Chapter 3

Carbonic Anhydrase and Epilepsy

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Abstract

The deficiencies of current antiepileptic drugs (AEDs) demand the search of new active compounds through novel strategies of drug discovery. Particularly, the design of AEDs based on molecular targets constitutes a promising alternative to empirical screening, the traditional method to detect anticonvulsant action in new structures. In this chapter we described the advances in the dynamic field of carbonic anhydrases, with emphasis in the development of selective inhibitors as anticonvulsants. We first detailed the 3D architecture of carbonic anhydrases and the mechanism of action of classical inhibitors. Then we reviewed the known anticonvulsant drugs that present carbonic anhydrase inhibition and the progress made in the design of selective inhibitors of CAVII, the isoform implicated in the generation of febrile seizures.

Key words Epilepsy, Carbonic anhydrase, Sulfonamides, Sulfamides, Sulfamates

1 New Targets for Antiepileptic Drugs

Epilepsy is a complex chronic brain disorder. The International League Against Epilepsy (ILAE) defines and quantifies over 15 different seizure types and more than 30 epilepsy syndromes that can be originated by a variety of pathological conditions [1]. Pharmacological experiments carried out over the last decades have increased our knowledge about the physiopathology of epilepsy, and they proved that mechanisms of generation of seizures are related with the imbalance between inhibitory and excitatory conductance in brain tissues [2]. Antiepileptic drugs (AEDs) work to reconstruct this balance and avoid the seizures, but they are efficient in about 70% of the patients. The remaining 30% of the people (who cannot completely control the seizures though drug therapy) suffer from refractory or intractable epilepsy [3, 4]. On the other hand, responsive patients experience medication-related side effects, which become more evident and dangerous when lifelong medication is necessary [3].

After four decades of research, numerous compounds have been introduced to the market with notable improvements in their absorption, distribution, metabolism, excretion, and toxicity

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(ADME/Tox) properties, relative to the first generation of AEDs. However, no significant progresses have been achieved in terms of the efficacy in resistant patients [3]. This fact demanded a conscientious analysis about the strategies employed to the design of anticonvulsant compounds.

Without a doubt, the approach more extensively addressed for the discovery of new AEDs has been the phenotypic screening in acute models of seizures [5]. The US National Institute of Health, through its Anticonvulsant Screening Project, proposes an initial protocol that includes in vivo/in vitro models to identify new active molecules [5]. Among them, the most employed assays are the maximal electroshock seizure test (MES test) and the pentylenetetrazol test (scMES test) in mice and rats [6]. The MES test is associated with the electrical induction of the seizure, whereas scMES test involves a chemical stimulus to generate the convulsion. Most of the marketed AEDs are capable of suppressing the seizures induced with at least one of these two tests.

The screening methodology does not detect AEDs with a specific mechanism of action. In fact, the molecular targets of the new compounds are elucidated after the identification of the activity. Accordingly, screening methods might ignore compounds with novel mechanisms of anticonvulsant effect, if they cannot avoid the seizures caused by classical test [7]. To partially solve this limitation, other assays have been recently included in the program, which detected drugs that are ineffective into the classical models [5].

The restrictions of the empirical screening, the progress in the knowledge of the mechanisms involved in ictogenesis (or epileptogenesis), and the lack of efficiency of known AEDs in refractory patients have supported the search of alternative methods of drug discovery, like the design of anticonvulsants based on molecular targets [3, 8]. Successful examples of this rational methodology are vigabatrin, tiagabine, and perampanel [9]. Vigabatrin and tiagabine potentiate the GABA-ergic neurotransmission, whereas perampanel impedes the glutamatergic excitation [9]. However other less explored molecular targets that affect in some way over the GABA-ergic neurotransmission or glutamatergic excitation have been pointed out as new alternatives for target-based drug design [8, 10]. In this context, carbonic anhydrase has emerged as an attractive enzyme for designing new active anticonvulsant compounds [11].

2 Carbonic Anhydrases

Carbonic anhydrases (CAs) belong to a family of metalloenzymes that are responsible for the reversible hydration of carbon dioxide and bicarbonate. They have been classified into five classes (named as α, β, γ, δ, and ε CAs). The α class is the most studied and it is found mainly in vertebrates. In fact, α-CAs is the only class found in mammals [12].

Up to now, 16 isoforms of α-CAs have been identified with different catalytic activities, cell/tissue distributions, and response to inhibitors [12, 13]. There are eight cytosolic proteins (CA I, CA II, CA III, CA VII, CA VIII, CA X, CA XI, CA XIII), two mitochondrial matrix proteins (CA VA, CA VB), one secreted protein (CA VI), two glycosylphosphatidylinositol (GPI)-anchored proteins (CA IV and CA XV), and three transmembrane proteins (CA IX, CA XII, CA XIV). Isoform CA XV is not expressed in primates, and isoforms CAVIII, CAX, and CAXI have no catalytic activity since they lack of histidine residues of the active sites [11]. They are also defined as CA-related proteins (CARPs).

Extensive experimental data showed the architecture of the catalytic forms of α-CAs, especially for CAII. The active site is well conserved in all the active isoforms, and it comprises a Zn^{2+} ion located at the bottom of a half hydrophilic and half hydrophobic conical cleft. The cavity is approximately 15 Å deep and the active site is accessible to water [13]. The metal ion then is coordinated by the imidazole rings of three histidine residues with the fourth position occupied by a water molecule at acidic pH (<8) and by a hydroxide ion at higher pH (Fig. 1).

2.1 Carbonic Anhydrase and Seizures

CAs are responsible for maintaining the intra- and extracellular balance between CO_2, H^+, and HCO_3^- ions [12]. They play important functions in crucial physiological processes for both normal and pathological conditions, such as the acid-base equilibrium, CO_2 and HCO_3^- transport across membranes, electrolyte secretion, calcification, biosynthetic reactions (gluconeogenesis, lipogenesis, and ureagenesis), signal transduction, and tumorigenicity [13].

Regarding their role in the control of pH, CAs represent a versatile system to balance the acid-base concentrations at the level of the blood–brain barrier, neurons, glia, and interstitial fluid in the brain, as well at levels of the whole organism (respiratory functions, energy metabolic functions, and renal functions) [14]. Of course, the modulation of pH is attached to the movements of many other ions, so this enzyme is indirectly involved in the flux control of other solutes [11, 14].

As mentioned at the beginning of this chapter, seizures are related with the imbalance between inhibitory and excitatory conductance in brain tissues, which may be related with fast alterations in the extracellular ionic compositions [15]. For example, epileptiform activity is caused by a rise in the extracellular potassium concentration [16], and neuronal excitability is affected by changes in pH [14]. Generally, the excitability of most central neurons and neuronal network is increased by alkalosis while it was suppressed by acidosis [11, 14, 17–20]. Hyperventilation is a standard practice employed in the clinic to generate respiratory alkalosis, causing precipitation of petit mal seizures [21]. It is also employed in children to trigger febrile

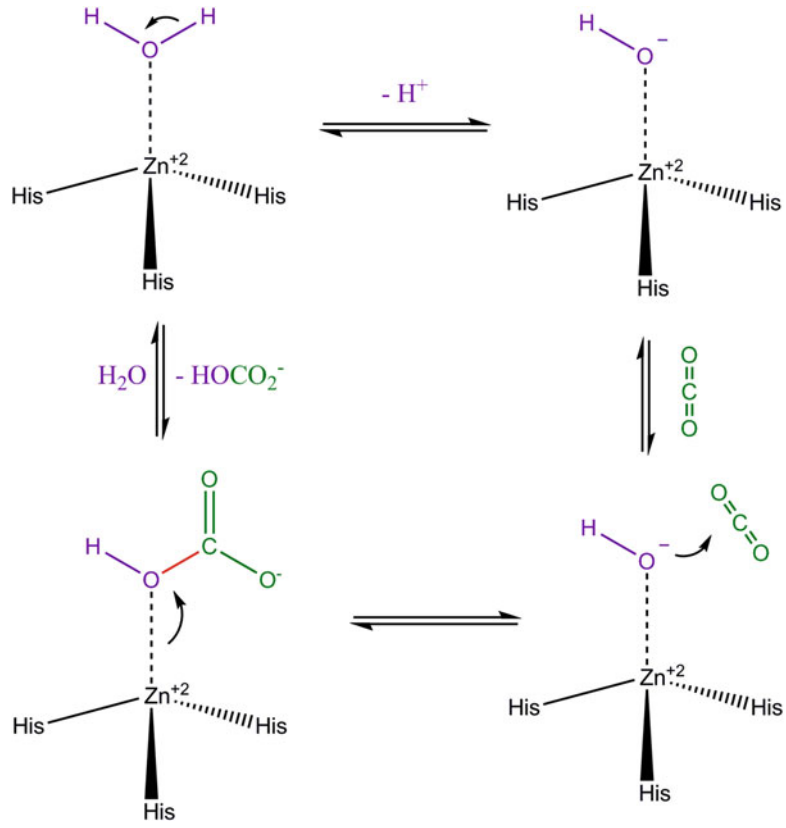

Fig. 1 Active site of catalytic α-CAs. The metal ion coordinates 3 histidine residues and a water molecule that generates the nucleophilic hydroxide anion necessary to trigger the catalytic cycle

seizures (as well in animal models of convulsions) [22, 23]. On the other hand, respiratory acidosis induced exogenously suppresses the neuronal excitability [14].

The pH is mainly balanced by the buffer CO_2/HCO_3^- (in the intracellular and extracellular space), so several links between CAs and the generation of seizures have been proposed [11]. Furthermore, the relation of CA and seizures is supported by the fact that CA inhibitors are (or were) employed to treat epilepsy. For example, in 1963, Esplin and Rosertain studied the inhibitor acetazolamide and they found that it decreased excitability in cat spinal cord [24]. After that, other authors proposed that the glial cells are more alkaline than neurons (due to higher concentrations of HCO_3^-), and the anticonvulsant action of the CA inhibitors could be related with extracellular acidosis [25]. A revision of the AEDs with inhibitory action against CA is given in the next section of this chapter.

3 CA Inhibitors as Anticonvulsant Drugs

CAs inhibitors have been the object of study in many fields of medicinal chemistry, as candidates to treat glaucoma, altitude sickness, obesity, pain, cancer, and epilepsy [12, 26–28].

Classical inhibitors of α-CAs exert their action by blocking the four position originally destined for water in the catalytic center (Fig. 1). They usually bind to the Zn^{2+} as anions, forming two different complexes depending on the presence/absence of water (Fig. 2). The most important functional group that serves as Zn^{2+} anchoring group of CA inhibitors is the sulfonamide function (and their bioisosteric partners, Fig. 2). However, it is worth pointing out that other inhibition mechanisms have been discovered for other families of compounds, such as polyamines and coumarins [12].

Some commercial AEDs are CA inhibitors [29]. However, their anticonvulsant action has not been totally attributed to the CA inhibition process in most of the compounds. In fact, the CA interactions represent a drug weakness in some cases, because it generates tolerance and/or important side effects.

Acetazolamide (Diamox®) was introduced to the market as a diuretic in 1953 (Fig. 3). Simultaneously, its anticonvulsant properties were discovered by Bergstrom et al. [30] and then other researchers probed their effect in animals and humans [31–34]. It has been employed in partial and generalized seizures, as well as in catamenial epilepsy [35, 36]. Regarding its safety, acetazolamide induces side effects as paresthesias, tinnitus, loss of appetite, and alterations of taste [37, 38]. Nowadays it is rarely indicated to treat convulsions, mainly due to the development of tolerance [39, 40]. Methazolamide is a structurally related compound that has been

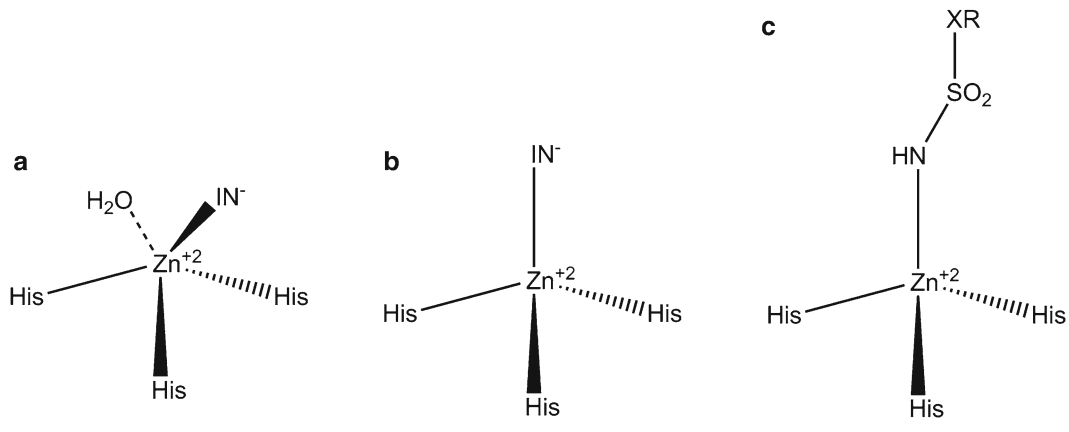

Fig. 2 Schematic representation of the interactions found for classical inhibitors and the active site [16]. (**a**) The Zn(II) ion is coordinated with a water molecule in addition to the inhibitor (IN^-). (**b**) The inhibitor (IN^-) substitutes the fourth position in the tetrahedral complex (originally available for water). (**c**) Sulfonamides (X = C atom), sulfamates (X = O atom) and sulfamides (X = N atom) bind to the zinc ion in their deprotonated form

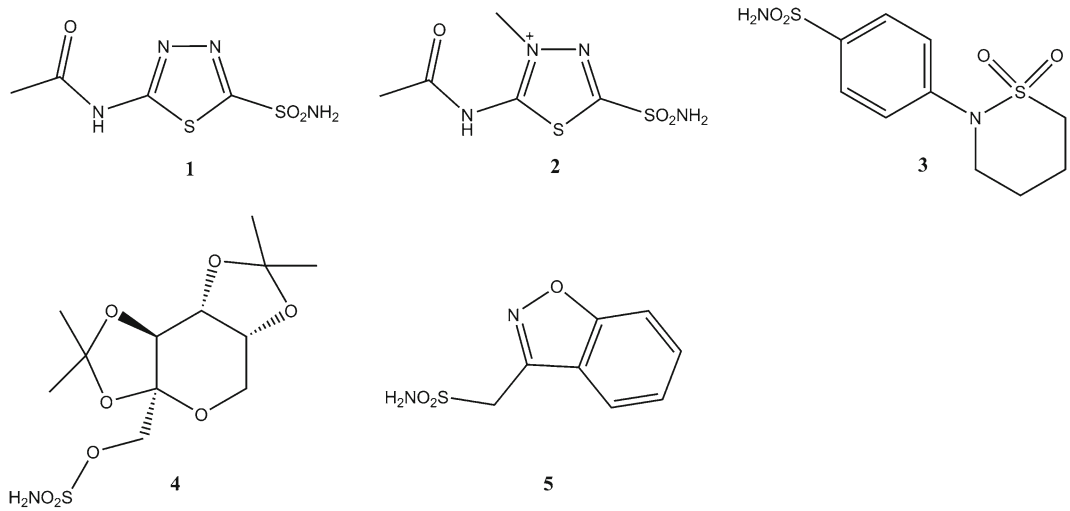

Fig. 3 Structures of anticonvulsant drugs with proved CA inhibition. **1**: Acetazolamide; **2**: Methazolamide; **3**: Sulthiame; **4**: Topiramate and **5**: Zonizamide

tested as anticonvulsant (Fig. 3), but it has gained more attention as antiglaucoma drug [29, 41, 42]. Sulthiame is another sulfonamide derivative employed as AED (Fig. 3). It is prescribed to treat partial epilepsy in children in Europe and Australia [43], but serious side effects related with deterioration in cognitive functions have been reported [44].

Topiramate (Topamax®) is a broad spectrum AED employed for the treatment of partial and generalized seizures, including Lennox–Gastaut syndrome and prophylactic treatment of migraine [45, 46]. It is also indicated in patients with bipolar and mood instability disorders, post-traumatic stress, eating disorders, addictions, and other pathologies [29, 47–50]. From a structural point of view, it is a substituted monosaccharide that contains sulfamate function (Fig. 3). The sulfamate group is a bioisosteric partner of sulfonamide moiety, and it serves as anchoring group to interact with CA active site [51].

The anticonvulsant action of topiramate is attributed to multiple mechanisms of action. It inhibits several CA isoforms [52], but it also blocks Na^+ and Ca^{2+} channels and AMPA/kainate receptors; and it enhances the GABA-ergic neurotransmission [29, 53–59]. On the other hand, CA inhibition contributes to the generation of several side effects like metabolic acidosis, hypocitraturia, hypercalciuria, and elevated urine pH, leading to an increased risk of kidney stone formation [10, 29, 60, 61].

Zonisamide is an AED effective for simple and complex partial seizures, generalized tonic–clonic seizures, myoclonic epilepsies, Lennox–Gastaut syndrome, and infantile spasms [29, 62–64]. Like acetazolamide, it has a sulfonamide function in its chemical structure

(Fig. 3), but a weaker inhibition profile: its CA-inhibiting activity in vivo is 100–1000 times weaker than acetazolamide [65]. Studies showed that its main mechanisms of anticonvulsant action are the blockade of voltage-sensitive sodium channels and the reduction of voltage-sensitive T-type calcium currents [29]. Zonisamide presents acceptable side effects like somnolence, dizziness, and weight loss but it develops tolerance very quickly [29, 65].

Regarding drugs in development, the search of new CA inhibitors as anticonvulsants is an active area of research [52, 66–70]. However, the limitations found in marketed and experimental CA inhibitors originated doubts about the potential of this target for designing efficient AEDs. Experts in the field have concluded that future inhibitors might overcome the difficulties of tolerance and toxicity by selective targeting specific isoforms involved in the pathological process [10]. Following this idea, in the last years, the drug design campaigns have focused on obtaining isoform-selective inhibitors of CAs. It represents a challenging objective because there are 13 active CAs in humans with similar architecture [12]. They share the active site characteristics (three residues of His bound to the zinc ion) and two residues highly conserved near the active site (identified as Thr 199 and Glu 106 in CAII), since they help with the coordination between water/hydroxide ion and zinc. Other common features are the physicochemical characteristics of the residues in the conical cleft, which presents a hydrophobic region opposite to one hydrophilic region [12].

However, there are environmental differences for the isozymes that provide the opportunity of designing selective inhibitors. For example, transmembrane isoforms CAIX and CAXII are targets for anticancer drugs, particularly in diseases associated with hypoxic tumors [12, 71]. Hydrophilic glycosyl sulfonamide inhibitors of CA IX and CA XII have been designed to minimize the diffusion through lipid membranes and to promote the selective inhibition of transmembrane CAs [12, 72]. Similarly, positively charged sulfonamide inhibitors were designed to minimize their transport through the lipophilic membranes [73].

There are also divergences in the identity of some amino acids in each isoform, mainly for those located at the middle and toward the exit of the active site cavity. It generates differences on the size and shape of the cleft, which finally affect the inhibition patterns of the ligands. For example, the inhibition constant (Ki) of the anticonvulsant topiramate is 210 times lower than its sulfamide analogue in CAII isoform [74]. However, this sulfamide effectively inhibits in the nanomolar range isozymes CA VA, VB, VII, XIII, and XIV [74]. The weak inhibitory properties of the sulfamide against CAII has been attributed to the unfavorable Van der Waals contacts between this ligand and one distinctive residue of CA II (Ala65) [74]. Additionally, the access to other protonation states of the sulfamide function relative to the sulfamate group has also been hypothesized as an extra source of selectivity [75].

On the other hand, the divergences in the identity of the amino acids at the entrance of the cavity provide the opportunity of designing elongated molecules that interact with the active site but also with the distinctive residues to be isoform selective [12]. This strategy is known as *the tail approach* and numerous successful examples are given in literature mainly for antiglaucoma and anticancer drugs [12]. Regarding epilepsy, there is a growing interest about the design of selective inhibitors for the isoform CAVII, which has gained attention as a promising target for AEDs.

3.1 Design of Selective CAVII Inhibitors as Anticonvulsants

As detailed previously in this chapter, CAs are responsible of preserving the intra- and extracellular balance between CO_2, H^+, and HCO_3^- ions, and these species influence the neuronal signaling in many different ways. Regarding neuronal excitability, numerous studies sustain the hypothesis that CA activity promotes depolarizing and excitatory GABA-ergic transmission through HCO_3^- currents [14, 76]. Moreover, important advancements have been recently reported about the influence of the isoform CAVII in the generation of febrile seizures via the activation of GABAA receptors [77].

Human CAVII is a cytosolic isoform composed of 263 amino acids that shares 56% of identity with the CAII isoform [78]. In contrast to ubiquitous CAII, CAVII is the unique isoform present mostly in the central nervous system. It is highly expressed in the cortex, hippocampus, and thalamus regions of humans' and rats' brain tissues and in the stomach, duodenum, colon, liver, and skeletal muscle of mice [79, 80].

In relation to CAVII inhibition, Gitto and coworkers rationally designed, synthesized, and evaluated ten isoquinoline-derived sulfonamides and other four substituted aryl sulfonamides as selective inhibitors [81]. They showed inhibitory efficacy at low nanomolar concentrations in some examples and selectivity against CAVII over CAII. The authors proposed that small substituents in the C1 position of the isoquinoline ring improve the inhibition in CAVII. Figure 4 shows the two more representative compounds (structures 1 and 2). After that, the authors tested other structurally related aryl sulfonamides, but the new compounds showed lower selectivity when compared to the previously reported ones [82].

Recently, De Luca and coworkers performed a virtual screening campaign in order to find new CAVII inhibitors [83]. They constructed two 3D pharmacophore models based on two experimentally available complexes of inhibitors with CAVII (protein data bank codes: 3ML5 and 3MDZ [84]). Later, a final pharmacophore model was made by superimposing the two structure-based hypotheses and taking out the overlapped chemical features. This pattern was employed as template for virtual screening into a focused library of 6313 sulfonamides available in the zinc database [85]. The 34 hits found were submitted to a docking simulation and finally two compounds were selected for experimental assays

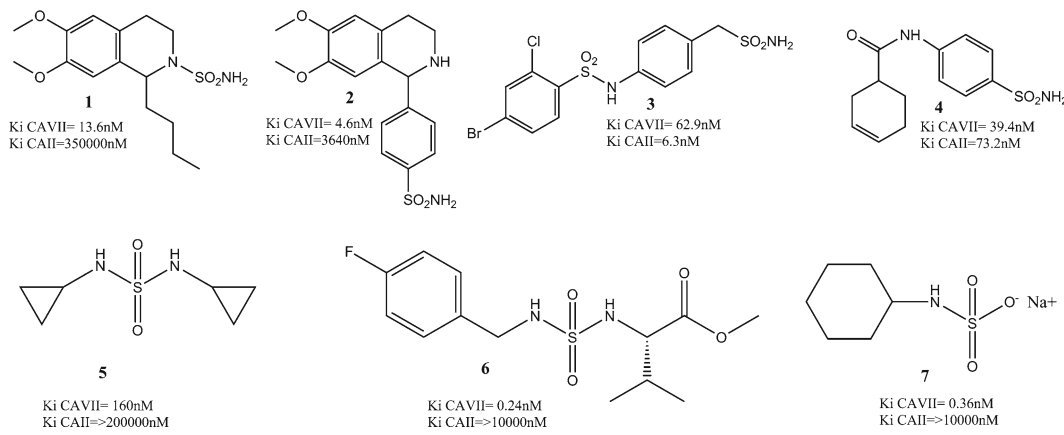

Fig. 4 Representative structures of CAVII inhibitors collected from literature [70, 81, 83, 86]

(Fig. 4, structures 3 and 4). Both molecules showed nanomolar inhibition against CAVII isoform but not selectivity against CAII.

We and other authors have also explored the applications of rational design for the discovery of new AEDs via CAVII inhibition [70, 86]. Particularly, we studied the inhibition pattern of sulfamides (–NH–SO_2–NH–), which are bioisosteres of the classic sulfonamide group [75]. Initially, we found that the *N*,*N*′-disubstitution of the sulfamide function causes a negative effect in the activity for the off-target CAII, but it provokes inhibitory action against CAVII in some cases. For example, we found that *N*,*N*′-dicyclopropylsulfamide (Fig. 4, structure 5) has a Ki value of 160 nM and it is inactive against CAII [70]. To explain the origin of the effects that may contribute to the different binding affinity, we simulated the interaction of this compound (among others) with CAII and CAVII using docking and molecular dynamics simulations.

The docking simulations for *N*,*N*-dicyclopropylsulfamide were performed using AutoDockTools 1.5.0 and AutoDock 4.0 docking softwares [87]. The starting CAII protein was prepared from the 0.99 Å resolution crystal structure of the CAII-sulfonamide complex deposited by Jude et al. (protein data bank code 2FOU) [88]. CA VII isoform was obtained from the complex provided by Opperman et al. (protein data bank 3MDZ) [89]. In both cases, the crystallographic water molecules, the ligand, and any cocrystallized molecule/ion were stripped. Hydrogen atoms were added using the LEaP module of AMBER11 [90].

We retained the default AutoDock parameters for all the variables but the charges of the ligands, for which AM1-BCC charges were calculated using Quacpac software [91]. We found this performs better in the docking for this particular system than the default Gasteiger charges [75]. The sulfamide was docked using

the Lamarckian genetic algorithm (LGA) in the "docking active site," defined through a grid centered on the ND2 atom of Asn67 residue for CA II and the ND2 atom of Asn64 for CA VII. In both isozymes, we employed 60, 50, and 60 grid points in X, Y, and Z dimensions respectively, with the default grid spacing (0.375 Å), and performed 50 docking runs.

The N,N'-dicyclopropylsulfamide was docked as anion, by removing the H atom of one NH group, since it is believed that classical inhibitors coordinate to the active site as negative species [75].

The conformations predicted by AutoDock for the complexes with CAII and CAVII were used as starting points for MD simulations with AMBER11 software [90]. The initial geometries were minimized (1000 cycles for the water molecules followed by 2500 cycles for the entire systems). After a 20 ps NTV equilibration period with a weak restraint (10 kcal/mol Å^2) for the complex and a NTP 200 ps without restraint, production runs larger than 12 ns were computed for each complex, for the coordinates saved every 1000 time steps. The ionizable residues were set to their normal ionization states at pH 7, except for the His residues coordinating the Zn metallocenter, which were modeled as Hid94, Hid96, and Hie119 (numbers relative to CA II). The protein atoms were surrounded by a periodic box of TIP3P32 water molecules that extended 10 Å from the protein. Counter ions were placed by the LEaP module of AMBER11 to neutralize the system [90]. The ff03 version of the all-atom AMBER force field was used to model the protein, and the GAFF force field was employed for the organic ligands [90, 92].

We derived our own nonstandard force fields for the Zn active site by means of geometry minimization (B3LYP/6-31G**, Gaussian03 software [93]), followed by calculation of the second derivatives and RESP charges for the active site supermolecule defined by three His residues, the Zn ion, and the sulfamide ligand [75].

Our molecular modeling studies suggested that the active site is more elongated in CAVII than in CAII, particularly in the hydrophilic region delimited by ASN64, HIS66, GLN69, LYS93, and GLN94 (GLN69 and LYS93 are distinctive residues for CAVII, and they are replaced by ASN67 and ILE91 in CAII). The larger space in the CAVII active site entrance allows it to accommodate bulkier sulfamides, like N,N'-disubstituted compounds. After that we design new amino acid-derived sulfamides with different alkyl/aryl chains with the purpose of improving the interactions with CAVII [86]. The idea was to construct a sulfamide with both polar and nonpolar chains, to promote the interactions with the elongated hydrophilic region close to the active site in CAVII as well with the other lipophilic half of the cavity. Snapshots of the molecular dynamic simulations previously described allowed us to access to elongated conformation of CAVII, and docking studies were also performed to analyze these new designed sulfamides. We employed the docking conditions described before for N,N'-dicyclopropylsulfamide.

However, in the simulations with the amino acid-derived sulfamides, two deprotonated forms can be constructed, since the substituents of the sulfamide function are different. Both anions were considered, and the most stable complex predicted by docking (lower binding energy) was conserved [86].

We found very interesting results in terms of CAVII activity and selectivity against CAII. Figure 4 shows one of the most promising structures (compound 6). It was also active against MES test in mice confirming the anticonvulsant activity in this classical model [94]. Compound 6 showed all animals protected in MES assay (3/3) at the lower dose tested (30 mg/kg), 4 h after administration [94].

We also studied the CA inhibition of the artificial sweetener sodium cyclamate (Fig. 4, structure 7). It showed important potency and selectivity against the off-target CAII [70]. This structure presents a sulfamate salt and it was previously tested by us in animal models of convulsion with positive results [95]. The selection of this compound was based on the results found for another sweetener, saccharine, which showed anticonvulsant action and high inhibitory potency and selectivity against CAVII [96].

4 Conclusions

Carbonic anhydrase is inhibited by drugs employed to treat several diseases, such as cancer, glaucoma, and also epilepsy. Nowadays, the main challenge in the design of CA inhibitors is to find compounds that act selectively and with high potency against specific isoforms. In this chapter, we have focused on successful examples of CAVII inhibitors, which prove its potentiality as anticonvulsant target. We are aware that more studies are needed to complete the anticonvulsant profile of the new compounds; but we consider that this isoform has much future in the treatment of febrile seizures and, perhaps, in other epileptic syndromes.

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