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Sulfonamide inhibition studies of the γ-carbonic anhydrase from the Antarctic bacterium *Pseudoalteromonas haloplanktis*

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ABSTRACT

The Antarctic bacterium *Pseudoalteromonas haloplanktis* encodes for a γ-class carbonic anhydrase (CA, EC 4.2.1.1), which was cloned, purified and characterized. The enzyme (PhaCAγ) has a good catalytic activity for the physiologic reaction of CO_2 hydration to bicarbonate and protons, with a k_{cat} of 1.4×10^5 s^{-1} and a k_{cat}/K_m of 1.9×10^6 $M^{-1} \times s^{-1}$. A series of sulfonamides and a sulfamate were investigated as inhibitors of the new enzyme. Methazolamide and indisulam showed the best inhibitory properties (K_Is of 86.7–94.7 nM). This contribution shed new light on γ-CAs inhibition profiles with a relevant class of pharmacologic agents.

Marine psychrophiles act as processors of the polar marine primary productivity constituting the base for the entire polar food web and, ultimately, feeding krill, fish, whales, penguins, and seabirds.[1,2] They play, in fact, a significant role in the so called 'substance turnover'. Moreover, a feature common to all psychrophiles are their remarkable ability to thrive under extremely cold and salty conditions.[3] Cold-adapted organisms have developed a number of adjustments at the molecular level to maintain metabolic functions at low temperatures, such as the production of enzymes, note as 'cold-enzyme'.[4–11] These enzymes are characterized by a specific activity at low and moderate temperatures higher than their mesophilic counterparts over a temperature range roughly covering 0–30 °C and by a relative instability.[6,7,11–13] Probably, in the case of psychrophilic microorganisms the selective pressure is essentially exerted towards the specific activity and not towards stability factors as happens in mesophilic or in thermophilic enzymes. The molecular structure of a 'cold-enzyme' is primarily characterized by an adequate plasticity of the molecule at the environmental temperature in order to accommodate the substrates with a minimum of energy expenditure.[14] 'Cold-enzymes' naturally achieved a good compromise between activity and stability. There is a continuum in the adaptation of a protein to its environment.[4–11,13,15–22] In fact, all known structural factors and weak interactions involved in protein stability are either reduced in number or modified in psychrophilic enzymes in order to increase their flexibility; but the same structural factors are also implicate for increasing the stability of the thermophilic proteins.[23–29]

Carbonic anhydrases (CAs; EC 4.2.1.1) are metalloenzymes that catalyze CO_2 hydration to bicarbonate and protons.[4,5,30–38] These enzymes are involved in a multitude of physiologic processes in organisms all over the phylogenetic tree, with six genetically distinct CA classes known to date: the α-, β-, γ-, δ-, ζ- and η-CAs.[26,39–50] Their biochemical features are known in detail for at least four classes, together with their distribution and role in various organisms.[32,33,41,48–62] Inhibition and activation studies of many such enzymes from vertebrates, protozoa, fungi and bacteria have shown that they are drug targets for obtaining pharmacological agents of the diuretic, antiglaucoma, antiobesity, antiepileptic, anticancer or anti-infective type.[55–58,60] Many such enzymes also possess biotechnologic applications for biomimetic CO_2 capture processes.[55–60] The cloning and characterization of many other

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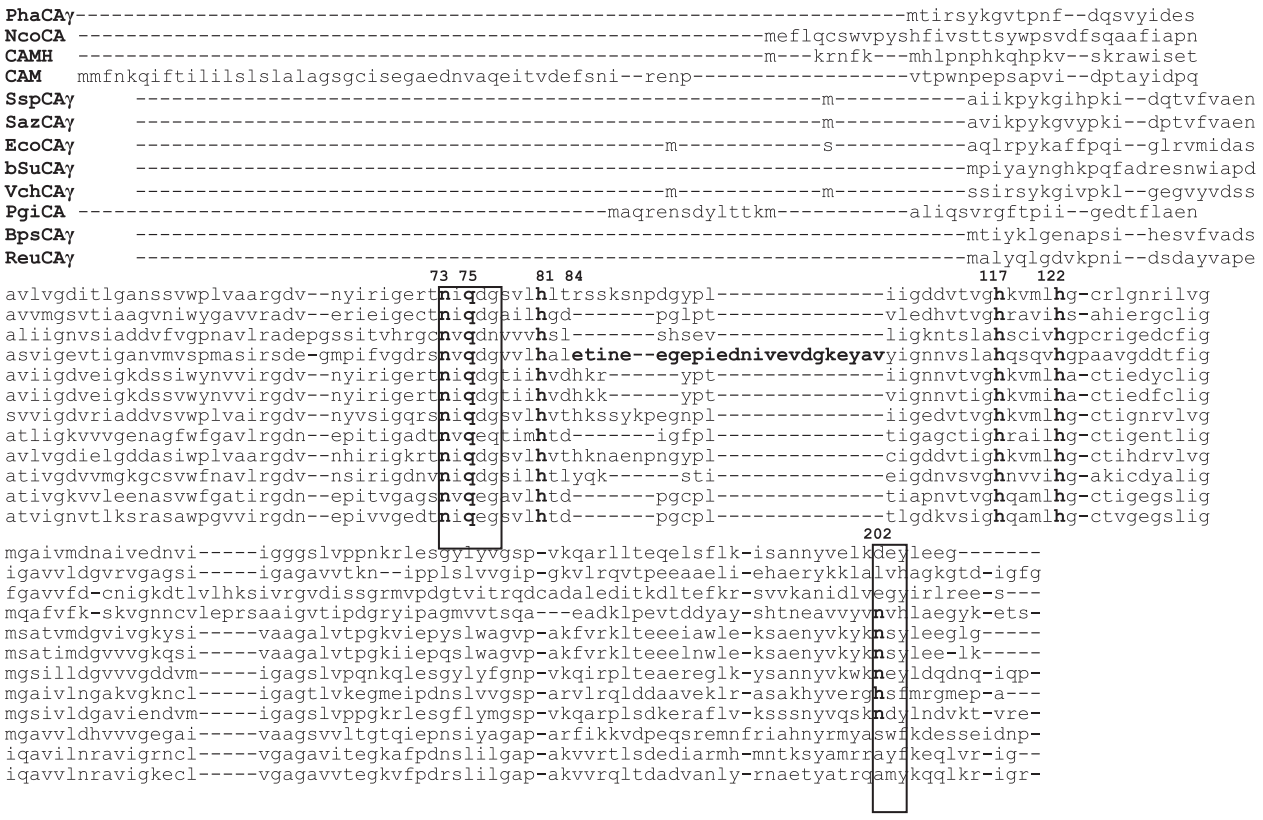


Figure 1. Multialignment performed with the program Clustal of the γ-CAs from different microorganisms. The metal ion ligands (His81, His117 and His122) are indicated in bold; the catalytically relevant residues of CAM (Asn73, Gln75 and Asn202), which participate in a network of hydrogen bonds with the catalytic water molecule, are boxed; the CAM acidic loop residues containing the proton shuttle residues Glu84 is missing in the other γ-CAs (CMA numbering system).

such enzymes will probably lead to the discovery of other CA families as well as enzymes with potentially important technologic applications.

CAs have been thoroughly investigated in mesophilic bacteria, but a limited number of studies are available on CAs from psychrophilic bacteria.[5,23,25–30,34,36–38,42,44,46–50] In the present paper, we describe the cloning, expression and purification of a γ-CA identified in the genome of *Pseudoalteromonas haloplanktis* (formerly *Alteromonas haloplanktis*), which is an obligate aerobic gram-negative rod-like bacterium that was isolated from seawater sampled along the Antarctic ice shelf. This species thrives permanently in seawater at temperature ranging between +2 °C and +4 °C, being able to survive in frozen conditions for a long period, when entrapped in the winter ice pack.

The analysis of genomic DNA from the aforementioned psychrophilic bacteria revealed that it encodes for CAs belonging to the β- and γ-classes. Here we report the cloning, purification and kinetic characterization of the recombinant γ-CA from *P. haloplanktis* and its inhibition profile with sulfonamides and their bioisosteres, such as the sulfamates. The new Antarctic γ-CA was named PhaCA. These studies are relevant at the level of the molecular structure because they allows the comprehension of the adaptative traits of the psychrophilic enzymes which have an unique goal: to improve the catalytic efficiency at low temperatures and possibly to gain conformational flexibility.

The *P. haloplanktis* PhaCAγ gene encodes a 177 amino acid polypeptide chain, which displays some identity with other such enzymes cloned and characterized recently, such as the γ-CA (NcoCA) from the Antarctic cyanobacterium *Nostoc commune* (37.3% identity; 69.0% similar); the mesophilic γ-CA (PgiCA) identified in the genome of the anaerobic bacterium *Porphyromonas gingivalis* (41.7% identity, 78.5% similar); as well as with the prototypical γ-CAs CAMH and CAM from the archaeon *Methanosarcina thermophila*.[61,62] Its alignment with the sequence of other γ-CAs (Fig. 1) shows that the metal ion ligands (His81, His117 and His122) are conserved in all these enzymes, as well as the catalytically relevant residues Asn73, Gln75 and Asn202 (first evidenced in CAM),[61] which participate in a network of hydrogen bonds with the catalytic water molecule. However the CAM acidic loop residues containing the presumed proton shuttle residue Glu84 is missing in the other γ-CAs investigated here (the CAM numbering system is used throughout this paper).

Table 1
Kinetic parameters for the CO_2 hydration reaction catalysed by the human cytosolic isozymes hCA I and II (α-class CAs) at 20 °C and pH 7.5 in 10 mM HEPES buffer and 20 mM Na_2SO_4, the cyanobacterial β-CA from *Coleofasciculus chthonoplastes*, and the γ-CAs CAM (*Methanosarcina thermophila*), PgiCA (*Porphyromonas gingivalis*) and NcoCA (*Nostoc commune*) measured at 20 °C, pH 8.3 in 20 mM TRIS buffer and 20 mM $NaClO_4$.

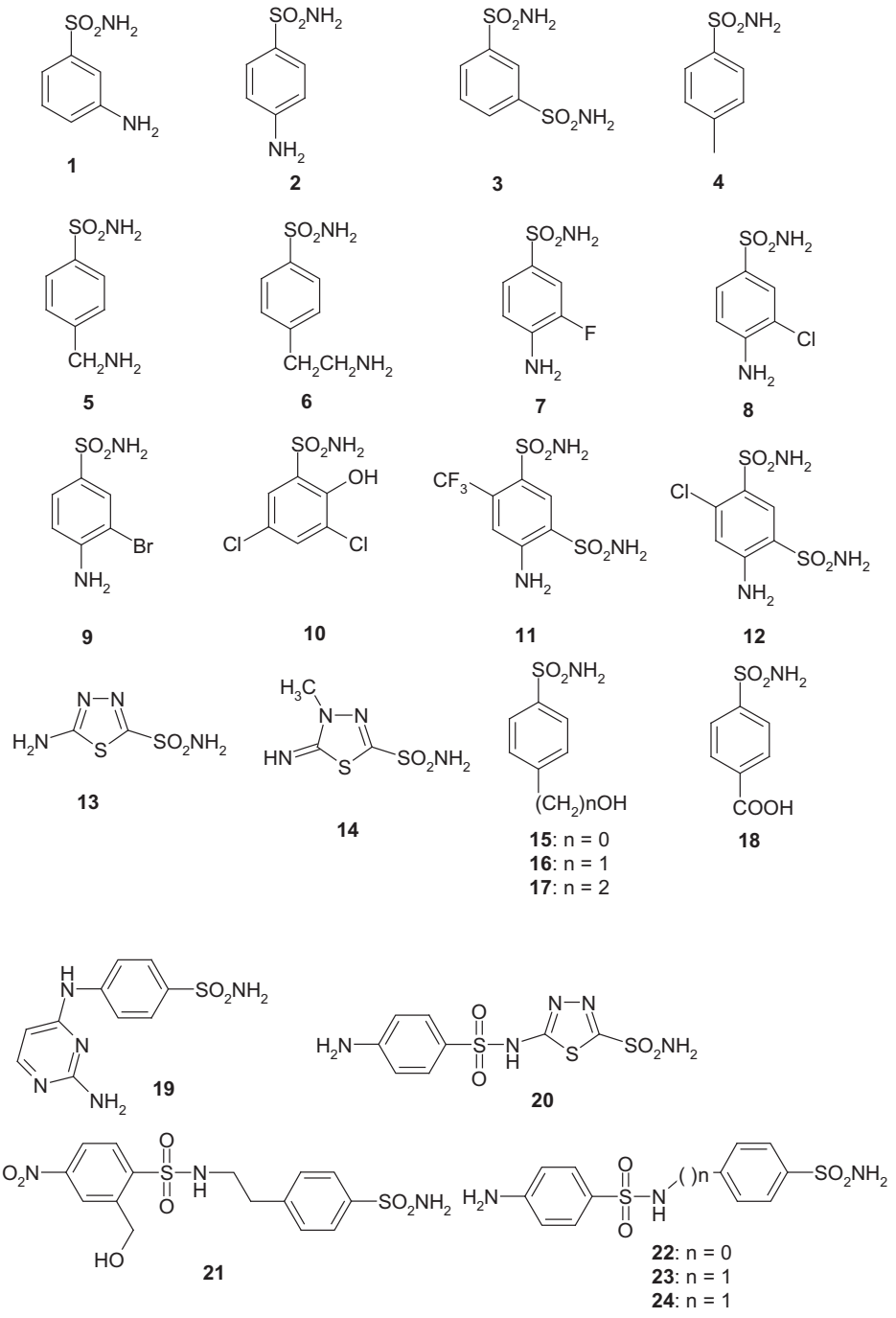
Enzyme	Activity level	Class	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \times s^{-1}$)	K_I (acetazolamide) (nM)
hCA I	Moderate	α	2.0×10^5	5.0×10^7	250
hCA II	Very high	α	1.4×10^6	1.5×10^8	12
CahB1	Low	β	2.4×10^5	6.3×10^7	76
CAM	Low	γ	6.1×10^4	8.7×10^5	63
PgiCA	Moderate	γ	4.1×10^5	5.4×10^7	324
NcoCA	High	γ	9.5×10^5	8.3×10^7	75.8
PhaCAγ	Medium–low	γ	1.4×10^5	1.9×10^6	403

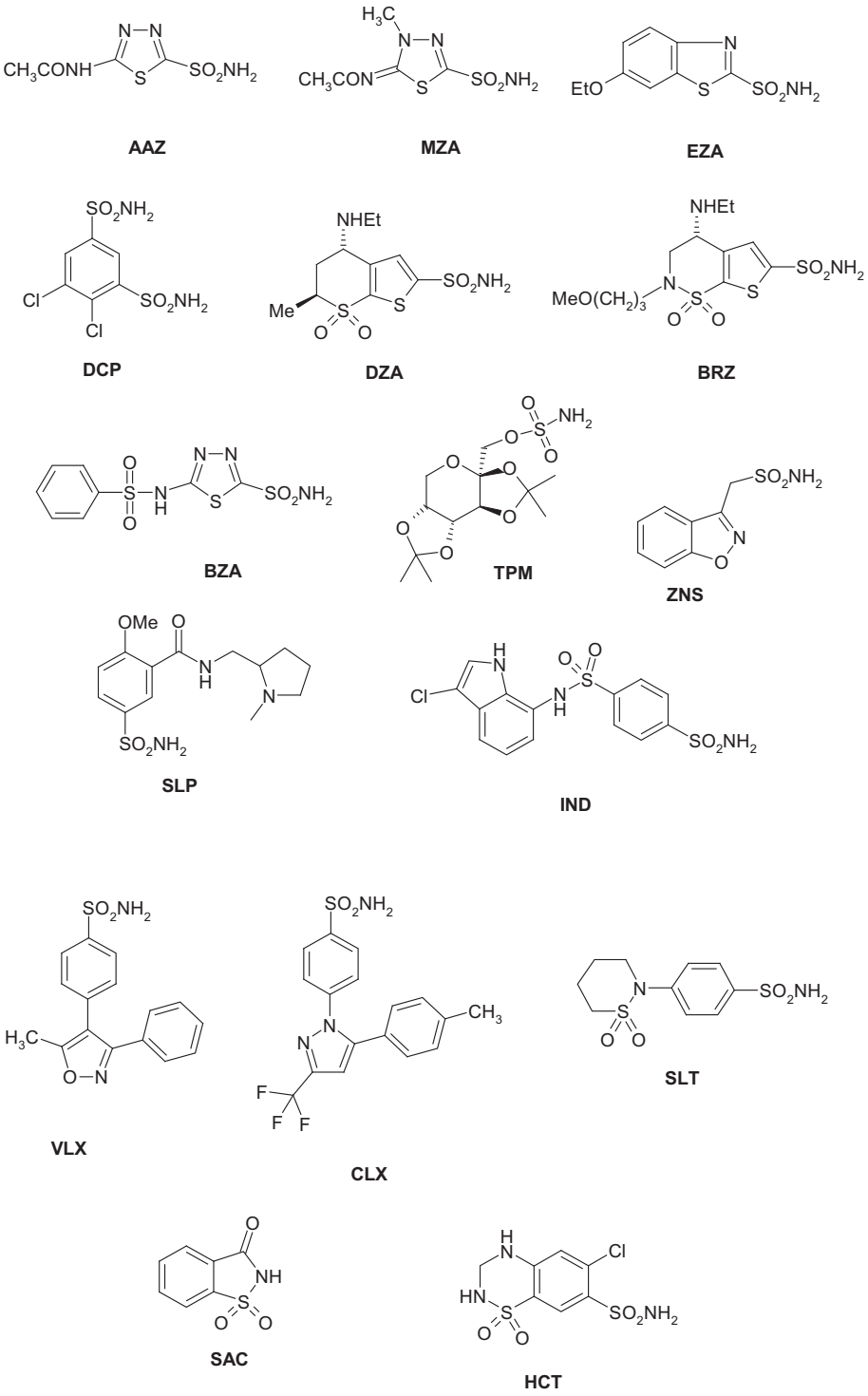
Inhibition data with the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided.

Indeed, as observed from data of Table 1, PhaCAγ shows a significant catalytic activity for the CO_2 hydration reaction, similar to other γ-CAs investigated earlier such as CAM, PgiCA and NcoCA.[57,58,23,31,60] The kinetic parameters for the PhaCAγ-catalyzed CO_2 hydration to bicarbonate and protons, were: k_{cat} of 1.4×10^5 s^{-1} and a k_{cat}/K_m of 1.9×10^6 $M^{-1} \times s^{-1}$. This activity is inhibited by the sulfonamide CA inhibitor (CAI) acetazolamide, with a K_I of 403 nM, in the same range as the γ-CA PgiCA from *P. gingivalis* or the human slow isoform hCA I (Table 1).

A phylogenetic tree was build for understanding the evolutionary relationship of the new enzyme with other γ-CAs present in the genomes of other organisms such as archaea and bacteria, but also with other CA classes such as for example the α-CAs present in vertebrates and bacteria (Fig. 2). The tree shown in Figure 2 clearly shows that the α- and γ-CAs are very distantly related to each other, with the two main branches clustering very distantly from each other. Among the γ-CAs (lower branches) the archaea enzymes CAMH and CAM are very distantly related to each other and to all other γ-CAs from bacteria. This is in fact expected as the separation of Archae and Bacteria is probably a very ancient event in the history of life on earth. It is interesting to note that all the bacterial γ-CAs (including PhaCAγ) clustered together on nearby branches, proving their similarity. The new enzyme characterized here seems to be the most similar with the γ-CAs from

Structures 1-24, AAX-HCT





Escherichia coli and *Vibrio cholerae* (Fig. 2). These enzymes were not yet investigated in detail or cloned, although they are present in one of the most investigated organisms in molecular biology or in a human pathogen provoking rather diffuse disease.

We investigated the susceptibility of PhaCAγ to inhibition with the main class of CA inhibitors (CAIs), the sulfonamides and their isosteres (sulfamates).[55–60] A panel of 40 such derivatives were included in this study. Derivatives **1**–**24** and **AAZ-HCT** are either simple aromatic/heterocyclic sulfonamides widely used as building blocks for obtaining new families of such pharmacological agents,[55–60] or they are clinically used agents, among which acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** and dichlorophenamide **DCP**, are the classical, systemically acting antiglaucoma CA inhibitors (CAIs). Dorzolamide **DZA** and brinzolamide **BRZ** are topically-acting antiglaucoma agents, benzolamide **BZA** is an orphan drug belonging to this class of pharmacological agents, whereas topiramate **TPM**, zonisamide **ZNS** and sulthiame **SLT** are widely used antiepileptic drugs. Sulpiride **SLP** and indisulam **IND** were also shown by our group to belong to this class of pharmacological agents, together with the COX2 'selective' inhibitors celecoxib **CLX** and valdecoxib **VLX**. Saccharin and the diuretic hydrochlorothiazide **HCT** are also known to act as CAIs. Inhibition

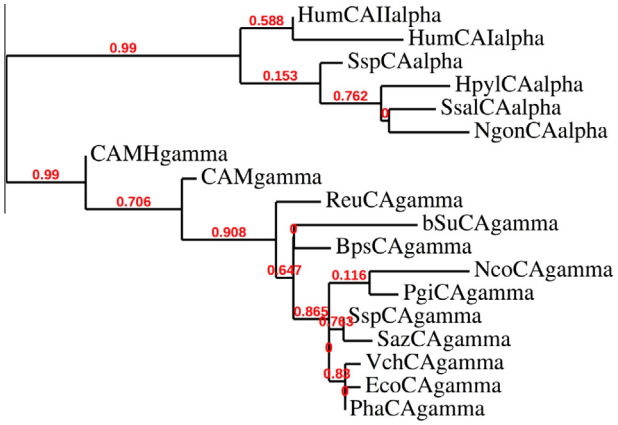


Figure 2. Phylogenetic trees of the γ-CA and α-CA amino acid sequences from different species. The tree was constructed using the program PhyML 3.0. Legend: **PhaCAgamma**, *Pseudoalteromonas haloplanktis* (Accession number: WP_016710385.1); **ReuCAgamma**, *Ralstonia eutropha* (Accession number: YP_725701.1); **bSuCAgamma**, *Brucella suis* (Accession number: NP_698263.1); **BpsCAgamma**, *Burkholderia pseudomalle* (Accession number: YP_108862.1); **SspCAgamma**, *Sulfurihydrogenibium yellowstonense* (Accession number: WP_007547159.1); **SazCAgamma**, *Sulfurihydrogenibium azorense* (Accession number: WP_012674376.1); **PgiCAgamma**, *Porphyromonas gingivalis* (Accession number: WP_012457873.1); **EcoCAgamma**, *Escherichia coli* (Accession number: CDL59494); **VchCAgamma**, *Vibrio cholerae*; **CAMgamma** and **CAMHgamma**, *Methanosarcina thermophila* (Accession numbers: P40881.1 for CAMgamma and ACQ57353.1 for CAMHgamma); **NcoCAgamma**, *Nostoc commune* (Accession number: YP_007048658.1); **HumCAIIalpha**, *Homo sapiens* (Accession number: AAH11949.1); **HumaCAIalpha**, *Homo sapiens* (Accession number: NP_001729.1); **SspCAalpha**, *Sulfurihydrogenibium yellowstonense*, (Accession number: WP_012459296.1); **HplyCAalpha**, *Helicobacter pylori* (Accession number: WP_010882609.1); **SsalCAalpha**, *Streptococcus salivarius*, (Accession number: WP_002888224.1); **NgoCAalpha**, *Neisseria gonorrhea* (Accession number: WP_003688976.1).

data of the human (h), possibly off target isoforms hCA I and II, and the only other γ-CAs investigated in detail so far, CAM, PgiCA and NcoCA, are also presented in Table 2, for comparison reasons. The following structure-activity relationship (SAR) data can be observed for the inhibition of NcoCA with this panel of sulfonamides/sulfamates:

(i) A number of sulfonamides, among which **3**–**5**, **7**, **8**, **17** and **SLP** were ineffective PhaCAγ inhibitors up to concentrations of 100 μM, They include simple benzenesulfonamides substituted in the 3, 4 or 3,4-positions with sulfamoyl, methyl, aminomethyl, hydroxyethyl or halogeno and amino moieties. Sulpiride is also a benzenesulfonamide incorporating however a more complex scaffold.

(ii) The largest majority of the investigated derivatives were weak, micromolar CAIs against PhaCAγ, with inhibition constants in the range of 2465–24,600 nM (Table 2. The compounds in this category include **1**, **2**, **6**, **9**–**16**, **18**–**24**, **DCP** and **BZA**. It is interesting to note that these weak inhibitors incorporate a variety of aromatic/heterocyclic sulfonamide scaffolds, belonging to heterogeneous classes of compounds. Furthermore, many of them show much better inhibitory effects against α- or other γ-CAs shown in Table 2 (e.g.; compare **12**, **18** and **19** against the various CAs from the Table).

(iii) A few of the clinically used derivatives were more effective PhaCAγ inhibitors, with K_Is ranging between 403 and 898 nM. They include **AAZ**, **EZA**, **DZA**, **BRZ**, **TPM**, **ZNS**, **VLX**, **CLX**, **SLT**, **SAC** and **HCT**. Although the activity of these compound as CAIs is not excellent, their large structural variability shows that probably this γ-CA, relatively not highly sensitive to sulfonamide inhibitors, may be better inhibited by some compounds belonging to this class. And indeed, this seems to be the case for the last two compounds discussed shortly.

(iv) Two inhibitors with efficacy <100 nM were detected in this study, **MZA**, with a K_I of 94.7 nM, and **IND** with a K_I of 86.7 nM. These compounds are rather different structurally between them, and still they show an effective inhibition profile of the enzyme. It should be also noted that the structural differences between MZA and AAZ is minimal (an extra CH2 group in MZA) and still the two compounds differ by a factor of 4.25 as PhaCAγ inhibitors (Table 2). Furthermore, the deacetylated MZA derivative (compound 14) is 64.4 times a weaker inhibitor compared to MZA. All these data suggest that small differences in the inhibitor scaffold lead to dramatic differences of activity.

Table 2
Inhibition of human isoforms hCA I and hCA II, of the bacterial enzymes from *Methanosarcina thermophila* (CAM) and *Porphyromonas gingivalis* (PgiCA)as well as the cyanobacterial one from *Nostoc commune* (NcoCA) with sulfonamides **1**–**24** and the clinically used drugs **AAZ**–**HCT**, by a CO_2 hydrase, stopped-flow assay

Inhibitor/enzyme class	K_I* (nM)				
	hCA II[a] α	CAM[b] γ	PgiCA[c] γ	NcoCA[d] γ	PhaCAγ γ
1	300	nt	4220	492	5460
2	240	250	893	488	9210
3	8	170	>100,000	683	>100,000
4	320	nt	945	785	>100,000
5	170	350	3600	825	>100,000
6	160	270	3840	742	6150
7	60	970	680	20,600	>100,000
8	110	140	662	23,750	>100,000
9	40	1720	201	872	7460
10	54	nt	218	2810	8450
11	63	830	711	632	8980
12	75	120	1040	269	8465
13	60	nt	510	84.7	6310
14	19	nt	595	480	6100
15	80	nt	326	7570	8760
16	94	nt	223	4034	9150
17	125	nt	178	777	>100,000
18	46	nt	560	74.6	8730
19	33	nt	685	78.3	5400
20	2	nt	1450	66.7	7690
21	11	nt	3540	402	8090
22	46	nt	4100	553	8465
23	33	180	4650	74.5	9290
24	30	nt	3400	40.3	8885
AAZ	12	63	324	75.8	403
MZA	14	140	343	191	94.7
EZA	8	200	613	264	679
DCP	38	190	1035	345	2465
DZA	9	410	685	67.4	831
BRZ	3	nt	722	81.3	735
BZA	9	nt	741	48.5	24,600
TPM	10	1020	>100,000	65.7	877
ZNS	35	nt	157	85.6	490
SLP	40	nt	418	60.5	>100,000
IND	15	nt	131	92.3	86.7
VLX	43	130	755	53.1	735
CLX	21	140	169	87.6	761
SLT	9	nt	424	82.4	898
SAC	5959	nt	273	408	867
HCT	290	nt	380	58.7	790

nt = not tested.
* Errors in the range of 5–10% of the shown data, from 3 different assays.
[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method, from Ref. 17.
[b] Recombinant protozoan enzyme, stopped flow CO_2 hydrase assay method, from Ref. [59].
[c] Recombinant bacterial enzyme, from Ref. [30].
[d] Recombinant bacterial enzyme, this work.

In conclusion, we investigated the Antarctic bacterium *P. haloplanktis*, which encodes for a γ-class CA. The enzyme (PhaCAγ) was cloned, purified and characterized in detail. It shows a good catalytic activity for the physiologic reaction of CO_2 hydration to bicarbonate and protons, with a k_{cat} of $1.4 \times 10^5\ s^{-1}$ and a k_{cat}/K_m of $1.9 \times 10^6\ M^{-1} \times s^{-1}$. A series of sulfonamides and a sulfamate were also investigated as inhibitors of PhaCAγ, considering that this is the main class of compounds possessing high affinity for CAs. Methazolamide and indisulam showed the best inhibitory properties (K_Is of 86.7–94.7 nM) followed by acetazolamide, zonisamide, ethoxzolamide, brinzolamide and valdecoxib, which showed inhibition constants in the range of 403–735 nM. Other aromatic/heterocyclic sulfonamides and topiramate were ineffective, micromolar PhaCAγ inhibitors. As the γ-CAs seem to be the most ancient class of such enzymes and they were poorly investigated up until now, this contribution may shed some light on their inhibition profiles with a relevant class of pharmacologic agents. Moreover, the inhibition study curried out on this Antarctic CA represents an excellent scientific tentative for searching and designing selective drug for structurally very similar CA classes. In fact, the identification of such class-selective compounds may led to novel drugs with less side effects compared to those used in a wide range of diseases.

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