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SHORT COMMUNICATION

Inhibition of the alpha- and beta-carbonic anhydrases from the gastric pathogen *Helycobacter pylori* with anions

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Abstract

The gastric pathogen *Helicobacter pylori* encodes two carbonic anhydrases (CAs, EC 4.2.1.1), an α- and a β-class one, hpαCA and hpβCA, crucial for its survival in the acidic environment from the stomach. Sulfonamides, strong inhibitors of these enzymes, block the growth of the pathogen, *in vitro* and *in vivo*. Here we report the inhibition of the two *H. pylori* CAs with inorganic and complex anions and other molecules interacting with zinc proteins. hpαCA was inhibited in the low micromolar range by diethyldithiocarbamate, sulfamide, sulfamic acid, phenylboronic acid, and in the submillimolar one by cyanide, cyanate, hydrogen sulfide, divanadate, tellurate, perruthenate, selenocyanide, trithiocarbonate, iminodisulfonate. hpβCA generally showed a stronger inhibition with most of these anions, with several low micromolar and many submillimolar inhibitors detected. These inhibitors may be used as leads for developing anti-*H. pylori* agents with a diverse mechanism of action compared to clinically used antibiotics.

Keywords: Carbonic anhydrase, anion, alpha/beta-class enzyme, inhibitor, *Helicobacter pylori*

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes which catalyze the hydration of carbon dioxide to bicarbonate and protons[1–3]. Many pathogenic bacteria encode such enzymes belonging to the α-, β-, and/or γ-CA families[1]. In the last decade, the α-CAs from *Neisseria spp.* and *Helicobacter pylori* as well as the β-class enzymes from *Escherichia coli, H. pylori, Mycobacterium tuberculosis*, *Brucella spp., Streptococcus pneumoniae*, *Salmonella enterica* and *Haemophilus influenzae* have been cloned and characterized in detail[1–13]. Most of these pathogenic enzymes show a very high catalytic efficiency for the physiological reaction. Recent studies detected various classes of CA inhibitors (CAIs) targeting these enzymes, most of which belong to the sulfonamide/sulfamate class. They were critical to establish the roles of these CAs in the pathogen life cycle, and whether CA inhibition may constitute and alternative pathway for finding novel types of antibiotics[1,14–16]. For some of these enzymes, the X-ray crystal structures were also reported, which can be helpful for drug design purposes[12,13]. As resistance to antibiotics belonging to several different classes is escalating and represents a worldwide problem[17,18], it is essential to explore alternative classes of compounds which inhibit crucial steps in pathogen's life cycles. Indeed, a high number of strains of Gram-negative/positive bacteria (such as *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Helycobacter pylori*, *Brucella suis, Streptococcus pneumoniae*, and so on) no longer respond to some classical antibiotics[17,18]. Cloning of the genomes of bacteria offers, however, the possibility to explore alternative pathways for inhibiting virulence factors or proteins essential for the pathogens. Among the many, new such possible drug targets recently explored, there are several CAs[1].

These metalloenzymes are found in various organisms all over the phylogenetic tree, as five different, genetically distinct families, the α-, β-, γ-, δ- and ζ-CAs[1–7]. The metal ion from the enzyme active site (which may be Zn(II); Fe(II); Cd(II) or Co(II) among others) is essential for the

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catalytic reaction and also for the binding of most (but not all) classes of CA inhibitors (CAIs) investigated so far[1–11].

The genome of the human carcinogenic pathogen *H. pylori* contains at least two CAs, an α-CA[12] (denominated hpαCA)[14–16] and a β-class enzyme, denominated hpβCA[14–16,19,20]. These two CAs show a different subcellular localization: a periplasmic one for the α-class CA[19] and a cytoplasmic one for hpβCA[15]. These enzymes were also shown to be catalytically efficient, with almost identical activity to that of the human (h) isoform hCA I, for the CO_2 hydration reaction, and highly inhibited by many sulfonamides/sulfamates, including acetazolamide, ethoxzolamide, topiramate and sulpiride, all clinically used drugs[14–16]. Furthermore, certain CAIs, such as the clinically used sulfonamides acetazolamide and methazolamide, were shown to inhibit the bacterial growth in cell cultures[15]. Since the efficacy of *H. pylori* eradication therapies currently employed has been decreasing due to drug resistance and side effects of the commonly used drugs[15,20,21], the dual inhibition of α- and/or β-CAs of *H. pylori* may serve as an alternative therapy in patients with *H. pylori* infection or for the prevention of gastroduodenal diseases provoked by this widespread pathogen[1,15]. In fact, a pilot study has demonstrated the efficacy of acetazolamide in the treatment of gastric ulcer[21]. Moreover, this compound (as well as ethoxzolamide) was widely used clinically as antiulcer agents in the 1970s and 1980s by Puscas's group, although its mechanism of action was not properly understood at that time as being due to the pathogen CA inhibition[20].

All these data show that hpαCA and hpβCA are promising drug targets and that sulfonamide CAIs may have clinical applications. However, their inhibition has been scarcely investigated to date, apart our earlier work on sulfonamide inhibitors[14–16]. Exploring alternative chemotypes to the sulfonamides as possible inhibitors of the α/β-CAs from this bacterial pathogen is thus of great interest. Here, we report an inhibition study of the two enzymes with a wide range of simple inorganic anions, as well as various small molecule compounds known to target the metal ion in metalloenzymes like CAs, such as among others sulfamides, sulfamic acid, boronic and arsonic acids, and so on.

Materials and methods

Chemistry

All anions/small compounds used here were commercially available, highest purity reagents, from Sigma-Aldrich (Milan, Italy).

Enzymology

hpαCA and hpβCA were recombinant enzymes obtained as described earlier[14–16].

CA catalytic activity and inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity[22]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10–20 mM Hepes (pH 7.5, for α-CAs) or TRIS (pH 8.3 for β-CAs) as buffers, and 20 mM Na_2SO_4 (for α-CAs) or 20 mM $NaBF_4$ – for β-CAs (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5%–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled–deionized water and dilutions up to 0.01 nM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver-Burk plots, as reported earlier[23–30], and represent the mean from at least three different determinations.

Results and discussions

Inhibition data of two human CA isoforms, hCA I and II (highly abundant proteins with important physiological functions)[2–4] as well as three bacterial CAs, PCA (a β-CA from *S. pneumoniae*) and hpαCA and hpβCA investigated here are shown in Table 1. The data of hCA I, hCA II and PCA were reported earlier by this group[9,25–27], and are provided here for comparisons reasons.

The following should be noted regarding the inhibition data of Table 1:

1. The α-class enzyme hpαCA was sensitive to this class of inhibitors, which showed inhibition constants in the range of 4.9 μM–10.1 mM. The only anion which did not show any notable inhibitory properties was tetrafluoroborate (K_I > 200 mM), known for its lack of interaction with most metal ions from metalloenzyme active sites[9]. The halogenides, thiocyanate, and perchlorate were the least effective inhibitors of hpαCA, with K_Is in the range of 2.41–10.1 mM. This is rather amazing, considering that perchlorate does not inhibit appreciably any other α- or β-CA investigated earlier[1–9]. The remaining anions were submillimolar hpαCA inhibitors, with K_Is in the range of 0.27–0.99 mM, except for four derivatives which were micromolar inhibitors (Table 1). Indeed, diethyldithiocarbamate, sulfamide, sulfamic acid and phenylboronic acid were the most effective inhibitors of this enzyme, with K_Is in the range 4.9–97 μM. It is interesting to observe that sulfate is a weak inhibitor (K_I of 0.82 mM) but replacing one or two oxygen

Table 1. Inhibition constants of anion inhibitors against α-/β-CAs from mammals (hCA I, and II, human isoforms) and bacteria: PCA (from *S. pneumoniae*), and hpαCA/hpβCA (from *H. pylori*) for the CO_2 hydration reaction, at 20 °C[22].

	K_I [mM][b]				
Inhibitor[a]	hCA I[d]	hCA II[d]	PCA[e]	hpαCA[c]	hpβCA[c]
F^-	> 300	>300	0.85	4.08	0.67
Cl^-	6	200	0.052	2.70	0.56
Br^-	4	63	0.046	2.41	0.38
I^-	0.3	26	0.054	6.05	0.63
CNO^-	0.0007	0.03	0.098	0.60	0.37
SCN^-	0.2	1.60	0.38	4.10	0.68
CN^-	0.0005	0.02	0.041	0.76	0.54
N_3^-	0.0012	1.51	0.35	0.83	0.80
HCO_3^-	12	85	0.33	0.75	0.50
CO_3^{2-}	15	73	0.53	0.66	0.42
NO_3^-	7	35	0.39	0.81	0.78
NO_2^-	8.4	63	0.66	0.93	0.67
HS^-	0.0006	0.04	0.35	0.69	0.58
HSO_3^-	18	89	0.57	0.99	0.63
SnO_3^{2-}	0.57	0.83	0.066	0.55	0.48
SeO_4^{2-}	118	112	0.044	0.72	0.65
TeO_4^{2-}	0.66	0.92	0.049	0.34	0.45
OsO_5^{2-}	0.92	0.95	0.060	0.48	0.89
$S_2O_7^{2-}$	0.99	0.97	0.048	0.71	0.61
$P_2O_7^{4-}$	25.77	48.50	0.45	0.66	0.75
$V_2O_7^{4-}$	0.54	0.57	0.038	0.27	0.18
$B_4O_7^{2-}$	0.64	0.95	0.32	0.56	0.68
ReO_4^-	0.110	0.75	0.039	0.88	0.82
RuO_4^-	0.101	0.69	0.036	0.36	1.10
$S_2O_8^{2-}$	0.107	0.084	0.046	0.92	0.93
$SeCN^-$	0.085	0.086	0.022	0.73	0.97
CS_3^{2-}	0.0087	0.0088	0.021	0.38	0.21
$Et_2NCS_2^-$	0.79	3.10	0.61	0.0049	0.0074
SO_4^{2-}	63	>200	4.15	0.82	0.57
ClO_4^-	>200	>200	>200	10.1	6.50
BF_4^-	>200	>200	>200	>200	>200
FSO_3^-	0.79	0.46	0.060	0.91	0.75
$NH(SO_3)_2^{2-}$	0.31	0.76	28.1	0.54	0.70
$H_2NSO_2NH_2$	0.31	1.13	4.25	0.073	0.072
H_2NSO_3H	0.021	0.39	6.68	0.080	0.094
Ph-B(OH)$_2$	58.6	23.1	6.47	0.097	0.073
Ph-AsO$_3$H$_2$	31.7	49.2	5.86	0.44	0.092

[a]As sodium salt.
[b]Errors were in the range of 3%–5 % of the reported values, from three different assays;
[c]This work.
[d,e]From references[9,25,26].

atoms from it with NH_2 moieties, as in sulfamic acid or sulfamides, leads to a dramatic increase of the inhibitory power. The same may be noted regarding trithiocarbonate, which is only slightly more inhibitory than carbonate (or bicarbonate). However, using this zinc-binding group and adding the diethylamino fragment to it, as in diethyldithiocarbamate, leads to a low nanomolar inhibitor. It is thus obvious that the dithiocarbamates, a class of recently discovered CAIs[24,28], constitute a novel class of inhibitors also for hpαCA. Among the other simple inorganic anions investigated here it should be noted also that tellurate, divanadate and perruthenate were among the most efficient submillimolar inhibitors, with K_Is in the range of 0.27–0.36 mM.

2. hpβCA was generally even more sensitive to this class of CAIs compared to the α-class enzyme discussed above, these compounds showing inhibition constants in the range of 7.4 μM–6.4 mM, again, except tetrafluoroborate which was not inhibitory, K_I > 200 mM (Table 1). The most effective hpβCA inhibitors were diethyldithiocarbamate, sulfamide, sulfamic acid, phenylboronic and phenylarsonic acid, with K_Is in the range 7.4–94 μM. All the remaining anions, except perchlorate and perruthenate (K_Is of 1.1–6.4 mM) were submillimolar inhibitors with K_Is in the range of 0.18–0.97 mM (Table 1). Among the effective anions were again trithiocarbonate and divanadate, with K_Is in the range 180–210 μM. Generally, most of these anions showed a slightly better inhibitory capacity against the β- over the α-class enzyme of *H. pylori*, although few anions (diethyldithiocarbamate, tellurate and perosmate among others) were better hpαCA than hpβCA inhibitors.
3. The inhibition profiles of the two *H. pylori* enzymes are very different both from those of the host enzymes considered here, hCA I and II, as well as from those of the bacterial enzyme used for comparison, PCA (Table 1). This is a very encouraging result being also a proof that it may be possible to develop CAIs which specifically target the *H. pylori* enzymes, without interfering with the human isoforms, highly abundant in many tissues and involved in critical physiological processes.

Conclusions

We evaluated a series of inorganic anions and similar small molecules known to bind to metalloenzymes, for the inhibition of hpαCA and hpβCA, the two CAs from the bacterial pathogen *H. pylori*. As other enzymes from these classes investigated earlier, they are highly sensitive to anion inhibitors. hpαCA was inhibited in the low micromolar range by diethyldithiocarbamate (K_I of 4.9 μM), sulfamide, sulfamic acid, phenylboronic acid, and in the submillimolar one by a wide range of anions including, cyanide, cyanate, hydrogen sulfide, divanadate, tellurate, perruthenate, selenocyanide, trithiocarbonate, and iminodisulfonate. hpβCA generally showed an even stronger inhibition with most of these anions compared to the α-class enzyme, with several low micromolar and many submillimolar inhibitors detected (among which diethyldithiocarbamate, sulfamide, sulfamic acid, phenylboronic/phenylarsonic acid). These new CA inhibitors detected here may be used as leads for developing anti-*H. pylori* agents with a diverse mechanism of action compared to clinically used antibiotics for which many strains exhibit a wide range of drug resistance.

Declaration of interest

The authors report no conflict of interest. This work was supported by an EU FP7 research grant (Metoxia project).

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