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# Carbonic anhydrase inhibitors: The β-carbonic anhydrase from *Helicobacter pylori* is a new target for sulfonamide and sulfamate inhibitors

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**Abstract**—DNA clones for the β-class carbonic anhydrase (CA, EC 4.2.1.1) of *Helicobactor pylori* (hpβCA) were obtained. A recombinant hpβCA protein lacking the N-terminal 15-amino acid residues was produced and purified, representing a catalytically efficient CA. hpβCA was strongly inhibited ($K_I$s in the range of 24–45 nM) by many sulfonamides/sulfamates, among which acetazolamide, ethoxzolamide, topiramate, and sulpiride, all clinically used drugs. The dual inhibition of α- and/or β-class CAs of *H. pylori* might represent a useful alternative for the management of gastritis/gastric ulcers, as well as gastric cancer. This is also the first study showing that a bacterial β-CA can be a drug target.


In a previous work, we have cloned, purified, and demonstrated the druggability of the α-carbonic anhydrase (α-CAs, EC 4.2.1.1) present in the gastric pathogen *Helicobacter pylori*.[1] Indeed, the α-CAs constitute a family of metalloenzymes that catalyze the reversible hydration of $CO_2$ to bicarbonate and a proton.[2–5] Our groups investigated the molecular cloning of some of the 15 presently known human CA (hCA) isoforms,[6–8] as well as screening analyses for inhibitory/activatory effects of a variety of compounds on most of them, showing that various such isozymes (e.g., hCA I, II, IV, VA, VB, VI, VII, IX, XII, XIII, and XIV) constitute valid targets for the development of novel antiglaucoma, antitumor, antiobesity or anticonvulsant drugs.[9–13] In addition to such α-CA enzymes, there are four other evolutionarily unrelated gene families encoding for CAs all over the phylogenetic tree, the β-ε-CAs.[1–5] Recently, α- and/or β-CA class representatives have been cloned and characterized in some pathogens such as *Plasmodium falciparum*,[14] *Mycobacterium tuberculosis*,[15] *Cryptococcus neoformans*[16] or *Candida* spp.[17] Some preliminary inhibition studies of the α-CA from *Plasmodium*[14] proved it to be critical for the growth of this pathogen.[14] Since many of these organisms are highly pathogenic and show different degrees of resistance to the currently available drugs, inhibition of their CAs may constitute novel approaches to treating affected patients.[14–17]

*Helicobacter pylori* plays a major role in the pathogenesis of peptic ulcer disease, chronic gastritis, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer.[18] In patients with such diseases and proven *H. pylori* infection, eradication of the bacteria has become the main therapeutic goal.[18] The recommended therapy consists of a proton pump inhibitor (PPI) and two antibiotics, mainly amoxicillin and clarithromycin, as first-line eradication triple therapy.[18–20] Although this treatment has been shown to be effective in a number of clinical trials, several meta-analysis revealed that the rates of eradication were widely variable (from 70% to 95%), due to increased resistance to antibiotics.[20–22] Following failure of the eradication by the first-line treatment, a second-line, quadruple therapy using PPI,

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bismuth salts, metronidazole, and tetracycline is used.[19] A recent meta-analysis showed that this treatment is effective,[23] but it has also been frequently associated with eradication failure in more than 20% of cases.[22–24] Reasons of eradication failure include *H. pylori* resistance to metronidazole[25,26] and considerable side-effect rates of metronidazole and tetracycline resulting in reduced patients' compliance.[27] Thus, there is a real need for the development of alternative therapies, eventually exploiting novel targets, that should be devoid of the problems arising with currently available drugs.

*Helicobacter pylori* has the unique ability among bacteria to grow in the stomach presenting highly acidic conditions, at pH values as low as 1.4.[28] Therefore, the pathogen has evolved specialized processes for survival in acid, which maintain its cytoplasmic pH around 6.4. At least two enzymes are involved in these processes: an urease[28] in the cytoplasm and an α-CA (designated as hpαCA) in the periplasm,[29,30] which separates an outer membrane and an inner membrane. However, a second CA belonging to the β-class has been found in the cytoplasm of *H. pylori* (designated throughout this paper as hpβCA), being postulated to play an important role in the urea and bicarbonate metabolism, as well as acid resistance of the bacterium.[30] hpαCA was cloned and purified in 2001 by Lindskog's group,[31] who showed that the enzyme had a catalytic activity similar to that of the human slow isoform hCA I (highly abundant in red blood cells and the gastro-intestinal tract).[1–3] We also obtained a DNA clone of hpαCA and successfully showed that the recombinant protein had significant catalytic CA activity and was inhibited by several types of sulfonamides and sulfamates.[1] hpβCA was discovered after the complete sequencing of *H. pylori* genome by Venter's group.[32] Subsequently, we successfully obtained DNA clones of hpβCA from 37 *H. pylori* strains and showed genetic polymorphisms of the clones.[33] In the present work, we report a study of inhibition profile with a panel of sulfonamides/sulfamates against hpβCA.

As previously reported,[33] the full-length hpβCA was observed to be toxic for the growth of *Escherichia coli* host cells and could not be obtained. Accordingly, we constructed an expression vector for the N-truncated form of hpβCA, lacking an N-terminal polypeptide of 15-amino acid residues, and thereafter successfully obtained the recombinant protein incorporating 206-amino acid residues.[33] The catalytic activity of recombinant, purified hpβCA for the physiologic reaction ($CO_2$ hydration), in comparison with that of hpαϖ CA, as well as several α-CAs of human origin, such as hCA I–III (cytosolic isozymes), hCA VA and VB (mitochondrial isoforms), and hCA XII and XIV (transmembrane isozymes) is shown in Table 1. It may be observed that hpβCA is a catalytically efficient CA, possessing an enzymatic activity 3.2 times higher than that of the α-CA from this bacterium investigated earlier.[33] Furthermore, this activity is almost identical (as $k_{cat}/K_m$ value) to that of hCA I, whereas the $K_m$ value of the bacterial enzyme is closer to that of hCA II than to that of hCA I. In fact, hpβCA is a medium efficiency CA, possessing a catalytic activity higher than that of hCA III, VA, XII, and XIV among others. Only hCA VB and especially hCA II, one of the best catalysts known in nature[1–4], show a better activity than hpβCA. It may be also observed that the activity of all these enzymes is inhibited by the CA inhibitor *par excellence*, the sulfonamide drug acetazolamide **AAZ** (Tables 1 and 2).

Inhibition data against hpβCA,[34] hpαCA, and the host, human enzymes hCA I and II are provided in Table 2, in order to compare the profiles for inhibiting these four unrelated enzymes. Data of Table 2 show that hpβCA is inhibited by all 47 derivatives (sulfonamides and one sulfamate) investigated here, with an inhibition profile completely distinct of those of the α-class enzymes of human (hCA I and II) or bacterial (hpαCA) origin. Thus, a number of aromatic/heterocyclic simple sulfonamides, such as compounds **1**–**10**, **13**, **24**, **25**, and **31** showed inefficient hpβCA inhibitory activity, with inhibition constants in the range of 1.1–24.8 μM. Weak inhibitory activity, with $K_I$s in the range of 128–973 nM, was then showed by compounds **10**, **11**, **14**, **21**–**23**, **MZA**, **BRZ**, **ZNS**, **IND**, **26**, **27**, **32**, and **33**. It may be observed that these compounds belong to heterogeneous classes of sulfonamides, possessing various substitution patterns. Potent hpβCA inhibitory action was then detected for many derivatives, among which:

**Table 1.** Kinetic parameters for $CO_2$ hydration reaction catalyzed by some human α-CA isozymes at 20 °C and pH 7.5, and hpCA isozymes belonging to the α- and β-CA class, and their inhibition data with acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used compound

| Isozyme | Activity level | $k_{cat}$ ($s^{-1}$) | $K_m$(mM) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) | $K_I$ (acetazolamide) (nM) |
|---|---|---|---|---|---|
| hCA I | Medium | $2.0 \times 10^5$ | 4.0 | $5 \times 10^7$ | 250 |
| hCA II | Very high | $1.4 \times 10^6$ | 9.3 | $1.5 \times 10^8$ | 12 |
| hCA III | Very low | $1.0 \times 10^4$ | 33.3 | $3 \times 10^5$ | 300,000 |
| hCA VA | Low | $2.9 \times 10^5$ | 10.0 | $2.9 \times 10^7$ | 63 |
| hCA VB | High | $9.5 \times 10^5$ | 9.7 | $9.8 \times 10^7$ | 54 |
| hCA XII | Medium | $4.2 \times 10^5$ | 12.0 | $3.5 \times 10^7$ | 5.7 |
| hCA XIV | Medium | $3.1 \times 10^5$ | 7.9 | $3.9 \times 10^7$ | 41 |
| hpαCA[a] | Low | $2.5 \times 10^5$ | 16.6 | $1.5 \times 10^7$ | 21 |
| hpβCA[b] | Medium | $7.1 \times 10^5$ | 14.7 | $4.8 \times 10^7$ | 40 |

h, human; hp, *Helicobacter pylori* enzyme.
[a] At pH 8.9 and 25 °C, from Ref. 1.
[b] At pH 8.3 and 20 °C, this work.

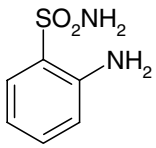
**15**–**17**, **DCP**, **DZA**, **BZA**, **28**, and **34**. These compounds showed $K_I$s in the range of 54–105 nM. The best hpβCA inhibitors ($K_I$s in the range of 24–45 nM) were the following derivatives: **18**–**20**, **AAZ**, **EZA**, **TPM**, **SLP**, **29**, **30**, **35**, and **36**. Some of them are clinically used drugs (the CA inhibitor *par excellence*, **AAZ**, as well as the structurally related **EZA**; the antiepileptic topiramate **TPM**, the antipsychotic sulpiride **SLP**, and the recently reported derivatives possessing lipophilic *tert*-butyl tails **29**, **30**, **35**, and **36**). As many of these derivatives were also quite effective hpαCA inhibitors (but also hCA I and II inhibitors, Table 2), dual inhibition of α- and/or β-class CAs of *H. pylori* could represent a useful and novel means for the management of gastritis/gastric ulcers, as well as gastric cancer.

To date, only one study regarding the inhibition of non-α-CAs has been published.[35] Zimmerman et al.[35] reported an inhibition study of the archaeal β- and γ-CAs from *Methanobacterium thermoautotrophicum* and

**Table 2.** Human (h) hCA I, II, and hpα/βCA inhibition data with compounds **1**–**24** and the clinically used derivatives **AAZ**–**IND**, and newly designed[1] inhibitors **25**–**36**

| Inhibitor | $K_I$[c] (nM) | | | |
|---|---|---|---|---|
| | hCA I[a] | hCA II[a] | hpαCA[a] | hpβCA[b] |
| **1** | 45400 | 295 | 426 | 16400 ± 820 |
| **2** | 25000 | 240 | 454 | 1845 ± 54 |
| **3** | 28000 | 300 | 316 | 8650 ± 62 |
| **4** | 78500 | 320 | 430 | 2470 ± 104 |
| **5** | 25000 | 170 | 873 | 2360 ± 170 |
| **6** | 21000 | 160 | 1150 | 3500 ± 61 |
| **7** | 8300 | 60 | 1230 | 1359 ± 37 |
| **8** | 9800 | 110 | 378 | 1463 ± 55 |
| **9** | 6500 | 40 | 452 | 1235 ± 60 |
| **10** | 6000 | 70 | 510 | 1146 ± 29 |
| **11** | 5800 | 63 | 412 | 973 ± 36 |
| **12** | 8400 | 75 | 49 | 640 ± 18 |
| **13** | 8600 | 60 | 323 | 2590 ± 74 |
| **14** | 9300 | 19 | 549 | 768 ± 38 |
| **15** | 6 | 2 | 268 | 64 ± 5 |
| **16** | 164 | 46 | 131 | 87 ± 7 |
| **17** | 185 | 50 | 114 | 71 ± 3 |
| **18** | 109 | 33 | 84 | 38 ± 2 |
| **19** | 95 | 30 | 207 | 39 ± 3 |
| **20** | 690 | 12 | 105 | 37 ± 2 |
| **21** | 55 | 80 | 876 | 236 ± 19 |
| **22** | 21000 | 125 | 1134 | 218 ± 16 |
| **23** | 23000 | 133 | 1052 | 450 ± 27 |
| **24** | 24000 | 125 | 541 | 15250 ± 605 |
| **AAZ** | 250 | 12 | 21 | 40 ± 3 |
| **MZA** | 50 | 14 | 225 | 176 ± 12 |
| **EZA** | 25 | 8 | 193 | 33 ± 1 |
| **DCP** | 1200 | 38 | 378 | 105 ± 9 |
| **DZA** | 50000 | 9 | 4360 | 73 ± 7 |
| **BRZ** | 45000 | 3 | 210 | 128 ± 11 |
| **BZA** | 15 | 9 | 315 | 54 ± 4 |
| **TPM** | 250 | 10 | 172 | 32 ± 2 |
| **ZNS** | 56 | 35 | 231 | 254 ± 18 |
| **SLP** | 1200 | 40 | 204 | 35 ± 3 |
| **IND** | 31 | 15 | 413 | 143 ± 14 |
| **25** | 12300 | 241 | 539 | 23500 ± 570 |
| **26** | 10750 | 210 | 316 | 241 ± 19 |
| **27** | 14250 | 133 | 79 | 158 ± 10 |
| **28** | 12300 | 241 | 539 | 101 ± 8 |
| **29** | 13270 | 127 | 62 | 44 ± 5 |
| **30** | 541 | 18 | 13 | 28 ± 2 |
| **31** | 14700 | 354 | 640 | 24800 ± 1200 |
| **32** | 9620 | 203 | 318 | 213 ± 17 |
| **33** | 13000 | 119 | 60 | 150 ± 9 |
| **34** | 12150 | 104 | 31 | 96 ± 8 |
| **35** | 12045 | 94 | 27 | 45 ± 4 |
| **36** | 338 | 15 | 12 | 24 ± 1 |

[a] Human/hp recombinant isozymes, stopped-flow $CO_2$ hydrase assay method, from Ref. 1.
[b] Recombinant hpCA, stopped-flow $CO_2$ hydrase assay method, this work, mean ± SE (from three different assays).
[c] Errors in the range of 5–10% of the shown data, from three different assays.



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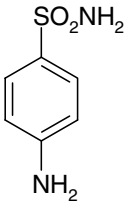

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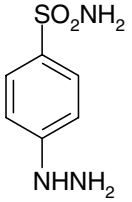

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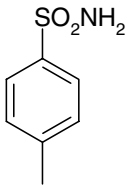

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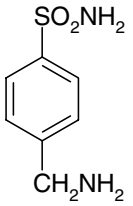

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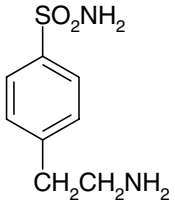

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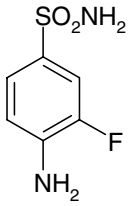

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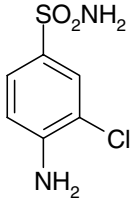

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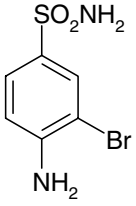

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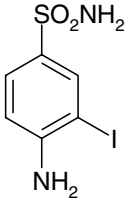

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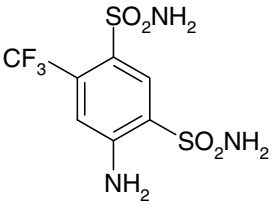

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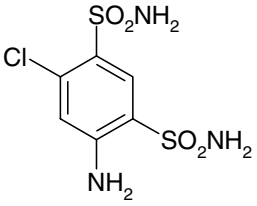

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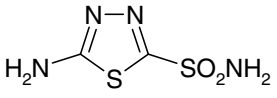

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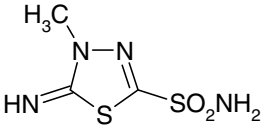

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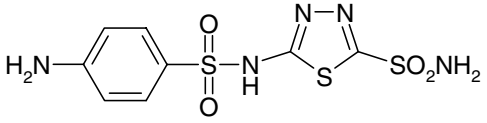

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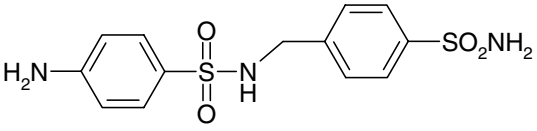

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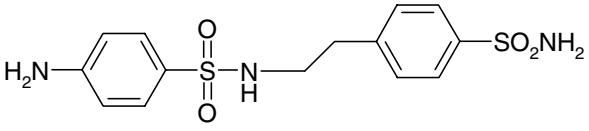

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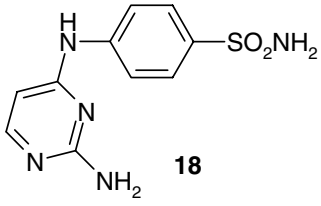

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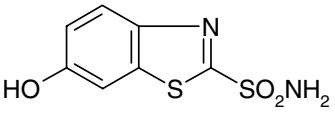

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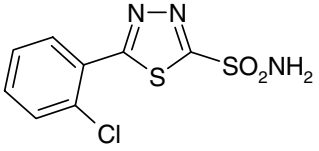

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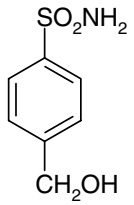

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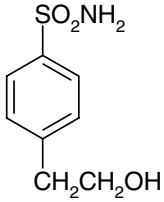

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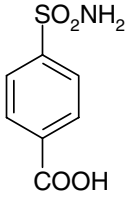

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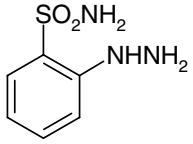

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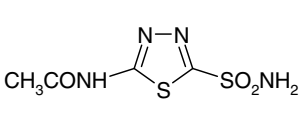
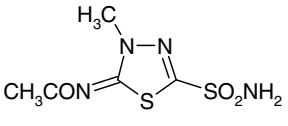



**AAZ**

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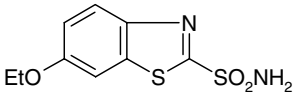


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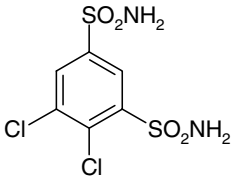
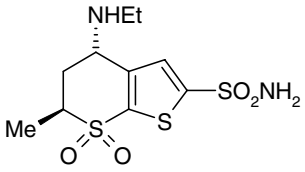



**DCP**

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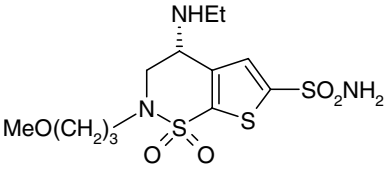


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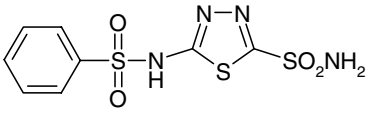


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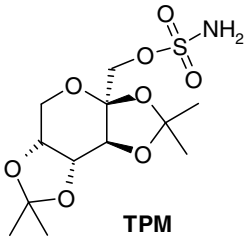


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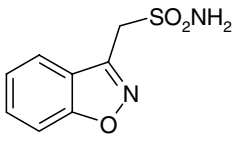


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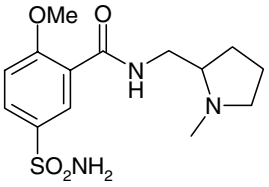


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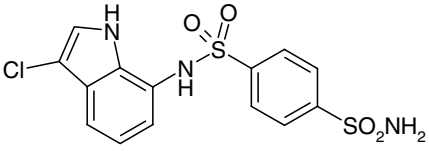


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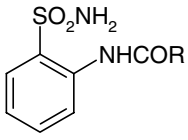


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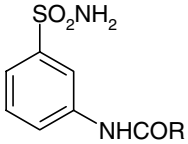


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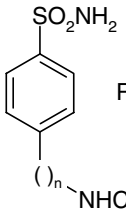


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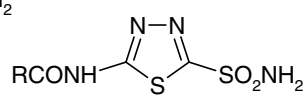
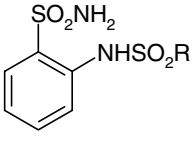
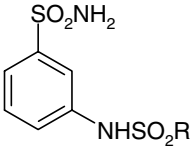
**29**: n=2



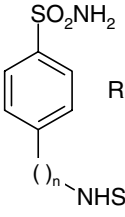

**30**




**31**




**32**

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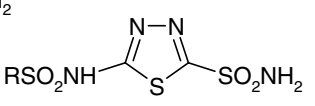


**36**

R = 4-t-Bu-$C_6H_4$

*Methanosarcina thermophila*, by a number of sulfonamide derivatives including the clinically used sulfonamide drugs such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, and brinzolamide. Although the *Archaea* are not pathogenic, the study of Zimmerman et al.[35] may be considered as a

first demonstration that non-α-CAs could also be targeted by sulfonamide inhibitors. Here we report the inhibition data of hpβCA with 11 clinically used sulfonamides/sulfamates (**AAZ**–**IND**), as well as other 36 sulfonamides of types **1**–**36**,[1–5,32,36,37] some of which are simple aromatic/heterocyclic derivatives that may be used as lead molecules, whereas others incorporate tails inducing increased lipophilicity,[1] provided that a compound that should inhibit hpβCA must cross two membranes, the outer and an inner membrane of *H. pylori*.

Figure 1 shows the consensus amino acid sequence of hpβCA (*H. pylori* cons.), which consists of the most commonly used amino acid residues among the 15 *H. pylori* strains from patients with gastritis,[33] aligned with other bacterial β-CAs as previously reported, including two β-CAs from *E. coli* (CynT, Accession No. AAB18063, and CynT2, Accession No. P36857), one from *Synechococcus elongatus* (strain PCC 7942, Accession No. P27134), one *Brucella suis* (strain 1330, Accession No. AAN33967), and *Haemophilus influenzae* (Accession No. P45148). Based on the results from the structural analysis of *E. coli* β-CAs (CynT2) previously reported,[38] secondary structural features shown above the sequences clearly place this new enzyme (hpβCA) in the β category. It has been reported earlier[38–43] that most bacterial β-CAs are composed of three sequential domains: (i) an N-terminal arm including two α-helices (H1 and H2), (ii) a zinc ion-binding core including three β-sheets (S1-S3) and two α-helices (H3 and H4), and (iii) a C-terminal subdomain including two β-sheets (S4-S5) and five α-helices (H5-H9) (Fig. 1).[39,40] In agreement with such reports,[38–43] it may be observed that the amino acid sequence of the zinc ion-binding core of hpβCA is highly conserved, similarly to other bacterial β-CAs sequenced so far (Fig. 1). Such conserved residues include the zinc(II)-coordinating amino acids Cys42, Asp44, His98, and Cys101 (indicated by triangles in Figure 1; residues numbering is based on the *E. coli* CynT2 numbering system) and Arg46, involved in a salt bridge with Asp44 when the active site of the enzyme is opened (see later in the text).[15,39]

X-ray structures for six β-CAs are available at this moment: the enzymes isolated from the red alga *Porphyridium purpureum*,[39] the enzyme from chloroplasts of *Pisum sativum*,[41] another prokaryotic enzyme, isolated from *E. coli*,[40] 'cab', the enzyme isolated from the archaeon *M. thermoautotrophicum*,[42,43] as well as two enzymes from *M. tuberculosis*.[15] The *P. purpureum* CA monomer was shown to be composed of two internally repeating structures, being folded as a pair of fundamentally equivalent motifs of an α/β domain and three projecting α-helices.[39] The motif is very distinct from that of either α- or γ-CAs.[39] This homodimeric CA appeared like a tetramer with a pseudo 2-2-2 symmetry. β-CAs are thus very different from the α-class enzymes (Fig. 2). The Zn(II) ion is essential for catalysis in both families of enzymes, but its coordination is different and rather variable for the β-CAs: thus, in most of the prokaryotic β-CAs the Zn(II) ion is coordinated by two cysteinate residues, an imidazole from a His residue, and a

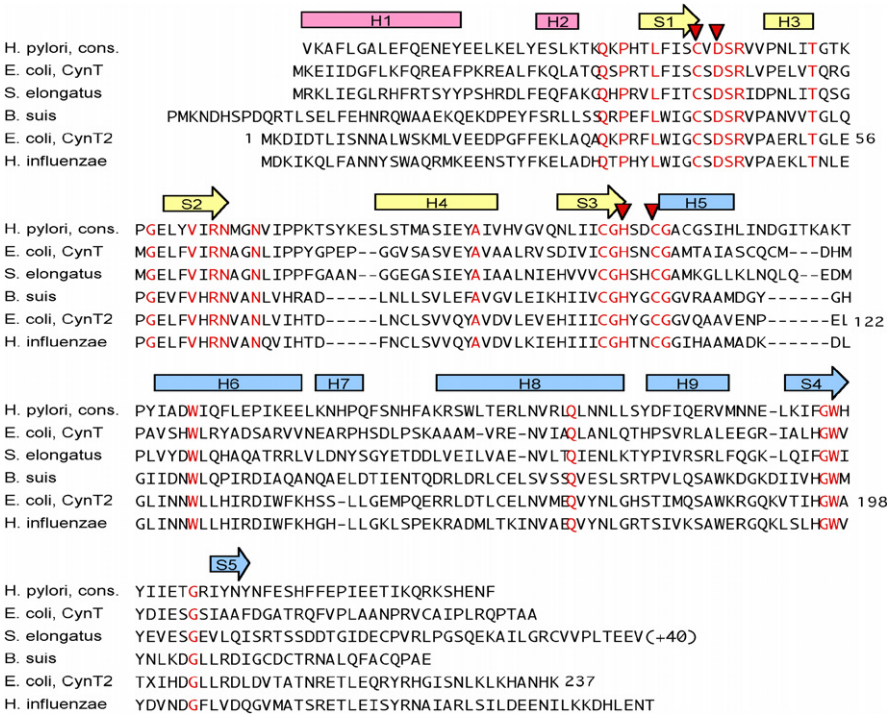


**Figure 1.** Consensus amino acid sequence of hpβCA (*H. pylori* cons.), which consists of the most commonly used amino acid residues among the 15 *H. pylori* strains from patients with gastritis, as compared to those of the β-CAs from *E. coli* (two gene products, CynT and cynT2), *S. elongatus, B. suis*, and *H. influenzae*. Amino acids conserved among all 6 β-CAs are indicated in red. According to previous reports[39,41], the secondary structural features are indicated above the alignment (helices as bars; strands as arrows) and colored according to the sequential subdomains. Arrowheads in red indicate the Zn(II)-coordinating amino acid residues: Cys42, Asp44, His98, and Cys101 (residue numbers are based on the *E. coli* CynT2 numbering system).[39]

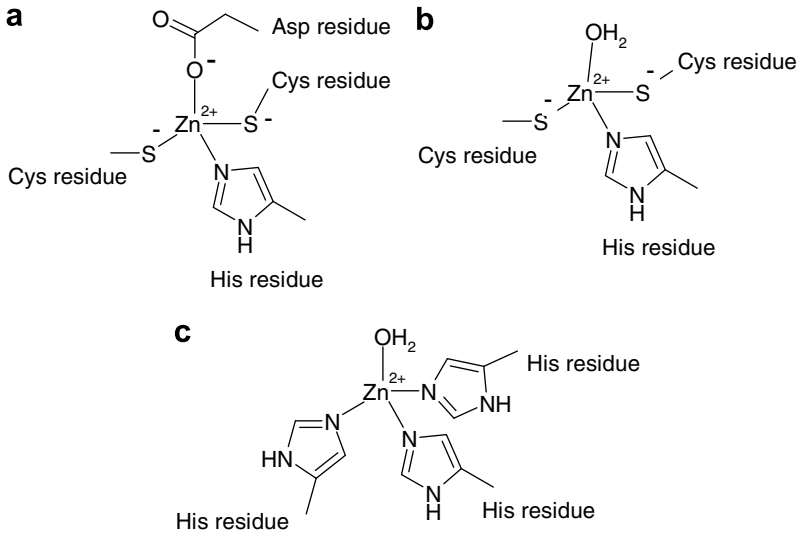


**Figure 2.** Schematic representation of the Zn(II) coordination sphere in β- and α-CAs for which X-ray crystallographic structures were reported. (a) *Porphyridium purpureum*,[39] *Escherichia coli*[38], and *Mycobacterium tuberculosis* Rv3588c[15] β-CA enzymes; (b) *Pisum sativum* chloroplast[41] and *Methanobacterium thermoautotrophicum*[42] β-CA enzymes; (c) α-CAs (all of them having the same coordination of the active site Zn(II) ion, by three histidines and a water molecule, all over the phylogenetic tree).[1–5]

carboxylate belonging to an Asp residue (Fig. 2a),[39,42] whereas the chloroplast enzyme has the Zn(II) ion coordinated by two cysteinates, the imidazole belonging to a His residue, and a water molecule (Fig. 2b).[38,40] The Zn(II) coordination in all α-CAs investigated up to now consisted always of three imidazoles belonging to His residues and a water molecule which can be deprotonated to hydroxide, with the generation of the nucleophilic, catalytically active form of the enzyme.[1–5,36] hpβCA has the Zn(II) ion coordinated by two cysteine residues, one histidine, and one aspartate, as the enzyme isolated from *P. purpureum*, *E. coli*, and one *Mycobacterium* enzyme, represented in Figure 2a. Since these enzymes do not have a hydroxide ion/water molecule coordinated to the Zn(II) ion, we propose the following catalytic mechanism (Fig. 3) of this efficient enzyme for the hydration of $CO_2$ (see Table 1), based on the recent crystallographic data of the *M. tuberculosis* β-CA possessing the same Zn(II) coordination as hpβCA.

Suarez Covarrubias et al.[15] recently showed that in *M. tuberculosis* Rv3588c[15] β-CA the active site is 'blocked'

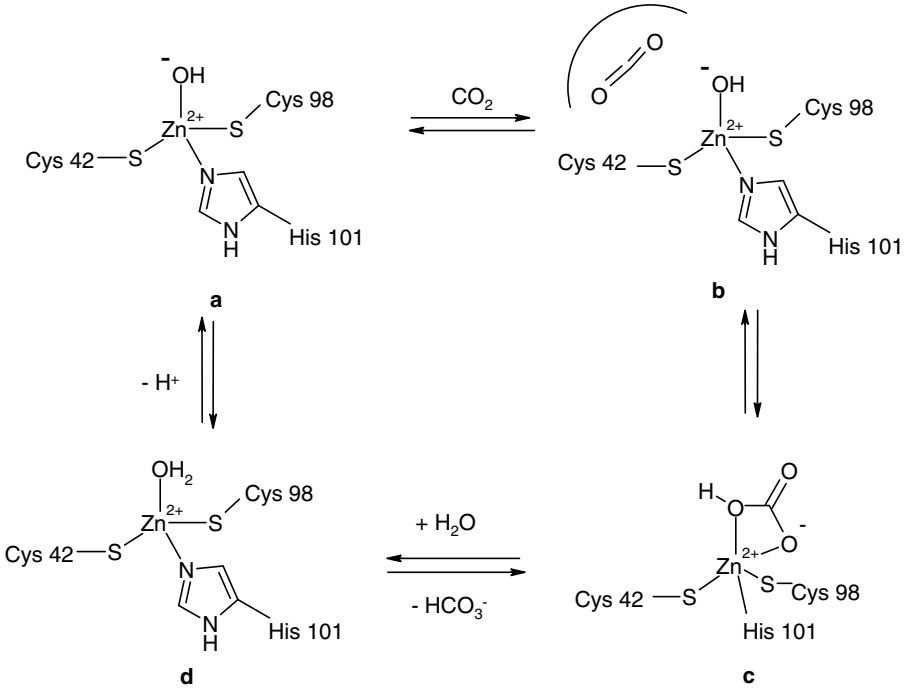


**Figure 3.** Proposed catalytic mechanism for hpβCA based on the crystallographic work of the related enzyme from *Mycobacterium tuberculosis* Rv3588c.[15]

at pH 7.5 or lower, when the carboxylate of an aspartic acid coordinates as the fourth ligand to the Zn(II) ion (as in Fig. 2a). However, at pH values over 8.3, an opening of the active site occurs, with the blocking aspartate forming a salt bridge with a conserved Arg residue in all β-CAs sequenced so far (Arg46 in the case of hpβCA, see Fig. 1), so that a water molecule/hydroxide ion has finally access to coordinate the metal ion for completion of its tetrahedral geometry (Fig. 3). The catalytic mechanism of this β-CA possessing the 'opened' active site is then rather identical to that of the α-class enzymes, with the substrate being probably bound in a hydrophobic pocket not far from the zinc-coordinated hydroxide (Fig. 3b), which attacks it with formation of bidentately coordinated bicarbonate (Fig. 3c). This is then displaced by a water molecule and liberated in solution, with formation of the acidic form of the enzyme, with water as the fourth zinc ligand (Fig. 3d). For generating the strong nuclophile with hydroxide coordinated to Zn(II), a proton transfer reaction must occur in the last step, with formation of the catalytically active enzyme species (Fig. 3a). The proton transfer step is not well investigated up to now in the β-class enzymes, this process being assisted by active site His residues in the α-CAs (e.g., His64 in hCA II and similar enzymes).[1–5]

No X-ray crystallographic data for any β-class CA in complex with organic inhibitors are available up to now. However, based on the strong inhibitory activity observed here for hpβCA with many sulfonamides/sulfamates, we hypothesize that similar to the α-CAs, these inhibitors bind directly to the Zn(II) ion within the enzyme active site, also participating to stabilizing interactions with various amino acid residues from the cavity (Fig. 4). It should be important to resolve X-ray structures of adducts of hpβCA with some of these inhibitors as such data might be highly relevant for the drug design of much stronger (and eventually selective) hpβCA inhibitors. However, what we consider as an even more important discovery in addition to unraveling low nanomolar hpβCA inhibitors, is the fact that we proved here that a non-α-CA from a pathogenic bacterium producing devastating diseases worldwide can be considered as a drug target, in addition to the well-studied α-class enzymes from vertebrates/protozoans. Since the genomes of many pathogenic bacteria/fungi contain one or more β-CAs which were shown to be essential for the growth/virulence of these pathogens,[14–17] we estimate that the approach presented here may be extended to other such enzymes, with the possibility to detect pharmacological agents possessing a novel mechanism of action, and fighting resistance to presently available drugs.

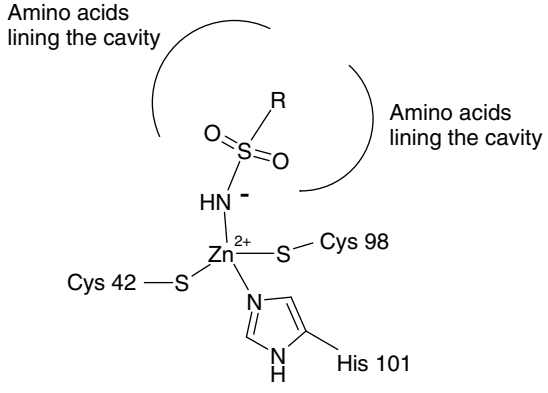


**Figure 4.** Proposed inhibition mechanism of hpβCA by sulfonamides. The deprotonated sulfonamide is coordinated to the catalytical Zn(II) ion as the fourth ligand, whereas the $SO_2$ moiety and the organic scaffold (R) of the inhibitor may interact with amino acid residues lining the active site cavity, as in the case of the α-CA—sulfonamide adducts.[10–13]

## Acknowledgments

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33. Morishita, S.; Nishimori, I.; Minakuchi,T.; Onishi, S.; Takeuchi, H.; Vullo, D.; Scozzafava, A.; Supuran, C. T. (*Helicobacter*, **2007**, submitted). DNA samples from a panel of *H. pylori* strains were subjected to polymerase chain reaction (PCR) amplification and sequencing of full-length DNA coding the hpβCA. The GenBank database search identified two DNA clones of hpβCA (gene symbol *icfA*). One clone (locus name jhp0004, Accession No. NC_000921) was obtained from *H. pylori* strain J99 by ASTRA research center (http://www.ncbi.nlm.nih.gov/entrez/query)and the other (locus name HP0004, Accession No. NC_000915) was obtained from *H. pylori* strain 26695 by TIGR sequencing center (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi). Based on 5′- and 3′-uncoding regions of the hpβCA sequence from the strain J99, in the present study, a primer pair was synthesized for PCR. The sequences of the primer pairs were as follows; 5′-GAAATGCAAGATTTTTAGCTATGA- 3′ and 5′-TCTAAACTCTCAATGACGCAT-3′. The PCR was hot-started with incubation for 3 min at 96 °C, consisted of 30 cycles of 30 s at 96 °C, 15 s at 55 °C, and 4 min at 60 °C. The PCR product was sequenced using an ABI PRISM Dye Termination Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA) and an ABI 370A DNA sequencer (ABI, Foster City, CA). The DNA fragment encoding the reading frame of hpβCA was amplified from a *H. pylori* strain isolated from a Japanese patient with gastritis. We further set two types of 5′-primers: one primer started from the first valine and another from glutamic acid at the 16th residue as lacking an N-terminal polypeptide 15-amino acid long. We substituted these N-terminal amino acid residues with methionine resulting in the Kozac sequence (ATGG, double-underlined in the following sequences) and further added EcoRI recognition sequences (underlined): 5′-CGGAATTCTGAGATTTAAGGGTTAAAGAATGGAA-3′ for the full-length protein, 5′-CGGAATTCCCATGGAGTTTAAAGAGCTTTATGAGAG3′ for the N-terminal truncated protein. The 3′-primer sequence including the SalI recognition site (underlined in the following sequences) was 5′-CGGTCGACTCTAAACTCTCAATGACGCAT-3′. The PCR was hot-started with incubation for 5 min at 94 °C and consisted of 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 90 s at 72 °C, and terminated with incubation for 10 min at 72 °C. The PCR products were cleaved with EcoRI and SalI, and then ligated in-frame into the pGEX-4T2 vector(Amersham, Tokyo, Japan). The proper DNA sequences of the hpβCA insert subcloned into the vector were reconfirmed by DNA sequencing. The constructs were then transfected into *E. coli* strain BL21 for production of the fusion protein with Glutathione *s*-transferase (GST) as previously reported.1,9,11 Following induction of protein expression by adding 1 mM isopropyl-β-D-thiogalactopyranoside, the bacteria were harvested and sonicated in PBS. The sonicated cell extracts were further homogenized twice with a Polytron (Brinkmann) for 30 s each at 4 °C. Centrifugation at 30 K*g* for 30 min afforded the supernatant containing the soluble proteins. The obtained supernatants were then applied to prepacked Glutathione Sepharose 4B columns (Amersham). The columns were extensively washed with buffer and then the

GST-CA fusion protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Finally the GST parts of the fusion proteins were cleaved with thrombin.32 The obtained hpβCA recombinant protein was further purified by sulfonamide affinity chromatography,1 the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments with $CO_2$ as substrate.

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