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To cite this article: Claudiu T. Supuran (2016) How many carbonic anhydrase inhibition mechanisms exist?, Journal of Enzyme Inhibition and Medicinal Chemistry, 31:3, 345-360, DOI: [10.3109/14756366.2015.1122001](https://doi.org/10.3109/14756366.2015.1122001)

To link to this article: <https://doi.org/10.3109/14756366.2015.1122001>



Published online: 30 Nov 2015.



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REVIEW ARTICLE

How many carbonic anhydrase inhibition mechanisms exist?

Claudiu T. Supuran

Neurofarba Department, Laboratorio Di Chimica Bioinorganica, Sezione Di Chimica Farmaceutica E Nutraceutica, Università Degli Studi Di Firenze, Florence, Italy

Abstract

Six genetic families of the enzyme carbonic anhydrase (CA, EC 4.2.1.1) were described to date. Inhibition of CAs has pharmacologic applications in the field of antiglaucoma, anticonvulsant, anticancer, and anti-infective agents. New classes of CA inhibitors (CAIs) were described in the last decade with enzyme inhibition mechanisms differing considerably from the classical inhibitors of the sulfonamide or anion type. Five different CA inhibition mechanisms are known: (i) the zinc binders coordinate to the catalytically crucial Zn(II) ion from the enzyme active site, with the metal in tetrahedral or trigonal bipyramidal geometries. Sulfonamides and their isosters, most anions, dithiocarbamates and their isosters, carboxylates, and hydroxamates bind in this way; (ii) inhibitors that anchor to the zinc-coordinated water molecule/hydroxide ion (phenols, carboxylates, polyamines, 2-thioxocoumarins, sulfocoumarins); (iii) inhibitors which occlude the entrance to the active site cavity (coumarins and their isosters), this binding site coinciding with that where CA activators bind; (iv) compounds which bind out of the active site cavity (a carboxylic acid derivative was seen to inhibit CA in this manner), and (v) compounds for which the inhibition mechanism is not known, among which the secondary/tertiary sulfonamides as well as imatinib/nilotinib are the most investigated examples. As CAIs are used clinically in many pathologies, with a sulfonamide inhibitor (SLC-0111) in Phase I clinical trials for the management of metastatic solid tumors, this review updates the recent findings in the field which may be useful for a structure-based drug design approach of more selective/potent modulators of the activity of these enzymes.

Keywords

Anchoring to zinc-coordinated water, carbonic anhydrase, inhibition mechanism, inhibitors, occlusion of the active site entrance, out of the active site binding, zinc binder

History

Received 11 November 2015

Revised 13 November 2015

Accepted 16 November 2015

Published online 30 November 2015

Introduction

Six distinct genetic families of carbonic anhydrases (CAs, EC 4.2.1.1), the superfamily of metalloenzymes which catalyze the interconversion between CO₂ and bicarbonate are known to date, the α -, β -, γ -, δ -, ζ -, and η -CAs^{1–11}. The catalytic/inhibition mechanisms for the physiologic reaction were reviewed in earlier papers^{12–21}, but the discovery of new classes of CA inhibitors (CAIs) possessing different inhibition mechanisms compared to the classical inhibitors of the sulfonamide/anion type, prompted me to review again this field.

The inhibition and activation of CAs are well-understood processes: most types of classical inhibitors bind to the metal center^{12–21}, 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^{22–24}. This leads to enhanced formation of the metal hydroxide, catalytically active species of the enzyme^{1,21–24}.

CO₂, bicarbonate, and protons are essential molecules/ions in many important physiologic processes in all life kingdoms (*Bacteria*, *Archaea*, and *Eukarya*), throughout the tree of life,

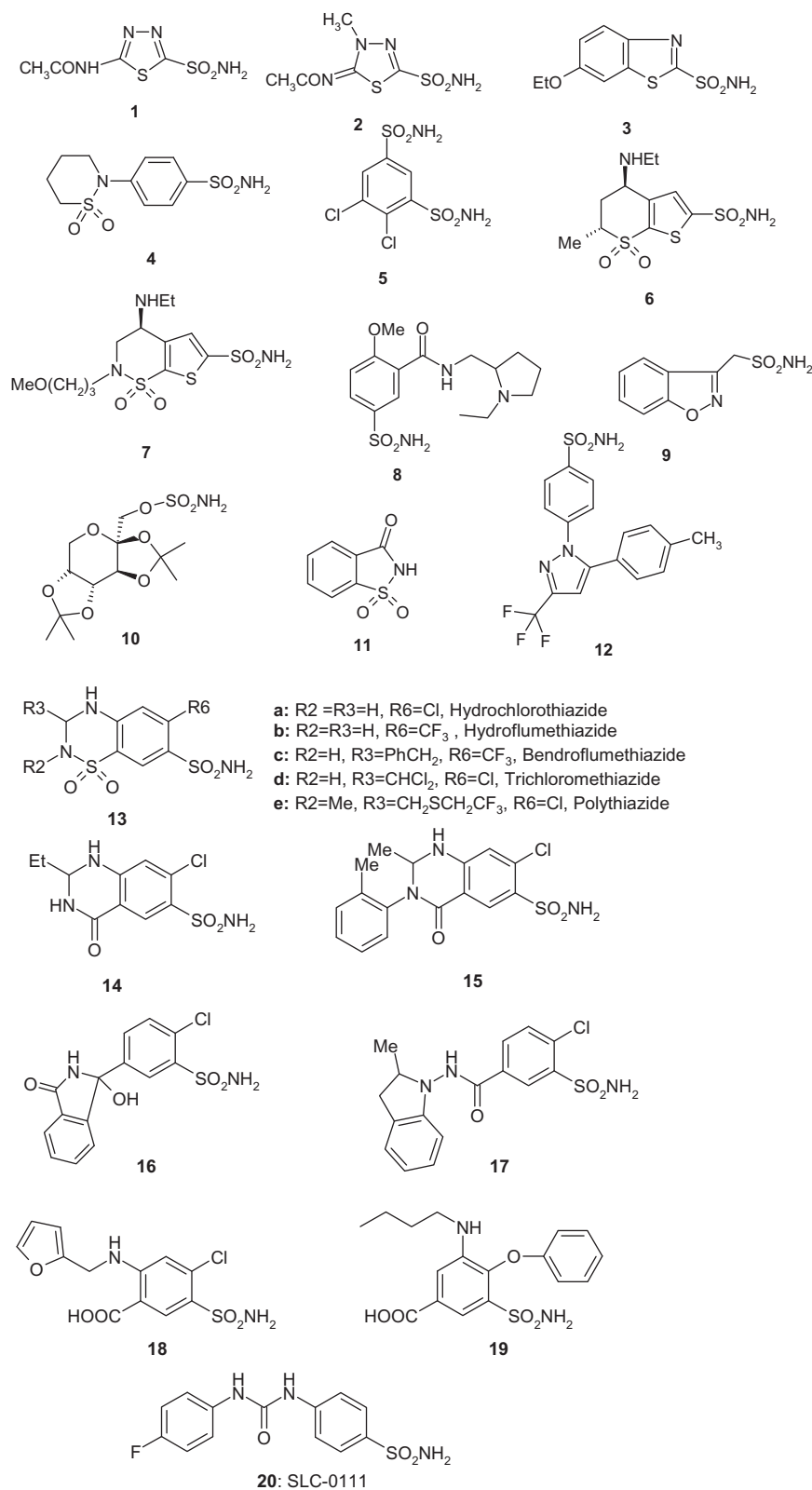
and for this reason, relatively high amounts of CAs are present in different tissues/cell compartments of most investigated organisms^{1–11}. The α -CAs are present in vertebrates, protozoa, algae, and cytoplasm of green plants and in some *Bacteria*^{1,3}; the β -CAs are predominantly found in *Bacteria*, algae and chloroplasts of both mono- as well as dicotyledons, but also in many fungi and some *Archaea*^{1–9}. The γ -CAs were found in *Archaea*, cyanobacteria, and most types of *Bacteria*^{3,25}, the δ - and ζ -CAs seem to be present only in marine diatoms¹¹, whereas the η -CAs in protozoa⁴. In many organisms these enzymes are involved in crucial physiological processes connected with respiration and transport of CO₂/bicarbonate, pH, and CO₂ 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}. In algae, plants, and some bacteria they play an important role in photosynthesis and other biosynthetic reactions^{3,5}. In diatoms δ - and ζ -CAs play a crucial role in carbon dioxide fixation, whereas in protozoa the role of the η -CAs is poorly understood for the moment¹¹. Many such enzymes from vertebrates, nematodes, fungi, protozoa and bacteria are well-known drug targets^{1,3,4,26–43}.

The zinc binders as CAIs

Sulfonamides are the most important class of CAIs^{1,2,5,6,8,16–19}, with at least 20 such compounds in clinical use for decades or

Address for correspondence: Claudiu T. Supuran, Neurofarba Department, Laboratorio di Chimica Bioinorganica, Sezione di Chimica Farmaceutica e Nutraceutica, Università degli Studi di Firenze, Via U.Schiff6, 50019 Sesto Fiorentino (Florence), Italy. Tel: +39-055-4573729. Fax: +39-055-4573385. E-mail: claudiu.supuran@unifi.it

Figure 1. Clinically used sulfonamide and sulfamate CAIs **1–19** and compound **20** in Phase I clinical development.



clinical development in the last period (Figure 1)^{44–79}. They include acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, sulthiame **4**, dichlorophenamide **5**, dorzolamide **6**, brinzolamide **7**, sulpiride **8**, zonisamide **9**, topiramate **10**, saccharin **11**, celecoxib **12**, chlorothiazide/highceiling diuretics of types **13–19**, including the widely prescribed hydrochlorothiazide **13a**, furosemide **18**, bumethanide **19**, compounds in clinical use for many years, as diuretics, antiglaucoma agents,

antiepileptics^{1,2,6–8,19,44–79}. Compound **20**, SLC-0111, is in Phase I clinical trials as an antitumor/antimetastatic agent, and was discovered in the author's laboratory^{19,54,63} (Figure 1). Most of the sulfonamides **1–19** act as potent CAIs and are in clinical use for decades, but their main problem is related to the fact that by inhibiting most of the catalytically active CA isoforms found in vertebrates (12 such isoforms are known to date^{1,21}), they show a wide range of side effects^{1,2,5,6,8,16–19}. Newer generation

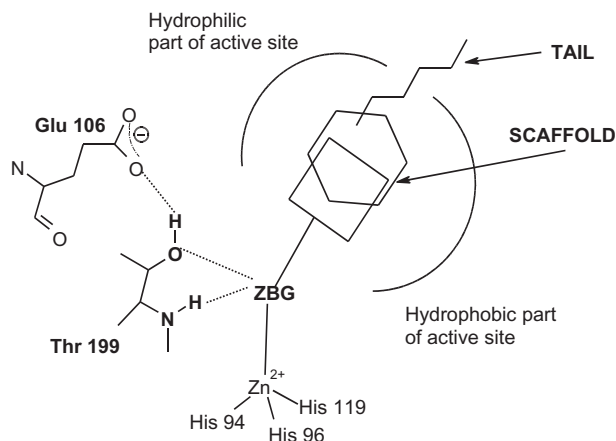


Figure 2. CAIs belonging to the zinc binders (sulfonamides, sulfamates, sulfonamides, carboxylates, hydroxamates, phosphonates). The ZBG is coordinated to the metal ion and participates in strong interactions with the gate keeper residues Thr199–Glu106. The scaffold may occupy either the hydrophilic or hydrophobic (or both) halves of the active site, whereas the tails generally are orientated toward the exit of the cavity where the most variable amino acid residues among the different mammalian isoforms are found^{1,21,80}.

inhibitors, among which **20** is a rather recent example, were designed in such a way as to act as isoform-selective inhibitors for the transmembrane, tumor-associated isoforms hCA IX and XII (h = human), and as thus, they probably will show less side effects compared to the classical CAIs of types **1–19** discussed above^{19,54,63}. Furthermore, sulfonamides and sulfamates strongly inhibit CAs belonging to most families, not only the α -class enzymes, and this may be exploited in the future for obtaining anti-infectives with a new mechanism of action^{1,3–6,9,11,13–18,21}.

A general CA inhibition mechanism with zinc binders, including sulfonamides, sulfamates, and sulfamides (which are in fact sulfonamide isosters) is shown in Figure 2. These CAIs bind in deprotonated form, as anions, to the Zn(II) ion from the enzyme active site, which is in a tetrahedral geometry, with the zinc-binding group (ZBG) (SO_2NH^- moieties for the three main classes of inhibitors mentioned above) directly interacting with the metal ion (Figure 2). There is overwhelming X-ray crystallographic evidence showing this type of binding for sulfonamides, sulfamates, and sulfamides belonging to a variety of chemical classes^{21,44–46,57–65}. In addition, the ZBG also interact with two other conserved residues in all α -CAs, acting as ‘‘gate keepers’’, i.e. Thr199 (hydrogen bonded through its OH group with the water molecule/hydroxide ion coordinated to the zinc in the uninhibited enzyme, and with the ZBG, as shown in Figure 2, in the enzyme-inhibitor adducts) and Glu106, which is hydrogen bonded to Thr199 through its carboxylate moiety (Figure 2)^{21,44–46,57–65}.

The main scope of the drug design campaigns in the last years was to obtain isoform-selective CAIs for the various isoforms involved specifically in different pathologies, as noted above^{1,21,44–46,57–65}. This is however not an easy task, considering that the 12 catalytically active isoforms present in primates, have an active site architecture quite similar with each other^{1,21–24,44–46}. In fact, all human isoforms have the three conserved His residues (His94, 96, and 119) acting as zinc ligands, as well as half the active site mainly lined with hydrophobic residues and the opposite one with hydrophilic residues^{1,21,80}. However, there are also important differences in amino acid residues mainly in the middle and toward the exit of the active site cavity^{1,21,80}. Most of the inhibitors mentioned above, of types **1–9**, do not make extensive contacts with them as their scaffolds are rather compact

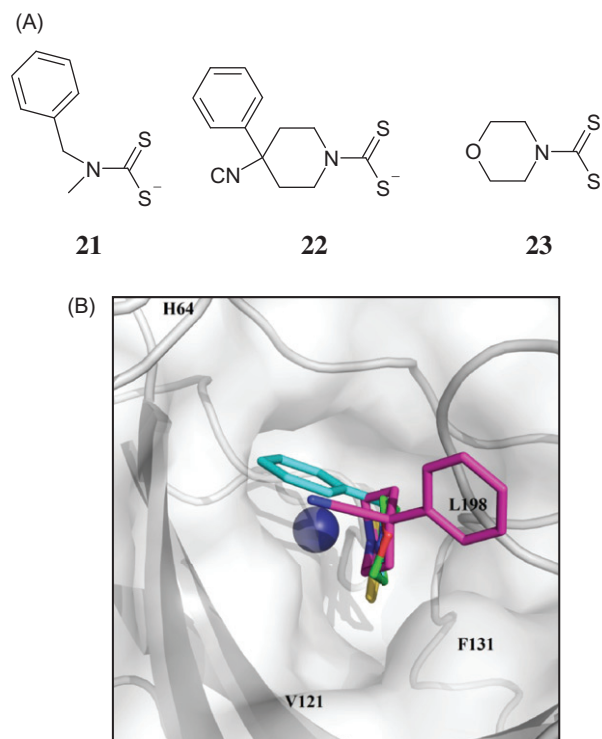


Figure 3. (A) Structure of DTCs **21–23**. (B) View of compounds **21** (cyan), **22** (magenta), **23** (green), superposed in the active site of hCA II. The zinc ion is shown as the central blue sphere and the amino acid residues involved in the binding are evidenced and numbered (hCA I numbering system)⁸³.

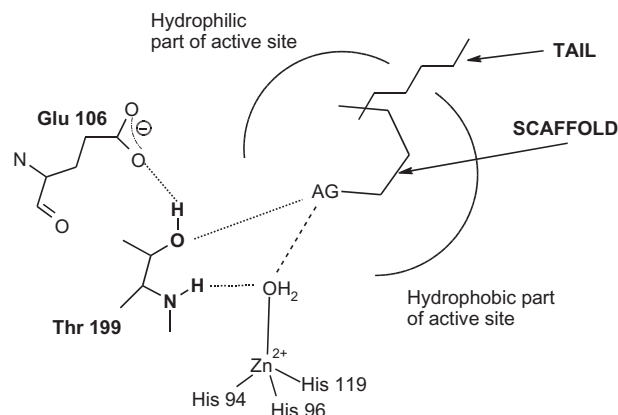
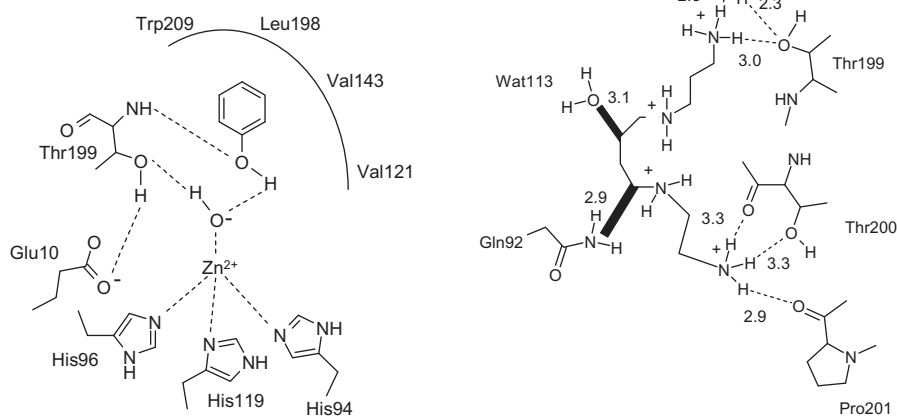


Figure 4. Compounds which anchor to the Zn(II)-coordinated water molecule/hydroxide ion. The anchoring group (AG) may be of the OH (phenol), amino (polyamine), ester (COOR), sulfonate (sulfocoumarin) type or just a simple sulfur atom (thioxocoumarin)^{103–114}.

and these sulfonamides bind deep within the active site cavity which is rather similar in all mammalian isoforms^{1,21,80}. 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’’^{1,21,48,81,82}. It consists in appending ‘‘tails’’ to the scaffolds of aromatic/heterocyclic sulfonamides possessing derivatizable 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 edge of the active site cavity, as shown in Figure 2 for a general zinc-binder CAI^{1,21,48,81,82}. By choosing tails with a diverse chemical nature, it was also possible

Figure 5. The first compounds which inhibit CAs by anchoring to the zinc-coordinated water molecule: (A) Phenol **24**¹⁰³; (B) Spermine **25**¹⁰⁴.



to modulate the physico-chemical properties of such CAIs, which are crucial for their biological activity^{1,21,48,81,82}. 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 (i.e. CA II, IV, and XII)^{48,81,82}. This approach was subsequently expanded to all classes of CAIs, such as among others to the dithiocarbamates (DTCs)^{28–30,83}, xanthates⁸⁴, carboxylates^{31,41,85,86}, phosphonates^{87–92}, and hydroxamates^{93–100}, etc. X-Ray crystal structures are available for adducts of various isoforms (mainly hCA I and II) with all these classes of CAIs except the xanthates for which computational methods were used to assess their inhibition mechanism^{30,83,88,100,101}. For example, DTCs **21–23** were crystallized bound to hCA II⁸³. As seen from Figure 3, one of the sulfur atoms of the inhibitor is coordinated to the Zn(II) ion and makes the canonical interactions of all ZBGs (Figure 2) with the gate keeper residues Thr199–Glu106. For these DTCs, the scaffolds have very diverse orientation within the active site, which may explain their high inhibitory activity against many CA isoforms^{28–30,83}. Using these derivatives as leads, new DTCs were reported recently, which showed better selectivity profiles compared to the first class of DTCs, including **21–23**¹⁰².

Many DTCs showed excellent intraocular pressure (IOP) lowering effects in animal models of glaucoma, being equivalent and superior to the sulfonamide CAIs, and due to their excellent water solubility (and high CA inhibitory action) this class of compounds might be exploited in the future for obtaining antiglaucoma agents^{30,74,102}.

CAIs anchoring to the zinc-coordinated water/hydroxide ion

The first compound for which this new CA inhibition mechanism, i.e. anchoring to the zinc-coordinated water molecule/hydroxide ion, was reported, is phenol **24**¹⁰³. The polyamine spermine **25** was the second one¹⁰⁵. Compounds inhibiting CAs by this mechanism (Figure 4) are characterized by a presence of an anchoring group (AG) which is attached to a scaffold possibly incorporating tails which can interact with the two halves of the active site (as for the zinc binders). The main difference between the zinc binders and the inhibitors anchoring to the zinc

nucleophile consists in the fact that the inhibitor is not in a direct contact with the metal ion. As reported in the last years by several groups, the AGs belong to a variety of chemical functionalities. They include phenolic OH^{104,106–113}, primary amine¹⁰⁵, COOH¹¹³, COOMe¹¹¹, and SO₃H¹¹⁴ moieties. The scaffolds of these compounds can be of the aromatic, aliphatic, heterocyclic, or sugar type^{114–121}, and many of them display efficient inhibitory activity against CAs belonging to the α - and β -CA classes^{105–122}.

As shown in Figure 5(A), phenol **24** is anchored to the non-protein zinc ligand (probably a hydroxide anion at the pH at which the experiments were carried out¹⁰³) by a hydrogen bond involving the H atom of the inhibitor and the donor OH from the zinc hydroxide. In addition, a second hydrogen bond involves the gate-keeping residue Thr199, whose NH group participates in a second hydrogen bond with the phenol (Figure 5A). Spermine **25** binds in a rather similar manner with phenol, although the hydrogen bonding network is slightly different (Figure 5B). Thus, one of the primary amine moieties of the inhibitor (probably as ammonium salt) anchors by means of a hydrogen bond to the zinc-coordinated water molecule/hydroxide ion, and also makes a second hydrogen bond with the OH (not NH as phenol) moiety of Thr199. The other terminal primary amine of spermine participates in hydrogen bonding with Thr200 and Pro201, whereas its main scaffold makes some clashes with a water molecule and Gln92, which explains why this is a rather weak hCA II inhibitor, but significantly inhibits other mammalian isoforms, such as for example hCA IV (K_i of 10 nM)¹⁰⁴.

As shown in Figure 6, X-ray crystallographic studies evidenced the same type of CA inhibition with resorcinol **26** (a diphenol)¹¹³, 2,5-dihydroxybenzoic acid **27** (compound for which the anchoring is assured not by the phenolic OH moieties but by the COOH one¹¹³), xylariamide **28** (the anchoring is assured by the ester moiety, COOMe, although the CAs possess esterase activity)¹¹¹, as well as the hydrolyzed sulfocoumarin derivative **29** (see discussion latter in the text for sulfocoumarins).

More recently a 2-thioxocoumarin derivative, **30**, was also observed to bind by anchoring to the zinc-coordinated water molecule¹²² (Figure 7), whereas iodide, an anion which was considered to be a zinc binder as all inorganic anions investigated so far¹², was also observed to anchor to the zinc-bound water/hydroxide ion, in the β -CA from the alga *Coccomyxa*¹²³. In the case of **30**, the exocyclic sulfur atom of the inhibitor is hydrogen bonded to the zinc-coordinated water molecule/hydroxide ion,

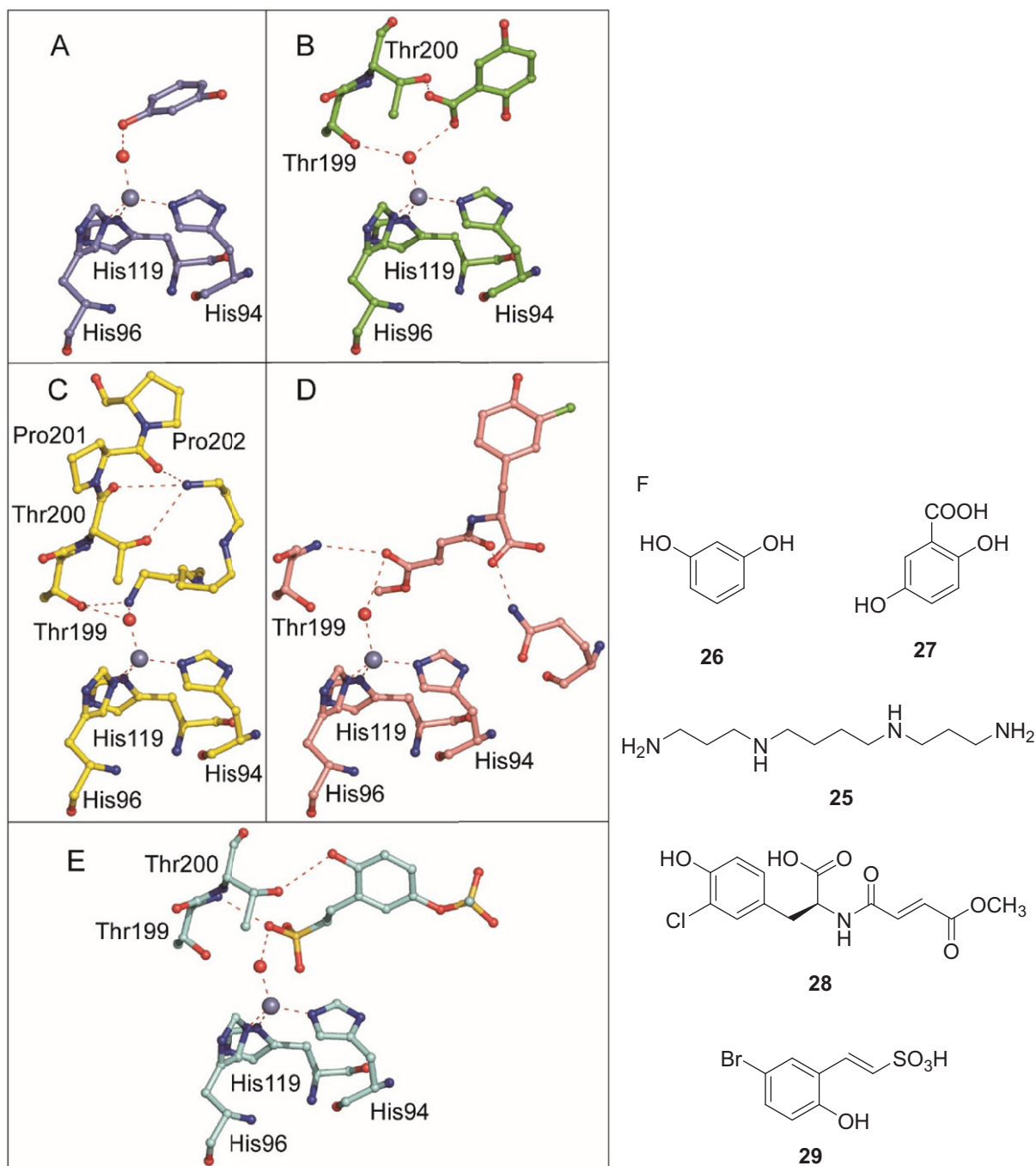


Figure 6. Other compounds which inhibit CAs by anchoring to the non-protein zinc ligand: (A) resorcinol **26**¹¹³; (B) 2,5-dihydroxybenzoic acid **27**¹¹³; (C) spermine **25** (stick view, not the schematic one as in Figure 5B)¹⁰⁴; (D) xylariamide A **28**¹¹¹; (E) hydrolyzed 6-bromo-sulfocoumarin **29**¹¹⁴. (F) Chemical structures of the discussed CAIs. All these adducts have been characterized by high resolution X-ray crystallography. The Zn(II) ion (gray sphere), its three His ligands and coordinated water molecule (red sphere) and amino acid residues involved in the binding of the inhibitors are shown.

whereas the organic scaffold makes favorable interactions with water molecules and amino acid residues from the active site¹²² (Figure 7). Thus, this CA inhibition mechanism seems to be much more general as initially considered when phenols were discovered as inhibitors. It should be also noted that CAs belonging to other genetic families than the α - and β -ones were not yet investigated for this type of alternative inhibition mechanism.

CA inhibition by occlusion of the active site entrance

Figure 8 shows schematically the third main CA inhibition mechanism, i.e. active site entrance occlusion. These inhibitors

bind even further away from the metal ion compared to the zinc binders (Figures 2 and 3) or the compounds anchoring to the zinc-coordinated water molecule (Figures 4–7), basically at the entrance of the active site cavity (which is the most variable region between various isoforms otherwise rather homologous with each other, as the 16 mammalian ones^{1,5–8}). Compounds acting by this mechanism of inhibition possess a sticky group (SG) attached to a scaffold which can be aromatic, heterocyclic, or aliphatic (Figure 8). They may also incorporate a tail, which can extend away from the active site, since, as mentioned above, these compounds bind in a rather external region of the cavity. This inhibition mechanism has been evidenced for the first time

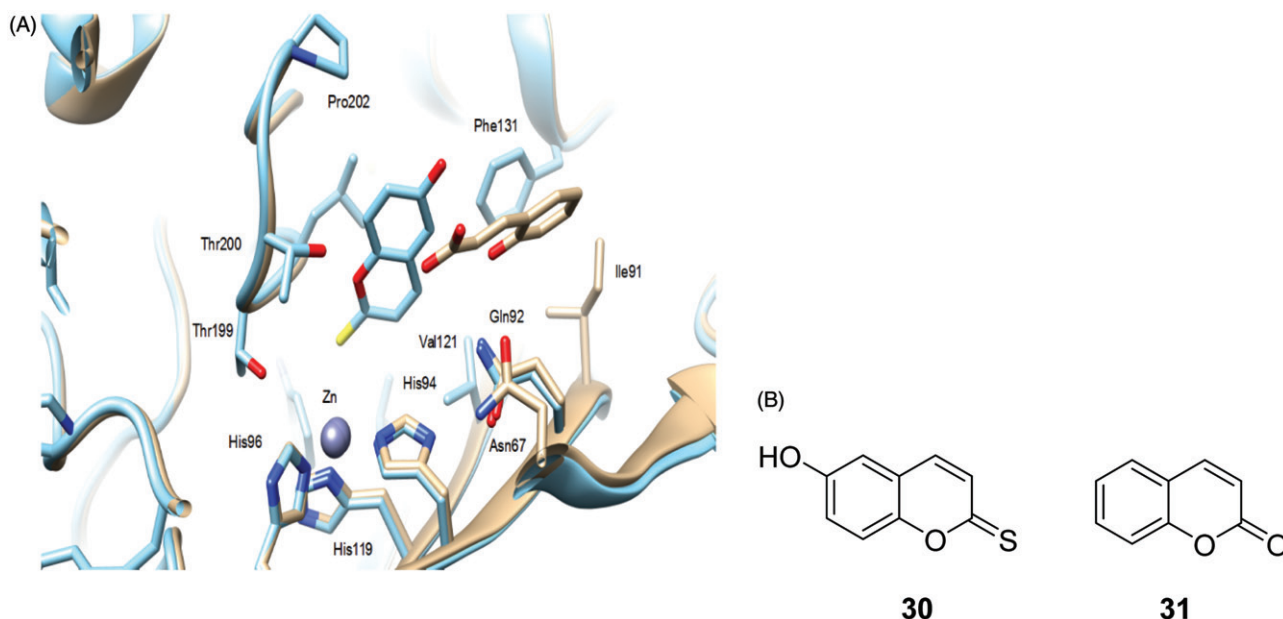


Figure 7. (A) Superposition of the adducts of hCA II with 6-hydroxy-2-thioxocoumarin **30** (sky blue, 4WL4) with the hCA II-hydrolyzed coumarin **31** adduct (5BNL) (silver). The zinc ion, its three His ligands and amino acid residues involved in the binding of inhibitors are shown in Ref. ¹²². (B) Chemical structures of compounds **30** and **31**.

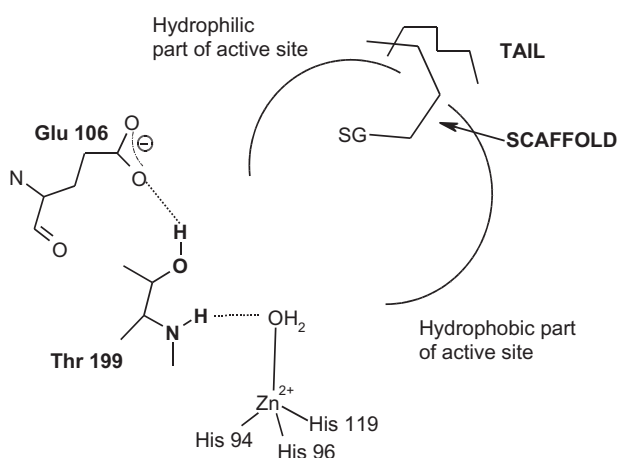


Figure 8. Compounds which inhibit the CAs by occluding the entrance to the active site. SG represents a sticky group, of the phenol, carboxylic acid or amide type, the scaffold binds at the entrance of the active site cavity, occluding it, and a tail may be also present, which will interact with residues on the surface of the protein^{115–117}.

for coumarins^{124,125} but thereafter other compounds such as the antiepileptic drug lacosamide¹²⁶, substituted coumarins^{127–131}, 5- and 6-membered lactones and thiolactones¹³² or quinolinones¹³³ (many of them display some structural similarity to coumarin **31**, the simplest such derivative) were observed to possess significant CA inhibitory properties and probably share a common mechanism of action.

The first compound with this interesting CA inhibition mechanism was the natural product coumarin **32**, isolated from the Australian plant *Leionema ellipticum*¹²⁴. When this natural product, 6-(1S-hydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one **32** as well as the simple, unsubstituted coumarin **31** were cocrystallized with hCA II, the electron density data showed the presence of the hydrolyzed derivatives **32a** and **31a**, respectively, bound within the CA active site (Figure 9). The formation of the 2-hydroxy-cinnamic acids **31a** and **31b**, which represent the *de facto* enzyme inhibitors

is shown schematically in Figure 10. The α -CAs possess esterase activity which is responsible for the hydrolysis of the lactone ring of coumarins or thiocoumarins (these last compounds were also shown to act as CAIs¹²⁵). However, the rather bulky nature of the obtained hydrolysis products interferes with their binding in the neighborhood of the metal ion. Thus, the obtained 2-hydroxy-cinnamic acids may bind as *cis* isomers (as for **32a**¹²⁴) or as *trans* isomers (as for **31a**¹²⁵) depending on how bulky the R and R' moieties present on the scaffold are. Indeed, for less bulky groups (R = R' = H), the *trans* isomer was observed¹²⁵, whereas for bulkier such groups the isomerization did not occur¹²⁴. This difference is well observed in the superposition of the two hCA II – hydrolyzed coumarin adducts shown in Figure 9(A). The most notable aspect of this inhibition mechanism is the fact that the inhibitors bind in an active site region which is the most variable between the various isoforms, i.e. the entrance to the cavity^{1,5–8}. This has important consequences for the drug design of CAIs, since compounds binding in that region should in principle interact differently with the various CAs and thus show isoform-selective inhibitory profiles. In fact, this was indeed the case when a larger series of diversely substituted coumarin/thiocoumarin derivatives of types **33–51** were investigated¹²⁵ (Figure 11), which showed a high level of isoform selective behavior against many isoforms such as CA IX, XII, XIII, XIV, etc.¹²⁵. Subsequent work from our group showed this situation to be the case for many classes of coumarins/thiocoumarins as well as structurally related derivatives which were investigated as CAIs against a large number of human isoforms^{125–133}.

It is also interesting to note that considering coumarins as leads, the sulfocoumarins were reported (in which the CO moiety of the lactone was replaced by a SO₂ group)¹¹⁴. Although the coumarins and the sulfocoumarins have a different inhibition mechanism (coumarins bind in hydrolyzed form at the entrance of the cavity, as discussed here, whereas sulfocoumarins also bind in hydrolyzed form but by anchoring to the zinc-coordinated water) many sulfocoumarins reported so far showed a very high degree of isoform selectivity toward the *trans*-membrane tumor-associated isoforms hCA IX and XII compared to the cytosolic ones hCA I and II^{114,134–138}.

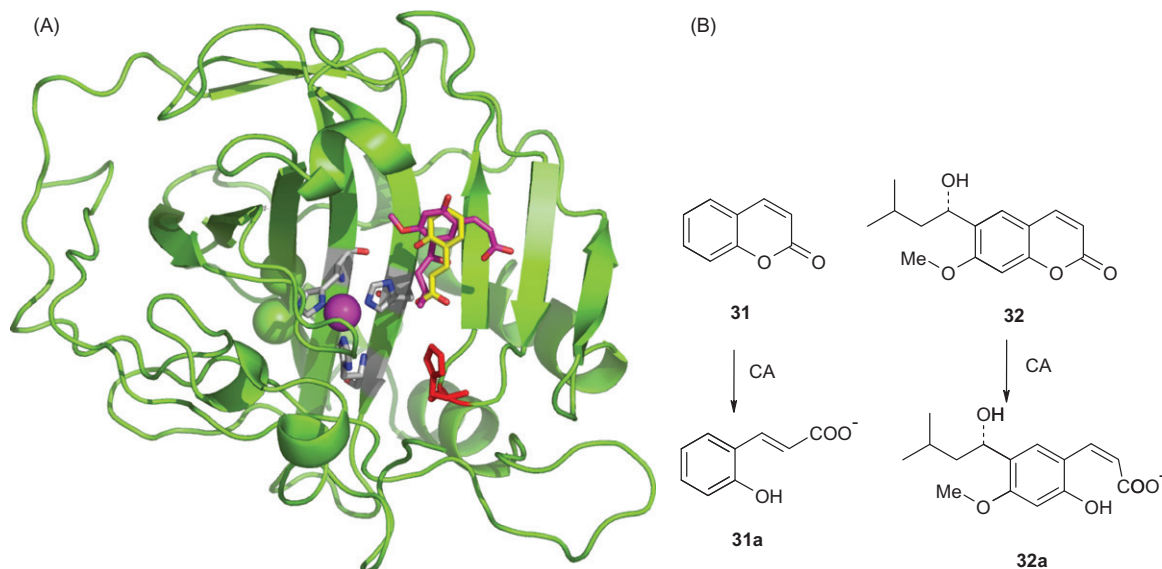


Figure 9. (A) Binding of the coumarin **31** hydrolysis product (*trans*-2-hydroxy-cinnamic acid **31a**, in yellow) and coumarin **32** hydrolysis product (*cis*-2-hydroxycinnamic acid **32a**, magenta) to the hCA II active site. The protein backbone is shown as green ribbon, the catalytic Zn(II) ion as violet sphere, with its three protein ligands (His94, 96, and 119, CPK colors) also evidenced. The proton shuttle residue (His64) is shown in red. (B) Coumarins **31** and **32** and their CA-mediated hydrolysis to **31a** and **32a**, respectively^{124,125}.

Figure 10. Proposed inhibition mechanism of CAs by coumarins/thiocoumarins, leading to *cis*- or *trans*-2-hydroxy/mercapto-cinnamic acids. (A) Hydrolysis of the lactone ring. (B) Movement of the hydrolysis product (as *cis* stereoisomer) toward the entrance of the active site cavity. (C) *Cis-trans* isomerization of the hydrolysis product^{124,125}.

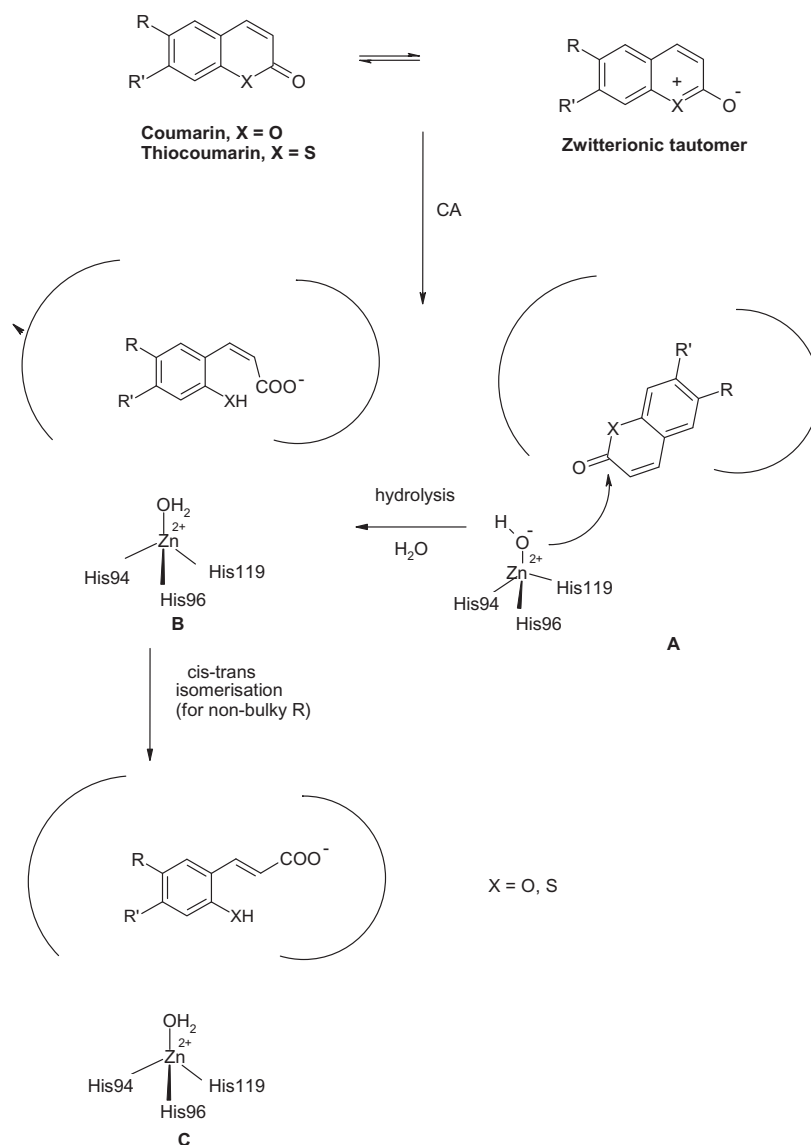
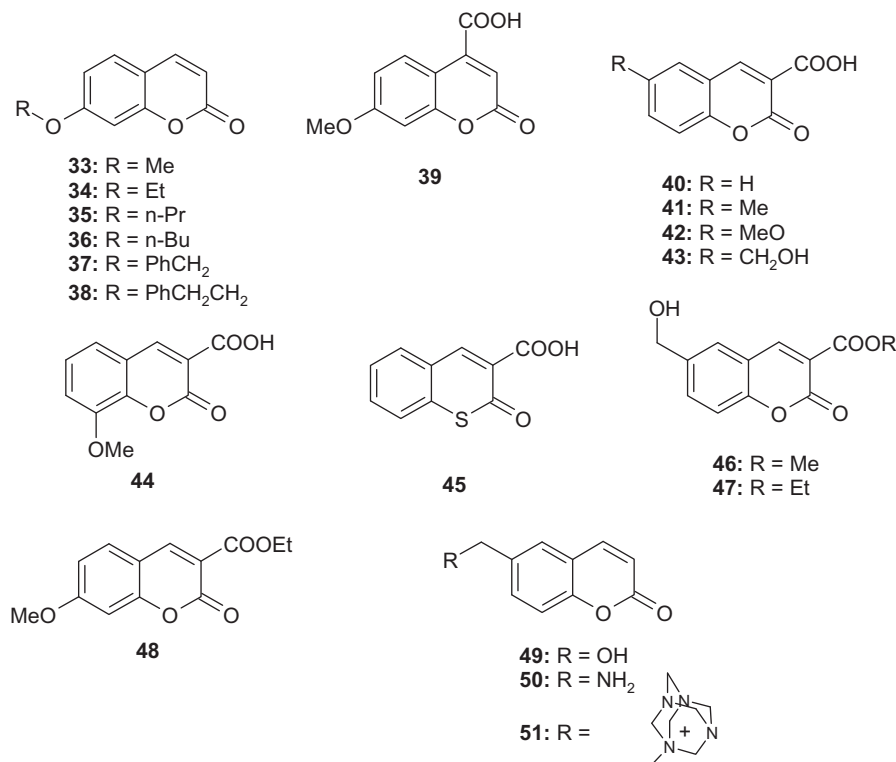


Figure 11. Some of the investigated coumarins **33–51** as CAIs which showed highly isoform-selective inhibition profile against various human CA isoforms^{124,125}.



Out of the active site binding as a CA inhibition mechanism

The CA inhibition mechanism shown in Figure 12(A) is the last one to be discovered, quite recently¹³⁹. When the 2-(benzylsulfonyl)-benzoic acid **52** was co-crystallized with hCA II, the electronic density of the inhibitor was not observed within the active site but in an adjacent binding pocket to the active site, where other inhibitors were never observed earlier (Figure 12B). Thus, this compound binds quite far away, outside the active site of the enzyme. But how does it inhibit the CA activity when the binding is in an outside region of the active site pocket? The same crystallographic study¹³⁹ showed that **52** blocks the proton shuttle of the enzyme, His64, in its *out* conformation, interfering thus with the rate determining step of the entire catalytic cycle, which is indeed the transfer of a proton from the zinc-coordinated water molecule to the environment, with generation of the zinc hydroxide species of the enzyme^{1,5–10,139}. This amino acid residue (His64) has a high flexibility with two main conformations, the *in* one (closer to the metal ion) and the *out* one, toward the exit of the active site cavity. By accepting a proton from the zinc-coordinated water, the imidazole moiety of this residue becomes protonated (in its *in* conformation) whereas when it is in the *out* one, the same proton may be transferred to the environment. Interfering with this process may block the entire catalytic cycle, and this exactly how compound **52** inhibits the enzyme, blocking the proton shuttle through a network of hydrogen bonds showed in detail in Figure 12(C)¹³⁹.

Compounds acting as CAIs with an unknown mechanism of action

The four inhibition mechanisms of the compounds discussed up until now are well understood as they were thoroughly documented by means of kinetic and more importantly, X-ray crystallographic studies. However in the last period relevant evidence accumulated on other classes of compounds, which show various levels of CA inhibitory properties, but for which the

precise mechanism of action is not known due to the fact that they were not yet crystallized in adducts with the enzyme. This is most of the time due to solubility problems for some of these derivatives, but it should be also stressed that many classes of CAIs only recently started to be investigated^{140–146}.

The secondary sulfonamide moiety is present in a large variety of clinically used drugs, among which the sulfa drugs are the best-known examples^{17,140}. Alp et al.¹⁴¹ investigated a panel of such derivatives, of type **53–70** (Figure 13) – including sulfadiazine **69** and sulfapyridine **70** – for their interaction with the physiologically dominant isoforms hCA I and II, proving that many of them show sub-micromolar affinity for both enzymes. The investigated derivatives incorporated both secondary sulfonamide (**53–62**, **69**, and **70**) as well as tertiary sulfonamide (**63–68**) functionalities in their molecules, as well as quite bulky substituents at the sulfonamide group, and clearly are unable to bind to the metal ion from the CA active site due to steric hindrance. In fact the available space in the active site funnel, at its bottom, is rather restricted to allow such sterically impaired compounds^{1,5,140–146}. It has been hypothesized that similar to lacosamide¹²⁶ and the hydrolyzed coumarins^{124,125}, these CAIs may bind at the entrance of the active site, in the coumarin-binding site (discussed in the section “CA inhibition by occlusion of the active site entrance” of this review), but this hypothesis has not yet been confirmed by X-ray crystallography. However many such secondary/tertiary sulfonamides were thereafter investigated as CAIs^{142–153} as well as structurally related sulfanates/sulfamides¹⁵³, some of which are shown in Figure 14. By using superacid chemistry Thibaudeau’s group reported a wide range of halogenated secondary/tertiary derivatives (e.g. **71–73**), some of which showed selective inhibitory action against the tumor-associated isoforms hCA IX/XII, without significant inhibition of hCA I and II^{145–148}. By using saccharin **11** as lead⁴⁶, Carradori’s group reported a range of N-substituted saccharins, among which **74–77** (and some of their congeners) showed CA XII selective inhibitory action¹⁴⁹. Acesulfame¹⁵⁰ or probenecid **78** derivatives of type **79**, incorporating a variety of aliphatic, aromatic, or amino acid

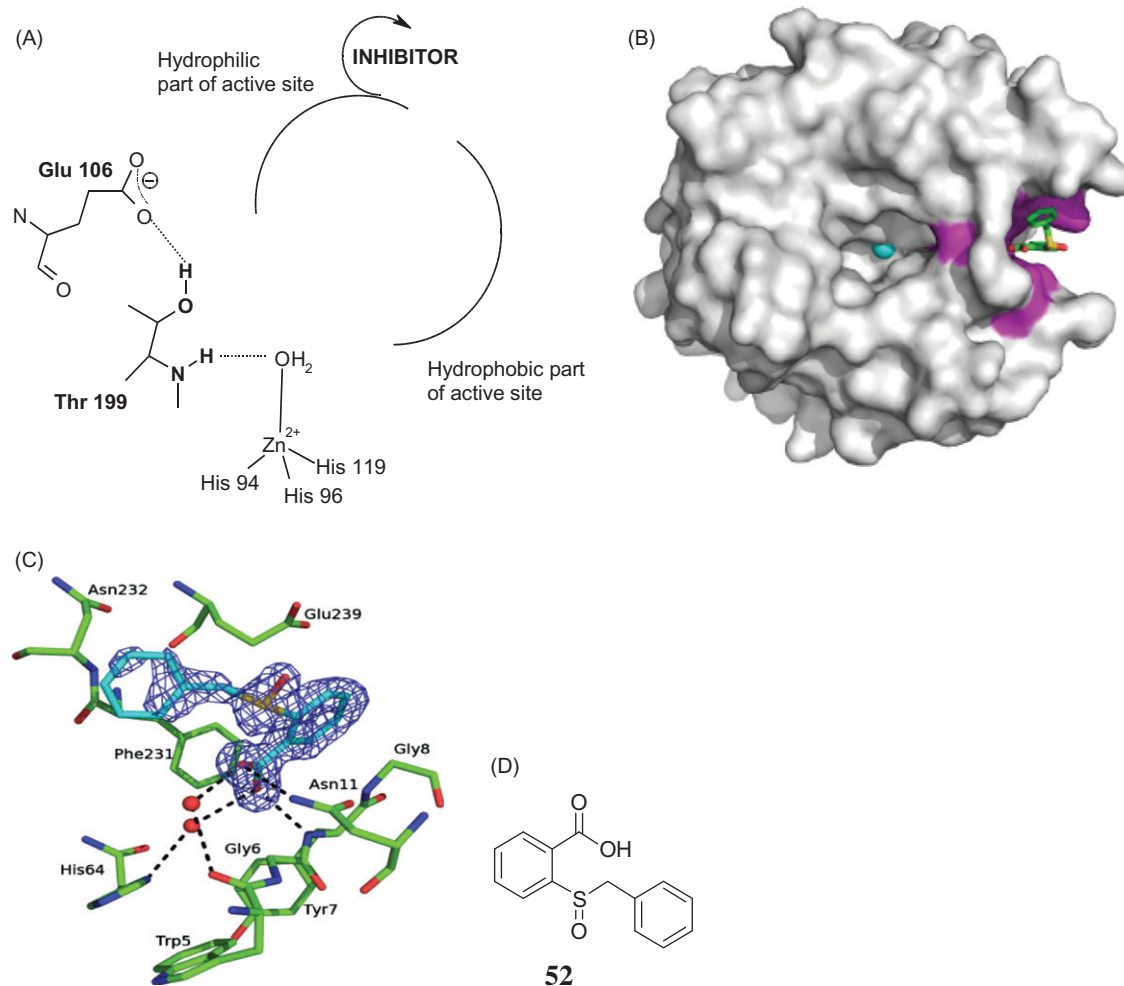
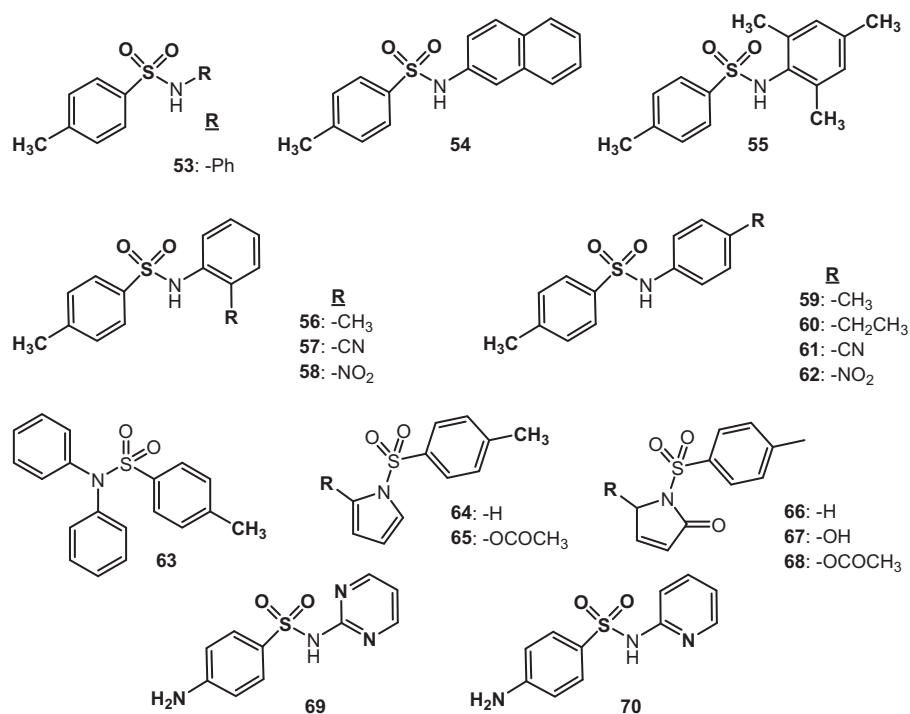


Figure 12. Out of the active site CA inhibition mechanism. (A) Schematic representation of the inhibitor binding site. (B) hCA II complexed with the carboxylic acid **52**. (C) Interactions between the inhibitor **52** and amino acid residues from the binding pocket. (D) Chemical structure of **52**¹³⁹.

Figure 13. Secondary/tertiary sulfonamides **53–70** initially investigated as CAIs¹⁴¹.



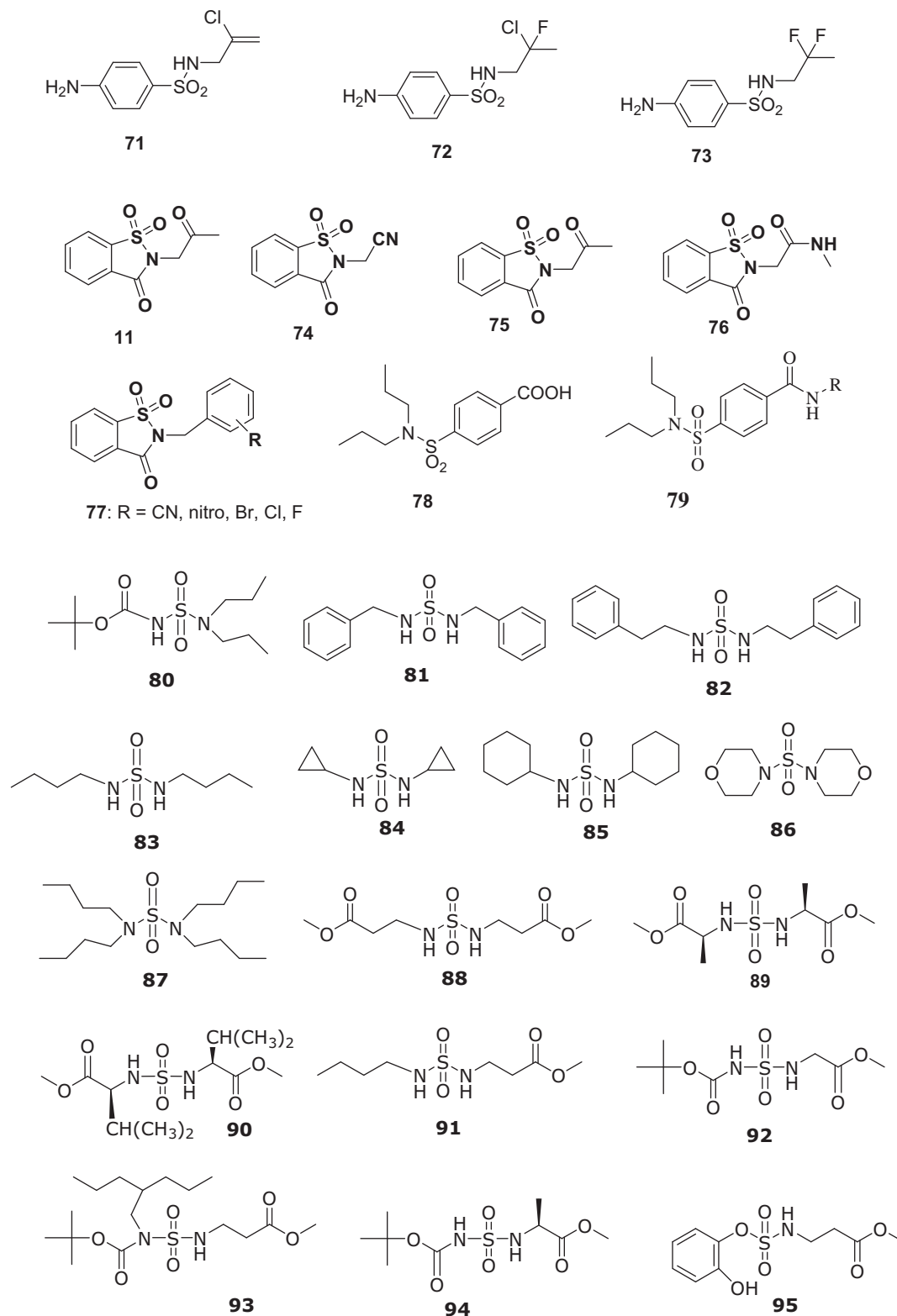


Figure 14. Secondary/tertiary sulfonamides, sulfamates, and sulfamides investigated as CAIs^{145–154}.

moieties derivatizing the carboxylic acid moiety of **78**^{151–153} were also prepared and investigated as inhibitors of at least four relevant isoforms, hCA I, II; IX and XII; by the same groups. Again notable CA selective inhibitory activity has been observed for many such tertiary sulfonamide derivatives, although their inhibition mechanism is unclear at this moment.

Gavernet et al.¹⁵⁴ explored *N,N'*-disubstituted sulfamides of types **80–94** or the *N,O*-disubstituted sulfamate **95**, which showed

a broad range of inhibitory activity and selectivity: some aromatic sulfamides were active against hCA I, hCA II and/or hCA VII, while they were less active against hCA XII and XIV. On the other hand, several bulky sulfamides were selective hCA VII inhibitors¹⁵⁴. The inhibitory mechanism of these compounds is also unknown, but it is rather probable that their bulky substituents at the NHSO₂X (X = NH or O) fragment of the molecule impairs their binding to the metal ion

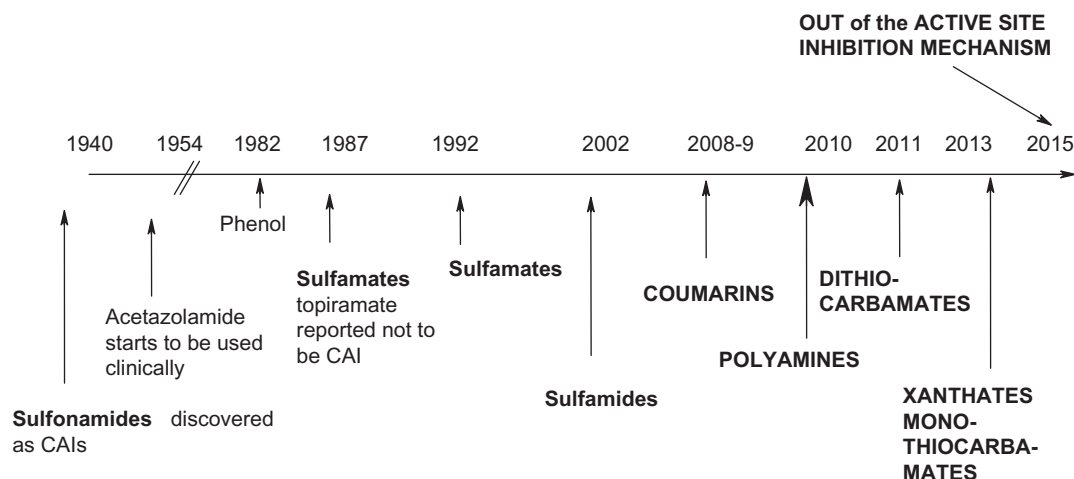


Figure 15. Historic overview of the CAI drug design panorama.

within the CA active site, as for derivatives **53–79** discussed earlier.

Effective, nanomolar inhibitory activity against several CA isoforms was also reported for the protein tyrosine kinase inhibitors and clinically used drugs imatinib and nilotinib¹⁵⁵, for some amino acid derivatized fullerenes¹⁵⁶ as well as for the murine CA inhibitor mICA, a protein belonging to the transferrin family¹⁵⁷. Also in these cases the inhibition mechanism is unknown.

Fabricated data and scientific misconduct issues of the CA field

As with all successful fields there is however also the reverse side of the medal, as the CA inhibitors field showed ultimately an increase in the number of fabricated data and similar scientific misconduct issues, some of which I will deal with in the last part of the review. I have already mentioned in an earlier review⁶ that the “two-prong” approach¹⁵⁸ (based on our tail approach published several years earlier^{81,82}) is probably fabricated. According to this approach, ideated by Srivastava’s group, iminodiacetic (IDA) moieties (which complexate Cu^{2+}) were attached to benzenesulfonamides via different chain length spacers, leading to the so-called “two-prong” inhibitors. In these CAIs one prong (the SO_2NH^- moiety) would coordinate the zinc ion, whereas the second one (containing the copper ions) would bind to the various His residues from the histidine cluster comprising His3, 4, 10, 15, and 64 (at least in the hCA II active site)²³. The His cluster was reported by Briganti et al.²³ whereas Zn(II), Cu(II), Co(II) and many other metal complexes of aromatic/heterocyclic sulfonamides incorporating polyamino-polycarboxylic acids (including IDA) in 2002 by Scozzafava et al.⁴⁸ In all cases in which such metal-containing CAIs were investigated by our group, we observed only binding of the metal ion to His64 and not to the other His residues from the His cluster mentioned above⁴⁸. Compounds sent by Srivastava in our laboratory in 2005–2006, did not show the inhibition pattern published in their paper¹⁵⁸ and the X-ray crystallography performed with some of these compounds (unpublished data from this and De Simone laboratory) showed the normal binding of the sulfonamide to the zinc ion and the Cu(II) always bound to His64. A subsequent crystallographic work from the same group¹⁵⁹ again evidenced binding of the Cu(II) only to His64, but the conclusion of the work was that the two-prong approach was working. Finally, we investigated some compounds prepared by Vomasta et al.¹⁶⁰ following exactly the two-prong approach,

and their inhibition data against four CA isoforms with various His residues in their cluster, among which hCA I, II, IX, and XII. They did not show any two-prong effects, as both mono- and two-prong compounds showed rather similar inhibitory properties¹⁶⁰. We thus clearly demonstrated that this apparently interesting approach, unfortunately does not work, probably because His64, unlike the other His residues from the cluster, has a high mobility, with its imidazole ring not only participating in the proton transfer processes as mentioned earlier in this review, but also having higher affinity for complexing metal ions (e.g. Cu^{2+}) compared to the other His residues from the cluster (which are less exposed to the solvent).

The second unfortunate example that I shall present here is from a research group in Shanghai, China^{161–164}. In several papers this group presented interesting scaffolds of aromatic/heterocyclic sulfonamides and their inhibition data mainly against hCA II and IX (as this last isoform is an attractive target due to its involvement in tumorigenesis^{1,2,19}). Initially I was rather enthusiastic of these papers^{161–164} as they were mainly based on the tail approach developed by my group in the late 90s^{81,82}. However when I read their experimental parts I realized that the enzyme inhibition procedure was basically exactly the one from our papers. Furthermore, all (but really all) the instrumentation that this group claimed to possess was identical to the one from my laboratory in Florence (e.g. stopped-flow spectrophotometer, UV-VIS spectrophotometer, etc.) and the enzymes were claimed to have been cloned exactly by our methods, although these authors did not ask the plasmids or the cDNAs encoding those CAs from me or my other associates. I thus started to suspect misconduct and contacted the group, asking just one of their published inhibitors to be sent to my laboratory. Initially they told me that it impossible to send compounds from China to Europe (which is not true as we received samples from other Chinese laboratories), and afterwards that they need to re-prepare the compounds (there were many of them in the papers, and I asked whatever one to be sent). I concluded that this are all fabricated data, and informed the Editors of the journals in which these papers were published, whom however did not take any action. It is of course a pity that this type of scientific misconduct pollutes our wonderful field. What is even worse is that many of these dishonest authors act thereafter as reviewers of our or other groups’ papers (which indeed present high-quality science) and create problems for the publication of the genuine data (whereas the falsified ones are still there in the literature). This is also one of the reasons why I raised this problem in the present review. Unfortunately there are many other similar examples which I will not present here for lack of space.

Conclusions

By catalyzing the simple but highly important hydration of carbon dioxide to bicarbonate and protons, CAs are involved in critical steps of the life cycle of many organisms, including eukaryotes, *Bacteria* and *Archaea*. In fact a large number of new such enzymes are constantly being cloned, purified, and characterized in detail from many pathogenic as well as nonpathogenic organisms. Modulation of the activity of these enzymes, mainly by using selective inhibitors, has been used pharmacologically for decades for various applications (diuretics, antiglaucoma agents, antiepileptics, antiobesity drugs and more recently anti-tumor therapies/diagnostic agents as well as novel anti-infectives^{1–10,14–19,21,165–167}). One of the main problems with the CAIs was however the lack of selectivity of the sulfonamides for the inhibition of the many mammalian (human) CAs, which led to a range of side effects for the first and second generation inhibitors of types **1–19**^{1–10,14–19,21,165–167}. However, this situation changed substantially in the last decade, when a large number of isoform-selective primary sulfonamides^{63,64,70,71} were reported by using the tail approach^{47–49,80–82} and with the discovery of new classes of CAIs, such as the coumarins and their isosters, the polyamines, DTCs, xanthates, etc., as shown in detail in this review. An overview on the history of CAI drug discovery is shown in Figure 15.

As mentioned throughout this review, the sulfonamides are the most important class of CAIs, discovered already in 1940, with the first clinically approved drug, acetazolamide **1**, launched in 1954^{1,5–9}. For the next 5 decades, there were only variations on the sulfonamide theme with the sulfamates and sulfamides the most logical class of compounds to be considered as CAIs¹⁶¹. Indeed, such an activity has been demonstrated for the simple, inorganic sulfamide and sulfamate¹⁶⁸, although Maryanoff et al.¹⁶⁹ reported that some sugar sulfamates (one of which later became the antiepileptic drug topiramate **10**) were devoid of CA inhibitory properties. We later showed that topiramate is a low nanomolar CAI against many isoforms¹⁷⁰. Indeed, a wide range of primary sulfamates and sulfamides were shown to possess potent such properties, in all aspects similar to the isosteric sulfonamides, considering the binding to the enzyme, mechanism of action, pharmacology, etc.^{1,5,6,167}.

However the breakthrough in the field was represented by the discovery of coumarins as a totally new chemotype with a particular inhibition mechanism, which has been discussed in detail in this review article^{124,125}. As shown here, coumarins were also used as lead compounds, leading to the discovery of a large number of structurally related chemotypes possessing prodrug-like CA inhibitory properties, among which the thiocoumarins, thioxocoumarins, coumarin oximes, 5-/6-membered ring lactones and thiolactones, sulfocoumarins, etc.^{114,127–138}. Polyamines were then discovered as CAIs in 2010¹⁰⁴ (Figure 15), followed shortly thereafter by DTCs and xanthates^{28–30,83,84}. Finally the out of the active site CA inhibition mechanism was reported in 2015¹³⁹. A particular feature of the new classes of CAIs discovered in the last years is that they show a much higher isoform-selectivity profile compared to most sulfonamides and sulfamates (of which **1–19** are the typical representatives, see Figure 1). Thus, there is enthusiasm and optimism that the new generations of CAIs will show less side effects and more efficacy for the treatment of the classical pathologies associated with these enzymes but also to newer ones such as neuropathic pain¹⁷¹, cerebral ischemia^{172,173}, and obviously tumors (with SLC-0111 **20** in clinical trials, as mentioned earlier^{2,19,54}).

Despite the problem of the fabricated data and similar scientific misconduct issues described in paragraph 7, I will conclude this article in an optimistic manner¹⁷⁴. The review

presented here is a continuation of the highly cited ones from 2008¹ and 2012⁵. Moreover, the field of the CAs and their inhibitors lived a great period in the last decade, with a lot of important discoveries being made. Several important mammalian isoforms (e.g. CA VA in obesity; CA VII in neuropathic pain; CA IX and XII in tumors) have been validated as drug targets and a large number of highly potent and selective inhibitors, belonging to a large number of diverse classes, reported and characterized in detail. The classical sulfonamide inhibitors are still massively used clinically but the hope is that in the nearby future novel CAIs belonging to the newly identified classes will reach the clinics and the patients in need for them.

Declaration of interest

The author declares conflict of interest, being author of several patents in the field of CA inhibitors. Research from my laboratory was financed by several EU projects (Euroxy, Metoxia, DeZnIt, and Dynano).

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