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DNA Cloning, Characterization, and Inhibition Studies of an α-Carbonic Anhydrase from the Pathogenic Bacterium *Vibrio cholerae*

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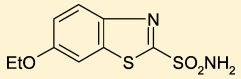
Supporting Information

ABSTRACT: We have cloned, purified, and characterized an α-carbonic anhydrase (CA, EC 4.2.1.1) from the human pathogenic bacterium *Vibrio cholerae*, VchCA. The new enzyme has significant catalytic activity, and an inhibition study with sulfonamides and sulfamates led to the detection of a large number of low nanomolar inhibitors, among which are methazolamide, acetazolamide, ethoxzolamide, dorzolamide, brinzolamide, benzolamide, and indisulam (K_I values in the range 0.69−8.1 nM). As bicarbonate is a virulence factor of this bacterium and since ethoxzolamide was shown to inhibit the in vivo virulence, we propose that VchCA may be a target for antibiotic development, exploiting a mechanism of action rarely considered until now.



Ki =0.69 nM

INTRODUCTION

Vibrio cholerae is a Gram-negative bacterium that causes cholera, a disease characterized by massive loss of water and electrolytes, leading to severe dehydration and hypovolemic shock if the condition is not treated.[1] Other bacteria belonging to the genus *Vibrio*, such as *V. parahemolyticus*, *V. vulnificus*, *V. owensii*, *V. fluvialis*, etc., are widespread organisms in the sea and in nonsalted water, infecting various organisms such as fish, oyster, corals, and mammals, in which they can provoke serious or even mortal disease.[2−4] Cholera is endemic in many countries, causing more than 120 000 deaths each year.[4] Significant antibiotic resistance to *V. cholerae* infection has been reported worldwide.[4]

Cloning of the genomes of many bacterial pathogens offers the possibility of exploring alternative pathways for inhibiting virulence factors or proteins essential for their life cycle, and such an approach started to be applied systematically for bacterial carbonic anhydrases (CAs, EC 4.2.1.1) lately.[5] CAs catalyze a simple but physiologically relevant reaction in all life kingdoms, carbon dioxide hydration to bicarbonate and protons.[6] These enzymes are involved in many physiologic processes in bacteria or other microorganisms, such as photosynthesis, respiration, CO_2 transport, and even metabolism of xenobiotics (e.g., cyanate in *Escherichia coli*).[7−10]

Five genetically distinct CA families are known to date, the α-, β-, γ-, δ-, and ζ-CAs, all of them being metalloenzymes that use Zn(II), Cd(II), or Fe(II) within their active sites.[11,12] Bacteria, the most successful organisms on earth, encode CAs belonging to the α-, β-, and/or γ-CA families.[5,11,12] Many such enzymes were investigated in detail lately in pathogenic (as well as nonpathogenic) bacteria such as *Brucella* spp., *Mycobacterium tuberculosis*, *Streptococcus* spp., *Helicobacter pylori*, *Salmonella enterica*, *Sulfurihydrogenibium* spp., etc., in the search for antibiotics with a novel mechanism of action, since it has been demonstrated that in many of them, CAs are essential for the life cycle of the organism.[5,6]

An inspection of the genome[13] of *V. cholerae* led us to the observation of three putative CAs (never investigated until now) belonging to each bacterial class: an α-CA originating from the *cah* gene VC0395_0957; a β-CA derived from the gene VC0395_A 0118; a γ-CA encoded by the gene VC0395_A2463. Furthermore, ethoxzolamide, a potent sulfonamide CA inhibitor (CAI) of mammalian/bacterial enzymes,[5] was recently demonstrated to inhibit the bicarbonate-mediated virulence induction in *V. cholerae*,[2] suggesting that conversion of CO_2 into bicarbonate by one or more CAs found in this organism plays a role in virulence induction.[2a] pH also regulates gene expression in this pathogenic bacterium,[2b] and CAs are known to be involved in pH regulation in many organisms.[5] In fact, bacteria can increase cytosolic bicarbonate levels using at least two mechanisms: (i) by means of transporters that directly bind to and import bicarbonate (one example being the bicarbonate transport system of *Synechococcus elongatus* PCC 6301)[8] and (ii) through the action of specific enzymes, such as the CAs, which convert into

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Table 1. Kinetic Parameters for CO_2 Hydration Reaction Catalyzed by Some Human α-CA Isozymes (hCAs I and II) and the Bacterial Enzymes hpαCA (*Helicobacter pylori*) and VchCA (*Vibrio cholerae*) at 20 °C and pH 7.5 and Their Inhibition Data with Acetazolamide AAZ (5-Acetamido-1,3,4-thiadiazole-2-sulfonamide), a Clinically Used Drug

enzyme	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	K_I(acetazolamide) (nM)
hCA I[a]	2.00×10^5	4.0×10^{-3}	5.0×10^7	250
hCA II[a]	1.40×10^6	9.3×10^{-3}	1.5×10^8	12
hpαCA[b]	2.5×10^5	16.6×10^{-3}	1.5×10^7	21
VchCA[c]	8.23×10^5	11.7×10^{-3}	7.0×10^7	6.8

[a]Human recombinant isozymes, stopped flow CO_2 hydrase assay method, from refs 2a and 11a. [b]From ref 11a. [c]Recombinant enzyme, stopped flow CO_2 hydrase assay method, this work.[15]

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hCAI          -----------------MASPDWGYDD-KNGPEQWSKLYP----IANGNNQSPVDIKTSE
hCAII         -----------------MSHHWGYGK-HNGPEHWHKDFP----IAKGERQSPVDIDTHT
hpαCA         MKKTFLIALALTASLIGAENTKWDYKNKENGPHRWDKLHKDFEVCKSGKSQSPINIEHYY
VchCA         MKKTTWVLAMAASMSFGVQASEWGYEG-EHAPEHWGKVAP---LCAEGKNQSPIDVSQS-
                                .*.*   .:.*.:* *         .*: ***:::.
                                          64                        94 96
hCAI          TKHDTSLKPISVSYNPATAKEIINVGHSFHVNFEDNDNRSVLKGGPFSDSYRLFQFHFHW
hCAII         AKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGPLDGTYRLIQFHFHW
hpαCA         HTQD--KADLQFKYAASKPKAVFFTHHTLKASFEPTNHINYRG-----HDYVLDNVHFHA
VchCA         VEAD--LQPFTLNYQG-QVVGLLNNGHTLQAIVSGNNPLQIDG-----KTFQLKQFHFHT
                 *     : ..*       ::   *:::. .. .:            : * :.***
                       106         119
hCAI          GSTNEHGSEHTVDGVKYSAELHVAHWNSAKYSSLAEAASKADGLAVIGVLMKVGEANPKL
hCAII         GSLDGQGSEHTVDKKKYAAELHLVHWN-TKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGL
hpαCA         P------MEFLINNKTRPLSAHFVHKD-----------AKGRLLVLAIGFEEGKENPNL
VchCA         P------SENLLKGKQFPLEAHFVHAD-----------EQGNLAVVAVMYQVGSENPLL
                     *  :.    . . *..* :             . * *:.:  : *. :* *
                                                    199
hCAI          QKVLDALQAIKTKGKRAPFTNFDPSTLLPSSLDFWTYPGSLTHPPLYESVTWIICKESIS
hCAII         QKVVDVLDSIKTKGKSADFTNFAARGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPIS
hpαCA         DPILEGIQ--KKQN----LKEVALDAFLPKSINYYHFNGSLTAPPCTEGVAWFVIEEPLE
VchCA         KALTADMP--TKGNSTQLTQGIPLADWIPESKHYYRFNGSLTTPPCSEGVRWIVLKEPAH
              . :   :   .. .       .     :*.* .:: : **** **  * * *:: :*.

hCAI          VSSEQLAQFRSLLSNVEGDNAVPMQHNNRPTQPLKGRTVRASF----
hCAII         VSSEQVLKFRKLNFNGEGEPEELMVDNWRPAQPLKNRQIKASFK---
hpαCA         VSAKQLAEIKKRMKN---------SPNQRPVQPDYNTVIIKSSAETR
VchCA         VSNQQEQQLSAVMG-----------HNNRPVQPHNARLVLQAD----
              ** :*  ::                * **.**     :  :
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Figure 1. Multialignment of the amino acid sequences of α-CAs from different sources was performed with the program Clustal W, version 2.1: hCA I, *Homo sapiens*, isoform I (accession no. NP_001158302.1); hCA II, *Homo sapiens*, isoform II (accession no. AAH11949.1); hpαCA, *Helicobacter pylori* J99 (accession no. NP_223829.1); VchCA, *Vibrio cholera* (accession no. AEA79886.1). The zinc ligands (His94, His96, and His119, indicated in red), the gatekeeper residues (Glu106 and Thr199, indicated in blue), and the proton shuttle residue (His64, indicated in green) are conserved in all these enzymes. hCAI numbering system was used. The asterisk (*) indicates identity at all aligned positions. The symbol (:) relates to conserved substitutions, while (.) means that semiconserved substitutions are observed.

bicarbonate the metabolic CO_2 and/or atmospheric CO_2 entered into the cell by diffusion.[5] As no bicarbonate transporters were reported so far in *V. cholerae*, we decided to investigate in some detail the CAs encoded by this organism, in the search of novel proteins involved in the virulence/survival of these bacteria and thus new targets of antibiotics. Here we report the cloning, purification, and characterization of the α-CA of *V. cholerae* (referred by us as VchCA from now on), which has been identified by translated genome inspection. This investigation aimed to study the biochemical properties of this enzyme, as well as its inhibition profile with a range of sulfonamides and sulfamates known to act as CAIs against other α-CAs, and to provide preliminary insights in the field of this pathogen virulence, for which some interesting novel approaches for drug design have been recently proposed.[4]

RESULTS AND DISCUSSION

Purification of Recombinant VchCA. The recombinant VchCA prepared as described in the Experimental Section was isolated and purified to homogeneity at room temperature from *Escherichia coli* (DE3) cell extracts. CA activity was recovered in the soluble fraction of cell extract obtained after sonication and centrifugation. The heterologously expressed VchCA enzyme was purified 1.4-fold with the ammonium sulfate precipitation step. By use of the affinity column (His-select HF nickel affinity gel), VchCA was purified 11-fold to apparent homogeneity, as indicated by a single protein band after SDS−PAGE (see Supporting Information Figure S1, lane 4). The molecular weight estimated by SDS−PAGE was 26.0 kDa. A subunit molecular mass of 26.4 kDa was calculated on the basis of the amino acid sequence translated from the gene.

VchCA Catalytic Activity. The VchCA catalytic activity for the CO_2 hydration reaction is shown in Table 1, where data for

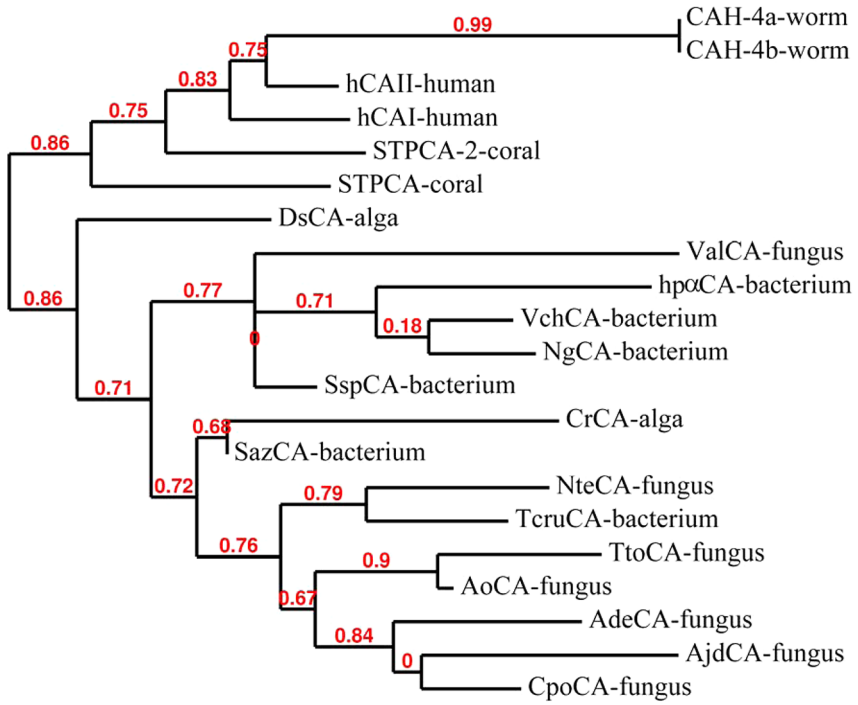


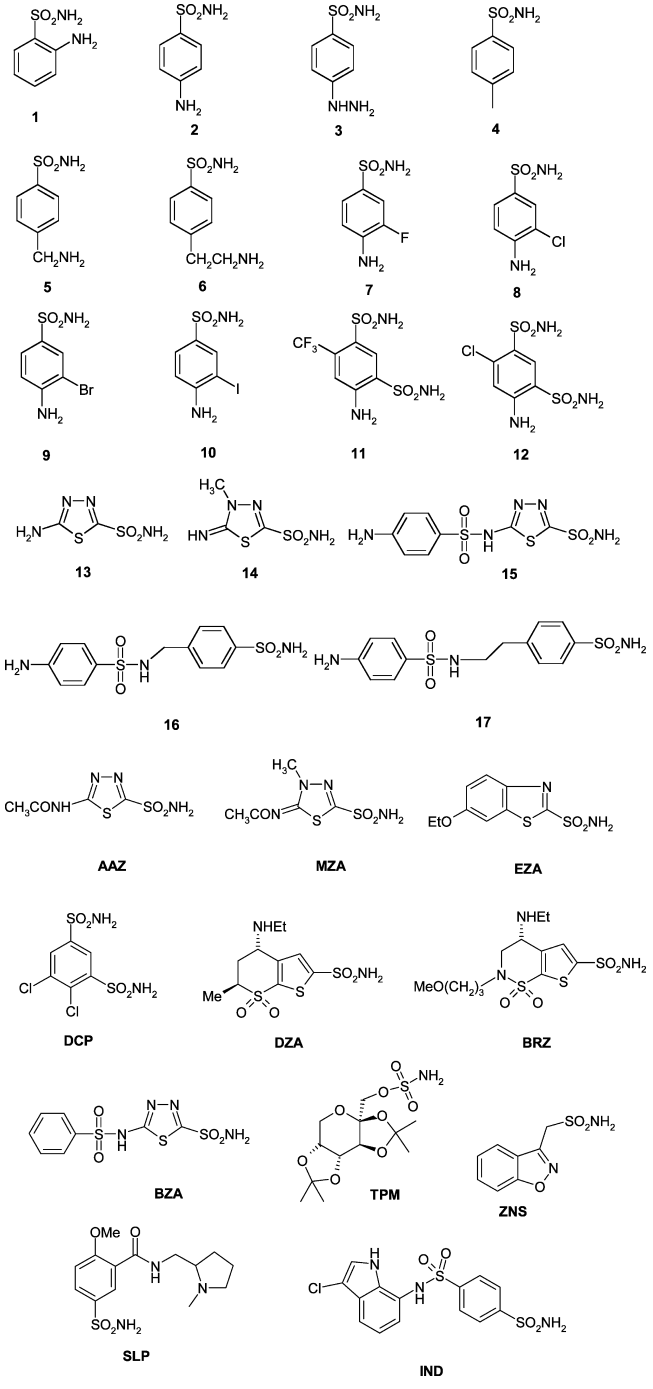
Figure 2. Phylogenetic analysis of VchCA and other α-CAs from different sources. The phylogenetic tree was constructed using the program PhyML 3.0. Branch support values, displayed in red, are reported at branch points. Definitions are as follows: CAH-4a-worm, *Caenorhabditis elegans,* isoform a (accession no. NP_510265 STPCA); CAH-4b-worm, *Caenorhabditis elegans,* isoform b (accession no. NP_510264); hCA I-human, *Homo sapiens,* isoform I (accession no. NP_001158302.1); hCA II-human, *Homo sapiens,* isoform II (accession no. AAH11949.1); STPCA-coral, *Stylophora pistillata* (accession no. ACA53457.1); STPCA-2-coral, *Stylophora pistillata* (accession no. ACE95141.1); DsCA-alga, *Dunaliella salina* (accession no. AAC49378.1); ValCA-fungus, *Verticillium albo-atrum* VaMs.102 (accession no. EEY23154); hpαCA-bacterium, *Helicobacter pylori* J99 (accession no. NP_223829.1); VchCA-bacterium, *Vibrio cholera* (accession no. AEA79886.1); NgCA-bacterium, *Neisseria gonorrhoeae* (accession no. CAA72038.1); SspCA-bacterium, *Sulfurihydrogenibium yellowstonense* YO3AOP1 (accession no. ACD66216.1); CrCA-alga, *Chlamydomonas reinhhardtii* (accession no. BAA14232.2); SazCA-bacterium, *Sulfurihydrogenibium azorense* (accession no. ACN99362.1); NteCA-fungus, *Neurospora tetrasperma* FGSC 2509 (accession no. EGZ68375); TcruCA-bacterium, *Thiomicrospira crunogena* XCL-2 (accession no. ABB42137.2); TtoCA-fungus, *Trichophyton tonsurans* (accession no. EGD97351.1); AoCA-fungus, *Aspergillus oryzae* (accession no. EIT75832.1); AdeCA-fungus, *Auricularia delicata* TFB-10046 SS5 (accession no. EJD34060); AjdCA-fungus, *Ajellomyces dermatitidis* ER-3 (accession no. EEQ91916.1); CpoCA-fungus, *Coccidioides posadasii* str. Silveira (accession no. EFW17342.1).

other α-CAs (such as the widespread and highly investigated human (h) isoforms hCAs I and II and the *Helicobacter pylori* hpαCA)[14] are also included for comparison. A stopped-flow CO_2 hydrase assay has been used to measure the catalytic activity of these enzymes in identical conditions.[15] It may be observed that VchCA has kinetic parameters quite similar to those of the human isoform hCA I, with a k_{cat} of 8.23×10^5 s^{-1} and a K_M of 11.7 mM, which leads to a k_{cat}/K_M of 7.0×10^7 $M^{-1}\cdot s^{-1}$ (compared to 5.0×10^7 $M^{-1}\cdot s^{-1}$ for hCA I). VchCA is thus slightly more active than hCA I but also 4 times more active compared to the other bacterial enzyme considered in Table 1, i.e., hpαCA. VchCA is about half as active as hCA II, one of the best catalysts known in nature (which has a k_{cat}/K_M of 1.5×10^8 $M^{-1}\cdot s^{-1}$; see Table 1). Similar to all these α-CAs considered here, VchCA was also strongly inhibited by acetazolamide, a sulfonamide in clinical use, with a K_I of 6.8 nM (thus being almost twice more sensitive to this inhibitor than hCA II and 3 times more sensitive than the *H. pylori* enzyme).[14]

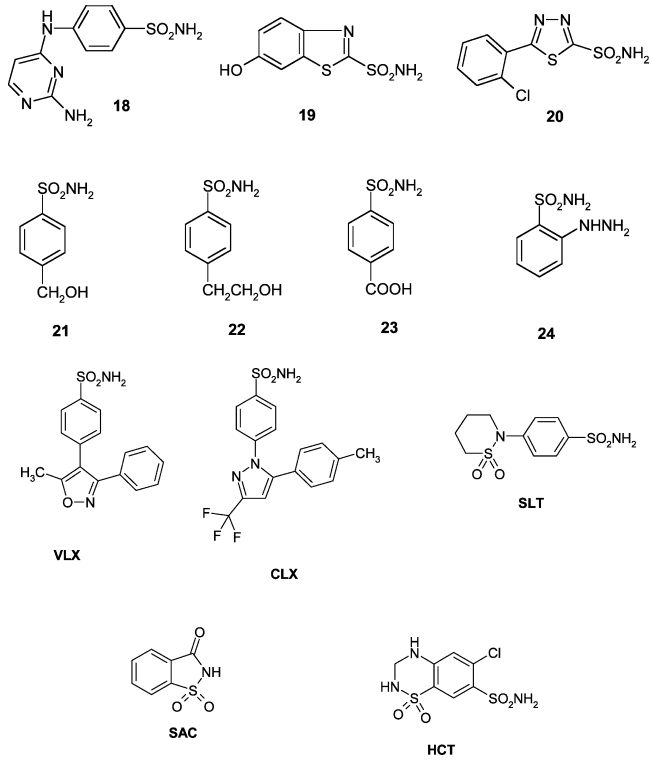
Sequence Analysis. An alignment of the amino acid sequences of VchCA and other α-CAs (such as hCAs I and II and hpαCA) is shown in Figure 1 in order to identify salient features of this bacterial enzyme. It may be observed that, like the other investigated α-CAs, VchCA has the conserved three His ligands, which coordinate the Zn(II) ion crucial for catalysis (His94, His96, and His119, hCA I numbering system). The proton shuttle residue (His64) is also conserved in all these enzymes. This residue assists the rate-determining step of the catalytic cycle, transferring a proton from the water coordinated to the Zn(II) ion to the environment with formation of zinc hydroxide nucleophilic species of the enzyme. VchCA has also the gate-keeping residues (Glu106 and Thr199), which orientate the substrate for catalysis and are also involved in the binding of inhibitors. The only unique macrofeature in the primary structure of the bacterial α-CAs with respect to the mammalian enzymes was the absence of four amino acid loops indicated in Figure 1 by the "- - -" symbols. This feature is typical of all bacterial α-CAs investigated so far. Deletion of these loops probably makes the bacterial proteins smaller and more compact, thus leading to a higher stability to heat and other denaturing agents.[16−18]

Phylogenetic Analysis. A phylogenetic analysis of VchCA and other α-CAs (such as human, coral, fungal, algal, and bacterial α-CAs) is shown in Figure 2 in order to understand the relatedness of the new enzyme reported here with other members of the α-class family, from the evolutionary viewpoint. It may be observed that VchCA is most closely related to the *Neisseria gonorrhoeae* (NgCA)[19] and to the *H. pylori* enzymes.[14] All of them cluster together on two neighboring branches of the tree of Figure 2. The next closest relatives of these enzymes are

the thermophilic bacterium enzyme SspCA, isolated from the extremophile *Sulfurihydrogenibium yellostonense*,[16−18] and unexpectedly, the fungal enzyme from the fungus *Verticillium alboatrum* (ValCA). The human, coral, and some algal CAs were the least related enzymes to VchCA, whereas many of the bacterial, fungal, and some algal CAs all clustered together in the lower branches of the tree of Figure 2.



Chemistry and CA Inhibition. Sulfonamides and sulfamates are well-known inhibitors of α-CAs, many of them possessing clinical applications as diuretics, antiglaucoma, antiobesity, anticonvulsant, or antitumor agents.[5,6,20−22] A large number of such derivatives was investigated for the inhibition of the new bacterial enzymes VchCA. Simple aromatic and heteroaromatic sulfonamides of types **1**−**24** were among them, as well as derivatives AAZ-IND, which are



clinically used drugs or agents in clinical development. Acetazolamide AAZ, methazolamide MZA, ethoxzolamide EZA, and dichlorophenamide DCP are the classical, systemically acting CAIs.[5,6] 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 and zonisamide ZNS are widely used antiepileptic drugs. Sulpiride SLP and indisulam IND were recently shown by this group to belong to this class of pharmacological agents.[5] Sulfonamides **1**−**24** and the clinically used agents investigated in this study were either commercially available or prepared as reported earlier by our group.[20−22]

Table 2 shows inhibition data for this panel of sulfonamides (and one sulfamate, TPM) against VchCA. For comparison reasons, inhibition data of the same compounds against another bacterial (hpαCA) and two mammalian (hCA I and hCA II) enzymes are also shown. These data were reported earlier by our group.[14] The following should be noted regarding VchCA inhibition with the compounds investigated in this study:

(i) Topiramate TPM, a sulfamate, sulpiride SLP, a primary sulfonamide, and saccharin SAC, an acylsulfonamide, were ineffective VchCA inhibitors (K_I > 1000 nM), although these compounds (except saccharin) generally act as good inhibitors of other bacterial or mammalian α-CAs.[5,14] Zonisamide, ZNS, an aliphatic primary sulfonamide, was also a very weak inhibitor (K_I = 982 nM).

(ii) A large number of simple aromatic sulfonamides, such as derivatives **1**−**9**, showed moderate VchCA inhibitory properties with inhibition constants in the range 125−440 nM (Table 1). It may be observed that all these derivatives are benzenesulfonamides with one or two simple substituents in ortho-, para-, or 3,4-position of the aromatic ring with respect to the sulfamoylzinc-binding moiety. The most ineffective CAIs in this subgroup were sulfanilamide and orthanilamide and the *p*-aminomethyl- and *p*-aminoethylbenzenesulfonamides (com-

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Table 2. Inhibition of *Vibrio cholerae* CA (VchCA) and of the Human Isoforms hCA I and hCA II, as Well as *H. pylori* Enzyme (hpαCA) with Compounds 1−24 and the Clinically Used Sulfonamides/Sulfamates AAZ−HCT

	K_I[a] (nM)			
inhibitor	hCA I[b]	hCA II[b]	hpαCA[c]	VchCA
1	45400	295	426	440
2	25000	240	454	471
3	28000	300	316	125
4	78500	320	450	219
5	25000	170	873	447
6	21000	160	1150	402
7	8300	60	1230	199
8	9800	110	378	139
9	6500	40	452	133
10	6000	70	510	99.1
11	5800	63	412	62.9
12	8400	75	49	45.3
13	8600	60	323	23.5
14	9300	19	549	12.1
15	6	2	268	4.2
16	164	46	131	42.7
17	185	50	114	30.3
18	109	33	84	59.8
19	95	30	207	4.7
20	690	12	105	0.59
21	55	80	876	54.5
22	21000	125	1134	56.7
23	23000	133	1052	71.5
24	24000	125	541	52.1
AAZ	250	12	21	6.8
MZA	50	14	225	3.6
EZA	25	8	193	0.69
DCP	1200	38	378	37.1
DZA	50000	9	4360	6.3
BRZ	45000	3	210	2.5
BZA	15	9	315	4.2
TPM	250	10	172	>1000
ZNS	56	35	231	982
SLP	1200	40	204	>1000
IND	31	15	413	8.1
VLX	54000	43	nt	89.7
CLX	50000	21	nt	>1000
SLT	374	9	nt	88.4
SAC	18540	5959	nt	>1000
HCT	328	290	nt	79.5

[a]Errors in the range of 5−10% of the shown data, from three different assays; nt = not tested. [b]Human recombinant isozymes, stopped flow CO_2 hydrase assay method, from ref 14. [c]Recombinant enzyme, stopped flow CO_2 hydrase assay method, this work.[15]

pounds **2**, **1**, **5**, and **6**), with K_I of 402−471 nM. Replacement of the amino group from the para position from sulfanilamide **2** by a hydrazine or methyl moiety enhanced the inhibitory power (with respect to the lead **2**), with compounds **3** and **4** showing K_I of 125−219 nM. The same effect was observed when a halogen atom was present in position 3 of the sulfanilamide scaffold, as in derivatives **7**−**10**, which showed K_I in the range 99.1−199 nM. The inhibitory power increased with an increase of the atomic weight of the halogen in a quite linear manner. This was not observed for the inhibition of the *H. pylori* enzyme with the same type of derivatives (but a rather similar effect has been observed for hCAs I and II, although the inhibition range is totally different from that of the VchCA enzyme; see Table 2).

(iii) Most of the sulfonamides investigated here showed a potent inhibitory effect against VchCA, with inhibition constants in the range 23.5−99.1 nM. These derivatives include compounds **10**−**13**, **16**−**18**, **21**−**24**, DCP, VLX, SLT, and HCT. This is a heterogeneous group of compounds having in common only the presence of the primary sulfonamide group. The scaffold present in this group of inhibitors includes simple benzenesulfonamides substituted with one or two moieties (generally in para or in the 3,4-position, relative to the sulfamoyl moiety), such as 3-fluorosulfanilamide **10** or the hydroxyalkyl-, carboxy-, or hydrazine-substituted derivatives **21**−**24**. Compounds **11**, **12**, and DCP are 1,3-disulfamoylbenzene derivatives, whereas **16**−**18** incorporate an elongated sulfanylated sulfonamide or pyrimidinyl substituted benzenesulfonamide scaffold. The same is true for valdecoxib VLX and celecoxib CLX, although the scaffolds of these COX-2 inhibitors are slightly more complex compared to the other sulfonamides from this group. Finally, only two heterocyclic derivatives are in this group of CAIs, the deacetylated acetazolamide precursor **13** and hydrochlorothiazide HCT.

(iv) Several very potent VchCA inhibitors were detected, such as compounds **14**, **15**, **19**, **20**, AAZ, MZA, EZA, DZA, BRZ, BZA, and IND, which showed K_I values in the range 0.59−12.1 nM (Table 2). Again a large structural variability characterizes these very potent CAIs targeting the bacterial enzyme. However, all of them are heterocyclic sulfonamides incorporating either five-membered rings (1,3,4-thiadiazole, 1,3,4-thiadiazoline) or bicyclic ring systems (benzothiophene, thienothiopyran, thienothiazine, etc). Two subnanomolar inhibitors were detected, the orphan drug chlorazolamide **20** and ethoxzolamide EZA. These compounds show K_I values of 0.59−0.69 nM (Table 2). Only indisulam IND had K_I > 10 nM, all the other derivatives in this subgroup being extremely effective VchCA inhibitors. Many of these compounds have a higher affinity toward VchCA, although they appreciably inhibit other α-CAs, such as the *H. pylori* enzyme or the human isoforms hCAs I and II. Small structural changes in the scaffolds of these inhibitors lead to important differences of activity. For example, AAZ and MZA differ only by a methylene group but MZA is almost twice as effective compared to AAZ as a VchCA inhibitor. Compound **19** is the EZA precursor, lacking an ethyl moiety compared to the clinical drug, and the difference of activity between them is almost 6.8-fold. All these data show that the structure−activity relationship for this group of CAIs is rather complicated and poorly understood, but it is important to note that a large number of effective or very effective CAIs were detected.

(v) The inhibition profile of VchCA is different from that of the other bacterial or mammalian CAs investigated until now, proving that it will probably be possible to design VchCA-selective inhibitors using the scaffold of leads detected here.

CONCLUSIONS

A new α-CA has been cloned, purified, and characterized from the human pathogenic bacterium *Vibrio cholerae*, designed here VchCA. This new enzyme showed significant catalytic activity, being more active than the human isoform hCA I or the *H. pylori* α-class enzyme. An inhibition study with a panel of sulfonamides and one sulfamate led to the detection of a large number of low nanomolar VchCA inhibitors, including

methazolamide, acetazolamide, ethoxzolamide, dorzolamide, brinzolamide, benzolamide, and indisulam (with K_I in the range of 0.69−8.1 nM). As it was proven that bicarbonate is a virulence factor of this bacterium and since ethoxzolamide was shown to inhibit this virulence in vivo, we propose that VchCA may be a target for antibiotic development, exploiting a mechanism of action rarely considered up until now, i.e., interference with bicarbonate supply as a virulence factor.

EXPERIMENTAL SECTION

Identification of the α-CA Gene Encoding VchCA. The identification of *V. cholerae* CA (VchCA) was performed at the link http://www.ncbi.nlm.nih.gov/protein using "*Vibrio cholerae* and carbonic anhydrase" as keyword. The α-CA of *V. cholerae* was identified running the "BLAST" program.

Cloning and Protein Expression. The GeneArt Company, specializing in gene synthesis, designed the synthetic *V. cholerae* gene encoding for the α-CA, lacking the signal peptide (i.e., the first 20 amino acid residues of the peptide sequence) and containing a *NdeI* and *XhoI* site at the 5′ and 3′ ends of the VchCA gene, respectively. The resulting plasmid was amplified into *E. coli* DH5 α cells. The *V. cholerae* DNA fragments were separated on 1% agarose gel. The recovered *V. cholerae* gene and the linearized expression vector (pET15-b) were ligated by T4 DNA ligase to form the expression vector pET15-b/Vch. In order to confirm the integrity of the *V. cholerae* gene and the fact that no errors occurred at the ligation sites, the vector containing the fragment was sequenced. Competent *E. coli* BL21 (DE3) cells were transformed with pET15-b/Vch, grown at 37 °C, induced with 1 mM IPTG, and grown for 5 h.

Protein Preparation. After additional growth for 5 h, cells were harvested and disrupted by sonication at 4 °C. Following centrifugation, the cell extract was placed in conical flasks and ammonium sulfate gradually added to obtain saturation of 45%. After resting for 14 h at 4 °C, the sample was centrifuged at 1200*g* at 4 °C for 30 min. The precipitate was resuspended in 20 mM buffer phosphate, pH 8.0, and loaded onto a His-select HF nickel affinity gel. The protein was eluted with 250 mM imidazole. At this stage of purification the enzyme was at least 95% pure and the obtained recovery was 30 mg of the recombinant bacterial CA.

SDS−PAGE. Sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE) was performed as described previously using 12% gels.

Sequence and Phylogenetic Analysis. Multialignment of nucleotide sequences was performed using the programs PileUp (GCG Wisconsin)[23] and ClustalW, version 1.7. A most parsimonious tree was constructed with the program PhyML.[24]

CA Inhibition Assay. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[15] Phenol red (at 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm with 10 mM Hepes (pH 7.5) as buffer and 0.1 M $NaClO_4$ (for maintaining constant ionic strength), at 20 °C, following the CA-catalyzed CO_2 hydration reaction for a period of 10−100 s (the uncatalyzed reaction needs around 60−100 s in the assay conditions, whereas the catalyzed ones are of around 6−10 s). The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters. For each inhibitor tested in the concentration range between 0.01 nM to 100 μM, 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 (1 mM) were prepared in distilled−deionized water with 10−20% (v/v) DMSO (which is not inhibitory at these concentrations), and dilutions up to 0.01 nM were done thereafter with the assay buffer. 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 nonlinear least-squares methods using PRISM 3. The curve-fitting algorithm allowed us to obtain the IC_{50} values (working at the lowest concentration of substrate of 1.7 mM), from which K_I values were calculated by using the Cheng−Prusoff equation, as reported earlier for other CAs.[14] Enzyme concentrations in the assay system were 9.2 nM for hCA I, 7.6 nM for hCA II, 10.3 nM for hpCA, and 8.6 nM for VchCA.

Chemistry. Compounds **1**−**24** and AAZ−HCT used in the present work were either commercially available or reported earlier by this group.[20−22]

ASSOCIATED CONTENT

Supporting Information

Figure S1 of SDS−PAGE results, showing the purification of VchCA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CA, carbonic anhydrase; CAI, carbonic anhydrase inhibitor; hCA, human carbonic anhydrase; hpαCA, *Helicobacter pylori* α-carbonic anhydrase; VchCA, *Vibrio cholerae* carbonic anhydrase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis

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