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Carbonic Anhydrase Inhibitors. Inhibition of Cytosolic Isozymes I and II and Transmembrane, Cancer-associated Isozyme IX with Anions

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Except for sulfonamides, metal complexing anions represent the second class of inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1). The first inhibition study of the transmembrane, tumor-associated isozyme CA IX with anions is reported here. Inhibition data of the cytosolic isozymes CA I and CA II with a large number of anionic species such as halides, pseudo-halides, bicarbonate, nitrate, hydrosulfide, arsenate, etc., are also provided for comparison. Isozyme IX has an inhibition profile by anions different in some aspects from those of CA I and CA II, that may have interesting physiological consequences.

Keywords: Carbonic anhydrase; Isozyme I, II, IX; Anions; Sulfonamide

INTRODUCTION

At least 14 different α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms have been isolated in higher vertebrates, where these zinc enzymes play crucial physiological roles.[1–3] Some of these isozymes are cytosolic (CA I, CA II, CA III, CA VII), others are membrane-bound (CA IV, CA IX, CA XII and CA XIV), CA V is mitochondrial and CA VI is secreted in saliva.[1–3] Three acatalytic forms are also known, which are designated CA related proteins (CARP), CARP VIII, CARP X and CARP XI.[1–3] Representatives of the β- and γ-CA family are highly abundant in plants, bacteria and archaea.[4] These enzymes are very efficient catalysts of the reversible hydration of carbon dioxide to bicarbonate, but at least the α-CAs possess a high versatility, being able to catalyze different other hydrolytic processes such as the hydration of cyanate to carbamic acid, or of cyanamide to urea; the aldehyde hydration to *gem*-diols; the hydrolysis of carboxylic, or sulfonic acids esters, as well as other less investigated hydrolytic processes, such as hydrolysis of halogeno derivatives, arylsulfonyl halides, etc.[1,2] It is not known whether reactions catalyzed by CAs other than the hydration of CO_2/dehydration of HCO_3^- may have physiological relevance in systems where these enzymes are present. The catalytic mechanism of the α-CAs is understood in great detail: the active site consists of a Zn(II) ion co-ordinated by three histidine residues and a water molecule/hydroxide ion. The latter is the active species, acting as a potent nucleophile.[1,2] For β- and γ-CAs, the zinc hydroxide mechanism is valid too, although at least some β-class enzymes do not have water directly coordinated to the metal ion.[4] CAs are inhibited primarily by two main classes of inhibitors: the inorganic anions (such as cyanide, cyanate, thiocyanate, azide, hydrogensulphide, etc) and the unsubstituted sulfonamides possessing the general formula RSO_2NH_2 (R = aryl; hetaryl; perhaloalkyl).[1,2] Several important physiological and physio-pathological functions are played by the CA isozymes present in organisms all over the phylogenetic tree, related to respiration and transport of CO_2/bicarbonate between metabolizing tissues and

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the lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions, such as the gluconeogenesis and ureagenesis among others (in animals), CO_2 fixation (in plants and algae), etc.[1,2,4] The presence of these ubiquitous enzymes in so many tissues and in so different isoforms, represents an attractive goal for the design of inhibitors or activators with biomedical applications.[1,2]

Some of the isozymes mentioned above, such as CA IX and CA XII, are predominantly found in cancer cells.[5] The first tumor-associated CA isozyme discovered was CA IX, a transmembrane protein with a suggested function in maintaining the acid-base balance and intercellular communication.[6] This protein consists of a N-terminal proteoglycan-like domain that is unique among the CAs, a highly active CA catalytic domain, a single transmembrane region and a short intracytoplasmic tail.[6] CA IX is particularly interesting for its ectopic expression in a multitude of carcinomas derived from cervix uteri, kidney, lung, esophagus, breast, colon etc., contrasting with its restricted expression in normal tissues, namely in the epithelia of the gastrointestinal tract.[6–12]

It has recently been demonstrated that such tumor-associated CAs (mainly CA IX) may be of considerable value as markers of tumor progression.[6–12] This is mostly due to their induction by hypoxia, a clinically important factor of tumor biology that significantly affects treatment outcome and disease progression.[8] Strong association between CA IX expression and intratumoral hypoxia (either measured by microelectrodes, or detected by incorporation of a hypoxic marker pimonidazole, or by evaluation of the extent of the necrosis) has been demonstrated in the cervical, breast, head and neck, bladder and non-small cell lung carcinomas (NSCLC).[9–12] Moreover, in NSCLC and breast carcinomas, correlation between CA IX and a constellation of proteins involved in angiogenesis, apoptosis inhibition and cell–cell adhesion disruption has been observed, possibly contributing to strong relationship of this enzyme to a poor clinical outcome.[12] Hypoxia is linked with acidification of the extracellular milieu that facilitates tumor invasion and CA IX is believed to play a role in this process via its catalytic activity.[13] Thus, inhibition of this enzyme may constitute a novel approach to the treatment of cancers in which CA IX is expressed.[13,14]

Recently, the first CA IX inhibition study by aromatic/heterocyclic sulfonamides has been reported by this group,[14] but no data regarding the interaction of this isozyme with anions are available so far. Here we present the first CA IX inhibition study by anions, the second class of inhibitors of these zinc enzymes.

MATERIALS AND METHODS

Chemistry

Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, perchlorate, nitrate, hydrogen sulfide and arsenate) were from Sigma–Aldrich (Milan, Italy) of the highest purity available and were used without further purification.

CA Inhibition

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog's group.[15] Cell growth conditions were those described in Ref. [16] and enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[17] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of $49\,mM^{-1}\,cm^{-1}$ for CA I and $54\,mM^{-1}\,cm^{-1}$ for CA II, respectively, based on $M_r = 28.85$ kDa for CA I, and 29.3 kDa for CA II, respectively.[18,19]

The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek *et al.*[6]) was amplified by PCR using specific primers for the vector pCAL-n-FLAG (from Stratagene, Milan, Italy). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100, as described by Wingo *et al.*[20] The homogenate thus obtained was extensively centrifuged (11,000 × g) in order to remove soluble and membrane associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione and 1 mM oxidized glutathione. Active hCA IX was extensively dialysed using a solution of 10 mM Hepes (pH 7.5), 10 mM Tris HCl, 100 mM Na_2SO_4 and 1 mM $ZnCl_2$. The amount of protein was determined by spectrophometric measurements and its activity by stopped-flow enzymatic assays, with CO_2 as substrate.[21]

An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA I, II and IX CO_2 hydration activity.[21] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with

TABLE I Inhibition constants of anionic inhibitors against isozymes CA I, II and IX, for the CO_2 hydration reaction, at 20°C

	K_I [mM]		
Anion	hCA I	hCA II	hCA IX
F^-	>300*	>300*	48
Cl^-	6†	200†	33
Br^-	4*	63‡	16
I^-	0.3†	26† (35)¶	7
CNO^-	0.0007§	0.03§	0.043 (0.046‖)
SCN^-	0.2*	1.6¶	0.13
CN^-	0.0005	0.02	0.004
N_3^-	0.0012	1.5¶	0.005
HCO_3^-	12*	85*	13
ClO_4^-	3.6*	1.3*	8
NO_3^-	7*	35*	46
HS^-	0.0006	0.04	0.007
AsO_4^{3-}	0.6	23	19

*From Ref. [24]. †From Ref. [25]. ‡From Ref. [26]. ¶From Ref. [27]. §From Ref. [28]. ‖From Ref. [20].

10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining a constant ionic strength—this anion is not inhibitory anyhow),[22] following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20°C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10–50 mM (in the assay buffer) and dilutions up to 0.1 μM done with the assay buffer mentioned above. Enzyme concentrations were 0.09 μM for CA I, 0.06 μM for CA II and 0.1 μM for CA IX; inhibition constants were calculated as described in Ref. [21].

RESULTS AND DISCUSSION

Although CA inhibition by anions has been known for some time,[23] very few quantitative and accurate data are presently available in the literature.[24–28] For CA IX, only the inhibition by cyanate has been investigated,[20] being shown that this anion is a potent inhibitor. Considering the relatively high concentration of some anions in body fluids (for example blood contains 80 mM of chloride and 15 mM of bicarbonate)[24] it appeared of interest to perform a detailed inhibition study with anions of the tumor-associated isozyme CA IX. Anions included in the study were the physiological ones, such as Cl^- and HCO_3^-, but also the poisonous, metal complexing anions known to interact with many metallo-enzymes, such as halides and pseudo-halides (iodide, bromide, azide, cyanide, cyanate and thiocyanate), hydrosulfide, and arsenate, together with the relatively non-toxic fluoride, nitrate and perchlorate (Table I). It should be mentioned that literature data for the inhibition of the cytosolic isozymes CA I and II are also presented in Table I, for comparison and where such data was not available (for example for cyanide, hydrosulfide or azide) it was determined in this study.

The data in Table I show anions as a class of inhibitors of CA IX being generally intermediate between that of the anion-resistant CA II and that of the anion susceptible CA I. Thus, the best inhibitors (inhibition constants in the range of 4–43 μM) were cyanide, azide, hydrosulfide and cyanate (our data for this last anion are very similar to the data reported by Wingo *et al.*[20]). These are generally also the best anionic inhibitors of CA I and CA II. For example, although it is well-known that cyanide is a strong CA I/CA II inhibitor,[24] no quantitative data are available in the literature regarding the interaction of this poison with these enzymes. Here we measured the inhibition constants of this and other such anions (azide, hydrosulfide) also for the red cell isozymes CA I and II. As seen from the data in Table I, cyanide is a particularly strong CA I inhibitor (K_I of 0.5 μM), whereas CA II (K_I of 0.02 mM) and CA IX (K_I of 4 μM), showed lower affinity for it. A similar behaviour was also observed for the other metal-coordinating anions such as azide, hydrosulfide, cyanate and thiocyanate, which all strongly inhibited CA I, and to a less extent CA IX or CA II. Returning to the CA IX inhibition data, the next most inhibitory anion was thiocyanate, with an inhibition constant of 130 μM. Actually, this is the only anion investigated here (together with fluoride, see below in the text) showing a higher affinity for CA IX than for the other two isozymes, although the order of magnitude of the K_I-s is the same for the three CAs investigated here. Other anions, such as iodide, bromide, bicarbonate, perchlorate and arsenate showed inhibition constants in the range of 7–19 mM against hCA IX, most of them being generally stronger CA I inhibitors, and weaker CA II inhibitors. It is particularly relevant to analyze the behaviour of bicarbonate, present in high concentration in many tissues, and also a possible substrate for these enzymes (for the dehydration reaction, leading to carbon dioxide). It may be seen that the inhibition constant of CA IX with bicarbonate (13 mM) is very similar to that of isozyme I (12 mM), and much lower than that of isozyme II (85 mM). Thus, it may be suggested that as for CA I in red blood cells,[24] the activity of CA IX present in hypoxic tumours that usually display decreased levels of extracellular bicarbonate may be compromised when bicarbonate levels increase. This is in agreement with the proposed role of CA IX in acidification of the tumor environment,[7–13] predominantly by catalyzing the hydration reaction of CO_2 to bicarbonate, and not the dehydration one, since bicarbonate (the substrate for the dehydration reaction) shows an appreciable inhibitory effect against this isozyme.[29] Finally, other anions, such as fluoride, chloride and nitrate showed quite weak inhibitory effects against

CA IX, with inhibition constants in the range of 33–48 mM. Still, they may have physiological significance (for example for Cl^-) since the physiological concentration of this anion in the blood is very high (80 mM),[24] as mentioned earlier. It is also interesting to note that fluoride, which has practically no inhibitory effects against CA I and II, shows a weak inhibitory effect on CA IX, with an inhibition constant of 48 mM (this anion is the weakest inhibitor in the series investigated here). It should also be mentioned that anions as an entire class are several orders of magnitude weaker inhibitors as compared to the sulfonamides, against the three investigated CA isozymes discussed here.[14]

In conclusion, CA IX has high affinity for metal poisons such as cyanide, azide, hydrosulfide and cyanate, being less inhibited by thiocyanate, iodide, perchlorate and bicarbonate. Interesting differences between the inhibition of CA IX and that of the cytosolic isozymes CA I and CA II with this class of inhibitors were also noted.

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