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Carbonic anhydrase inhibitors. Inhibition of the newly isolated murine isozyme XIII with anions

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Abstract—The inhibition of the newly discovered cytosolic carbonic anhydrase (CA, EC 4.2.1.1) isozyme XIII of murine origin (mCA XIII) has been investigated with a series of anions, such as the physiological ones (bicarbonate, chloride), or the metal complexing anions (cyanate, cyanide, azide, hydrogen sulfide, etc), nitrate, nitrite, sulfate, sulfamate, sulfamide as well as with phenylboronic and phenylarsonic acids. The best mCA XIII inhibitors were cyanate, thiocyanate, cyanide and sulfamide, with K_I-s in the range of 0.25 μM–0.74 mM, whereas fluoride, iodide, azide, carbonate and hydrogen sulfide were less effective (K_I-s in the range of 3.0–5.5 mM). The least effective inhibitors were sulfate, chloride and bicarbonate (K_I-s in the range of 138–267 mM). The affinity of mCA XIII for anions is very different from that of the other cytosolic isozymes (hCA I and II) or the mitochondrial isozyme hCA V. This resistance to inhibition by the physiological anions bicarbonate and chloride suggests an evolutionary adaptation of CA XIII to the presence of high concentrations of such anions (e.g., in the reproductive tract of both female and male), and the possible participation of this isozyme (similarly to CA II, CA IV and CA V) in metabolons with proteins involved in the anion exchange and transport, such as the anion exchangers (AE1-3) or the sodium bicarbonate co-transporter (NBC1 and NBC3) proteins, which remain to be identified.

1. Introduction

We have recently cloned, purified and characterized a novel member of the α-carbonic anhydrase (CA, EC 4.2.1.1) family, that is, the isozyme CA XIII, both in the mouse (mCA XIII) and humans (hCA XIII).[1] Indeed, these metalloenzymes catalyze the reversible hydration of carbon dioxide to bicarbonate, handling in this way one of the simplest and most important biomolecules (CO_2), generated in many physiological processes.[1–6] An increasing number of CAs are constantly being discovered both in higher vertebrates, humans included, as well as in many other organisms, such as archaea, bacteria, protozoa, plants, etc.[2,7] CA XIII, the last member of the α-family characterized in higher vertebrates,[1] was also demonstrated to be a druggable target,[8] with some aromatic/heterocyclic sulfonamides being shown to act as very potent inhibitors, with affinities in the low nanomolar range. It has also been proved that CA XIII has a cytosolic localization and shows catalytic activity similar to that of the mitochondrial isozyme V and the cytosolic isozyme I, with k_{cat}/K_M of $4.3 \times 10^7\,M^{-1}s^{-1}$ and k_{cat} of $8.3 \times 10^4\,s^{-1}$.[1] Immunohistochemical and PCR data indicated that the distribution of CA XIII is clearly unique when compared to the other cytosolic CA isozymes (mainly CA I and II).[1] The most distinct differences between CA XIII and II (the major cytosolic isoform)[1–6] were observed in the human testis and uterus, organs in which pH and ion balance has to be tightly regulated to ensure normal fertilization.[1] CA XIII was found to be expressed in all stages of developing human sperm cells. In contrast, CA II was confined to the mature sperm cells as shown earlier by some of us.[9] The bicarbonate present in the ejaculate has been proposed to maintain the sperm motility until the cells enter the lumen of the uterus

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through the cervical canal.[9] In the female genital tract, the endometrial and oviductal epithelium produce an alkaline environment for maintaining the sperm motility. In the uterine endometrium and cervical glands, the presence of CA XIII could thus explain the early histochemical and biochemical results showing CA activity in the epithelial cells that was not due to CA I or II.[9] Thus, it seems that CA XIII is a key factor contributing to the appropriate bicarbonate concentration in the cervical and endometrial mucus needed for normal fertilization processes.[1,9] Being such a widely expressed isozyme, CA XIII could compensate other CAs, and thus, needs to be considered when any CA-deficient animal model is tested in phenotypic analyses, or when inhibitors are clinically used in the treatment and prevention of diverse disorders.[1–6] All these findings suggests that CA XIII plays a major role in reproduction, and perturbation of its function could potentially lead to developmental abnormalities.[1,9] Furthermore, topically acting CA XIII inhibitors might be developed that may lead to novel contraceptives, devoid of the side effects of the hormonal therapies now in clinical use.

Considering the above-mentioned role of CA XIII in maintaining an appropriate alkaline pH and rather high bicarbonate concentrations both in the male and female reproductive tracts, as well as the fact that CAs, as many other metalloenzymes are inhibited by anions,[10–13] we investigate here for the first time the inhibition of mCA XIII with a large number of anionic species such as halides, pseudohalides, bicarbonate, carbonate, nitrate, nitrite, hydrosulfide, sulfate, bisulfite, sulfamate, sulfamide, as well as with phenylboronic acid and phenylarsonic acid among others. Our data suggest an evolutionary adaptation of CA XIII to the presence of high concentrations of bicarbonate and chloride, which act as very weak CA XIII inhibitors, and the eventual participation of this isozyme (similarly to CA II, CA IV and CA V) in metabolons with proteins involved in anion exchange and transport, such as the anion exchangers (AE1-3) or the sodium bicarbonate co-transporter (NBC1 and NBC3) proteins.[14–16]

1.1. Chemistry

Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite and sulfate) were of highest purity available, and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid and phenylarsonic acid are also commercially available.

1.2. CA inhibition data

Inhibition data against four CA isozymes, that is, hCA I, hCA II, hCA V and mCA XIII,[1,17–19] with the above mentioned anions are shown in Table 1. In addition to the physiological anions (chloride, bicarbonate) and the 'metal poisons' (i.e., metal-complexing anions), we also investigated sulfamic acid and sulfamide, as these are the simplest compounds incorporating a sulfonamido moiety, present in the potent CA inhibitors (the sulfonamides) previously investigated.[2,8] It also has been shown[20] that they bind to the Zn(II) ion of the human isozyme hCA II in a way that allows their use as lead mole-

Table 1. Inhibition constants of anionic inhibitors against human isozymes hCA I, II, V and murine isozyme mCA XIII, for the CO_2 hydration reaction, at 20 °C[20]

Inhibitor	K_I [mM]			
	hCA I[a]	hCA II[a]	hCA V[b]	mCA XIII
F^-	>300	>300	241	3.0
Cl^-	6	200	156	138
Br^-	4	63	50	45.0
I^-	0.3	26	25	5.4
CNO^-	0.0007	0.03	0.028	0.00025[c]
SCN^-	0.2	1.6	0.74	0.74
CN^-	0.0005	0.02	0.015	0.065
N_3^-	0.0012	1.5	0.30	4.8
HCO_3^-	12	85	82	140
CO_3^{2-}	15	73	95	5.5
NO_3^-	7	35	16	36
NO_2^-	8.4	63	16	12.6
HS^-	0.0006	0.04	0.023	5.2
HSO_3^-	18	89	65.0	75.5
SO_4^{2-}	63	>200	680	267
H_2NSO_3H*	0.021	0.39	0.12	21.5
$H_2NSO_2NH_2$	0.31	1.13	0.84	0.14
$PhB(OH)_2$	58.6	23.1	4.51	2.85
$PhAsO_3H_2$*	31.7	49.2	7.43	1.65

Errors were in the range of 3–5% of the reported values, from three different assays.
* As sodium salt.
[a] From Ref. 11.
[b] From Ref. 10.
[c] From Ref. 1.

cules for the development of potent, nonsulfonamide CA inhibitors (the X-ray structure of the adducts of these two compounds with hCA II have recently been reported).[21] Furthermore, phenylboronic and phenylarsonic acids were also included in our study, as we recently showed that these two compounds act as potent inhibitors of archaeal CAs belonging to the β- and γ-class.[22] Inhibition data for the other major cytosolic isozymes, hCA I and II, as well as the mitochondrial isozyme hCA V (which shows a catalytic activity rather similar to mCA XIII)[1] are also provided in Table 1 for comparison, in order to allow a better rationalization of our results.

Data of Table 1 allow us to draw the following conclusions regarding mCA XIII inhibition with anions: (i) the most potent mCA XIII inhibitors were the metal poisons cyanate, cyanide and thiocyanate, as well as sulfamide, which showed inhibition constant in the range of 0.25 μM–0.74 mM. It may be observed that mCA XIII shows a quite similar behaviour with hCA I and hCA V in its interaction with these inhibitors, whereas hCA II is generally more resistant to them (except for cyanide which is a stronger hCA II than mCA XIII inhibitor); (ii) another group of anions, such as fluoride, iodide, azide, carbonate, hydrogen sulfide, as well as phenylboronic and phenylarsonic acids act as efficient mCA XIII inhibitors, showing K_I-s in the range of 1.65–5.5 mM. Considering in more detail these data, the potent inhibitory property of fluoride is unique for CA XIII, since it acts as an extremely weak inhibitor for other investigated isozymes (such as hCA I, II and V—Table 1, but also against hCA IV or hCA IX—data not shown). We also note important differences between hCA I and mCA XIII in their behaviour towards such metal complexing anions as azide and hydrogen sulfide, which are low micromolar inhibitors of the first isozyme, and act as much weaker inhibitors of isozyme XIII. On the other hand, carbonate is an effective mCA XIII inhibitor (K_I of 5.5 mM), having a much lower affinity for the other investigated isozymes (in the range of 15–95 mM). It is rather difficult to explain these differences of affinity between these isozymes in their behaviour towards such anions at this moment, since the X-ray crystal structure of mCA XIII is unknown. The two compounds investigated for the first time, that is, phenylboronic and phenylarsonic acids, act as good mCA XIII inhibitors (K_I-s in the range of 1.65–2.85 mM), whereas their affinity for the other cytosolic isozymes are much lower (K_I-s in the range of 23.1–58.6 mM). These two compounds show an intermediate behaviour as hCA V inhibitors, with an affinity in the range of 4.5–7.4 mM for the mitochondrial isozyme; (iii) weak mCA XIII inhibitory properties were shown by the following anions: bromide, nitrate, nitrite, bisulfite and sulfamic acid, which showed K_I-s in the range of 12.6–75.5 mM. It should be noted the very large difference between the mCA XIII inhibitory properties of sulfamide and sulfamic acid (much larger as for other isozymes), which allow us to hypothesize that CA XIII—specific inhibitors may be obtained considering sulfamide as lead molecule (or eventually the two compounds discussed previously, i.e., phenylboronic and phenylarsonic acids). It is also interesting to note that the halides showed a very unexpected affinity for this isozyme, which distinguish it from all other investigated CAs. Thus, generally fluoride showed very low or no affinity at all for other CAs (e.g., hCA I, II and V—see Table 1), whereas inhibitory properties increased with the increase of the atomic weight of the halogen, the best inhibitor being always iodide for all other investigated isozymes. In the case of mCA XIII, the best inhibitor is fluoride, as already stressed above, bromide is an ineffective inhibitor, whereas iodide shows good inhibitory properties (chloride will be discussed in the next paragraph); (iv) very ineffective mCA XIII inhibitory properties were shown by chloride, bicarbonate (K_I-s around 140 mM) and sulfate (K_I of 267 mM). Data of sulfate are as expected, since this anion shows weak hCA I inhibitory properties, and very low affinity for the other investigated isozymes, generally with K_I-s higher than 200 mM (Table 1). The most unexpected data are those of bicarbonate, which acts as a good hCA I inhibitor (K_I of 12 mM) and a much more ineffective inhibitor of isozymes II and V (K_I-s around 80–85 mM). Unexpectedly, the affinity of mCA XIII for bicarbonate is almost two times lower, with a K_I of 140 mM. Considering on the other hand the tissue localization of this isozyme and its physiological functions, briefly commented in the introduction, we note that this is a logical result, as CA XIII is believed to provide the appropriate bicarbonate concentration in the male and female reproductive tracts (as well as in other tissues where it is present). Thus, this enzyme must catalyze the hydration of CO_2 to bicarbonate in the presence of very high concentrations of bicarbonate, and our finding that mCA XIII is insensitive to this inhibitor, strongly supports the proposed physiological function of this enzyme, as discovered from immunohistochemical experiments.[1] This finding also explains why another cytosolic isozyme 'has been invented' during the evolution of mammals, in addition to the highly abundant cytosolic isozymes I and II: clearly the sensitivity of CA XIII to physiological anions such as bicarbonate is very different as compared to that of isozymes I and II, and it may be stated that CA XIII is the cytosolic isozyme resistant to bicarbonate inhibition, acting as an efficient CO_2 hydration catalysts in the presence of high amounts of the reaction product, that is, bicarbonate. Another interesting point relates to the sensitivity of CA XIII to the other physiologically relevant anion, that is, chloride. It may be observed that only hCA I is sensitive to this anion (K_I of 6 mM) whereas isozymes II, V and XIII are much more resistant to inhibition by chloride (K_I-s in the range of 138–200 mM). Why are these isozymes resistant to chloride inhibition? A possible answer may be found by considering the association of diverse CA isozymes with proteins involved in the transport of anions, such as the anion exchangers (AE) or the sodium bicarbonate co-transporter proteins NBC1 and NBC3, with the formation of metabolons, recently investigated in detail by Casey and co-workers.[14–16] For example, a physical interaction has been identified between hCA II, and the erythrocyte membrane Cl^-/HCO_3^- anion exchanger, AE1, mediated by an acidic motif in the AE1 carboxy-terminus.[14–16,23] It has been proven that the presence of hCA II attached to AE1 accelerates AE1 HCO_3^- transport activity, as AE1

moves bicarbonate either into or out of the cell.[14–16,23] In efflux mode the presence of CA II attached to AE1 increased the local concentration of bicarbonate at the AE1 transport site. As bicarbonate is transported into the cell by AE1, the presence of CA II on the cytosolic surface accelerates transport by consumption of bicarbonate (which is enzymatically dehydrated to CO_2), thereby maximizing the transmembrane bicarbonate concentration gradient experienced by the AE1 molecule.[14–16] Functional and physical interactions were also shown to occur between CA II or CA IV and Na^+/HCO_3^- co-transporter isoforms NBC1 and NBC3 by the same group.[14–16,23] Another metabolon was also recently reported by Sly et al. for the mitochondrial isozyme CA V,[24] which interacts with some gluconeogenic enzymes such as malate dehydrogenase and pyruvate carboxylase (for example the real substrate of pyruvate carboxylase is not CO_2, but bicarbonate, and this explains why the mitochondrial isozyme CA V plays such an important physiological function).[25] Returning at data of Table 1, it may be seen that both CA II as well as CA V are resistant to inhibition by bicarbonate and chloride. Considering the involvement of these anions in the metabolons discussed above, it appears obvious why these CAs should not be inhibited by the two anions with which they are in contact at high concentrations. For CA XIII no metabolons have been found up to now, but observing the high inhibition constants of both chloride and bicarbonate towards this isozyme, we hypothesize that CA XIII is involved in such metabolons, which hopefully will be identified in the near future.

2. Conclusion

We report here the first detailed inhibition study of the newly isolated cytosolic isozyme CA XIII with anions. This enzyme is expressed in many different organs including the salivary glands, kidney, brain, lung, gut, uterus and testis. Interestingly, CA XIII showed a very different affinity for anions as compared to the other cytosolic isozymes investigated (hCA I and II) or the mitochondrial isozyme hCA V. The best mCA XIII inhibitors were cyanate, thiocyanate, cyanide and sulfamide, with K_I-s in the range of 0.25 μM–0.74 mM, whereas fluoride, iodide, azide, carbonate and hydrogen sulfide were less effective (K_I-s in the range of 3.0–5.5 mM). The least effective inhibitors were sulfate, chloride and bicarbonate (K_I-s in the range of 138–267 mM). This resistance to inhibition by the physiological anions bicarbonate and chloride suggests an evolutionary adaptation of CA XIII to the presence of high concentrations of such anions (e.g., in the reproductive tract of both female and male, but probably in other tissues too where this isozyme is present), and the possible participation of CA XIII (similarly to CA II, CA IV and CA V) in metabolons with proteins involved in anion exchange and transport, such as the anion exchangers (AE1-3) or the sodium bicarbonate co-transporter (NBC1 and NBC3) proteins. Such metabolons remain to be identified, in order to better understand the physiological roles of this new isozyme.

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17. To amplify mouse *CA13* full-length cDNA, two primers (Sigma Genosys) were chosen based on the mouse *CA13* sequence published in GenBank™ (AF231123); forward (F1) 5′-GTCCCTGCCACAGGCTCT-3′ (nucleotides 3–20) and reverse primer (R2) 5′-ATACATT-GGGGCAAATCT-3′ (nucleotides 993–1010) generated a full-length 1008-bp amplification product.

The PCR amplifications for mouse spleen and 17-days-old embryo with primers F1 and R2 were performed using a three-step method consisting cycling parameters of denaturation at 94 °C for 2 min, followed by 33 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min 30 s, followed by final extension at 72 °C for 10 min. *E. coli* TOP 10 strain transformed with pTrcHis plasmid containing mCA XIII cDNA was grown at 37 °C in 50 mL LB medium containing 50 μg/mL ampicillin to an OD of 0.6 at 600 nm. The inducer, IPTG, was then added to a final concentration of 1 mM and then the culture was incubated at 37 °C. The cells were harvested after 5 h by centrifugation and the pellet was frozen at −20 °C for later use. For large scale expression the bacteria were grown in 1 L of LB medium. The bacterial cell pellet was resuspended in 160 mL of lysing buffer (50 mM Na_2HPO_4, 0.5 M NaCl, pH 8.0) and 160 mg lysozyme (Sigma) was added. The resuspended cells were incubated on ice 30 min and lysed by pipetting. Bacterial cell components were removed by centrifugation (13,000 rpm, 15 min, +4 °C). The supernatant was directly applied onto Invitrogen's ProBond™-purification system. The ProBond™ purification was performed under native conditions according to Invitrogen's protocol using the ProBond™ resin.

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