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Invited article review

A Simple Yet Multifaceted Enzyme

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Abstract*. Carbonic anhydrases (CAs) are involved in many physiological and pathological .events in organisms all over the phylogenetic tree. Over the last three decades I was involved in unravelling the biochemical basic phenomena connected to these enzymes and in drug design of modulators of their activity (inhibitors and activators). The various approaches I developed were applied to many types of such enzymes and allowed the discovery of many classes of highly isoform-selective inhibitors. This afforded new applications of the inhibitors for the management of hypoxic tumors, neuropathic pain, cerebral ischemia, arthritis, degenerative disorders apart the classical ones connected with these drugs (diuretics, antiglaucoma, antiepileptic and antiobesity action). The study of CA activators showed that these enzymes may represent a crucial family of new targets for improving cognition as well as in therapeutic areas, such as phobias, obsessive-compulsive disorder, generalized anxiety, and post-traumatic stress disorders, for which few efficient therapies are available.*

Keywords*: carbonic anhydrase; inhibition mechanism; inhibitor; activator; tail approach; anticancer drug; diuretic; neuropathic pain; neurodegeneration; memory therapy*

1. Introduction

I started to work on the metalloenzyme carbonic anhydrase (CAs, EC 4.2.1.1) in 1987 and in the first 15 years of my research the constant leitmotiv was "when will you stop working on such a simple and well-known enzyme"? Such queries came from friends, collaborators and sometimes professors with a much more important experience in chemical/biomedical research than myzelf. Indeed, CAs are present in all living organism, acting as catalysts for the reversible hydration of CO_2 to bicarbonate and protons, an exquisitely simple chemical transformation [1-5]. Indeed, the enzyme was already reported in the '30s of the last century, its inhibitors (primary sulfonamides) were known since the '40s and the first clinically used agents based on CA inhibitors (CAIs) were launched in the 50s [1-6]. Thus, the expressed doubts were in fact quite reasonable, but fortunately did not stop me for continuing my trip in exploring CAs, their inhibitors, activators and their pharmacological/ biotechnological applications [7-10]. Why are CAs relevant may be understood from the fact that these enzymes act on carbon dioxide and water, two neutral molecules, which are very efficiently converted to bicarbonate and H^+ ions, generating a weak base and a very strong acid [5-10]. As a consequence, and due to the high availability of CO_2 from metabolic processes, this reaction constitutes the basis of pH regulation in all living organism [1-4]. Furthermore, CAs are metabolic enzymes [10], being involved in many other processes apart pH regulation, as I proposed and demonstrated recently, mainly but not exclusively in the tumor metabolism [1-3].

Eight genetically distinct CA families were reported to date in various organisms, the α-, β-, γ-, δ-, ζ-, η, θ, and ι-CAs. The last three classes were only recently discovered [11-17] and the η-CA class was reported by my group [11,14]. The CA-classes distribution is rather variegated in most organisms investigated so far, with many of them (except animals) possessing more than one genetic family [1-11].

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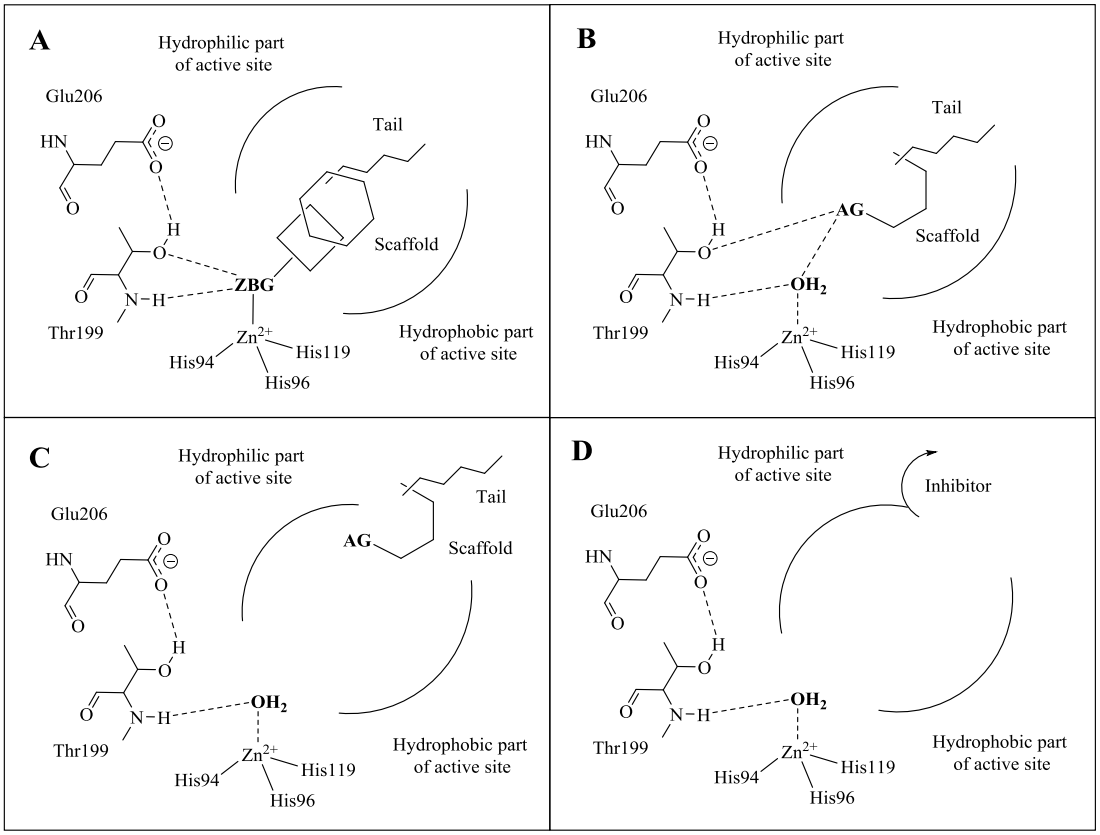
These enzymes, as I mentioned above, are drug targets for almost 7 decades, with virtually all modern diuretics being developed from acetazolamide **1**, the first drug belonging to the CAIs to enter into clinical use in 1954 [1-3]. Nowadays CAIs are still used as diuretics [18,19], systemic and topically acting antiglaucoma agents [20-22] but also for the management of epilepsy [23], and obesity [24,25], whereas some compounds are in clinical development for the management of hypoxic, metastatic cancers [26-29]. However, the last years saw many other interesting developments, all of which reported in proof-of-concept studies from my group, showing that some types of CAIs improve conditions for which few or no therapeutic opportunities are available, such as neuropathic pain [30,31], cerebral ischemia [32], and some forms of arthritis [33-35]. All these developments were only possible due to the fact that highly isoform-selective inhibitors were developed, based on a series of discoveries related to the mechanism of action of novel classes of such modulators of activity [2-8,36]. Crucial to these discoveries were the rationalization of the various CA inhibition mechanism as well as the discovery of the CA activation mechanism, which were achieved in our laboratory in the last 30 years and which may explain why I continued to be interested in this simple but multifaceted enzyme.

2. CA inhibition/activation mechanisms

Four CA inhibition mechanisms are known to date, being characterized in details by X-ray crystallography, kinetic and biophysical studies, together with an activation mechanism (Figure 1).



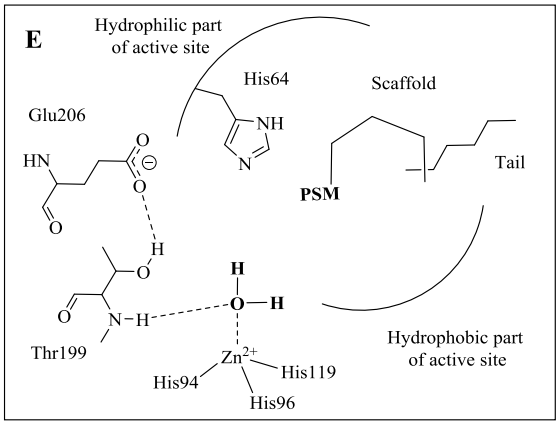


Figure 1. CA inhibition (**A-D**) and activation (**E**) mechanisms. The zinc binders incorporate a ZBG (**A**); the compounds anchoring to the nucleophile an AG that interacts with the zinc-coordinated water (**B**). The inhibitors occluding the active site entrance (**C**) also contain AG moieties but bind more externally, whereas the inhibitor binding outside the active site are shown in **D**. The activators bind in the middle of the active site and contain a proton shuttle moiety (PSM) of the amine, imidazole or carboxylate type (**E**). All these modulators incorporate various scaffolds and tails in their molecule.

(i) CA inhibitors acting as zinc binders

This is the classical CA inhibition mechanism (Fig. 1A). Being a metalloenzyme, the Zn(II) ion from the active site of α-CAs (or other cations which may be present in other CA genetic families, e.g., Fe(II), Cd(II), Co(II) or Mn(II) [1,6-8,13]) may bind metal complexing anions known to have affinity for cations such as but not only cyanide, thiocyanate, azide, halides, etc. [36]). The same situation is observed for compounds possessing a zinc-binding group (ZBG) possibly attached to a scaffold [37,38] – Fig. 1A. Sulfur-based, carbon- [38] or phosphorus- [39] based ZBGs were investigated, whereas some boron-containing derivatives (e.g., the benzoxaboroles [40]) were only recently shown to afford highly effective inhibitors [41]. There are many chemical classes showing effective CA inhibitory activity with this mechanism of action: sulfonamides, sulfamates and sulfamides, which are the super-classical CAIs known to date [1-3,37,38]. Acetazolamide **1** is the archetypical example, and it binds the Zn(II) ion from the CA active site as shown in Figure 1A: the ZBG coordinates to the zinc ion (through the deprotonated nitrogen of the sufonamide moiety), participates in hydrogen bond networks with conserved amino acid residues, a Thr and a Glu residue), whereas the scaffold interacts with the two halves of the active site, one hydrophobic, one hydrophilic (Figure 1A). Other CAIs belonging to the zinc binders are the *N*-substituted sulfonamides incorporating small substituting groups on the nitrogen (OH, NH_2, Me, etc.) [38], the benzenephosponamidates [39], benzoxaboroles [40,41], dithio-carbamates [42], monothiocarbamates [43], xanthates [44], thiols and selenols [45,46], some aromatic, aliphatic or heterocyclic carboxylates [47-50], hydroxamates [51] and even carbamates [52]. Except sulfonamides which were reported in the 40s, and sulfamates in the 80s [1], all these classes of CAIs and their inhibition mechanism were reported by my group.

(ii) CA inhibitors acting by anchoring to the zinc-coordinated water

Phenol (C_6H_5OH) was the first compound for which this binding mode has been reported [53]. The OH moiety of phenol (abbreviated as AG, anchoring group, in Fig. 1B), is anchored through a strong hydrogen bond to the zinc coordinated water molecule whereas a second hydrogen bond involves the NH of Thr199 [53]. A large number of synthetic or natural product phenols were thereafter investigated as CAIs and for some of them the crystal structures were reported in complex with hCA II, making phenols a rather well studied class of CAIs [54-58]. Furthermore, the same inhibition mechanism though anchoring to the zinc-coordinated water molecule was observed through X-ray crystallography and kinetic techniques, for several other classes of compounds among which the

polyamines [59], sulfocoumarins [60], thioxocoumarins [61,62] and some carboxylates [47]. Again these new classes of CAIs were discovered by us in Florence.

(iii) CA inhibitors occluding the entrance to the active site

A natural product coumarin was the starting point leading to the discovery of this CA inhibition mechanism [63] as well as a wealth of new types of CAIs belonging to various chemical classes [73-81]. When this coumarin was co-crystallized with hCA II, the original compound was not found in the electronic density; instead, its hydrolysis product, a *cis*-2-hydroxy-cinnamic acid derivative was found bound in a region of the CA active site where inhibitors were never observed to bind, the entrance of the cavity [63] – Figure 1C. This type of inhibitor was thereafter termed as a "prodrug CA inhibitor", since the esterase activity of the enzyme is needed to generate the real inhibitor, a 2-hydroxy-cinnamic acid derivative, form the original coumarin [63]. Furthermore, no direct interaction with the metal ion or with the zinc coordinated water was observed, as the inhibitor was located far away from the bottom of the active site, occluding its entrance. This binding mode and inhibition mechanisms are rather extravagant *per se*, considering just the structural features mentioned above, but it was thereafter observed that they lead to a highly desired inhibition profile for this type of compounds: the possibility to design highly isoform-selective inhibitors for the different human isoforms [1,6-8,63-71]. In fact, the binding site of these CAIs is situated in the most variable region of the CA active sites among the 15/16 α-CA isoforms found in vertebrates, humans included [1-8]. Indeed, considering only the active site, the zinc coordinating residues as well as most of the bottom and mid-active site amino acid residues are conserved among the different CA isoforms, whereas the highest variability is observed in residues at the entrance of the cavity, where the hydrolyzed coumarins bind [1,6-8,63,64]. As a consequence, many drug design studies of coumarin [63-71] and coumarin-like compounds [72 - 77] were reported, all of them leading to isoform-selective CAIs. These new classes of prodrug inhibitors include among others, 5- and 6-ring lactones/thiolactones [72], 3,4-dihydro-1H-quinoline-2-ones [73], heterocoumarins, such as selenocoumarins, thioselenocoumarins, tellurocoumarins and variously substituted quinoline-2(1H)-ones [77].

(iv) CA inhibitors binding out of the active site

A benzoic acid derivative (2-(benzylsulfinyl)benzoic acid) was observed (by means of X-ray crystallography) bound outside the active site cavity (Figure 1D) of hCA II, in an adjacent hydrophobic pocket at the entrance of the active site [78]. This is a very intriguing binding mode, which does not involve the active site of the enzyme where the catalytic processes occur. Indeed, the COOH moiety of the inhibitor was observed to be orientated towards the active site entrance, more precisely towards His64 which acts as the proton shuttle residue in the catalytic cycle of these enzymes [1,2]. This COOH bridges the imidazole of His64 by a water molecule with which both of them make hydrogen bonds. In this way the side chain of His64 is blocked in its *out* conformation [1,2], being unable to participate to the catalytic cycle, which collapses [78].

(v) CA activation mechanism

The carbonic anhydrase activators (CAAs) belong to the biogenic amines (histamine, serotonin, and catecholamines), amino acids, oligopeptides, or small proteins classes [79]. The general mechanism of action for the CA activators (CAAs) is shown in equation *1* [79,80] and was proposed by myself already in 1990 and then confirmed in 1997 when the first X-ray crystal structure of an activator bound to CA was reported (Figure 1E) [80].

$$EZn^{2+}\text{—}OH_2 + A \rightleftharpoons \underbrace{[EZn^{2+}\text{—}OH_2 \text{ - } A] \rightleftharpoons [EZn^{2+}\text{—}HO^- \text{ - } AH^+]}_{\text{enzyme - activator complexes}} \rightleftharpoons EZn^{2+}\text{—}HO^- + AH^+ \quad (1)$$

The activator binds within the enzyme active site with the formation of enzyme – activator complexes in which the activator molecule (which incorporates a proton shuttling moiety, PSM, Figure 1E) participates to the rate-determining step of the catalytic cycle, i.e., transfer of protons from the zinc-coordinated water to the external reaction medium, similar to the natural proton shuttle, which is residue His64 (in many CA isoforms) [-3]. In such enzyme-activator complexes, the proton transfer becomes intramolecular, being more efficient compared to the intermolecular transfer to buffer molecules, not bound within the enzyme cavity [81-84]. Many X-ray crystal structures with amines and amino acid activators were reported, among which histamine, L- and D-His bound to hCA II and hCA I, L- and D-Phe, D-Trp and L-adrenaline bound to hCA II, which confirmed this general CA activation mechanism [79-84]. The thirteen catalytically active mammalian CAs, (e.g., CA I-VA, VB, VI, VII, IX, XII-XV) were investigated for their interaction with a library of amino acids and amines. It is worth mentioning that CA targeted drug design studies on CAAs are in their infancy: most of the known activators were identified by screening libraries of amines and amino acids followed eventually by the subsequent derivatization of such compounds (e.g., histamine, histidine, etc.) [80-84]. Administration of CAAs (such as D-Phe) to animal models of various diseases, was shown to lead to enhanced discrimination learning [85]. CAAs-induced increased ERK phosphorylation which is necessary for memory consolidation, and recent data suggest that these enzymes may represent a crucial family of new targets for improving cognition as well as in therapeutic areas, such as phobias, obsessive-compulsive disorder, generalized anxiety, and post-traumatic stress disorders, for which few efficient therapies are available [85].

3. Clinically used CAIs

There is a large number of CAIs in clinical use (Figure 2), some discovered directly as inhibitors of these enzymes (acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, sulthiame **4**, dichlorophenamide **5,** dorzolamide **6** and brinzolamide **7**) [2,3,6-8], whereas for other derivatives the CA inhibitory activity was documented by us, after those compounds were developed as drugs for other purposes, such as antagonists of dopamine D_2 receptors, for sulpiride **8** [86], antiepileptics, for topiramate **9** [87] and zonisamide **10** [88], sweeteners, for saccharine **11** [89], COX-2 selective inhibitors, for celecoxib **12** [90] and valdecoxib **13** [91], histamine H_2-receptor antagonists, for famotidine **14** [92], tyrosine kinase pan-inhibitors, for pazopanib **15** [93], etc. SLC-0111 (compound **16**) is in Phase II clinical trials as an antitumor/antimetastatic agent was developed in my group [94-97], whereas the indoleamine-2,3-dioxygenase inhibitor epacadostat **17**, in Phase III clinical trials as an antitumor drug, was reported again by us to act as a CAI [98]. It should be mentioned that all these CAIs are zinc binders, as they possess as ZBG the sulfonamide, sulfamate or sulfamide moieties, discussed in the preceding paragraph. However, most of them behave as pan-inhibitors, being effective binders to most of the 12 active CA isoforms, thus provoking side effects when used as drugs [1-8]. Only the last generation compounds, with an elaborate scaffold, such as the derivatives **15-17**, possessing a more extended conformation, were observed to interact with external parts of the enzyme active site, such as for example its entrance or the middle part, and indeed they do show isoform-selective behavior [94-98]. These phenomena were extensively documented in the X-ray crystal structures of adducts of **1-17** with various isoforms, such as CA I, II, and IX reported by my group over the last two decades [86-98]. In all compounds in which the scaffold participates in important interactions with amino acid residues from the external part of active site, selective inhibition of various isoforms was observed, which explains why CAIs possessing non classical inhibition mechanisms (e.g., mechanism shown in Fig 1B-1D) lead to isoform-selective inhibitors devoid of the serious side effects of the sulfonamides or more generally, first generation inhibitors [1-8]. However, all compounds shown in Fig. 2 are still in clinical use for various pathologies [18-28].

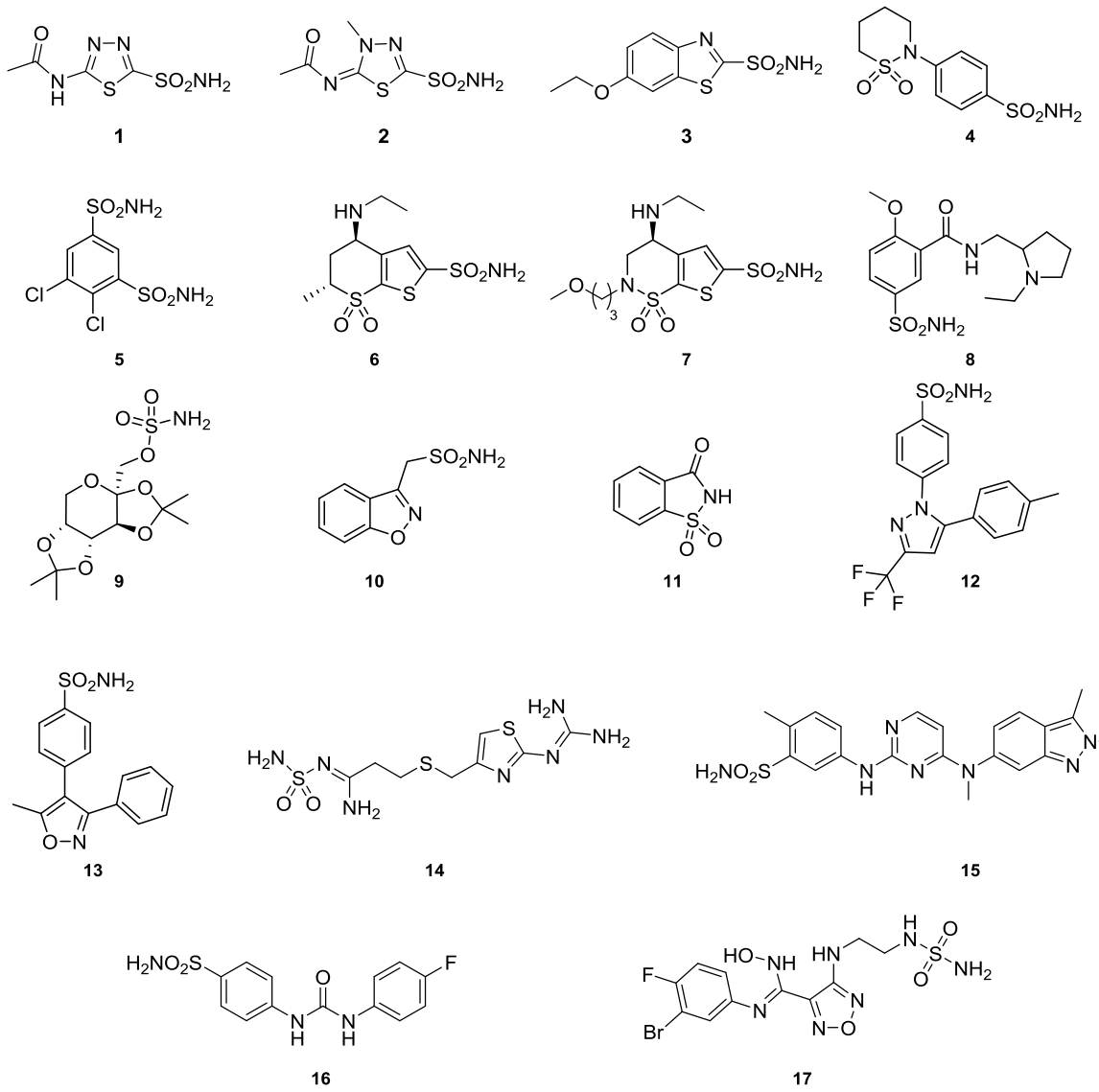


Figure 2. Clinically used CAIs **1-15,** and compounds in clinical trials, SLC-0111 **16** and epacadostat **17**.

Probably one of the most unexpected development in the field of CAI drug design was the discovery of the tail approach in 1999 [99-102]. My initial idea was to attach to the scaffold of simple sulfonamides (such as 4-aminobenzenesulfonamide and its derivatives or 5-amino-1,3,4-thiadiazole-2-sulfonamide, the deacetylated acetazolamide derivative) moieties that may induce enhanced hydrosolubility and presumably also interactions with more external parts of the active site, e.g., the region at the entrance of the cavity [99]. It should be mentioned that although the X-ray structures of many human CA isoforms was already known in that period, around 2000, the number of inhibitor adducts was rather reduced. Thus, the tail approach was initially more of an intuition than a structure-based drug design campaign, but it soon thereafter became one. Indeed, a large number of X-ray structures of chemically heterogeneous sulfonamides bound to CA II (and more rarely to other isoforms) started to appear in the next decade after the tail approach was reported [103-112], which allowed for a rationalization of these structural and kinetic data, also obtained by assaying large homologous series of sulfonamides/sulfamates/sulfamides against all the catalytically active isoforms in my group in Florence. The tail approach was thereafter successfully applied to other classes of CAIs, such as the dithiocarbamates and their derivatives [42-44], the selenols [45,46], to some carboxylic acids [47-50] – although these CAIs show multiple binding modes, being able to inhibit

CAs by all four mechanisms (i)- (iv) mentioned above – and more recently to the benzoxaboroles [40,41]. Virtually, all drug design studies worldwide were inspired by the tail approach in the last two decades after it has been reported.

4. Novel applications of the isoform-selective CAIs targeting human enzymes

(i) Cancer and metastasis

At least three CA isoforms, the transmembrane CA IX and XII, and the cytosolic CA II, are involved in tumorigenesis both by regulating intra- and extracellular pH [4,9,113-115] and tumor metabolism [10,116]. CA IX and XII are overexpressed in tumors secondary to hypoxia through activation of the pathway regulated by the transcription factor HIF-1α (hypoxia inducible factor-1α) [1-4,113]. Starting with 2004 [113], a lot of evidence accumulated that inhibiting the transmembrane isoforms IX/XII leads to an impaired growth of the tumors and metastases [94], which culminated with the proof of concept study of Lou et al. [95] and the discovery and development of SLC-0111 in our laboratory [97]. The main challenges for arriving to CA IX-selective inhibitors were not few, as the active site of this isoform is rather similar to that of CA II [1,117]. However, the determination of its X-ray crystal structure by De Simone's group in 2009 [117] was the breakthrough which helped the rational drug design of more effective CA IX inhibitors [1-8]. The emergence of coumarins already in 2009 as new CA inhibitory chemotype [73] with a significant selectivity for CA IX/XII over other isoforms [64-77], as well as the progress done in sulfonamide chemistry with the tail approach, led to a considerable number of highly CA IX/XII-selective inhibitors belonging to various classes [1-10]. Thus, the study of the multiple binding modes of various types of inhibitors to the three isoforms mentioned here, CA II, IX and XII, vas decisive for validating CA IX/XII as antitumor/antimetastatic drug targets. Nowadays, the largest number of new publications in the field are those dealing with novel classes/types of CA IX/XII inhibitors [27].

(ii) Cerebral ischemia

Another condition characterized by hypoxia, as for tumors, see discussion above, is that of cerebral ischemia when impaired or insufficient blood supply to the brain leads to overexpression of HIF-1α downstream targets, such as CA IX and XII [32]. In a proof-of-concept study, our group [32] demonstrated that both sulfonamide and coumarin CA IX/XII selective inhibitors were able to increase the neurological score (up to 40 %) in rats with permanent middle cerebral artery occlusion as an animal model of cerebral ischemia. Considering the fact that the therapeutic opportunities for this disease affecting a considerable number of patients worldwide are quite limited, this research [32] opens interesting possibilities for the applications of CA IX/XII-selective inhibitors in this therapeutic area.

(iii) Neuropathic pain

Neuropathic pain affects up to 8 % of the world population and the only effective treatment to date is constituted by gabapentin, which however does not work in all patients [30,31]. The relationship between neuropathic pain and CA inhibition was discovered by Kaila's group [118] who demonstrated that spinal GABAergic networks are responsible of the bicarbonate concentration, which leads to depolarization via a reduction in the neuron-specific potassium-chloride (K^+-Cl^-) cotransporter (KCC2) activity. Furthermore, the same group showed that CAIs can reduce the bicarbonate-dependent $GABA_A$ receptors depolarization, which has as a consequence an analgesic effects [118], hypothesizing that probably more than one CA isoforms were involved, with hCA VII and hCA II, being the most relevant ones [119]. The inhibitor used in these pioneering studies of Kaila's group was acetazolamide **1**, which as mentioned above, is a pan-inhibitor showing a multitude of side effects when used for the treatment of various conditions [120,121]. Thus, a campaign to develop CA VII-selective inhibitors was started, which led to the design of compounds with a good selectivity ratios for inhibition of hCA VII over other isoforms. They belong again to various classes, among which the

sulfonamides [30,122-124] and the sulfamates [125] were the predominant and most effective ones. In many cases, the X-ray crystallography or computational methods were very useful for the design of the inhibitors and for rationalizing their selectivity for the target isoform hCA VII versus the off-target CAs [122-125]. Although many such compounds are presently available, and in animal studies of neuropathic pain they work quite effectively, no compound of this class progressed for the moment to clinical trials.

(iv) Arthritis

We reported that CA IX and XII are overexpressed in some forms of arthritis [33] and hypothesized that their inhibition with sulfonamides/coumarins acting as selective inhibitors may have a beneficial effect. This has been thereafter demonstrated in an animal model of the arthritis, providing the proof of concept study that these enzymes, and possibly also CA IV may be considered as drug targets for the management of arthritis [33-35]. Thus, several types of non-steroidal anti-inflammatory drugs belonging to the carboxylic acid derivatives, were conjugated to amino-tailed sulfonamides [34] or coumarins [35], affording hybrid derivatives, by the well-known tail approach discussed above. These compounds showed effective CA IX/XII inhibitory profiles and had a potent and long-lasting antihyperalgesic effects in a rat model of arthritis [34,35]. The same potent effects were observed for hybrids of CAIs of the sulfonamide/coumarin type which were decorated with carbon monoxide releasing moieties of the cobalt carbonyl type [126] in the same animal model of arthritis, reinforcing our hypothesize that CAIs may have a future for the management of this widespread disease.

(v) Neurodegenerative conditions (Alzheimer's disease)

Two recent studies from [127,128] showed that acetazolamide **1** and methazolamide **2** (Fig. 2) were effective in the prevention of mitochondrial dysfunction, caspase activation and cell death associated with amyloid β formation in animal models of Alzheimer's diseases. Methazolamide and acetazolamide were also shown to be effective in reducing memory impairment and amyloid pathology in a transgenic mouse model of amyloidosis, as reported by the same group [129]. Although no drug design study was reported so far for CAIs potentially useful for this devastating pathology, these findings mentioned above and a recent rationalization [130] of the possible mechanism behind these interesting effects of the CAIs may open the way to novel and potentially highly relevant applications for this class of pharmacological agents, considering the fact that no effective anti-Alzheimer's disease agents are available so far.

5. Conclusions

The wealth of possible binding modes for inhibitors/activators within the CA active site and the new generation isoform-selective inhibitors, most of which were discovered by me and my group, afforded interesting applications in new research fields, with relevant results being obtained for compounds involved in the management of hypoxic tumors [27,97], neuropathic pain [30,31], cerebral ischemia [32], arthritis [33-35] and neurodegenerative diseases, such as Alzheimer's disease [127-130]. The activator field, still in its infancy from the pharmacologic viewpoint, may lead to a better understanding of cognition but may as well lead to applications in therapeutic areas, such as phobias, obsessive-compulsive disorder, generalized anxiety, and post-traumatic stress disorders, for which few efficient therapies are available. Thus, my involvement for more than 30 years in the research field of just a single, yet highly multifaceted enzyme, may be considered of some relevance, although I was and am working on various other drug targets, such as metalloproteases [131-135], serine proteases [136,137] or viral proteases [138-140]. However, the work on CAs remain the most well-known one.

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with us, my gratitude to Prof. Mircea D. Banciu and to Prof. Costantin Luca, both from the Polytechnic Univ. of Bucharest, will remain a constant feeling due to their long-lasting mentorship and friendship. My present and past research group(s) are probably the most relevant scientific and human adventures I lived and that was and is very rewarding. The collaborations with research groups and the friendship with colleagues from all over the world is another relevant point in my career. To all of them I am extremely grateful.

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