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Cloning, Characterization, and Inhibition Studies of a β-Carbonic Anhydrase from *Brucella suis*

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A β-carbonic anhydrase (CA, EC 4.2.1.1) from the bacterial pathogen *Brucella suis*, bsCA 1, has been cloned, purified, and characterized kinetically. bsCA 1 has appreciable activity as catalyst for the hydration of CO_2 to bicarbonate, with a k_{cat} of 6.4×10^5 s^{-1} and k_{cat}/K_m of 3.9×10^7 $M^{-1} \cdot s^{-1}$. A panel of 38 sulfonamides and one sulfamate have been investigated for inhibition of this new β-CA. All types of activities have been detected, with K_Is in the range of 17 nM to 5.87 μM. The best bsCA 1 inhibitors were ethoxzolamide (17 nM), celecoxib (18 nM), dorzolamide (21 nM), valdecoxib, and sulpiride (19 nM). Whether bsCA 1 inhibitors may have application in the fight against brucellosis, an endemic disease and the major bacterial zoonosis, producing debilitating infection in humans and animals, warrants further studies.

Introduction

Infections caused by bacteria represent one of the main causes of mortality and morbidity worldwide.[1] Antibiotics are successfully used in fighting bacterial diseases, but the emergence of new pathogens, the reemergence of bacteria whose incidence had previously declined (such as *Mycobacterium tuberculosis* for example),[2] the antibiotic resistance of many common bacterial strains to several classes of antibiotics, as well as the potential of using bacteria as bioterrorism agents, led to considerable hurdles in fighting bacterial infections in recent years.[1−3] Such facts led to a renewed interest in the discovery of antibacterials able to act on novel molecular targets, circumventing the drug resistance problems.[2]

Brucella spp. are facultative intracellular pathogens responsible of the most widespread zoonosis worldwide, known as brucellosis or Malta fever.[3−5] *Brucellae* are small Gram-negative α-proteobacteria, with several species infecting various vertebrates, from fish to primates, among which *Brucella melitensis* is the least host specific and also the most infectious for humans.[3−5] The other highly infectious species are *Brucella suis* (the primary host being the pig) and *Brucella abortus* (cattle), but more recently human cases of infection with the whale pathogen, *Brucella cetaceae*, have also been reported.[5] Why such a re-emerging disease is so difficult to fight, is due to the fact that these pathogenic bacteria have developed strategies to hide from immune recognition.[3−5]

This bacterium is able to cause enormous losses in agriculture and is endemic in several areas such as the Mediterranean Europe, Middle East, and Latin America. The incidence of human brucellosis may be as high as 200 per 100 000 inhabitants.[6] Human brucellosis is a highly disabling disease which may last for weeks, resulting in chronic forms especially when not treated.[6] *Brucella* is extremely infectious by aerosol (10 bacteria are sufficient to contract disease) and is considered as a potential bioterrorism agent,[6] especially as strains resistant to antibiotics used in the treatment of human brucellosis may be easily obtained and no human vaccine is available.[7] Furthermore, it is worth pointing out the emergence of resistant strains in clinical isolates recently reported in Turkey and Balkanic countries.[7]

The growing information resulting from bacterial genomics[2,8−10] led to new targets for the design of mechanism-based drugs.[10] The genome sequences of several *Brucella sp.* have been reported to date.[8] Among the many proteins encoded in these genomes are also the carbonic anhydrases (CAs,[a] EC 4.2.1.1),[11] zinc enzymes present in many pathogens such as the protozoa *Plasmodium falciparum*,[12] the bacterium *Helicobacter pylori*,[13] fungi such as *Candida albicans*, and *Cryptococcus neoformans*,[14] and the widespread bacterial pathogen *Mycobacterium tuberculosis*.[15] Many of the α- or β-class enzymes present in these pathogens started to be investigated as new drug targets in the search of novel agents devoid of drug resistance problems.[11−15] By searching for metalloenzyme-specific sequence motifs within the *Brucella suis* genome,[8] two CA-encoding genes belonging to the β-class CA family (BR1829 and BRA0788) were identified. Considering these β-CAs as possible new targets, we report here the cloning, characterization, and inhibition studies with a panel of sulfonamides/sulfamates of the first β-CA from *B. suis*, denominated here bsCA 1.

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[a] Abbreviations: CA, carbonic anhydrase; bsCA, *Brucella suis* CA; hCA, human CA; CAI, CA inhibitor; SDS, sodium dodecyl sufate; PAGE, polyacrylamide gel electrophoresis; SAR, structure−activity relationship.

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Results

bsCA 1 Cloning, Purification, and Catalytic Activity. bsCA 1 was cloned and purified as His-tagged protein as reported for similar β-CAs from fungal or bacterial pathogens (see Experimental Protocols for details).[13−15] The protein is a homodimer constituted of two 25 kDa monomers (Figure 1), and its amino acid sequence and phylogenetic analysis shown in Figures 2 and 3, demonstrate it to be related to the β-CAs from *Escherichia coli*, *Haemophilus influenzae*, and *H. pylori*, some of which have been investigated in detail by this and other groups.[13−18]

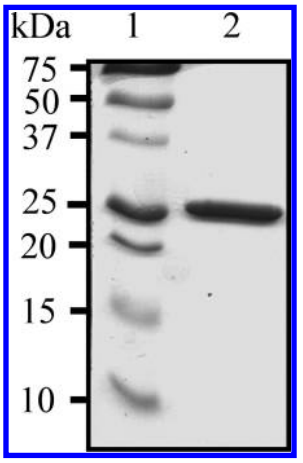


Figure 1. SDS-PAGE of the purified bsCA 1 (lane 2) compared to ladder (Biorad) (lane 1) stained with Coomassie blue, under denaturing conditions, when the monomeric 25 kDa β-CA is observed.

The catalytic activity of bsCA 1 for the physiologic reaction, i.e., CO_2 hydration to bicarbonate and protons,[11,19] is shown in Table 1, where the catalytic activity of α-CAs of human origin as well as that of β-CAs from several bacterial pathogens are also shown for comparison.

bsCA 1 Inhibition with Sulfonamides and Sulfamates. Table 2 shows bsCA 1 inhibition data with a panel of 38 sulfonamides and one sulfamate (obtained for the CO_2 hydration reaction catalyzed by CAs),[19] some of which are clinically used drugs,[11] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, zonisamide **ZNS**, sulpiride **SLP**, indisulam **IND**, celecoxib **CLX**, and valdecoxib **VLX** (Chart 1). The simpler derivatives **1**−**26** (Chart 2) were also included in the study as they represent the most extensively used scaffolds for designing potent or isoform-selective CAIs targeting human CAs (hCAs).[11] Data for the inhibition of the dominant isoforms hCA I and II[11] as well as those of another

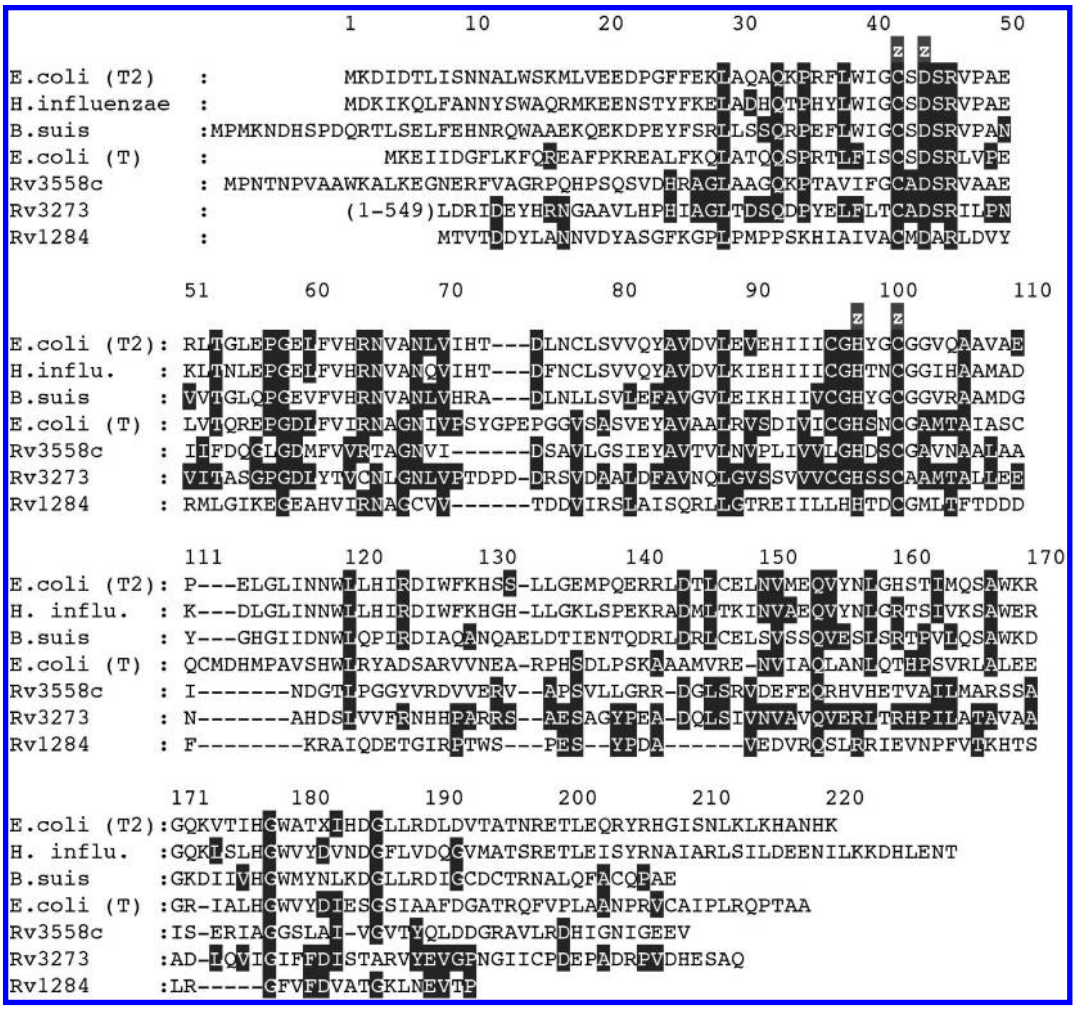


Figure 2. Amino acid sequence of bsCA 1 aligned with that of other bacterial β-CAs, including the two enzymes from *E. coli* (T and T2),[17] *H. influenzae* (P45148),[16] and the three β-CAs from *M. tuberculosis*, Rv3588c, Rv3273, and Rv1284.[15,20] Conserved amino acid residues in these β-CAs are indicated by black boxes. The four zinc-binding residues, [42]Cys, [44]Asp, [98]His, and [101]Cys, are indicated by the "z" sign (residue numbering is based on the *E. coli* CynT2 numbering system).[17]

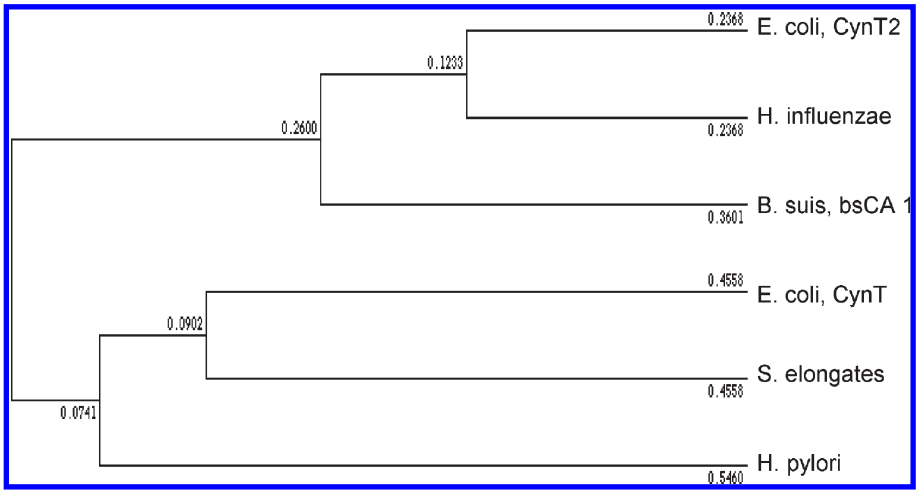


Figure 3. An evolutionary tree analysis subdivides six bacterial β-CAs into two groups, bsCA 1 clusters with the *H. influenzae* and *E. coli* cynT2 enzymes. Bootstrap consensus tree of 1000 replicates.

Table 1. Kinetic Parameters for CO_2 Hydration Reaction Catalyzed by Some Human α-CA Isozymes at 20 °C and pH 7.5, and β-CA Enzymes from *Brucella suis* (bsCA 1), *Helicobacter pylori* (hpβCA), and *Mycobacterium tuberculosis* (Rv1284 and Rv3273) at 20 °C, pH 8.3, in 20 mM TRIS·HCl Buffer and 20 mM NaCl, and Their Inhibition Data with Acetazolamide **AAZ** (5-Acetamido-1,3,4-thiadiazole-2-sulfonamide), a Clinically Used Drug

enzyme	class	activity level	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	K_I (acetazolamide) (nM)
hCA I[a]	α	medium	2.0×10^5	5.0×10^7	250
hCA II[a]	α	high	1.4×10^6	1.5×10^8	12
hCA VA[a]	α	low	2.9×10^5	2.9×10^7	63
hCA XII[a]	α	low	4.2×10^5	3.5×10^7	5.7
hCA XIV[a]	α	medium	3.1×10^5	3.9×10^7	41
hpβCA[b]	β	medium	7.1×10^5	4.8×10^7	40
Rv1284[b]	β	medium	3.9×10^5	3.7×10^7	480
Rv3273[b]	β	medium	4.3×10^5	4.0×10^7	104
bsCA 1[c]	β	medium	6.4×10^5	3.9×10^7	63

[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method (pH 7.5), from ref 11,13c. [b] Recombinant enzymes, stopped flow CO_2 hydrase assay method (pH 8.3), from ref 13c,15. [c] Recombinant bsCA 1, stopped flow CO_2 hydrase assay method (pH 8.3), this work.

bacterial β-class enzyme, *H. pylori* hpβCA reported earlier[13c] with these compounds, are also included in Table 2, for comparison.

Discussion

bsCA 1 Cloning, Purification, and Catalytic Activity. bsCA 1 was cloned as hexa-histidyl-tagged fusion protein from the BRA0788 gene (accession number NC_004311) identified as a putative β-CA,[8b] and presents an identical sequence to the clones previously deposited in GenBank (accession no. NP_699962). This gene encodes a protein of 219 amino acid residues, which belongs to the β-CA class, and has a molecular weight (as a monomer) of 25 kDa (Figure 1). bsCA 1 has 51.0% homology with the *E. coli* Cyn T2 enzyme, 45.6% homology with the *H. influenzae* enzyme, and 27.1% homology with the *E. coli* Cyn T β-CA.

Aligment of the amino acid sequence of bsCA 1 with that of other bacterial β-CAs recently investigated by this and other groups (Figure 2),[13−20] such as the *E. coli* T2 and T enzymes,[16] the CA from *H. influenzae*,[17] as well as the mycobacterial enzymes encoded by the genes Rv3558c, Rv1284, and Rv3273, evidenced that bsCA 1 possesses all the amino acids residues typical of β-CAs and involved in the catalytic cycle of this class of enzymes: i.e., the Zn(II) binding residues Cys52, Asp54, His108, and Cys111, as well as the Asp54-Arg56 dyad involved in the opening/closing of the active site.[20] Indeed, in the case of the β-CAs there are two types of metal ion coordination within the enzyme active site:

(i) The open-active site, with the Zn(II) ion coordinated by 2 Cys and one His residues, and the fourth zinc ligand being a water molecule/hydroxide ion, which is responsible for the catalysis.[13−20]

(ii) Closed-active site enzymes, with the Zn(II) ion coordinated by 2 Cys, one His and one Asp residues, in the tetrahedral geometry typical of Zn(II) in metalloenzymes.[13−20] For these enzymes, no water coordinated to the metal ion is present at pH values <8, as shown in an excellent crystallographic work from Jones' group on the mycobacterial enzymes Rv3558c and Rv1284.[20] However, at pH values >8, a conserved Arg residue in all β-CAs investigated so far (belonging to the catalytic dyad mentioned above)[18] makes a salt bridge with the Asp coordinated to Zn(II), liberating the fourth Zn(II) coordination position, which may be occupied by an incoming water molecule/hydroxide ion.[20] Thus, the catalytic activity of the β-CAs possessing the closed active site can be measured only at pH values >8 (and this is the reason why we measure the catalytic/inhibitory activity of enzymes from this class at pH values of 8.3, see Experimental Protocols for details). On the basis of the amino acid sequence, it is impossible to predict whether a β-CA will have a close or open active site. For example, the enzyme from the fungal pathogen *Cryptococcus neoformans*, Can2, was recently crystallized by one of our groups in collaboration with Steegborn's group.[14b] It has been shown that Can2

Table 2. Human (h) hCA I, II, and Bacterial Enzyme (hpβCA and bsCA I) Inhibition Data with Compounds **1**−**26** and the Clinically Used Derivatives **AAZ**−**VLX**

	K_I[a](nM)			
inhibitor	hCA I[b]	hCA II[b]	hpβCA[c]	bsCA I[d]
1	45400	295	16400	5870
2	25000	240	1845	2500
3	28000	300	8650	2400
4	78500	320	2470	1580
5	25000	170	2360	768
6	21000	160	3500	880
7	8300	60	1359	1070
8	9800	110	1463	800
9	9650	73	nt[e]	243
10	14000	124	nt	345
11	5800	63	973	4830
12	8400	75	640	940
13	8600	60	2590	1210
14	9300	19	768	1430
15	6	2	64	70
16	1.4	0.3	nt	186
17	40	5	nt	27
18	164	46	187	1050
19	185	50	71	745
20	109	33	38	21
21	95	30	39	48
22	690	12	37	33
23	55	80	236	754
24	21000	125	218	865
25	23000	133	450	340
26	24000	125	15250	1035
AAZ	250	12	40	63
MZA	50	14	176	54
EZA	25	8	33	17
DCP	1200	38	105	58
DZA	50000	9	73	21
BRZ	45000	3	128	26
BZA	15	9	54	75
TPM	250	10	32	57
ZNS	56	35	254	1850
SLP	1200	40	35	19
IND	31	15	143	50
CLX	50000	21	nt	18
VLX	54000	43	nt	19

[a] Errors in the range of 5−10% of the shown data, from three different assays. [b] Human recombinant isozymes, stopped flow CO_2 hydrase assay method, from ref 11,13c. [c] Recombinant hpCA, stopped flow CO_2 hydrase assay method, from ref 13c. [d] Recombinant bsCA 1, stopped flow CO_2 hydrase assay method, this work. [e] nt = not tested.

possesses an open active site, and the enzyme is an effective catalyst for the CO_2 hydration reaction.[14b] Thus, on the basis of these facts, we cannot establish what type of active site bsCA 1 possesses, i.e., whether Asp 54 is coordinated to the metal ion at pH < 8 (closed active site), or whether it is interacting permanently with Arg56 (in the Asp-Arg dyad mentioned above), which is critical for orientating the substrate and generating the nucleophilic species of the enzyme crucial for catalysis.

A brief phylogenetic analysis of bsCA 1 and other bacterial β-CAs (Figure 3) showed this new enzyme to be more closely related with the CynT2 and *H. influenzae* enzymes investigated earlier by Cronk's group,[16,17] both of which possess a closed active site, i.e., four protein ligands (2 Cys, 1 His, and 1 Asp residue). However, as shown by Cronk et al.,[16,17] Covarrubias et al.,[20] and by us,[13,15] this type of enzyme shows CO_2 hydrase activity at pH values over 8. Thus, it is rather probable that bsCA 1 may have a closed active site at pH < 8, but the full evidence may be obtained only by resolving the 3D structure of bsCA 1 by means of X-ray crystallography.

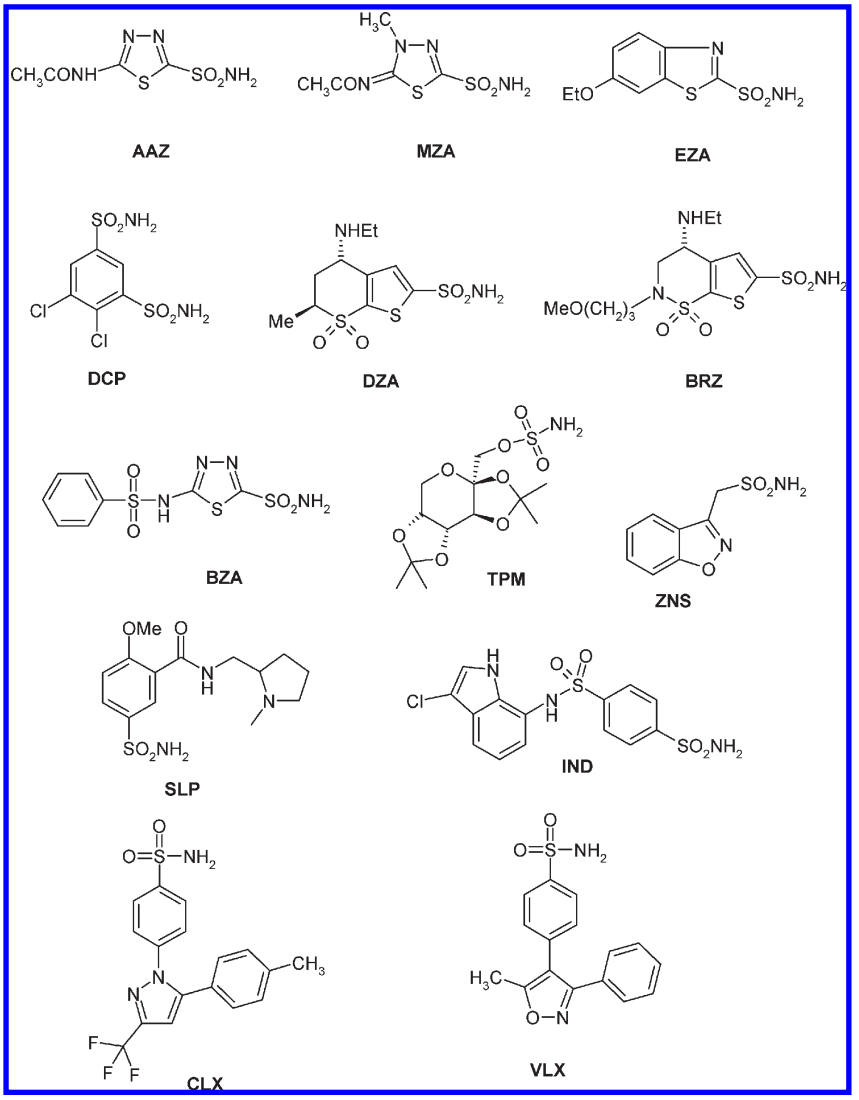
As shown in Table 1, we have measured the activity of bsCA 1 and compared it to that of other α-class enzymes of human origin, known to be drug targets, such as hCA I, II, VA, XII, and XIV as well as the β-CAs from bacterial pathogens, such as *H. pylori* and *M. tuberculosis* (2 isoforms, Rv1284 and Rv3273, were included in this comparison). These data show that similarly to the bacterial enzymes from *H. pylori* and *M. tuberculosis* investigated earlier,[13,15] bsCA 1 has a significant activity as catalyst for the conversion of CO_2 to bicarbonate (at pH 8.3), with a k_{cat} of 6.4×10^5 s^{-1} and k_{cat}/K_m of 3.9×10^7 $M^{-1} \cdot s^{-1}$. Thus, bsCA 1 was a better catalyst for the physiological reaction than the human isoforms hCA VA and hCA XII (know to be antiobesity[21] and anticancer[11,22] drug targets, respectively) and showed similar levels of activity with the bacterial β-class enzymes from *Helicobacter* and *Mycobacterium*, investigated earlier.[13,15] It can be also observed that all these enzymes are appreciably inhibited by the clinically used sulfonamide compound, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide (see Discussion later in the text).

bsCA 1 Inhibition with Sulfonamides and Sulfamates. Table 2 shows bsCA 1 inhibition data with a panel of 38 sulfonamides and one sulfamate (obtained for the CO_2 hydration reaction catalyzed by CAs),[19] some of which are clinically used drugs[11] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, zonisamide **ZNS**, sulpiride **SLP**, indisulam **IND**, celecoxib **CLX**, and valdecoxib **VLX**. The simpler derivatives **1**−**26** were also included in the study, as they represent the most extensively used scaffolds for designing potent or isoform-selective CAIs.[23,24] Data for the inhibition of the dominant isoforms hCA I and II[9] as well as those of the related enzyme from *H. pylori*, hpβCA, investigated earlier for its interaction with sulfonamides,[13c] are also included in Table 2, for comparison reasons. The following structure−activity relationship (SAR) can be observed from data of Table 2:

(i) A first group of sulfonamides, including **1**−**4**, **7**, **11**, **13**, **14**, **18**, and **ZNS**, showed modest, micromolar inhibitory activity against bsCA 1, with inhibition constants in the range of 1035−5870 nM. It may be observed that they include simple benzenesulfonamides substituted in ortho- or para- with compact groups such as amino, hydrazino, methyl (**1**−**4**, **26**), as well as derivatives possessing more than one substituent in various positions of the phenyl ring (e.g., **7** and **11**). The sulfanilyl-substituted homosulfanilamide **18** also belongs to this subgroup, proving that only the length of the inhibitor molecule is not a decisive factor influencing strong CA inhibitory activity. Furthermore, the heterocyclic sulfonamides **13**, **14**, and **ZNS** belong to the same subgroup, although they do not share any structural similarity with compounds mentioned above, except for the sulfamoyl group, present in all the investigated derivatives, as it is one of the best zinc-binding groups for the design of CA inhibitors (CAIs).[25]

(ii) Compounds such as **5**, **6**, **8**−**10**, **12**, **16**, **19**, and **23**−**25**, showed more effective bsCA 1 inhibitory activities,

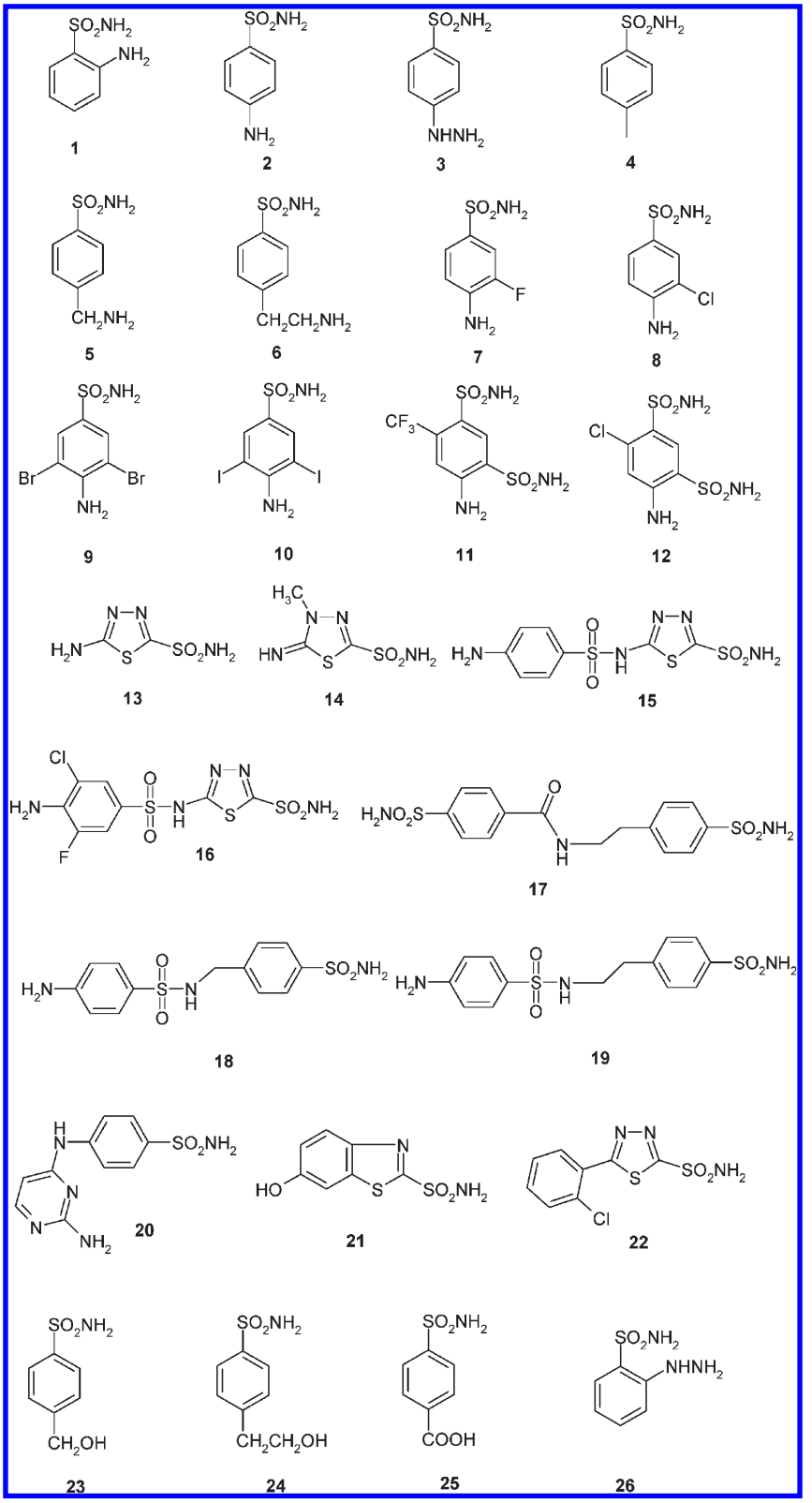
Chart 1. Structures **AAZ**−**VLX**



with K_Is in the range of 186−940 nM (Table 2). Again, these sulfonamides belong to rather heterogeneous chemotypes, with mono-para-substituted benzenesulfonamides incorporating aminoalkyl, hydroxyalkyl, or carboxyl moieties (**5**, **6**, and **23**−**25**), di-, and tri-, tetra-substituted benzenesulfonamides incorporating amino, halogeno, and another sulfamoyl group (**8**−**10** and **12**), as well as the heterocyclic, aminobenzolamide derivative **16** (the best CAI against bsCA 1 in this subgroup, with a K_I of 186 nM). Similarly to **18** discussed above, compound **19**, possessing an extra CH_2 moiety is a medium potency inhibitor (K_I of 745 nM) being slightly more effective compared to its congener **18** (K_I of 1050 nM).

(iii) The best bsCA 1 inhibitors reported here include derivatives **15**, **17**, **20**−**22**, and all the clinically used compounds except **ZNS**, which as discussed above, was a weak, micromolar inhibitor. These compounds showed inhibition constants in the range of 17−75 nM (Table 1). It may be observed that SAR is again rather complicated. Thus, **17** and **20** as well as **DCP**, **SLP**, **IND**, **CLX**, and **VLX**, are benzenesulfonamide derivatives possessing a rather bulky para-substituent, of the sulfamoyl-phenyl-carboxamidoethyl type for **17**, pyrimidinylamino for **20**, and the more complicated scaffolds incorporating the substituted-pyrazole/isoxazole from celecoxib and valdecoxib. Presumably these more complex scaffolds (compared to compounds discussed earlier) lead to more favorable contacts between the enzyme active site and the inhibitor, which has as a consequence the stabilization of the enzyme−inhibitor complex. **DCP** is a 1,3-benzenesulfonamide incorporating also two chlorine atoms as substituents of the benzene ring. It may be observed that **DCP** is a 83 times better bsCA 1 inhibitor compared to **11**, another benzene-1,3-disulfonamide derivative, possessing a not so different substitution pattern compared to **DCP**. The same may be said by comparing the **12** and **DCP**, with the last compound being 16 times a better bsCA 1 inhibitor compared to **12** (which is also a substituted benzene-1,3-disulfonamide). All these data show that minor modifications in the inhibitor scaffold have profound consequences for the interaction with the enzyme active site and thus the potency of the inhibitor. **SLP** has two substituents at the benzene ring, a compact one (MeO) in para and a bulkier one in meta. Indisulam, on the other hand, is a bis-sulfonamide

Chart 2. Structures **1**−**26**



which has a secondary sulfamoyl group in para to the SO_2NH_2 one, which also incorporates the bulky chloroaminoindole moiety.All other derivatives from this subgroup, except topiramate **TPM**, are heterocyclic sulfonamides incorporating 1,3,4-thiadiazole, 1,3,4-thiadiazoline, benzothiazole, thienothiopyran, or thienothiazine ring systems on which the SO_2NH_2 moiety and diverse other substituents are grafted. Again, this substitution pattern is critical for the bsCA 1 inhibitory activity of such derivatives. For example, **13**, the deacetylated precursor of acetazolamide, is a weak bsCA 1 inhibitor (K_I of 1210 nM). Its acetylation as in AAZ leads to a 19.2 times more effective inhibitor, whereas the benzenesulfonylated compound **BZA** is

16.1 times a better inhibitor (Table 2). Excellent inhibitory activity was observed for the bicyclic, fused-ring heterocyclic sulfonamides such as **21**, **EZA, DZA**, and **BRZ**, which had K_Is < 50 nM. The best bsCA 1 inhibitors were ethoxzolamide (17 nM), celecoxib (18 nM), valdecoxib, and sulpiride (19 nM both), as well as compound **20** and **DZA** (21 nM). Thus, a rather large number of sulfonamides (and one sulfamate, **TPM**) showed very effective bsCA 1 inhibitory activities, with K_Is in the range of 17−60 nM (Table 2).

(iv) bsCA 1 has an inhibition profile with sulfonamides quite distinct from that of the human α-class enzymes hCA I and II or the bacterial β-class enzyme from *H. pylori* hpβCA investigated earlier[11,13] (Table 2). Thus, all the investigated compounds inhibit all these CAs but with very diverse profiles. No bsCA 1-selective inhibitor has been detected so far in this study.

Conclusions

The β-carbonic anhydrase (CA, EC 4.2.1.1) from the bacterial pathogen *Brucella suis*, bsCA 1, has been cloned, purified, and characterized kinetically. bsCA 1 has appreciable activity as catalyst for the hydration of CO_2 to bicarbonate, with a k_{cat} of 6.4×10^5 s^{-1}, and k_{cat}/K_m of 3.9×10^7 $M^{-1} \cdot s^{-1}$. A panel of sulfonamides and one sulfamate have been investigated for inhibition of this new β-CA. All types of activities have been detected, with K_Is in the range of 17 nM to 5.87 μM. The best bsCA 1 inhibitors were ethoxzolamide (17 nM), celecoxib (18 nM), valdecoxib, and sulpiride (19 nM both), as well as dorzolamide (21 nM). Whether bsCA 1 inhibitors may have application in the fight against brucellosis, an endemic disease producing invalidating infection in humans and animals, warrants further studies.

Experimental Protocols

Chemistry. Compounds **1**−**26** and **AAZ**−**VLX** are either commercially available (Sigma-Aldrich) or were prepared as described earlier.[23,24]

Cloning, Protein Expression, and Purification of bsCA 1. The CA-encoding gene *BRA0788* (accession number NC_004311), described here as bsCA 1, was specifically amplified by PCR using *B. suis* 1330 chromosomal DNA as template and OPJ17-foward primer (5′-GCGGGCATATGCCCATGAAGAAC-GATC-3′) and OPJ18-reverse primer (5-GCGCGGGATCCT-TATTCTGCCGGTTGGCAGG-3′), which contain *Bam*HI and *Nde*I recognition sequences (underlined), respectively. The PCR products were digested with *Bam*HI and *Nde*I and ligated to *Bam*HI-and-*Nde*I-digested pET15b (Novagen) prior to introduction into *E. coli* strain DH5α. The integrity of the cloned gene was verified by sequencing, using primers OPJ17 and OPJ18 described above. The construct pET15bCA was then transformed into *E. coli* strain BL21(DE3) for production of the 6x(His)-CA fusion protein. *E. coli* BL21(DE3) harboring pET15bCA was grown at 37 °C in two liters of Luria−Bertani medium supplemented with 50 μg/mL ampicillin. When the culture reached an optical density at 600 nm (OD_{600}) of approximately 0.6, expression of 6x(His)-CA protein was induced by the addition of isopropyl-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM and growth was continued for 5 h. Cells were then harvested by centrifugation at 3500 rpm at 4 °C for 20 min and broken by sonication in buffer A (200 mM KCl, 50 mM Tris-Cl (pH 7.5), 10% glycerol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A) supplemented with 0.5 mM dithiothreitol and 0.2 mM disodium EDTA. All subsequent steps were performed at 4 °C. After centrifugation (13000 rpm, 20 min), the soluble extract was treated with streptomycin sulfate to remove ribosomes and nucleic acids. The suspension was then centrifuged at 13000 rpm for 15 min, and the supernatants were dialyzed against 2 L of sonication buffer A for 1 h. The dialyzed lysates were mixed with Talon Co^{2+}-affinity resin (Clontech) that has been equilibrated with buffer I (20 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, and 0.1% Nonidet P-40) supplemented with 150 mM KCl. The resin and bound His-tagged protein were collected by centrifugation and washed with buffer I containing 500 mM KCl and 10 mM imidazole. A subsequent wash was performed with buffer I supplemented with 125 mM KCl and 75 mM imidazole without Nonidet P-40. The 6x(His)-CA protein was eluted with buffer I containing 125 mM KCl and 150 mM imidazole without Nonidet P-40. Elution fractions were free of detectable contaminating proteins as determined by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels. The fractions containing the His-tagged proteins (estimated purity, >95%) were pooled and dialyzed prior to lyophilization. The purified bsCA 1 was dialyzed sequentially against the following buffer, buffer B (125 mM KCl, Tris-HCl (pH 7.5), imidazole 100 mM), buffer C (50 mM KCl, Tris-HCl (pH 7.5), imidazole 50 mM), and buffer D (10 mM Tris-HCl (pH 8.3)). The His_6 tag has been removed in a small sample of the protein by treatment with enterokinase (Sigma-Aldrich). However, the catalytic activity and acetazolamid inhibition of the His-tagged and untagged enzymes were identical (data not shown) so that the data of Tables 1 and 2 refer to the enzyme with the (6x)His-tag.

CA Catalytic Activity and Inhibition. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[19] 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 10−20 mM NaCl, 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 nonlinear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver−Burk plots, as reported earlier,[15] and represent the mean from at least three different determinations.

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