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Carbonic Anhydrase Inhibitors and Activators: Small Organic Molecules as Drugs and Prodrugs

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1. Introduction

This chapter concerns influences of inhibitors and activators on carbonic anhydrase isoenzymes of various living systems. Carbonic anhydrase (EC 4.2.1.1., CA) is a pH regulatory/metabolic enzyme in all life kingdoms, found in organisms all over the phylogenetic tree (Supuran, 2008a,b,c) catalyzing the hydration of carbon dioxide to bicarbonate and the corresponding dehydration of bicarbonate in acidic medium with regeneration of CO_2 (Sly and Hu, 1995). 16 isozymes have been described up to now in mammals, the most active ones as catalysts for carbon dioxide hydration being CA II and CA IX (Sly and Hu, 1995; Ozensoy et al., 2004; Bayram et al., 2008; Senturk et al., 2009; Hilvo et al, 2008). The sixteen isozymes differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. Some of them are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA VB), and one is secreted in saliva (CA VI). It has been reported that CA XV isoform is not expressed in humans or in other primates, but it is abundant in rodents and other vertebrates (Table 1.) (Nair et al., 1994; Parkkila et al., 1996; Pastorekava et al., 2004; Bayram et al., 2008; Innocenti et al., 2008a,b; Senturk et al., 2009; Ekinci et al., 2011). CAs are produced in a variety of tissues where they participate in several important biological processes such as acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and body fluid generation (Supuran, 2008a,b,c). CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited / activated for the treatment of a range of disorders such as edema, glaucoma, obesity, cancer, epilepsy and osteoporosis (Innocenti et al., 2008b; Ekinci et al., 2007; Ekinci et al., 2011).

Isozyme	Catalytic activity (CO_2 hydration)	Affinity for sulfonamides	Sub-cellular localization
CA I	Low (10%of thatof CAII)	Medium	Cytosol
CA II	High	Very high	Cytosol
CA III	Very low (0.3%of thatof CAII)	Very low	Cytosol
CA IV	High	High	Membrane-bound
CA VA	Moderate-high[a]	High	Mitochondria
CA VB	Moderate	High	Mitochondria
CA VI	Moderate	Medium-High	Secreted into saliva/milk
CA VII	High	Very high	Cytosol
CARP VIII	Acatalytic	*	Cytosol
CA IX	High	High	Membrane-bound
CARP X	Acatalytic	*	Cytosol
CARP XI	Acatalytic	*	Cytosol
CA XII	Medium-Low	Very high	Membrane-bound
CA XIII	LOW	High	Unknown
CA XIV	Moderate	High	Membrane-bound

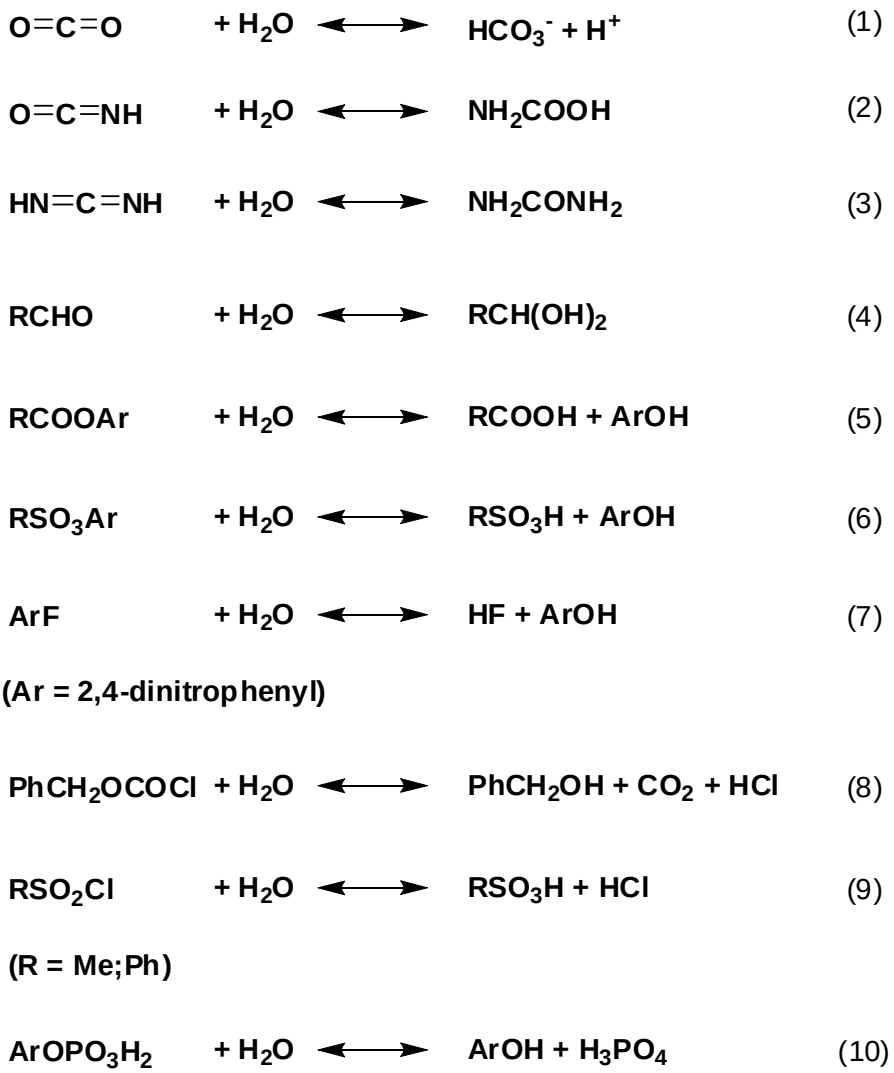
[a]Moderate at pH 7.4, high at pH 8.2 or higher.
[b]CA XIII has not been isolated as a protein but has been identified from an expressed sequence tag (EST) derived from a mouse mammary gland cDNA library.
*The native CARP isozymes do not contain Zn (II), so that their affinity for the sulfonamide inhibitors has not been measured. By site-directed mutagenesis it is possible to modify these proteins and transform them in enzymes with CA-like activity which probably are inhibited by sulfonamides, but no studies on this subject are available presently.

Table 1. Higher vertebrate α-CA isozymes, their Relative CO_2 hydrase activity, affinity for sulfonamide inhibitors, and sub-cellular localization

In addition to the physiological reaction, the reversible hydration of CO_2 to bicarbonate (reaction 1, Scheme 1), CAs catalyze a variety of other reactions, such as: the hydration of cyanate to carbamic acid, or of cyanamide to urea (reactions 2 and 3); the aldehyde hydration to gem-diols (reaction 4); the hydrolysis of carboxylic, or sulfonic (reactions 5, 6), as well as other less investigated hydrolytic processes, such as those described by equations 7-10 in Scheme 1 (Supuran et al., 1997; Supuran and Scozzafava, 2000b; Guerri et al., 2000). It should be mentioned that the previously reported phosphatase activity of CA III was recently proved to be an artefact (Kim et al., 2000). It is unclear at this moment whether CA catalyzed reactions other than the CO_2 hydration has physiological significance.

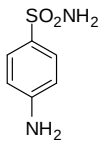
2. Carbonic anhydrase inhibitors

As will be discussed shortly, many of these isozymes are important targets for the design of inhibitors with clinical applications. CA inhibition with sulfanilamide (**1**) discovered by Mann and Keilin (Krebs, 1948) was the beginning of a great scientific adventure that led to important drugs, such as the antihypertensives of benzothiadiazine and high-ceiling diuretics type (Supuran 1994), the sulfonamides with CA inhibitory properties mainly used as antiglaucoma agents (Krebs, 1948; Supuran, 1994; Mansoor et al., 2000), some antithyroid drugs (Krebs, 1948), the hypoglycemic sulphonamides (Maren et al., 1983) and, ultimately,



Scheme 1. Reactions (1-10) catalyzed by α-CAs.

some novel types of anticancer agents (Supuran and Scozzafava, 2000). The report of Krebs (1948) that mainly the unsubstituted aromatic sulfonamides of type $ArSO_2NH_2$ act as strong CAIs, and that the potency of such compounds is drastically reduced by N-substitution of the sulfonamide moiety, constituted the beginning of extensive structure-activity correlations, which led to some valuable drugs during a short period of time. Among the active structures found by Krebs were also the azodyes **2** (prontosil red) and **3**, derived from sulfanilamide (Maren, 1976; Krebs, 1948; Supuran et al., 2003). The early stages of CAIs development have thouroughly been reviewed by Maren (Maren, 1976; Maren, 1967) whereas literature till 1993 was reviewed by Supuran (Supuran and Scozzafava, 2000; Supuran et al., 2003) and more incompletely (up to 1996) by Mansoor et al. (2000). Thus, in this review we will concentrate on the recent developments in this field that led to important advances in the design of topically acting antiglaucoma sulfonamides, isozyme-specific inhibitors, inhibitors with modified sulfonamide moieties, antitumor sulfonamides, as well as diagnostic tools and biosensors based on this class of pharmacological agents (Supuran et al., 2003).



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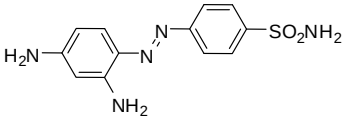

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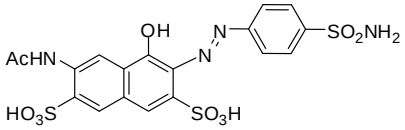

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CAs are inhibited by four main mechanisms: (*i*) coordination of the inhibitor to the Zn(II) ion by replacing the zinc-bound water/hydroxide ion and leading to a tetrahedral geometry of Zn(II) (e.g., the sulfonamides, Fig. 1A) (Supuran 2008a; Alterio et al., 2009). (*ii*) Addition of the inhibitor to the metal coordination sphere, when the Zn(II) ion is in a trigonal bipyramidal geometry (e.g., the thiocyanate adduct, Fig. 1B) (Supuran 2008a; Alterio et al., 2009). (*iii*) Anchoring of the inhibitor to the Zn(II)-bound solvent molecule, i.e., a water or hydroxide ion (e.g. phenol, Fig. 1C) (Nair et al., 1994) (*iv*) Occlusion of the CA active site entrance, with coumarins (Maresca et al., 2009; Maresca et al., 2010) lacosamide (Temperini et al., 2010) and fullerenes (Innocenti et al., 2010) binding in this way, as shown schematically in Fig. 1D for a hydrolyzed coumarin derivative (Maresca et al., 2009). All these binding modes have been demonstrated by means of X-ray crystallography of enzyme-inhibitor adducts (Nair et al., 1994; Supuran, 2008a; Maresca et al., 2009; Durdagi et al., 2011). It should be noted that passing from sulfonamides and their bioisosteres (sulfamates, sulfamides, etc.) to inhibitors occluding the entrance of the active site, the role of the Zn(II) ion is constantly diminishing in its interaction with the inhibitor molecule (Nair et al., 1994; Supuran, 2008a; Maresca et al., 2009; Durdagi et al., 2011). This phenomenon has important consequences for the drug design of CA inhibitors (CAIs), because the bottom of the active site cavity is very much conserved in the 16 CA isozymes described so far in mammals, whereas the regions with the highest variation in amino acid sequence, therefore the highest degree of structural diversity, are just those at the entrance of the active site (Supuran and Scozzafava, 2007b; Supuran, 2008a; Alterio et al., 2009). Indeed, phenols, (Ekinci et al., 2010; Durdagi et al., 2011) but also coumarins (Maresca et al., 2009; Maresca et al., 2010) and other types of non-zinc binder inhibitors (Alterio et al., 2009; Ekinci et al.,

2011) were recently shown to lead to isozyme-selective CAIs, a goal difficult to achieve with the classical sulfonamide/sulfamate inhibitors (Supuran, 2008a; Zu and Sly, 1990).

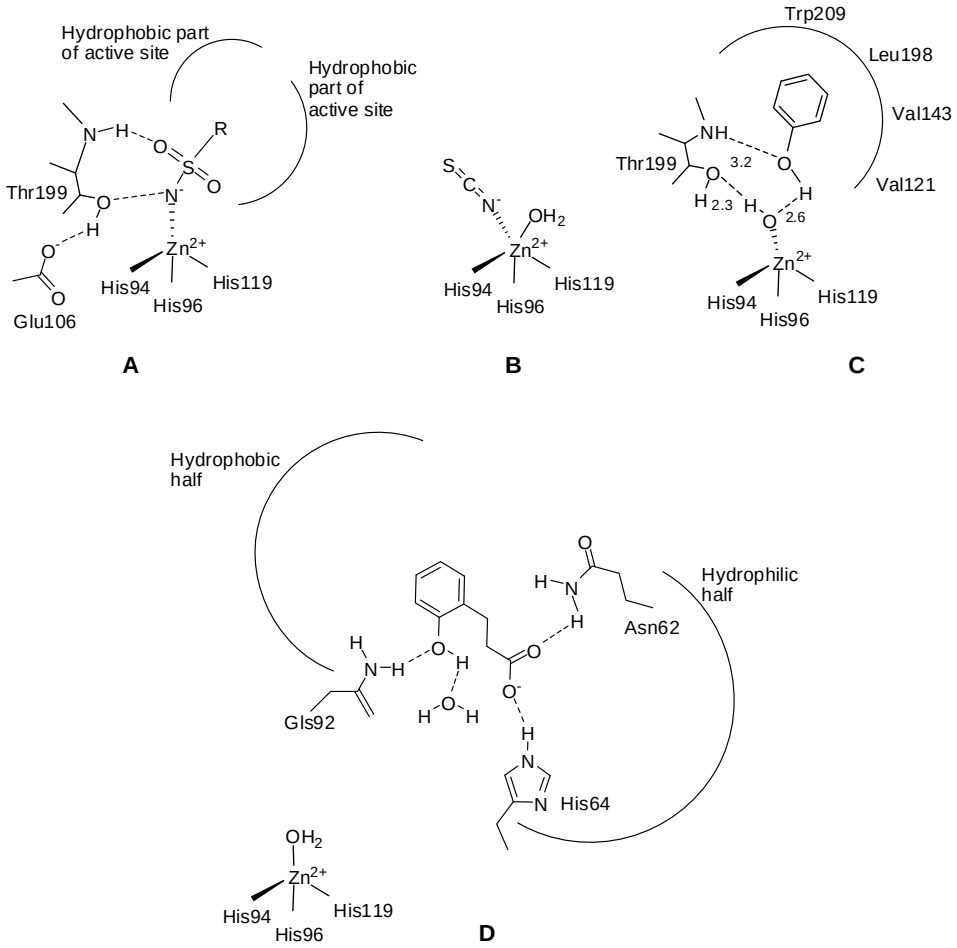


Fig. 1. Schematic representation for the three main CA inhibition mechanisms: (**A**) Sulfonamides (and their isosteres, sulfamate, and sulfamide) substitute the fourth zinc ligand and bind in tetrahedral geometry of the metal ion (Alterio et al., 2009); (**B**) Inorganic anion inhibitors (thiocyanate as an example) add to the metal ion coordination sphere leading to trigonal bipyramidal adducts (Alterio et al., 2009); (**C**) Phenols anchor to the Zn(II) coordinated water molecule/hydroxide ion (Nair et al., 1994); (**D**) Coumarins (hydrolyzed in situ to 2-hydroxycinnamic acids) occlude the entrance of the active site cavity, interacting both with hydrophilic and hydrophobic amino acid residues. The inhibitor does not interact at all with the catalytically crucial Zn(II) ion which is coordinated by three His residues and a water molecule (Maresca et al., 2009; Maresca et al., 2010).

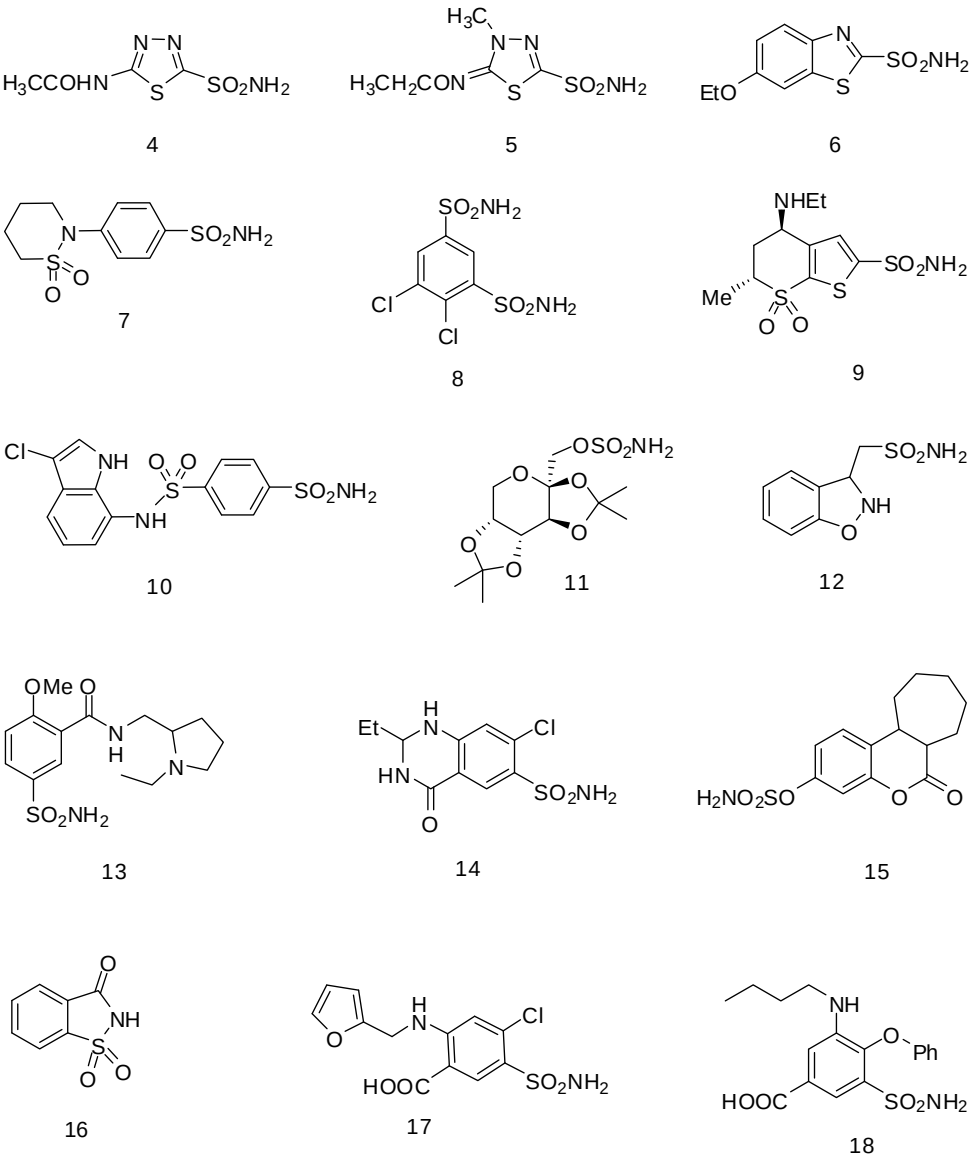


Fig. 2. CAIs include the classical inhibitors acetazolamide (**4**), methazolamide, (**5**) ethoxzolamide (**6**), sulthiame (**7**) and dichlorophenamide (**8**). CAIs also include more recent drugs/investigational agents such as dorzolamide (**9**), indisulam (**10**), topiramate (**11**), zonisamide (**12**), sulpiride (**13**), COUMATE (**15**), saccharin (**16**). Many of these compounds were initially developed years ago during the search for diuretics, among which the thiazides, compounds, as well as derivative **14** are stil widely clinically used (Supuran, 2008a). However, some of these enzyme inhibitors could also be used for the systemic treatment of glaucoma

(see below), and more recently, newer derivatives have been discovered that have the potential as topical antiglaucoma agents, as well as antitumour, anti-obesity or anti-infective drugs (Supuran, 2008a,b,c).

3. Carbonic anhydrase activators

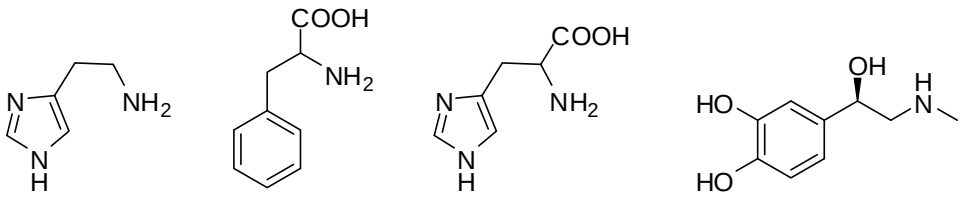
Carbonic anhydrase (CA, EC 4.2.1.1) inhibition with sulfonamides, discovered by Mann and Keilin (1940), and its activation by different classes of compounds, reported by Leiner (1940), although simultaneous, had completely different consequences for research of these enzymes and their modulators of activity. Whereas CA inhibitors (CAIs) were extensively studied, leading to a detailed understanding of the catalytic and inhibition mechanisms and also to several valuable drugs ((Supuran and Scozzafava, 2001), CA activators (CAAs) constituted a controversial issue immediately after they were first described (Kiese, 1941). Thus, activation of crude human red cell enzyme (a mixture of isozymes CA I and CA II) by different compounds, such as histamine, amino acids, and some purine derivatives, has been reported and retracted several times by the above mentioned and other authors (van Goor, 1948; Supuran and Scozzafava, 2000), without arriving at a clear-cut answer regarding the mere existence of such a class of CA activity modulators. This topic, then, received little attention from the scientific community for at least two reasons: (i) the statement by Clark and Perrin (1951) that activators of CA do not exist and (ii) the idea that the reported activation is not a phenomenon per se but an artifact generally due to restoration of CA activity possibly lost in the presence of adventitious metal ions or other impurities (or due to enzyme adsorption at interfaces, or even due to enzyme denaturation followed by renaturation in the presence of activators) (Maren, 1967) Leiner (1940), the researcher whose role in discovering this important class of modulators of CA activity should be completely revaluated, observed among others that the activation was readily detected when working with highly purified enzyme preparations, and this may explain the large discrepancies between the different early studies describing this phenomenon. Only recently Supuran's group reported the X-ray crystallographic structures of adducts of the human isozyme hCA II with different activators, proving undoubtedly the existence of this class of modulators of enzyme activity as well as elucidating their mechanism of action at the molecular level (Maren, 1967; Briganti et al., 1997; Briganti et al., 1998; Scozzafava and Supuran, 2002). The very recent report (Briganti et al., 1998) that some CAAs (such as phenylalanine and imidazole) administered to experimental animals may produce an important pharmacological enhancement of synaptic efficacy, spatial learning, and memory proves that this class of relatively unexplored enzyme modulators may have pharmacological applications in conditions in which learning and memory are impaired, such as for example Alzheimer's disease or aging. One must also mention that it was previously reported that the levels of CA are significantly diminished in the brain of patients affected by Alzheimer's disease (Sun and Alkon, 2001), and these facts strongly support the involvement of different CA isozymes in cognitive functions (Briganti et al., 1997; Briganti et al., 1998; Sun and Alkon, 2001; Meier-Roge et al., 1984; Scozzafava and Supuran, 2002).

The binding of CA activators (CAAs) to various isozymes, such as CA I, II and IV, was studied by kinetic and X-ray crystallographic techniques (the last techniques were applied only for the cytosolic isozymes I and II) (Ilies et al., 2002; Scozzafava and Supuran 2002a,b;

Temperini et al., 2007), which showed the activator to intervene in the rate-determining step of the catalytic cycle, that is, the shuttling of protons between the active site and the reaction medium, a process which in most CA isoforms is assisted by a histidine residue (His64, CA I numbering) placed in the middle of the active site cavity (Scozzafava and Supuran 2002a,b; Temperini et al., 2007). CAAs, there is the possibility of alternative proton transfer pathways, involving a protonatable moiety of the activator bound within the enzyme active site, which explains the enhanced overall catalytic efficiency, reflected in the augmentation of k_{cat}, without any effect on K_M, for all isoforms investigated up to now in detail (i.e., CA I, II, IV, VA, VII, XIII, and XIV) (Ilies et al., 2002; Scozzafava and Supuran 2002a,b; Temperini et al., 2007). X-Ray crystallography of adducts of human CA (hCA) II with histamine (**19**) (Birganti et al., 1997), L- and D-phenylalanine (**20**) (Temperini et al., 2005), and L- and D-histidine (**21**) (Temperini et al., 2006a), as well as the adduct of hCA I with L-His (**21**) (Temperini et al., 2006b), allowed a better understanding of the CA activation phenomenon at the molecular level, bringing also new insights into problems of ligand recognition by an enzyme active site, since it has been observed that enantiomers such as L-/D-His and L-/D-Phe bind in a very different manner to hCA II, interacting with different amino acid residues from the activator binding site. In addition, the interaction between the two best studied isozymes, that is, hCA I and II, with the same activator, L-His, was also very different, with the activator binding deep within the active site cavity in the case of hCA I, and toward the external of the cavity for hCA II. These studies are helpful for the design of better CAAs or for obtaining compounds with selectivity for an isozyme, and less affinity for another one which is not desirable to be activated (or inhibited) (Scozzafava and Supuran 2002a,b; Temperini et al., 2005a,b; Temperini et al., 2007).

L-Adrenaline (**22**), one of the neurotransmitter catecholamines released by the sympathetic nervous system and adrenal medulla in response to a range of stresses in order to regulate the host physiological functions, is involved in regulation of blood pressure, vasoconstriction, cardiac stimulation, relaxation of the smooth muscles (such as the bronchial ones) as well as in several metabolic processes (Hoffman, and Lefkowitz, 1996). As a consequence, 4 has a variety of clinical uses, such as among others for relieving respiratory distress in asthma, in treating hypersensitivity reactions due to various allergens, cardiac arrest, or as a topical hemostatic agent, etc . (Hoffman, and Lefkowitz, 1996; Rawas-Qalaji, et al., 2006).

The activating effects of adrenaline (**22**) on CA II (of bovine origin, bCA II) were first investigated by this group (Supuran and Puscas, 1994), being shown that the compound is a weaker CAA, as compared to histamine, aromatic/heterocyclic amino acids or other structurally related amines investigated in the same study. Temperini et al. (2007) decided to investigate in more detail its interaction with various physiologically relevant CA isozymes,1-5 such as CA I, II, IV, VA, VII, and XIV (all of them present among others in the brain).1 Temperini et al. (2007) reported kinetic investigations regarding the activation of the above-mentioned isoforms with L-adrenline (**22**), as well as an X-ray crystallographic study of the hCA II–4 adduct. Same study work may bring a better understanding of the CA activation processes, potentially useful for the design of pharmacological agents, whereas from the chemical point of view, it reveals a completely new interaction between the activator and the enzyme, which explains at the molecular level the lower efficacy of adrenaline as a CA II activator (Temperini et al., 2007).



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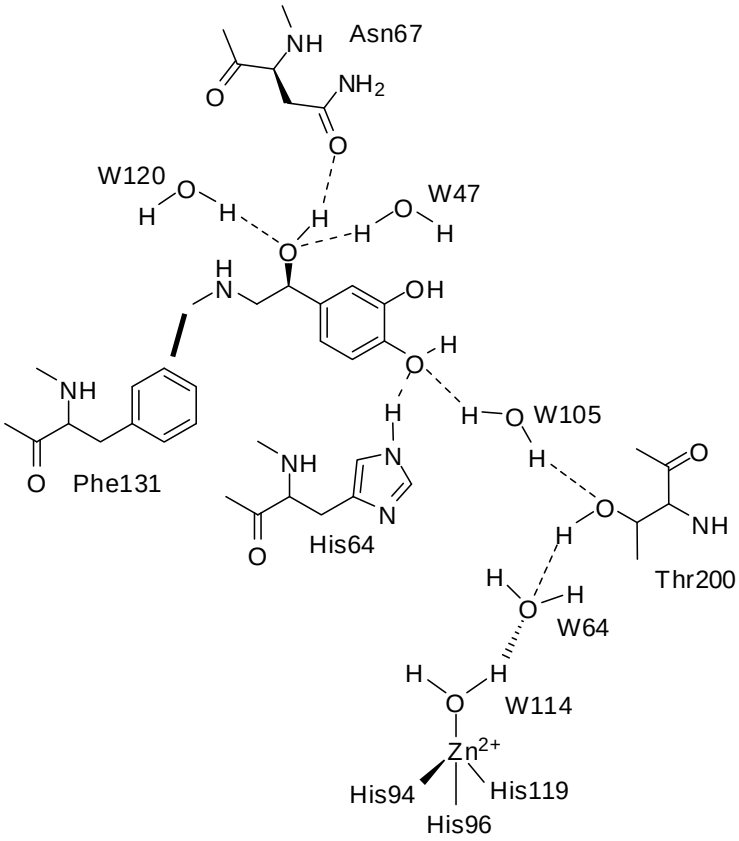

Fig. 3. Schematic representation for the binding of L-adrenaline to the hCA II active site. The Zn(II) ligands, hydrogen bonds connecting the Zn(II) ion and the activator molecule with other amino acid residues/water molecules through a network of hydrogen bonds, stabilizing the enzyme-activator complex, are also evidenced (dotted lines). His64 is shown only in the 'in' conformation, the only one making a hydrogen bond with the activator molecule. The 'out' conformation of His64 does not interact with the activator. The methylamino group of 4 does not participate in any polar interaction, being rather close to the phenyl ring of Phe131 (bold line) (Temperini et al., 2007).

Temperini et al. (2007) studied may also shed new light in the recognition processes by metalloenzymes of ligands which do not directly interact with the metal ion, phenomena far less investigated up to now, since the majority of ligands interacting with metalloenzymes usually directly coordinate the metal ion(s) from the enzyme active site.

The X-ray crystal structure of the CA II L-adrenaline adduct revealed the reason why this compound is a weaker activator as compared to histamine (**19**) and related biogenic amines/amino acids. Thus, in contrast to other activators investigated earlier, L-adrenaline (**22**) plugs the entrance of the active site cavity, obstructing it almost completely. In this conformation, it is unable to facilitate the shuttling of protons between the active site and the environment, also because the pKas of its protonatable moieties are in the range of 8.6–11.34. On the contrary, histamine bound to the enzyme active site adopts a conformation that allows its imidazolic moiety (with a pKa around 7) to easily participate in proton shuttling, similarly with residue His64, the natural proton shuttle amino acid in the CA II active site. These findings explain thus that both the steric requirements (orientation in which the activator binds within the active site) and electronic factors (pKa of the proton shuttle moiety) are important for a compound to act as an effective CA activator, and may shed new light in the recognition processes by metalloenzymes of ligands which do not directly interact with the metal ion (Temperini et al., 2007).

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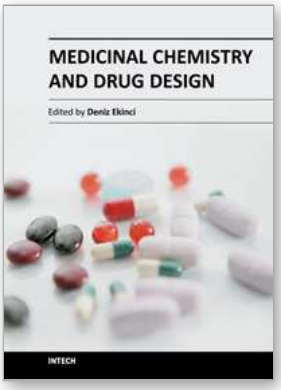

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Over the recent years, medicinal chemistry has become responsible for explaining interactions of chemical molecules processes such that many scientists in the life sciences from agronomy to medicine are engaged in medicinal research. This book contains an overview focusing on the research area of enzyme inhibitors, molecular aspects of drug metabolism, organic synthesis, prodrug synthesis, in silico studies and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in medicinal chemistry and drug design. Particular emphasis is devoted to both theoretical and experimental aspect of modern drug design. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in associated areas. The textbook is written by international scientists with expertise in chemistry, protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the textbook will enhance the knowledge of scientists in the complexities of some medicinal approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of medicinal chemistry and drug design.

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