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Carbonic Anhydrase Inhibitors: Inhibition of Human and Murine Mitochondrial Isozymes V with Anions

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Abstract—In addition to 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 mitochondrial isozyme CA V (of murine and human origin) with anions is reported here. Inhibition data of the cytosolic isozymes CA I and CA II as well as the membrane-bound isozyme CA IV with a large number of anionic species such as halides, pseudohalides, bicarbonate, nitrate, hydrosulfide, arsenate, sulfamate, and sulfamidate and so on, are also provided for comparison. Isozyme V has an inhibition profile by anions completely different to those of CA I and IV, but similar to that of hCA II, which may have interesting physiological consequences. Similarly to hCA II, the mitochondrial isozymes show micro-nanomolar affinity for sulfonamides such as sulfanilamide and acetazolamide.

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 denominated 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 for 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, and so on.[1,2] It is not known whether other reactions catalyzed by CAs 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 the lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions, such as the lipogenesis, gluconeogenesis and

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ureagenesis among others (in animals), CO_2 fixation (in plants and algae), and so on.[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]

Only CA V is present in mitochondria, among the many isoforms of CA from animals. This isozyme was shown to be involved in several biosynthetic processes, such as ureagenesis,[5] gluconeogenesis,[6] and lipogenesis, both in vertebrates (rodents) as well as invertebrates (locust).[7–10] Indeed, in several important biosynthetic prcesses involving pyruvate carboxylase, acetyl CoA carboxylase, and carbamoyl phosphate synthetases I and II, bicarbonate is the real substrate of these carboxylating enzymes, not carbon dioxide, and the provision of enough bicarbonate is assured mainly by the mitochondrial isozyme CA V.[8,11–14]

Considering the fact that inhibition of the mitochondrial enzyme with anions (and also sulfonamides) was not investigated in detail up to now, and that this information may be important for obtaining inhibitors with new pharmacological applications, we conducted such a study in detail, using both the murine as well as human isozymes CA V (mCA V, and hCA V, respectively). Both physiological anions (such as chloride, bicarbonate) as well as 'metal poisons' (cyanide, cyanate, thiocyanate, azide, etc.) were included in this study, together with sulfamide (as sodium salt) and sulfamate, the two simplest anions incorporating the sulfonamide moiety present in the other class of CA inhibitors, the aromatic/heterocyclic sulfonamides. Inhibition data for two sulfonamides (sulfanilamide and acetazolamide) are also provided, as well as anion inhibition data for isozymes I, II and IV, in order to compare them with those of the mitochondrial isoforms investigated in the present study.

Chemistry

Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, perchlorate, nitrate, hydrogen sulfide and arsenate) were of highest purity available, and were used without further purification. Sulfamide, sulfamic acid, sulfanilamide and acetazolamide are also commercially available. Recombinant human isozymes CA I and II were used,[15–22] whereas mCA V and hCA V were obtained by a new procedure described here.[23]

CA inhibition

The full length, mitochondrial isozymes of murine and human origin mCA V and hCA V have been obtained in this work by a novel procedure as compared to the one reported by Heck et al.[23] for the truncated form of mCA V, which is missing the first 21 amino acid residues. As seen from data of Table 1, the full-length mCA V and hCA V show similar catalytic activities with the truncated form of mCA V mentioned above, possessing the same K_m values for CO_2 as substrate, and slighly lower k_{cat} values.

Although CA inhibition by anions has been discovered quite early,[24] very few quantitative and accurate data on the subject are presently available in the literature.[25–29] For mCA V, only the inhibition by cyanate has been investigated, being shown that this anion is a potent inhibitor (K_i of 30 μM).[23] No inhibition data at all were reported up to now for hCA V. Considering the relatively high concentration of some anions in body fluids (for example blood contains 80 mM of chloride and 15 mM of bicarbonate)[25] it appeared of interest to perform a detailed inhibition study with anions of the mitochondrial isozymes mCA V and hCA V. 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 metalloenzymes, such as halides and pseudohalides (iodide, bromide, azide, cyanide, cyanate and thiocyanate), hydrosulfide, and arsenate, together with the relatively non-toxic fluoride, nitrate and perchlorate (Table 2). It should be mentioned that literature data for the inhibition of the cytosolic isozymes CA I and II and the membrane-bound isozyme CA IV are also presented in Table 2, for comparison, whereas when such data were not available (for example for cyanide, hydrosulfide or azide) they were determined in this study.

As seen from data of Table 2, the mitochondrial isozymes hCA V and mCA V are inhibited by anions, their affinity for this class of inhibitors being generally intermediate between that of hCA I (an isozyme quite susceptible to this class of inhibitors) and hCA II or hCA IV (isozymes more resistant to inhibition by anions, but very susceptible to be inhibited by sulfonamides).[1,2,25] Thus, strongest inhibition has been observed with the poisonous, metal complexing anions cyanide, hydrosulfide and cyanate, which showed inhibition constants in the range of 15–31 μM against hCA V and mCA V. These anions are on the other hand much stronger hCA I inhibitors, whereas their affinity for hCA II and hCA IV is of the same order of magnitude as that for the mitochondrial enzymes. The next potent inhibitors were thiocyanate, azide, sulfamate and sulfamidate, with inhibition constants in the range of 0.12–0.86 mM. Again hCAV and mCA V showed an affinity intermediate between that of hCA I on the one part, and hCA II/hCA IV on the other part, for these anions, some of which (sulfamide and sulfamic acid) are really important for the design of compounds possessing different zinc-binding functions, and thus leading to novel

Table 1. Activity (catalytic rate and K_m for CO_2 as substrate) of different CA V preparations

Enzyme	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	Ref
mCA V (truncated)	3.10^7	3.10^5	Heck et al.[23]
mCA V (full length)	$1.2.10^7$	$1.2.10^5$	This work
hCA V (full length)	$2.9.10^7$	$2.9.10^5$	This work

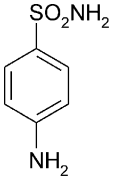
Table 2. Inhibition constants of anionic and sulfonamide inhibitors against isozymes CA I, II, IV and V, for the CO_2 hydration reaction, at 20 °C

Inhibitor	K_i (mM)				
	hCA I	hCA II	hCA IV	hCA V	mCA V
F^-	>300[a]	>300[a]	—	241	230
Cl^-	6[b]	200[b]	36[f]	156	164
Br^-	4[a]	63[c]	52[f]	50	52
I^-	0.3[b]	26[b] (35)[d]	11[f]	25	24
CNO^-	0.0007[e]	0.03[e]	0.03[f]	0.028	0.031 (0.03)[g]
SCN^-	0.2[a]	1.6[d]	—	0.74	0.80
CN^-	0.0005	0.02	—	0.015	0.016
N_3^-	0.0012	1.5[d]	—	0.30	0.33
HCO_3^-	12[a]	85[a]	44[f]	82	81
ClO_4^-	3.6[a]	1.3[a]	—	12	12
NO_3^-	7[a]	35[a]	—	16	18
HS^-	0.0006	0.04	—	0.023	0.025
AsO_4^{3-}	0.6	23	—	21	19
$H_2NSO_3^-$	0.021	0.39	0.34	0.12	0.12
$H_2NSO_2NH^-$	0.31	1.13	1.02	0.84	0.86
Sulfanilamide	0.028	0.0003	0.003	0.00135	0.00136
Acetazolamide	0.002	$1.0.10^{-5}$	0.00012	6.1×10^{-5}	5.8×10^{-5}

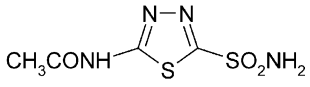
[a]From ref 25.
[b]From ref 26.
[c]From ref 27.
[d]From ref 28.
[e]From ref 29.
[f]From ref 20.
[g]From ref 23.

classes of CA inhibitors.[22,30,31] Indeed, after the recent report of the X-ray crystal structure of hCA II complexed with sulfamidate and sulfamate,[22] we have designed novel classes of inhibitors incorporating sulfamate[30] and sulfamide[31] zinc-binding functions, with nanomolar affinity for the enzyme, although the two lead molecules possess rather high inhibition constants. Thus, important lessons for the drug design may be learned even from quite ineffective lead molecules.[22,30,31]

Other anions, such as iodide, perchlorate, nitrate and arsenate were much less effective inhibitors of hCAV/mCAV, showing inhibition constants in the range of 12–25 mM, practically of the same order of magnitude as those for hCA II. Finally, the most ineffective anionic inhibitors were the other halides (fluoride, chloride and bromide), and bicarbonate, which showed K_i values in the range of 50–241 mM. The most interesting cases are obviously the physiologically relevant chloride and bicarbonate. In fact, these anions strongly inhibit hCA I, whereas they seem to have no effect on hCA II, hCA V and mCA V. Their effect on hCA IV is on the other hand intermediate between the two extremes mentioned above. These data clearly show that both hCA II as well as the mitochondrial isozymes hCA V/mCA V are not affected by the high chloride/bicarbonate concentrations present in body fluids, and this insensitivity is presumably due to the fact that these isozymes must generate high amounts of bicarbonate to be used thereafter in the carboxylating reactions involving pyruvate carboxylase, acetyl CoA carboxylase, and carbamoyl phosphate synthetases I and II mentioned above.



1



2

A last and very important aspect regards the interaction of these mitochondrial isozymes with the two sulfonamides investigated here, sulfanilamide **1** and acetazolamide **2**. The first compound was the lead for obtaining a large number of different classes of biologically active compounds, whereas acetazolamide was the first non-mercurial diuretic in clinical use.[1,2] This compound has at present clinical applications as an antiglaucoma drug and clinical tool in many types of non-invasive investigations.[1,2] As seen from data of Table 2, sulfanilamide is a strong CA V inhibitor, with an inhibition constant of around 1.35 μM, being also an effective hCA II (K_I of 0.3 μM) and hCA IV (K_I of 3 μM) inhibitor, but not as good a hCA I inhibitor. On the other hand, acetazolamide is the most effective CA V inhibitor investigated up to now, with an inhibition constant of 58–61 nM against hCA V and mCA V. Only hCA II has a higher affinity for this compound (K_I of 10 nM) among the different isozymes investigated here, whereas hCA IV and hCA I are inhibited to a much lower degree.

In conclusion, CA V has high affinity for metal poisons such as cyanide, hydrosulfide and cyanate, being less

inhibited by azide, thiocyanate, iodide, perchlorate and bicarbonate. It has also an affinity comparable to that of hCA II for sulfamide and sulfamic acid, two lead molecules for obtaining novel classes of CA inhibitors, but is insensitive to bicarbonate and chloride, the two physiological anions present in high concentrations in different tissues. Sulfanilamide shows low micromolar, whereas acetazolamide nanomolar affinity for both mCA V as well as hCA V.

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