloned and characterized. For some of these enzymes the X-ray crystal structures were determined at rather high resolution, allowing for a good understanding of the catalytic/inhibition mechanisms. However, no adducts with inhibitors of these enzymes have been characterized so far; although in vitro and in vivo inhibition studies with various classes of inhibitors, such as anions, sulfonamides/sulfamates, DTCs and carboxylates have been reported. Efficient in vitro inhibitors have been reported for many such enzymes, and for various chemotypes, but only for *Nessseria spp*., *H. pylori, B. suis*, *M. tuberculosis* and *S. pneumoniae* CAs it has been possible to evidence inhibition of bacterial growth in vivo. Thus, bacterial CAs represent at this moment very promising targets for obtaining antibacterials devoid of the resistance problems of the clinically used such agents but further studies are needed to validate these and other less investigated enzymes as novel drug targets, and more importantly, structure-based drug design approached are needed for these targets.

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