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Zinc and Carbonic Anhydrase Inhibitors -An Update

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Abstract: Carbonic anhydrase (CA) catalyzes a simple but essential reaction, CO_2 hydration to bicarbonate and protons. Sulfonamides are the most important class of carbonic anhydrase inhibitors. Several compounds, among which acetazolamide, methazolamide, ethoxzolamide, sulthiame, dichlorophenamide, dorzolamide, brinzolamide, sulpiride and zonisamide, are in clinical use for years, as diuretics, antiglaucoma agents, as well as antiepileptics. All these compounds directly coordinate to the zinc ion from the enzyme active site in a deprotonated form of their sulfonamide/sulfamate zinc binding groups. This review thus highlights the importance of carbonic anhydrase and its structural variants, inhibitors, and the potential aspects of zinc. This may lead to a deeper understanding of the CA active sites and permit the design of isoform specific inhibitors.
Keywords: Carbonic anhydrase, Inhibitors, Metalloenzymes, Zinc.

Introduction

Carbonic anhydrases (CA; EC 4.2.1.1) form a super family of enzymes that catalyzes the reversible hydration of carbon dioxide to bicarbonate and a proton in the absence of a catalyst [1] and thus played an important role in respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues and organs, biosynthetic reactions (such as gluconeogenesis and lipid and urea synthesis), bone resorption, calcification, tumorigenicity and many other physiological or pathological processes [2]. The isoforms of CA vary in location and tissue distribution, thus cytosolic (I, II, III, VII, and XIII), membrane-bound (IV, IX, XII, and XIV), mitochondrial (VA and VB), and secreted (VI) forms have been described [3,4]. The isozymes CA IX and XII have been known as the membrane CAs associated with cancers, which were also found in a very limited number of normal tissues, such as gastrointestinal mucosa and gastrointestinal related structures [5, 6]. These enzymes contain zinc metal, which helps in catalyzing the reversible reaction between CO_2 hydration and bicarbonate dehydration. CA plays a prominent role in transport of CO_2 and protons across the biological membranes such as intercellular and extracellular spaces [7, 8].

These enzymes are found in all the kingdoms of life and are involved in respiration, photosynthesis in eukaryotes and cyanate degradation in prokaryotes. In plant cells, these enzymes are related with the photosynthetic fixation of CO_2 in the presence of chloroplasts [9]. CAs is involved in diverse physiological functions including pH regulation, ion transport, bone resorption and secretion of gastric, cerebrospinal fluid and pancreatic juices [10, 11]. The most important function of CA is related to the respiration and transport of CO_2/bicarbonate in various metabolizing tissues. This enzyme is also involved in electrolyte secretion, CO_2 and pH homeostasis, CO_2 fixation and biosynthetic reactions such as gluconeogenesis and ureagenesis [12-14]. The membrane-bound CAs have unique pattern of functional relationships with membrane transporters. This association depends on the physiological demands of the tissue to smooth the HCO_3 movement process in and outside the cell [15].

The growing information on the virulence mechanisms of pathogens and the relationship with their hosts has led to increased interest among scientists for virulence factors as novel targets for anti-infectious agents, so-called anti-virulence drugs [16, 17]. Metabolic enzymes, also directly linked to virulence in certain cases, represent other potential targets [18]. Among the metalloenzymes, carbonic anhydrase has attracted attention of researchers working in the field of human health, opening new insights into drug discovery. Metalloenzymes are proteins widely distributed in nature which are involved directly in essential biosynthetic processes both in eukaryotic and prokaryotic organisms. They are considered as belonging to the most attractive targets of modern drug therapy.

Mechanism of Action of Carbonic anhydrase

The waste CO_2 released from cells into the capillary blood diffuses across the erythrocyte membrane. In its gaseous form, CO_2 dissolves poorly in aqueous solution, such as blood plasma, and the carbonic anhydrase inside the erythrocyte converts the CO_2 to water-soluble bi carbonate (HCO_3^-) anion. The active site of most carbonic anhydrases contains a zinc ion; they are therefore classified as metalloenzymes. They share a common reaction mechanism: a nucleophilic attack on CO_2 by a Zn^{2+} bound hydroxide, followed by displacement of the product bicarbonate with water, and deprotonation of water to regenerate the hydroxide [19]. A zinc prosthetic group in the enzyme is coordinated in three positions by histidine side-chains. The fourth coordination position is occupied by water. This causes polarisation of the hydrogen-oxygen bond, making the oxygen slightly more negative, thereby weakening the bond. A fourth histidine is placed close to the substrate of water and accepts a proton, in an example of general acid - general base catalysis (see the article "Acid catalysis"). This leaves a hydroxide attached to the zinc. The active site also contains specificity pocket for carbon dioxide, bringing it close to the hydroxide group. This allows the electron-rich hydroxide to attack the carbon dioxide, forming bicarbonate.

The reaction catalyzed by carbonic anhydrase is:

$$H_2CO_3 \quad \text{Carbonic anhydrase} \quad CO_2 + H_2O \text{ (in tissues)}$$

$$HCO_3^- + H^+ \longrightarrow H_2CO_3 \longrightarrow CO_2 + H_2O \text{ (in lungs and kidneys)}$$

Zinc is the key to this enzyme reaction. The water bound to the zinc ion is actually broken down to a proton and hydroxyl ion. Since zinc is a positively charged ion, it stabilizes the negatively charged hydroxyl ion so that it is ready to attack the carbon dioxide. Zinc directs the transfer of this bound hydroxyl to carbon dioxide, forming a bicarbonate ion. Histidine 64 swings towards and away from the zinc ion in each cycle of enzyme action while helping the zinc to recharge with a new hydroxyl ion. As soon as the zinc is reloaded with a new water molecule and the bicarbonate ion has been released, the enzyme will be ready for action on another carbon dioxide molecule The active center normally comprises metal ions in tetrahedral geometry, with three protein ligands in addition to the water molecule/hydroxide ion, but Zn(II) or Co(II) were also observed in trigonal bipyramidal or octahedral coordination geometries, at least in γ-CAs [20, 21].

Functions of Carbonic anhydrase

Carbonic anhydrase is a central enzyme for both transport and metabolic processes at the cellular level. The ability of CAs to facilitate this reaction has linked their activity to many physiological and pathological processes, such as maintaining respiration and transport of CO2 and bicarbonate, pH and CO_2 homeostasis, electrolyte secretion in various tissues and organs; and on the other hand, mitochondrial CA is known to supply HCO3 for the initial reactions of biosynthetic reactions (i.e.gluconeogenesis, lipogenesis, and ureagenesis), bone resorption; calcification; and tumorigenicity and CA inhibitors have the therapeutic potential to treat disorders ranging from cancer to glaucoma to epilepsy [22]. This CA facilitates cellular ammonia transport by providing H^+ ions for the protonation of NH3, thus maintaining the transmembrane ammonia gradient. It was suggested that CA presumably evolved as an enzyme to facilitate the CO_2 transport across the membrane and attains a secondary metabolic role later in the course of evolution. The limited capacity of CO_2 (gas) to be retained by water as well as the slow diffusion rate of CO_2 (aq) which is 10^4 times that in the atmosphere [23] makes the marine environment poor in CO_2 which is required for photosynthesis. Carbonic anhydrases play a

key role in this process: subsequent to the active transport of HCO_3 into the cell, they catalyze the dehydration of HCO_3 to CO_2 required in the first step of Calvin cycle [24].

Inhibitors of Carbonic anhydrase

Carbonic anhydrase inhibitors are a class of pharmaceuticals that suppress the activity of carbonic anhydrase. Several clinically used drugs belonging to the sulfonamide, sulfamate or sulfamide classes possess significant CA inhibitory properties. Sulfonamides/sulfamates compounds show a wide range of inhibitory activity for both the Zn(II) and Cd(II)-containing fragments [25]. It has been known that primary sulfonamides act as carbonic anhydrase inhibitors (CAIs) by binding to the catalytic zinc ion in the active site of the enzyme and blocking its function. Carbonic anhydrases inhibitory property of sulfanilamide has led to the discovery of important drugs, such as the antihypertensives (benzothiadiazines) and high-ceiling diuretics. The sulphonamides' CA inhibition has mainly been used to treat glaucoma, diabetics, and ultimately different types of cancer [26]. The discovery that CA enzymes are over-expressed in many pathological conditions has led to the belief that inhibitors of the CAs could be useful for the treatment of a range of inflammatory disorders. Hence, their usefulness in the treatment of rheumatoid arthritis, osteoarthritis, Crohn's disease, ulcerative colitis, peridontitis, gingivitis, psoriasis, dermatitis, Alzheimer'sdisease, multiple sclerosis, cardiovascular disorders as well as in cancer can be utilized as new therapeutic motif [27].

CA enzymes have been investigated in detail in pathogenic (as well as nonpathogenic) bacteria such as Brucella spp., Mycobacterium tuberculosis, Streptococcus spp., Helicobacter pylori, Salmonella enterica, Sulfurihydrogenibium spp., Vibrio cholera etc., in the search for antibiotics with a novel mechanism of action, since it has been demonstrated that in many of these organisms CAs are essential for their life cycle. For some of them, X-ray crystal structures of the encoded CAs was also determined, and in vitro and in vivo inhibition studies with various classes of inhibitors, such as anions, sulfonamides and sulfamates have been reported [28-31].

Porphyromonas gingivalis is a Gram-negative oral anaerobe involved in the pathogenesis of periodontitis, an inflammatory disease provoking destruction of the tissues supporting the tooth, leading to gums and tooth loss. Recently it was identified that γ-carbonic anhydrase in the P. gingivalis genome, denominated PgiCA, which consists of 192 amino acid residues and displays a low homology (30–33% identity), when compared to the prototypical γ-CAs, CAM and CAMH, reported earlier by Ferry's group. CAM and CAMH are the best known enzymes belonging to the γ -CA class of the P. gingivalis enzyme produced as a recombinant protein in Escherichia coli [32-34].

γ-CA (denominated here PgiCA) in the genome of the pathogenic bacterium Porphyromonas gingivalis, which is a Gram-negative oral anaerobe involved in the pathogenesis of periodontitis. This inflammatory disease destroys the tissues supporting the tooth, eventually leading to tooth loss [35, 36]. Because of the pleiotropic physiological roles, they have wide-ranging of therapeutic potentials, as exemplified by the use of sulfonamides and their bioisosteres, such as sulfamates and sulfamides are the most investigated types of organic inhibitors, having various biomedical applications as diuretics or as drugs for the treatment or prevention of a variety of disorders such as antiglaucoma drugs, anticonvulsants, antiobesity, anticancer, antipain and antiinfective agents in the management of mountain sickness, gastric and duodenal ulcers, neurological disorders, or osteoporosis [37-40].

The inhibition and activation of CAs are well understood processes, with most types of inhibitors binding to the metal center, whereas the activators bind at the entrance of the active site cavity where they participate in the proton shuttling between the metal coordinated water molecule and the environment [41]. Sulfonamides and their bioisosteres bind to the metal ion from the CA active site in deprotonated form, as anions, by replacing the metal-coordinated water molecule/hydroxide ion which is necessary for catalysis, and thus exerting their inhibitory mechanism. Other anions, such as the inorganic metal-complexing ones or more complicated species such as the carboxylates, are also known to bind to the CAs, but generally with less efficiency compared to the sulfonamides [42].

Acetazolamide is an inhibitor which binds the active centre of carbonic anhydrase. It is used for glaucoma, epilepsy (rarely), idiopathic intracranial hypertension, and altitude sickness [43]. It can act as a mild diuretic by reducing NaCl and bicarbonate reabsorption in the proximal tubule. However, the distal segment

partially compensates for the sodium loss, and the bicarbonaturia will produce a metabolic acidosis, further reducing the effect. Methazolamide is also a CA inhibitor, with a longer elimination half-life than acetazolamide and is less connected with unfavourable effects to the kidney [44]. Both the drugs are used against calcium metabolism and its response to citrate metabolism showed a metabolic acidosis, hypocitraturia and increased incidence of nephrolithiasis.

Dorzolamide is a sulfonamide and a specific inhibitor of the CO_2 hydration and tropical CAII inhibitor [45]. It is used to reduce the elevated intraocular pressure in patients with open-angle glaucoma or ocular hypertension. It increases the CA-catalyzed production of vasoactive nitric oxide from nitrite at a significant extent (2 to 6-fold) [46]. Inhibition of carbonic anhydrase II in the ciliary processes of the eye decreases aqueous humor secretion, presumably by slowing the formation of bicarbonate ions with subsequent reduction in sodium and fluid transport.

Sulfocoumarins (1,2-benzoxathiine 2,2-dioxides) were recently reported to act as efficient inhibitors of the zinc enzyme carbonic anhydrase and to possess a novel mechanism of inhibition [60]. Sulfocoumarins undergo a CA-mediated hydrolysis leading to 2- hydroxyphenyl-ω-ethenylsulfonic acids which thereafter bind to the zinc-coordinated water molecule from the enzyme active site. More than 700 bacterial species colonize the oral cavity, but only few of them are involved in disease. P. gingivalis is the species mainly associated with the chronic form of periodontitis. Furthermore, like most enzymes belonging to the CA superfamily, PgiCA was also inhibited by acetazolamide (AZA, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a standard, clinically used sulfonamide CA inhibitor.

However, there are still some poorly understood aspects of the catalytic cycle of the γ-CAs, related to the proton transfer residue which generates the nucleophilic, catalytically active species of the enzyme (i.e., with a hydroxide ion coordinated to the Zn(II) ion). In γ-CAs this step is assisted by a His residue, whereas in the -class enzymes a catalytic dyad constituted of an Asp–Arg pair (conserved in all such enzymes studied so far) participates in generating the zinc hydroxide form of the enzyme. In the γ-CAs, or at least for CAM, the residue acting as a proton shuttle seems to be a glutamic acid (Glu84), probably in its anionic form, as glutamate [47].

However, at the level of primary structure, there are net differences between CAM and CAMH (the second γ-CA identified by Ferry's group in M. thermophila), which are outlined here: (a) the presence of an acidic loop which contains the proton shuttle residue (identified as the first glutamic acid residue of the amino acid sequence of the loop, which is present in CAM but not in CAMH; (b) an additional N-amino terminal residue with characteristics of the signal peptides in secretory proteins is present in CAM (but not CAMH), suggesting that this enzyme is located outside the cytoplasmic membrane. PgiCA showed a good catalytic activity for the CO_2 hydration reaction, comparable to that of the human isoform hCA I. Inorganic anions such as thiocyanate, cyanide, azide, hydrogen sulfide, sulfamate and trithiocarbonate were effective PgiCA inhibitors. The role of this enzyme as a possible virulence factor of P. gingivalis is poorly understood at the moment but its good catalytic activity and the possibility to be inhibited by a large number of compounds may lead to interesting developments in the field [48].

The enzyme–ligand complex structures are required for the rational development of CA specific inhibitors which may function as probes for cellular studies, and potential therapeutic agents e.g. for the prevention of caries. In conclusion, it was found that the first inhibition study of PgiCA was with sulfonamides and one sulfamate. Dichlorophenamide, topiramate and many simple aromatic/heterocyclic sulfonamides were ineffective as PgiCA inhibitors whereas the best inhibition was observed with halogenosulfanilamides incorporating heavy halogens, 4-hydroxy- and 4-hydroxyalkyl-benzenesulfonamides, acetazolamide, methazolamide, zonisamide, indisulam, celecoxib, saccharin and hydrochlorothiazide. The inhibition profile of PgiCA was very different from that of CAM, hCA I and II or the β-CA from a protozoan parasite. Identification of potent and possibly selective inhibitors of PgiCA may lead to pharmacological tools useful for understanding the physiological role(s) of this enzyme.

Conclusions

Carbonic anhydrase is a primitive enzyme present in virtually every tissues, cell type, subcellular organelles, and in organisms ranging from unicellular cyanobacteria to mammals. These isozymes differ widely in their kinetics, susceptibility to different inhibitors, subcellular localization and tissue-specific distribution.

The structural and functional analysis of human CAs revealed a better understanding of its importance in living organisms. Both inhibitors and activators of CAs are essentially useful, therefore CAs are considered as suitable therapeutic target for the structure based rational drug design. The development of inhibitors containing a metal binding functionality with greater affinity for Cd(II) rather than Zn(II) could allow to achieve a greater selectivity for ζ- compared to α- and β- CAs. In the light of the previous and current findings, pharmacological handling of mitochondrial carbonic anhydrases to defend diseases seems even more hopeful.

Conflict of Interests

The author has no conflict of interests to declare.

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