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A new β-carbonic anhydrase from *Brucella suis*, its cloning, characterization, and inhibition with sulfonamides and sulfamates, leading to impaired pathogen growth

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ABSTRACT

A β-carbonic anhydrase (CA, EC 4.2.1.1) from the bacterial pathogen *Brucella suis*, bsCA II, has been cloned, purified, and characterized kinetically. bsCA II showed high catalytic activity for the hydration of CO_2 to bicarbonate, with a k_{cat} of 1.1×10^6, and k_{cat}/K_m of 8.9×10^7 M^{-1} s^{-1}. A panel of sulfonamides and sulfamates have been investigated for inhibition of this enzyme. All types of activities, from the low nanomolar to the micromolar, have been detected for these derivatives, which showed inhibition constants in the range of 7.3 nM–8.56 μM. The best bsCA II inhibitors were some glycosylated sulfanilamides, aliphatic sulfamates, and halogenated sulfanilamides, with inhibition constants of 7.3–87 nM. Some of these dual inhibitors of bsCA I and II, also inhibited bacterial growth in vitro, in liquid cultures. These promising data on live bacteria allow us to propose bacterial β-CA inhibition as an approach for obtaining anti-infective agents with a new mechanism of action compared to classical antibiotics.

1. Introduction

Bacterial infections constitute an alarming health problem worldwide. The widespread emergence of resistance and multi-resistance to antibiotics among bacterial pathogens represents a major threat and necessitates a permanent race for new molecules. The challenge at present is to identify and to validate novel pharmaceutical targets in bacteria, starting point for the discovery of new classes of antibacterial agents that could circumvent the established mechanisms of resistance.[1]

Among the many antibacterial drug targets available so far from bacterial genomics studies, metalloenzymes are highly attractive as they provide an excellent opportunity for mechanism-based drug discovery of novel classes of antibiotics.[2] In this framework, carbonic anhydrases (CAs, EC 4.2.1.1) have recently emerged as promising anti-infective targets.[3,4] Indeed, several bacterial β-CA class representatives have been cloned and characterized in some pathogens such as, among others, *Helicobacter pylori* and *Mycobacterium tuberculosis*.[5,6] Inhibition or genetic silencing studies of the β-CA from *H. pylori* proved it to be critical for the growth and virulence of this pathogen.[5,7] Thus, inhibition of β-CAs[8] can be considered as a new possible approach for designing antibacterial agents possessing a different mechanism of action than classical pharmacological agents in clinical use for a long period, for which many pathogenic bacteria developed various degrees of resistance.[3,4]

By searching for metalloenzyme-specific sequence motifs within the *Brucella suis* genome[9] (the pathogen responsible of brucellosis, the most widespread bacterial zoonosis worldwide),[10] we identified two genes encoding carbonic anhydrases belonging to the β-CA superfamily[8] (BR1829 and BRA0788). Indeed, these enzymes are widespread in organisms all over the phylogenetic tree, with five different families encoding them, the α-, β-, γ-, δ-, and ζ-CAs.[2–4] All of them are metalloenzymes, but whereas α-, β-, and δ-CAs use Zn(II) ions at the active site,[2–4] the γ-CAs are probably Fe(II) enzymes (but they are active also with bound Zn(II) or Co(II) ions), whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis.[2–4] The 3D folds of the five enzyme classes are very different from each other, as is their

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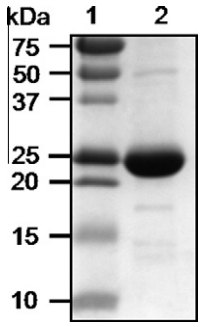

Figure 1. SDS–PAGE of the purified bsCA II—lane 2 compared to ladder (Biorad) lane 1—stained with Coomassie blue, under denaturing conditions, when the monomeric 25 kDa β-CA is observed.

oligomerization state: α-CAs are normally monomers and rarely dimers; β-CAs are dimers, tetramers or octamers; γ-CAs are trimers, whereas the δ- and ζ-CAs are probably monomers but in the case of the last family, three slightly different active sites are present on the same protein backbone which is in fact a pseudotrimer.[2–8] Many representatives of all these enzyme classes have been crystallized and characterized in detail, with the exception of the δ-CAs.[2–8]

Recent contributions from this group,[11] presented the cloning, kinetic characterization and inhibition studies of the first *B. suis* carbonic anhydrase, termed bsCA I. We showed that bsCA I (which is encoded by the gene BRA0788 of *B. suis*) has moderate catalytic activity for CO_2 hydration (k_{cat}/K_m of 3.9 10^7 M^{-1} s^{-1}), comparable to other α- or β-CAs which are well-established drug targets.[4] Furthermore, some clinically used sulfonamides/sulfamates investigated by us as CA inhibitors (CAIs) targeting mammalian α-CAs, showed promising inhibitory activity against bsCA I.[11]

We report here the characterization and the study of the second *B. suis* CA, denominated here bsCA II (encoded by the gene BR1829), its kinetic characterization, as well as an in vitro inhibition study with sulfonamides and sulfamates. We also investigated the influence of inhibition of both bsCAs on the growth of this pathogen, demonstrating that in liquid cultures, inhibition of the two enzymes leads to an impaired growth of the bacteria.

2. Results and discussion

2.1. bsCA II cloning, purification, and catalytic activity

bsCA II was cloned and purified as a His-tagged protein expressed from gene *BR1829* (GenBank accession number NP699962) as previously reported for bsCA I from the same pathogen (see Section 4 for details).[11] The gene encodes a protein of 213 amino acids, yielding a molecular mass of 25 kDa (Fig. 1). The alignment of the amino acid sequences of bsCA II with those of bsCA I and other bacterial β-CAs, recently investigated by us and others,[5,6] evidenced that bsCA II possesses all the conserved amino acid residues typical of β-CAs involved in the catalytic cycle (indicated by a red arrow on Fig. 2), that is, the four zinc-binding residues, Cys42, Asp44, His98, and Cys101 and the catalytic dyad Asp44–Arg46.[8] Indeed, unlike α-, γ-, and δ-CAs, for which the Zn(II) is coordinated by three His-residues and a water molecule/hydroxide ion, in the case of the β-class CAs the metal ion coordination is more complicated, being generally achieved by one His- and two Cys-residues.[4,5,11,12] However, some β-class enzymes have four

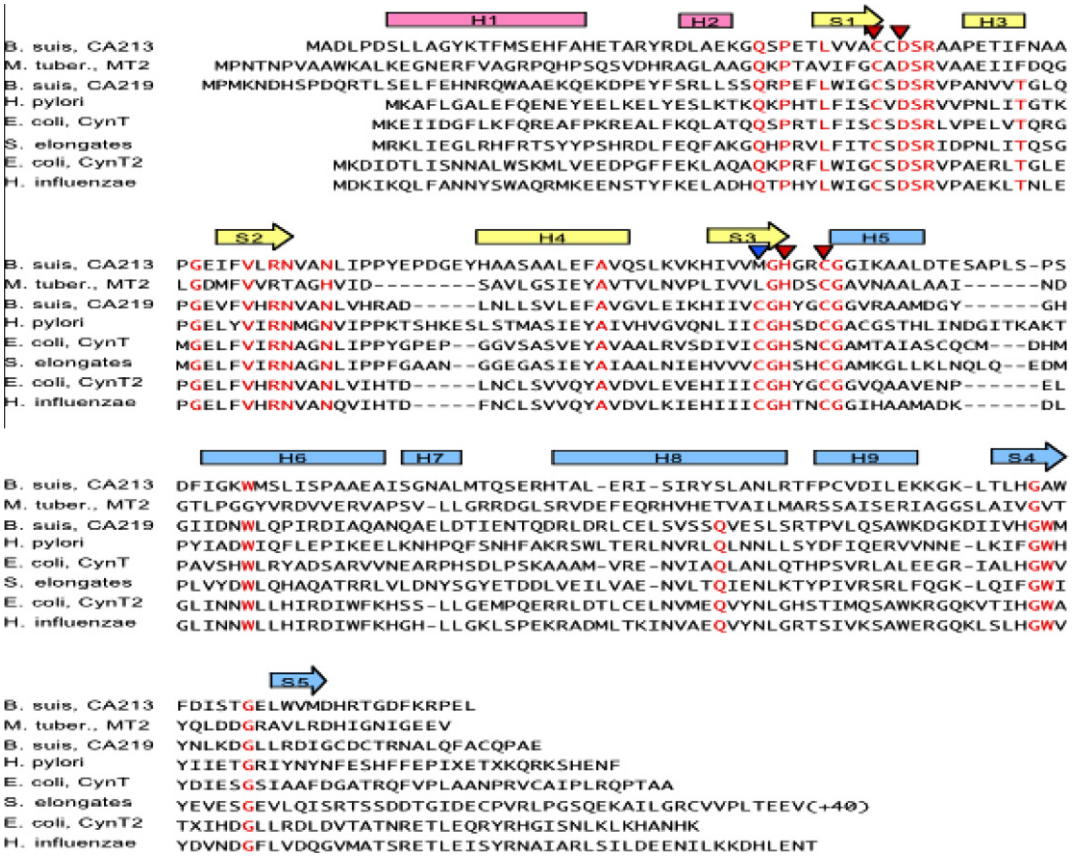

Figure 2. Consensus amino acid sequence of bsCA I (*B. suis* 219) and bsCA II (*B. suis* 213) aligned with that of other bacterial β-CAs including the two enzymes from *E. coli* (T and T2), *H. influenza*, one of the three β-CAs from *M. tuberculosis*, and the β-CAs from *H. pylori* and *S. elongates*.

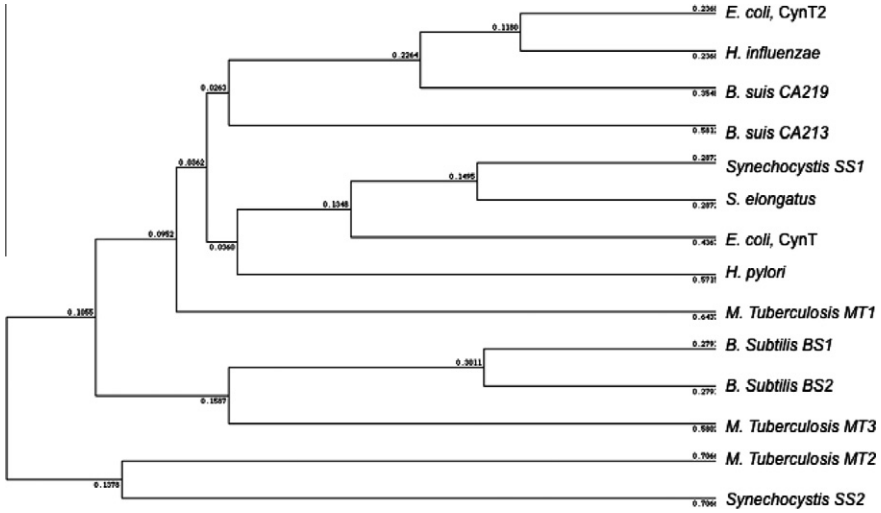

Figure 3. Evolutionary tree of some bacterial β-CAs among which the two *Brucella suis* enzymes bsCA I (*B. suis* 219) and bsCA II (*B. suis* 213).

protein zinc ligands, that is, one His, two Cys, and one Asp coordinated to Zn(II).[12] For these enzymes, no water coordinated to the metal ion is present at pH values <8, as shown in the crystallographic work from Jones' group on the mycobacterial enzymes Rv3558c and Rv1284.[12] However, at pH values >8, a conserved Arg-residue in all β-CAs investigated so far (belonging to the above-mentioned catalytic dyad)[12] makes a salt bridge with the Asp coordinated to Zn(II), liberating the fourth Zn(II) coordination position, which is then occupied by an incoming water molecule/hydroxide ion, acting thereafter as nucleophile in catalyzing CO_2 hydration to bicarbonate.[12] Based only on the amino acid sequence (Fig. 2), it is however difficult to predict whether bsCA II will have an open or closed active site. In the open form, Cys42, Cys44, His98, and a catalytic water molecule/hydroxide ion would coordinate the Zn(II) ion, allowing the enzyme to be active over a broad pH-range. In the closed form, Asp44 would additionally coordinate to the Zn(II) ion as well, thereby replacing the water molecule/hydroxide ion needed for the catalytic activity. Only at pH values >8, when the conserved Arg46 residue makes a salt bridge with Asp38, the fourth Zn(II) coordination position may be available for the catalytic water molecule/hydroxide ion.[14] This is also the reason why we measured the catalytic activity of bsCA II (as well as other β-CAs investigated earlier)[6,7,11] at a pH of 8.3, when the active site of these enzymes is open.

A brief phylogenetic analysis of the two CAs from *B. suis* together with other bacterial β-CAs (Fig. 3) demonstrates that bsCA II is on a branch distinct from that containing bsCA I, being however related to the β-CAs from *Escherichia coli, Haemophilus influenzae* and to bsCA I (which cluster all together, but without bsCA II). bsCA II shows some relatedness to *H. pylori* and *Synechococcus/Synechocystis* CAs, but this second enzyme is however quite distinct from the first isoform, bsCA I, that was characterized earlier.[11]

The catalytic activity of bsCA II has been determined by measurement of the physiologic reaction, that is, CO_2 hydration to bicarbonate and protons, and is shown in Table 1. The catalytic activity of bsCA I, α-CAs of human origin as well as that of β-CAs from *H. pylori*, are also shown for comparison reasons. bsCA II was a better catalyst than bsCA I for the conversion of CO_2 to bicarbonate (at pH 8.3). Thus, bsCA II demonstrates high catalytic activity (k_{cat} = 1.1.10^6 s^{-1}) with a turnover number 1.85 times higher than that of bsCA I (k_{cat}/K_m of 8.9 × 10^7 M^{-1} s^{-1}). Moreover, bsCA II exhibits a catalytic activity higher than that of hCA I, VA, XII, and hpβCA. Only hCA II, one of the most effective catalysts known in nature,[4] showed a better activity than bsCA II.

2.2. bsCA II inhibition with sulfonamides and sulfamates

Table 2 shows bsCA II inhibition data with a series of sulfonamides and sulfamates, some of which are clinically used drugs,[4] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, zonisamide **ZNS**, sulpiride **SLP**, indisulam **IND**, celecoxib **CLX**, and valdecoxib **VLX**. The simpler derivatives **1–31** 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).[4]

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 I and bsCA II) at 20 °C, pH 8.3 in 20 mM Tris buffer and 20 mM NaCl, and their inhibition data with acetazolamide **AAZ**

Enzyme	Class	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} 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	Medium–low	4.2 × 10^5	3.5 × 10^7	5.7
hpβCA[b]	β	Medium	7.1 × 10^5	4.8 × 10^7	40
bsCA I[c]	β	Medium	6.4 × 10^5	3.9 × 10^7	63
bsCA II[c]	β	High	1.1 × 10^6	8.9 × 10^7	303

[a] Human recombinant isozymes, stopped-flow CO_2 hydrase assay method (pH 7.5).[4]
[b] Recombinant enzymes, stopped-flow CO_2 hydrase assay method (pH 8.3).[5]
[c] Recombinant bsCA I and bsCA II, stopped-flow CO_2 hydrase assay method (pH 8.3), this work.[11,12]

Table 2
Human hCA I, II, and *Brucella suis* enzyme (bsCA I and bsCA II) inhibition data with compounds **1**–**31** and the clinically used derivatives **AAZ**–**VLX**

Inhibitor	K_I* (nM)			
	hCA I[a]	hCA II[a]	bsCA I[b]	bsCA II[c]
1	45,400	295	5870	5048
2	25,000	240	2500	5080
3	28,000	300	2400	4763
4	78,500	320	1580	5560
5	25,000	170	768	5910
6	21,000	160	880	8560
7	8300	60	1070	83
8	9800	110	800	79
9	9650	73	243	60
10	14,000	124	345	84
11	5800	63	4830	87
12	8400	75	940	691
13	8600	60	1210	847
14	9300	19	1430	11.2
15	6	2	70	108
16	1.4	0.3	186	663
17	40	5	27	90
18	164	46	1050	94
19	185	50	745	68
20	109	33	21	769
21	95	30	48	83
22	690	12	33	166
23	55	80	754	758
24	21,000	125	865	5130
25	23,000	133	340	697
26	24,000	125	1035	5639
27	1000	25	9.2	10.1
28	840	16	8.9	14.6
29	510	10	19	7.3
30	4.6	1.1	79	215
31	378	14.6	75	193
AAZ	250	12	63	303
MZA	50	14	54	642
EZA	25	8	17	420
DCP	1200	38	58	112
DZA	50,000	9	21	923
BRZ	45,000	3	26	625
BZA	15	9	75	117
TPM	250	10	57	99
ZNS	56	35	1850	406
SLP	1200	40	19	84
IND	31	15	50	130
CLX	50,000	21	18	128
VLX	54,000	43	19	612

Data for hCA I and II inhibition are from Ref. 5.
* Errors in the range of 5–10% of the shown data, from three different assays.
[a] Human recombinant isozymes, stopped-flow CO_2 hydrase assay method.[5]
[b] Recombinant bsCA I, stopped-flow CO_2 hydrase assay method, from Ref. 11.
[c] Recombinant bsCA II, stopped-flow CO_2 hydrase assay, this work.

The chemical structures of the investigated inhibitors are shown in Figures 4 and 5.

Data for the inhibition of the dominant isoforms hCA I and II as well as those of bsCA I reported earlier[11] with these compounds, are also included in Table 2, for comparison. The following structure–activity relationship (SAR) may be drawn from data of Table 2:

(i) Most of the clinically used sulfonamides and sulfamates showed interesting inhibitory activity against bsCA II, with inhibition constants in the submicromolar ranges (K_Is of 84–923 nM). The best inhibitors were **TPM** and **SLP** with K_Is of 84–99 nM, whereas **DCP**, **BZA**, **IND**, and **CLX** showed inhibition constants in the range of 100–130 nM. The remaining derivatives were medium potency inhibitors, with K_Is of 303–923 nM (Table 2). It may also be observed that in general, these clinically used compounds acted as much stronger bsCA I inhibitors (except zonisamide **ZNS**),[11] whereas their activity against bsCA II is generally very high, in the low nanomolar range. On the other hand, the activity against hCA I is normally in the micromolar range except for **MZA**, **EZA**, **BZA**, **ZNS**, and **IND** which are nanomolar hCA I inhibitors.

(ii) An excellent inhibitory potency against bsCA II has been also observed for compounds **7–10**, **14**, **15**, **17–20**, **22**, and **27–29** with K_Is in the range of 7.3–108 nM. Thus, all the halogenated sulfanilamides investigated here (**7–10**) show good bsCA II inhibitory activity although their effects against the first isoform, bsCA I, are much weaker, with inhibition constants of 243–4830 nM. This is also a proof that the two β-CAs from this pathogen are rather different, as already inferred from the phylogenetic analysis mentioned above. On the other hand, with K_Is of 7.3–14.1 nM, the imino derivative **14** together with the three glycosidic sulfanilamides **27–29** were the best bsCA II inhibitors detected so far. The latter compounds are also particularly active against bsCA I with K_Is of 8.9–19 nM. Moreover, it is clear that these compounds belong to a heterogeneous category of sulfonamides, which makes SAR rather complicated.

(iii) The remaining sulfonamides and sulfamates investigated here, of types **1–6**, **12**, **13**, **16**, **20**, **22–26**, **30**, and **31**, were moderate-weak bsCA II inhibitors, with K_Is of 166–8560 nM. Again, these are derivatives possessing a variety of scaffolds, being aromatic, aliphatic or heterocyclic derivatives, possessing the sulfonamide or sulfamate zinc-binding groups. Many of them show very good hCA II inhibitory activity while being less effective as inhibitors of bsCA I or hCA I (Table 2). All these data demonstrate that bsCA II is a druggable target, with many compounds acting as effective inhibitors, whereas other are less effective or ineffective bsCA II inhibitors.

(iv) The inhibition profile of bsCA II is distinct from that of the related enzyme bsCA I, and also from those of the offtarget human isoforms hCA I and II (Table 2). Despite the fact that bsCA II was generally less prone to be inhibited by sulfonamides and sulfamates than bsCA I, effective inhibitors against both isoforms were detected in this study. We also observed cases in which an inhibitor was much more effective against bsCA II compared to bsCA I (for example sulfonamide **14**, which was 127.7 times a better bsCA II than bsCA I inhibitor).

2.3. Inhibition of bacterial growth with sulfonamides acting as bsCA inhibitors

The effect of several inhibitors such as **AAZ** and molecules **29–31**, which showed interesting activity on the purified enzymes, was also investigated on the growth of *B. suis* in minimal medium, in order to establish whether inhibition of the two β-CAs has antibacterial effects. Data of Figure 6 show inhibition of bacterial growth in the presence of **AAZ** (a medium potency inhibitor of bsCA I and II, with inhibition constants of 63 and 303 nM, respectively) or the much stronger bsCA I/II inhibitors **27**–**29** (K_Is of 7.3–189 nM against the two bacterial enzymes, Table 2). It may be observed that acetazolamide **AAZ** had a much weaker inhibitory growth effect compared to the glycosylsulfonamide **29** or sulfamates **30 and 31** (also included in this study due to their more lipophilic properties compared to acetazolamide), which were very effective in inhibiting the growth of *B. suis* in the above-mentioned assay. The best inhibitor of *Brucella* growth was the glycosylsulfanilamide **29**, which is also a potent inhibitor of the enzyme activity (K_Is of 7.3–19 nM) and which has a balanced hydro- and liposolubility allowing easy penetration through the bacterial membranes. These studies thus show that the dual inhibition of the two β-CAs from this pathogen may lead to inhibition of the bacterial growth. Further studies are needed to better understand the role of the two CA isoforms on the pathogenicity and growth of this bacterium and to determine whether this approach is valid for obtaining anti-infectives with a new mechanism of action.

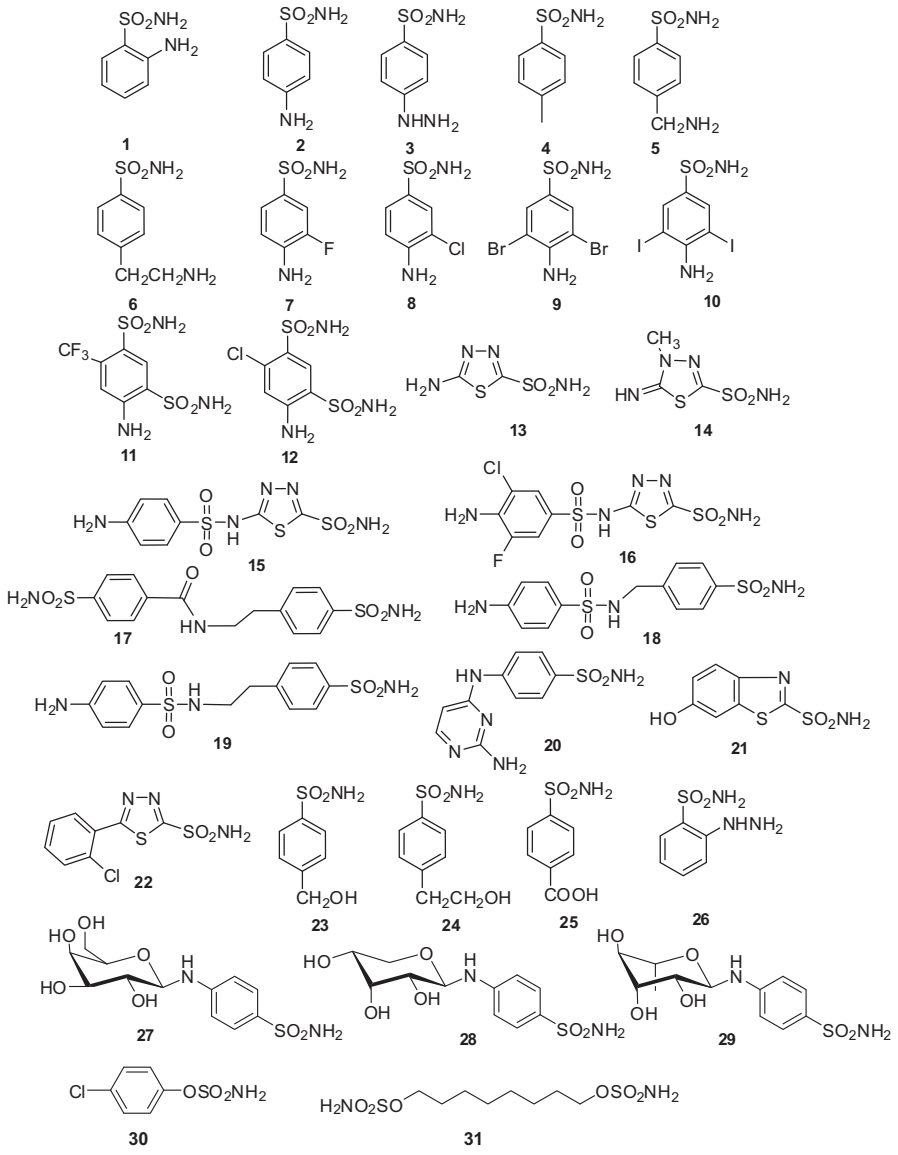

Figure 4. Sulfonamide/sulfamate inhibitors tested against bsCA I and bsCA II.

3. Conclusions

A second β-carbonic anhydrase (CA, EC 4.2.1.1) from the bacterial pathogen *B. suis*, bsCA II, has been cloned, purified and characterized kinetically. bsCA II showed high catalytic activity for the hydration of CO_2 to bicarbonate, with a k_{cat} of 1.1×10^6, and k_{cat}/K_m of $8.9 \times 10^7\ M^{-1}\ s^{-1}$. A panel of sulfonamides and sulfamates have been investigated for inhibition of this new enzyme. All types of activities, from the low nanomolar, to the micromolar, have been detected for these compounds, with K_Is in the range of 7.3 nM–8.56 μM. The best bsCA II inhibitors were some glycosylated sulfanilamides, aliphatic sulfamates, and halogenated sulfanilamides, with inhibition constants of 7.3–87 nM. Some of these good dual inhibitors of bsCA I and II also inhibited bacterial growth in liquid cultures. Whether dual bsCA I and II inhibitors may have applications in the fight against brucellosis, an endemic disease yielding serious infections in humans and important economical loss in agriculture, warrants further studies. At this stage, these promising data on live *Brucella* allow us to propose such an approach for obtaining anti-infectives with a new mechanism of action compared to classical antibiotics in clinical use.

4. Experimental protocols

4.1. Chemistry

Compounds **1**–**31** and **AAZ**–**VLX** are either commercially available (Sigma–Aldrich) or were prepared as described earlier.[13–17]

4.2. Cloning, protein expression, and purification of bsCA II

The CA-encoding gene BR1829 (GenBank accession number AAN30724.1), described here as bsCA II, was specifically amplified by PCR using *B. suis* 1330 chromosomal DNA as template and OPJ15-foward primer (5′-GCGGGCATATGGCTGATCTTCCAGATTCAC-3′). The OPJ16-reverse primer (5-GCGCGGGATCCTCAAAGTTCAGGGCGTTTG-3′) which contain *Nde*I and *Bam*HI recognition sequences (underlined) was also used, 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 OPJ15 and OPJ16 described above. The construct pET15bCA2 was then transformed into *E. coli* strain

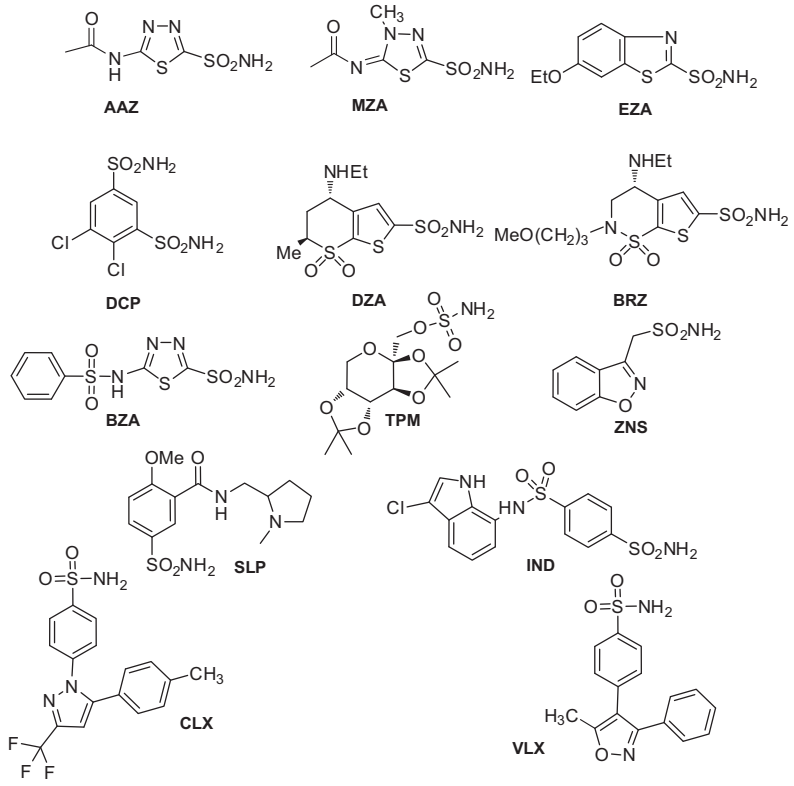

Figure 5. Clinically used drugs tested for their inhibitory activity against bsCA I and bsCA II.

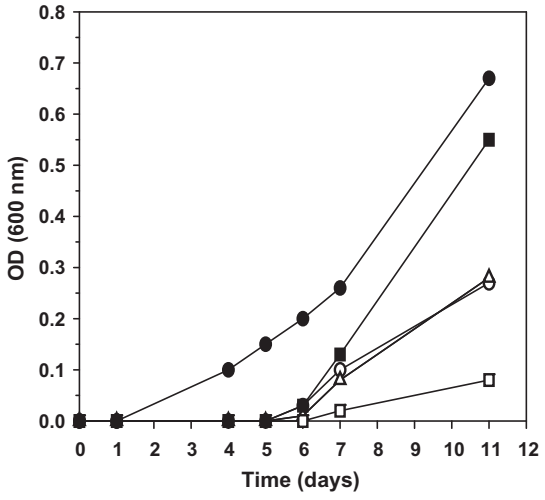

Figure 6. Inhibition of *B. suis* growth in minimal media. Growth of untreated bacteria (●), and of bacteria treated with 100 μM of **30** (○), **31** (△), **29** (□) or **AAZ** (■), was monitored over an incubation period of 11 days in minimal medium at 37 °C with shaking as described previously.[19]

BL21(DE3) for production of the 6×(His)-bsCA II fusion protein. *E. coli* BL21(DE3) harboring pET15bCA2 was grown at 37 °C in 2 L of Luria–Bertani medium supplemented with 50 μg/ml ampicillin. At an optical density at 600 nm (OD_{600}) of approximately 0.6, expression of 6×(His)-bsCA 2 protein was induced in the culture by the addition of isopropyl-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM and growth was continued for 4 h. Cells were then harvested by centrifugation at 3500 rpm at 4 °C for 20 min and broken by sonication in buffer A (50 mM Tris–Cl (pH 7.5), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). All subsequent steps were performed at 4 °C. After centrifugation (13,000 rpm, 20 min), the pellet containing bsCA II in inclusion bodies was washed once in buffer A and resuspended in extraction buffer (25 mM Tris–Cl (pH 7.5), 6 M guanidinium chloride, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for overnight incubation. The solubilized pellet was then mixed with Talon Co^{2+}-affinity resin (Clontech) that has been equilibrated with buffer I (20 mM Tris–HCl (pH 8.0), 6 M guanidinium chloride, 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 6×(His)-bsCA II protein was eluted with buffer 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 II protein was dialyzed sequentially against the following buffers: buffer B (125 mM KCl, 20 mM Tris–HCl (pH 7.5), 150 mM imidazole, 10% glycerol), buffer C (50 mM KCl, 20 mM Tris–HCl (pH 7.5), 50 mM imidazole, 5% glycerol) and buffer D (20 mM Tris–HCl (pH 8.3)).

4.3. CA catalytic activity and inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[18] 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 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 down to 0.01 nM were made 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 were obtained from Lineweaver–Burk plots, as reported earlier,[6] and represent the mean from at least three different determinations.

4.4. Inhibition of *B. suis* growth

B. suis was grown in Gerhardt's minimal medium[19] at 37 °C to an optical density ($OD_{600\ nm}$) of 0.7. The culture was then diluted 1:100 in fresh minimal medium in the absence or presence of carbonic anhydrase inhibitors acetazolamide (**AAZ**), **29**, **30** or **31**, respectively, at concentrations of 100 μM. Bacterial growth at 37 °C and under shaking was followed over a period of 11 days by $OD_{600\ nm}$ measurements.

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