The Catalytic Activity and the inhibition profile of the carbonic anhydrase CynT2

Subjects: Others

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CAs catalyze the physiologically crucial reversible reaction of the carbon dioxide hydration to bicarbonate and protons. Herein, we investigated the sulfonamide inhibition profile of the recombinant β-CA (CynT2) identified in the genome of the Gram-negative bacterium, *Escherichia coli*. This biocatalyst is indispensable for the growth of the microbe at atmospheric pCO_2. CynT2 was strongly inhibited by some substituted benzene-sulfonamides, and the clinically used inhibitor sulpiride (K_Is in the range of 82–97 nM). This study may be relevant for identifying novel CA inhibitors, as well as for another essential part of the drug discovery pipeline, such as the structure-activity relationship for this class of enzyme inhibitors.

Keywords: Carbonic anhydrase ; sulfonamides ; inhibitors

The first wholly sequenced microbial genomes were obtained in 1995 from two pathogenic bacteria, *Haemophilus influenzae* and *Mycoplasma genitalium* [1,2]. From 1995 onward, genomes belonging to 11,691 eukaryotes, 247,392 prokaryotes, and 34,747 viruses have been sequenced (Data from National Center for Biotechnology Information, May 2020). The extensive DNA sequencing has opened a new era to contrast human, animal, and plant diseases [3]. Two main reasons support this. The first is that most of the sequenced genomes belong to pathogens, and the second is that the knowledge of the genome of harmful microbes offers the possibility to identify gene encoding for protein targets, whose inhibition might impair the growth or virulence of the prokaryotic and eukaryotic pathogens [4,5]. Proteins as drug targets are prevalent. Among them, enzymes represent a significant group, since most of them catalyze reactions essential for supporting the central microbe metabolism and, as a consequence, the vitality of the pathogen [6]. The basis of the drug target approach is supported by the following criteria: (a) to identify metabolic pathways which are absent in the host and indispensable for the survival of the pathogen; (b) to recognize enzymes of the metabolic pathway whose inhibition compromise the microbe lifecycle; and, finally, (c) to find compounds which, in vitro (as the first investigation), can interfere with the activity of the identified enzymes [7]. In this context, the genome exploration of pathogenic and non-pathogenic microorganisms has revealed genes encoding for a superfamily of metalloenzymes, known as carbonic anhydrases (CAs, EC 4.2.1.1) [8,9,10,11,12]. CAs catalyze the physiologically crucial reversible reaction of the carbon dioxide (CO_2) hydration to bicarbonate (HCO_3^-) and protons (H^+) according to the following chemical reaction: $CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+$ [13,14,15]. Many CA inhibitors (CAIs) exist and efficiently inhibit, in vitro, the activity of the CAs encoded by the genome of several pathogens [13,16,17,18]. It has been demonstrated that CAIs are also effective in vivo, impairing the growth and virulence of several pathogens responsible of human diseases, such as *Helicobacter pylori* [19,20,21], *Vibrio cholerae* [22], *Brucella suis* [23,24,25,26], *Salmonella enterica* [27], and *Pseudomonas aeruginosa* [28]. Considering the three major criteria typifying the drug-target approach, it is evident that CAs meet the criteria (b) and (c) entirely. Instead, the criterion (a) is satisfied partly because CAs are ubiquitous metalloenzymes involved in the balance of the equilibrium between dissolved CO_2 and HCO_3^- in all living organisms. Even if CAs are not species-specific enzymes, they are considered promising drug targets because they offer the possibility to design specific and selective inhibitors for the microbial CAs [13,16,17,18]. For example, the enzyme dihydrofolate reductase (DHFR), although it is ubiquitously expressed in all kingdoms, is a target of several drugs, such as the antibacterial trimethoprim [29]. This enzyme is responsible for the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of 5,6-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF), an essential cofactor used in the biosynthetic pathways of purines, thymidylate, methionine, glycine, pantothenic acid, and *N*-formyl-methionyl tRNA. The bacterial DHFR amino acid sequence has an identity of 30% with the corresponding human protein [29]. Nevertheless, trimethoprim selectively inhibits the bacterial enzyme but not the human DHFR [29].

The CA superfamily is grouped into eight genetically distinct families (or classes), named with the Greek letters α, *β*, γ, δ, ζ, η, θ, and ι [13,14,15,30,31]. In mammalian, for example, 15 CAs are expressed, 12 of which are catalytically active, and all belong to the *α*-class [9,16,32,33,34,35,36,37]. It is interesting to stress that the genome of most pathogens does not

encode for a *α*-CA [12,13,14,34,38,39]. This is a unique advantage in finding inhibitors with no inhibitory effect on the CAs from humans and animals. However, when the genome of a pathogen encodes for a *α*-CA, such enzyme (amino acid sequence identity of about 35% respect to the mammalian protein) shows structural differences in the amino acid residues surrounding the catalytic pocket, offering the possibility to tune the CA inhibitors and, hence, a higher probability to inhibit selectively the *α*-CA identified in the pathogen [40,41,42]. Recently, our groups focused on the in vitro inhibition of recombinant *β*-CA (CynT2) from *Escherichia coli* because this CA, localized in the cytoplasm, is indispensable for the growth of the microbe at atmospheric pCO_2 [43,44]. *E. coli* is a Gram-negative bacterium that, as a commensal microorganism, colonizes the lower intestine of warm-blooded organisms [45,46,47]. In some cases, *E. coli* can act as a severe pathogen able to generate disease outbreaks worldwide [48,49,50], or, as an opportunistic pathogen, which can cause diseases if the host defenses are weakened [51]. Surprisingly, although this enzyme was reported and crystallized two decades ago [43], no inhibition study with any class of CAIs was reported so far. Here, we compare the inhibition profiles of CynT2 with those determined for the *β*-CA from *Vibrio cholerae* and the two human *α*-CA isoforms (hCA I and hCA II), using the sulfonamides and their bioisosteres, which, among the groups of the classical CAIs, generally inhibit the other CAs in the range of nanomolar and have been clinically used for decades as antiglaucoma [29], diuretic [35], antiepileptic [32], anti-obesity [52,53], and anticancer [37] agents.

The goal of the present manuscript is to identify putative compounds, which can eventually go through the other phases of the drug discovery pipeline, such as the structure–activity relationship (SAR), in vitro cell based-tests, in vivo studies, and, finally, the clinical trials, leading to the discovery of new antibacterials.

Results:

The recombinant enzyme resulted in an excellent catalyst for the CO_2 hydration reaction with a k_{cat}= 5.3 x 10^5 s^{-1} and a k_{cat}/K_M= 4.1 x 10^7 M^{-1} s^{-1}. (Table 1)

Table 1. Kinetic parameters for the CO_2 hydration reaction catalyzed by the human α-CAs and bacterial CAs (α-, β-, γ- and ι-CAs).

Organism	**Acronym**	**Class**	**k_{cat} (s^{-1})**	**k_{cat}/K_m (M^{-1} x s^{-1})**	**K_I (acetazolamide) (nM)**
Homo sapiens [a]	hCA I	α	2.0 x 10^5	5.0 x 10^7	250
	hCA II	α	1.4 x 10^6	1.5 x 10^8	12
Vibrio cholerae	VchCAα	α	8.2 x 10^5	7.0 x 10^7	6.8
Escherichia coli	CynT2	β	5.3 x 10^5	4.1 x 10^7	227
Vibrio cholerae	VchCAβ	β	3.3 x 10^5	4.1 x 10^7	451
Porphyromonas gingivalis	PgiCAβ	β	2.8 x 10^5	1.5 × 10^7	214
Helicobacter pylori	HpyCAβ	β	7.1 x 10^5	4.8 x 10^7	40
Porphyromonas gingivalis	PgiCAγ	γ	4.1 x 10^5	5.4 × 10^7	324
Vibrio cholerae	VchCAγ	γ	7.3 x 10^5	6.4 x 10^7	473

Burkholderia territorii	BteCAı	ı	3.0×10^5	9.7×10^7	65

In Table 2 is reported the inhibition profile of CynT2. The comparative analysis was carried out analyzing the CynT2 inhibitory behavior with those obtained for the enzyme VchCAβ (β-CA form *Vibrio cholerae*) and the two human γ-CA isoforms, hCA I and hCA II.

Table 2. Inhibition of the human isoforms hCA I and hCA II and the two bacterial β-CAs (CynT2 and VchCAβ) with sulfonamides **1-24** and the clinically used drugs **AAZ-EPA**.

Inhibitor	**K_I^*(nM)**			
	hCA I[a]	**hCA II[a]**	**CynT2**	**VchCAβ[a]**
1	28000	300	705	463
2	25000	240	790	447
3	79	8	457	785
4	78500	320	3015	>10,000
5	25000	170	2840	>10,000
6	21000	160	3321	>10,000
7	8300	60	>10000	>10,000
8	9800	110	>10000	9120
9	6500	40	2712	>10,000
10	7300	54	8561	>10,000
11	5800	63	6246	879
12	8400	75	4385	4450
13	8600	60	4122	68,1
14	9300	19	440	82,3
15	5500	80	6445	349
16	9500	94	2340	304
17	21000	125	502	3530
18	164	46	205	515

19	109	33	416	2218
20	6	2	726	859
21	69	11	473	4430
22	164	46	93	757
23	109	33	322	817
24	95	30	82	361
AAZ	250	12	227	4512
MZA	50	14	480	6260
EZA	25	8	557	6450
DCP	1200	38	>10000	2352
DZA	50000	9	629	4728
BRZ	45000	3	2048	845
BZA	15	9	276	846
TPM	250	10	3359	874
ZNS	56	35	3189	8570
SLP	1200	40	97	6245
IND	31	15	2392	7700
VLX	54000	43	2752	8200
CLX	50000	21	1894	4165
SLT	374	9	285	455
SAC	18540	5959	6693	275
HCT	328	290	5010	87
FAM	922	58	2769	-
EPA	8262	917	2560	-