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# Inhibition studies of the β-carbonic anhydrases from the bacterial pathogen *Salmonella enterica* serovar Typhimurium with sulfonamides and sulfamates

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ABSTRACT

The two β-carbonic anhydrases (CAs, EC 4.2.1.1) from the bacterial pathogen *Salmonella enterica* serovar Typhimurium, stCA 1 and stCA 2, were investigated for their inhibition with a large panel of sulfonamides and sulfamates. Unlike inorganic anions, which are weak, millimolar inhibitors of the two enzymes [Vullo et al., *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3591], sulfonamides and sulfamates are effective micro-to nanomolar inhibitors of the two enzymes. Various types of inhibitors have been detected among the 38 investigated sulfonamides/sulfamates, with $K_I$s in the range of 31 nM–5.87 μM. The best stCA 1 inhibitors were acetazolamide and benzolamide-based compounds, whereas the best stCA 2 inhibitors were sulfonylated benzenesulfonamides and amino-benzolamide derivatives ($K_I$s in the range of 31–90 nM). 3-Fluoro-5-chloro-4-aminobenzolamide showed an inhibition constant of 51 nM against stCA 1 and of 38 nM against stCA 2, being the best inhibitor detected so far for these enzymes. As many strains of *S. enterica* show extensive resistance to classical antibiotics, inhibition of the β-CAs investigated here may be useful for developing novel antibacterials, targeting β-CAs which may be involved in pathogenicity and invasion of some bacteria.



## 1. Introduction

*Salmonella* belongs to the *Enterobactericiae* group, which has pathogenic characteristics, being one of the most common causes of enteric infections worldwide.[1] They were named after the scientist Daniel Salmon who isolated the first organism, *Salmonella* 'Choleraesuis' (now considered a serovar of *Salmonella enterica*), from the intestine of a pig.[1–3] There are now two recognized *Salmonella* species, *Salmonella bongori* and *S. enterica*,[1] the last of which possesses a high number of subspecies and serovars, some of which are highly pathogenic for specific hosts.[2,3] Certain serovars of *S. enterica* are responsible of serious diseases in humans, such as typhoid fever (*S. enterica* serovar Typhi), whereas most of the food borne infections are due to the serovar Typhimurium.[1–5] Although such enteric/systemic infections can be treated with antibiotics, there are reports worldwide, on the extensive resistance of *Salmonellae* to many classes of such agents, among which ampicillin, chloramphenicol, streptomycin, antibacterial sulfonamides and tetracyclines.[6–9] Furthermore, the antibiotic resistance is a general problem with many species of other bacteria, including *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Brucella suis*, etc.[7,10]

The carbonic anhydrases (CAs, EC 4.2.1.1),[11] are metalloenzymes catalyzing a crucial physiologic reaction, hydration of carbon dioxide to bicarbonate and protons, which is essential for many organisms, including bacteria and fungi. CAs, of which five genetically different families are known,[11] are present in many human pathogens such as the malaria provoking protozoa *Plasmodium falciparum*,[12] bacteria such as *H. pylori*,[13] *M. tuberculosis*,[14,15] *B. suis*,[16] *Streptococcus pneumoniae*,[17] and fungi/yeasts such as *Candida albicans*,[18] *C. glabrata*,[18] *Saccharomyces cerevisiae*,[19] and *Cryptococcus neoformans*[19] among others. Many of the α- or β-class enzymes present in these pathogens started to be investigated as possible new drug targets in the search of novel agents devoid of drug resistance problems observed with classical antibiotics/antifungal agents.[11–20] Most of the time, inhibition of these enzymes has been investigated with the classical CA inhibitors (CAIs) belonging to the sulfonamide/sulfamate class, but other chemotypes were also explored, such as boronic acids, metal complexing anions and similar small molecules, naturally occurring phenols, etc.[11–20] It should be mentioned that for some bacteria (*H. pylori* and *B. suis*)[13,16b] it has been possible to evidence inhibition of the pathogen growth in vivo, following CA inhibition with pharmacological agents of the sulfonamide type, whereas in other cases (e.g., *M. tuberculosis*) this has not been possible so far, probably due to problems of penetration of the pharmacological agent through the bacterial cell walls.[21] Furthermore, pharmacologic inhibition of the fungal β-CAs was also correlated with antifungal

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activity in niches of low $CO_2$ availability,[22] whereas sulfonamide CAIs showed a powerful antimalarial activity in experimental models of the disease, in rodents.[13,23]

By searching for metalloenzyme-specific sequence motifs within the *S. enterica* serovar Typhimurium genome,[4] two CA-encoding genes belonging to the β-CA family (STM0171 and PSLT046) were identified. Considering that β-CAs are the most widespread such enzymes all over the phylogenetic tree in microorganisms,[11,24] and the fact that they are not found in mammals (including humans),[11] such enzymes may be possible new drug targets. We reported in a preliminary work about the cloning and inhibition of two CAs from this pathogen with inorganic anions and structurally related small molecules (sulfamide, sulfamic acid, phenylboronic acid).[24] Here we report the first inhibition study with a panel of sulfonamides/sulfamates of the two β-CAs from *S. enterica*, serovar Typhimurium, stCA 1 and stCA 2 cloned and characterized kinetically earlier by our groups.[24]

## 2. Results

### 2.1. stCA 1 and 2 cloning, purification and catalytic activity

Cloning and sequencing of the two β-CA clones from *Salmonella typhimurium LT2* showed identical DNA sequences with those previously reported in the GenBank database.[4] Some modifications of the DNA cloning and incubation conditions (see Experimental) led to the successful production of the two recombinant proteins stCA 1 and 2 tagged with GST, as for other bacterial β-CAs reported earlier.[12–17] SDS–PAGE of these proteins showed only one band, which was induced by addition of IPTG, and in agreement with the molecular weight of a GST-CA fusion protein, (Fig. 1a) whereas after the removal of the GST tag the purified enzymes had the SDS–PAGE shown in Fig. 1b, with molecular weights of 24.8 and 26.6 kDa, respectively (as monomeric enzymes, since the gels were run in denaturing conditions, see discussion later in the text). In the preliminary communication[24] in which we reported the two enzymes, these details were not included.

The catalytic activity of stCA 1 and 2, for catalysis of the physiologic reaction, that is, $CO_2$ hydration to bicarbonate and protons,[11] shown in Table 1, where the catalytic activity of several α-CAs of human origin as well as those of β-CAs from bacterial pathogens[25–29] investigated earlier[12–18] are also shown for comparison.

### 2.2. stCA 1 and 2 inhibition with sulfonamides and sulfamates

Table 2 shows stCA 1 and stCA 2 inhibition data with a panel of sulfonamides and one sulfamate (obtained for the $CO_2$ hydration reaction catalyzed by CAs),[30] 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**, valdecoxib **VLX**, sulthiame **SLT**, saccharin **SAC** and hydrochlorothiazide **HCT**. 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 targeting human CAs (hCAs)[31–34] as well as compounds targeting bacterial/fungal β-CAs.[12–19]

## 3. Discussion

### 3.1. stCA 1 and 2 catalytic activity

We cloned and purified two β-CAs encoded in the genome of *S. enterica* serovar Typhimurium, strain LT2, whose genome has been sequenced.[4] Only these two β-CAs were evidenced so far in the genome of this bacterium. The two enzymes were initially obtained as GST-fusion proteins (Fig. 1a), as this procedure offers an easy way for purification of high amounts of recombinant protein, necessary for enzymatic kinetic and inhibition studies.[13–16] The GST part of the fusion protein has been thereafter cleaved,[13] leading to pure stCA 1 and 2, with molecular weight of 24.8 kDa for stCA 1 and 26.6 kDa for stCA 2 (Fig. 1b), which are in excellent agreement with the expected molecular weight of these proteins based on their amino acid sequence, of 24.821 and 26.644 kDa, respectively.

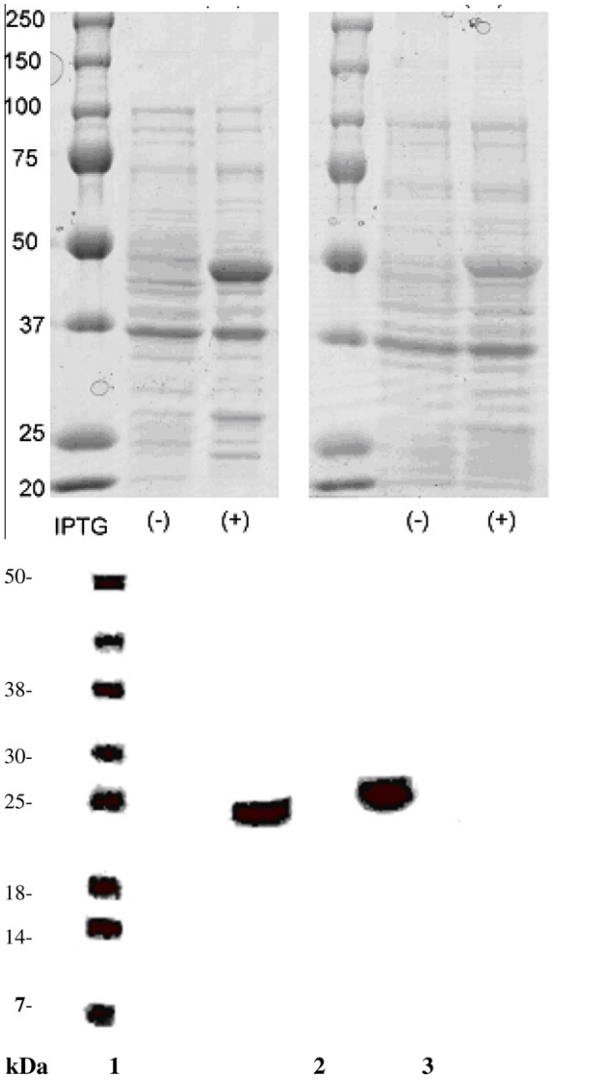



**Figure 1.** SDS–PAGE of: a. stCA 1-GST fusion protein (left lanes) and stCA 2-GST fusion proteins (right lanes), with and without IPTG added in the culture medium, and b: ladder (lane 1) and purified stCA 1 (Mw = 24.8 kD, lane 2) and stCA 2 (Mw = 26.6 kDa, lane 3) enzymes.

Unlike α-CAs, in which the catalytic Zn(II) ion is coordinated by three His residues and a water molecule/hydroxide ion (essential for catalysis), in the β-class enzymes the zinc ion may be coordinated by four amino acid residues (2Cys, one His and one Asp) or by three residues (2Cys and one His, with a water as the fourth ligand).[11–18] This is also the case with the enzymes investigated here. Indeed, since we started our work on the *Salmonella* CA enzymes, a crystallographic consortium[29] reported and released the coordinates of the X-ray crystal structure of stCA 1, which are freely available (PDB file 3QY1).[29] According to these data, stCA 1

**Table 1**
Kinetic parameters for $CO_2$ hydration reaction catalysed by some human α-CA isozymes at 20 °C and pH 7.5 (hCA I, and II), and β-CA enzymes from *Mycobacterium tuberculosis* (Rv1284 and Rv3273), *Brucella suis* (bsCA 1 and bsCA 2) and *Salmonella typhimurium* (stCA 1 and stCA 2) 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 | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) | $K_I$ (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 |
| 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 [b] | β | Medium | $6.4.10^5$ | $3.9.10^7$ | 63 |
| bsCA 2 [b] | β | High | $1.1.10^6$ | $8.9.10^7$ | 303 |
| stCA 1[c] | β | High | $1.0.10^6$ | $8.3.10^7$ | 59 |
| stCA 2 [c] | β | Medium | $7.9.10^5$ | $5.2.10^7$ | 84 |

[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 enzymes, stopped flow $CO_2$ hydrase assay method (pH 8.3), from Ref. 24.

is a homodimeric enzyme similar to all other bacterial/fungal β-CAs investigated so far.[13–19,21–27] The Zn(II) ion from the two active sites was shown to be coordinated by the four predicted amino acid residues found in other bacterial β-CAs, that is, Cys42, Asp44, His98, and Cys101, being found at the bottom of a long and narrow channel. These data thus envisage that stCA 1 (and presumably also stCA 2) may be druggable targets, considering that they have a rather deep active site cavity where structurally different inhibitors may bind effectively.

We have recently reported[24] that the catalytic activity of the purified two new enzymes stCA 1 and 2 is comparable to those of other α- and β-CAs known to be drug targets, such as the human enzymes hCA I, and II,[11] or the bacterial enzymes from *M. tuberculosis* (Rv1284 and Rv3273)[14] and *B. suis* (bsCA 1 and 2)[16]—Table 1. The catalytic activity of these β-CAs was very low or absent at pH values < 8 (data not shown). Both enzymes from *Salmonella* had appreciable activity as catalysts for the hydration of $CO_2$ to bicarbonate and protons, with a $k_{cat}$ of $7.9 \times 10^5$–$1.0 \times 10^6$ $s^{-1}$, and $k_{cat}/K_m$ in the range of $(5.2–8.3) \times 10^7$ $M^{-1}$ $s^{-1}$ (Table 1).[24]

### 3.2. stCA 1 and 2 inhibition with sulfonamides and sulfamates

In the preliminary report[24] we have investigated stCA 1 and stCA 2 inhibition with 37 inorganic anions and small molecules such as sulfamide, sulfamic acid and diethyldithiocarbamate. Whereas inorganic anions were ineffective millimolar inhibitors of these enzymes, both sulfamide and sulfamate, incorporating the classical zinc binding function (ZBF) of the $SO_2NH_2$ type,[11] showed effective, low micromolar affinity for both bacterial enzymes.24 Thus, we have decided to explore in details organic compounds incorporating such ZBFs, of the sulfonamide and sulfamate type.

Indeed, sulfonamides and their bioisosteres (sulfamates, sulfamides, etc.) constitute the main class of CAIs.[11,35] They bind to the catalytic zinc ion in deprotonated state, as sulfonamidate anions, substituting the nucleophilic hydroxide ion necessary for catalysis.[11,35] Many representatives of such derivatives have clinical applications as diuretics, antiglaucoma, antiobesity and anticancer agents.[11,20,35,36]

Data of Table 2 show the inhibition of stCA 1 and 2 with a pannel of 42 such derivatives, among which the simple aromatic/heterocyclic derivatives **1–26**, together with the clinically used compounds AAZ-HCT. Inhibition of the human physiologically relevant isoforms hCA I and II[11] is also provided in Table 2, for comparison reasons. The following structure–activity relationship (SAR) can be evidenced from data of Table 2, regarding inhibition of the new bacterial enzymes investigated here with sulfonamides/sulfamates:

(i) A large number of compounds, such as **1–12**, **23–26** and **ZNS, SLP, IND, CLX, VLX, SLT, SAC** and **HCT**, inhibited stCA 1 in the micromolar range, with inhibition constants in the range of 5.43–26.7 μM. It should be observed that most of them are simple aromatic mono- or dibenzenesulfonamides incorporating amino, hydrazino, methyl, aminoalkyl, carboxy, hydroxyalkyl and halogeno moieties (derivatives **1–12** and **23–26**). The same is true for the clinically used derivatives (**ZNS-HCT**) although they usually incorporate slightly more complex scaffolds, which are anyhow based on the benzenesulfonamide core (except for zonisamide and saccharin). It is interesting to note the close parallelism for the inhibition of the second enzyme, stCA 2, with these derivatives, which showed $K_I$s in the range of 4.22–14.8 μM. Sar is thus quite similar for the inhibition of both stCA 1 and stCA 2 with most of these sulfonamides. In most cases stCA 1 was slightly more inhibited than stCA 2 with these derivatives (althoough many exceptions were observed, e.g., **4, 7, 9, 12, VLX, SLT** and **SAC** were slightly better stCA 2 than stCA 1 inhibitors, Table 2).

(ii) A second group of derivatives, including **13, 14, 17, 20–22, MZA, EZA, DZA, BRZ** and **TPM**, were submicromolar stCA 1 inhibitors, with $K_I$s in the range of 124–770 nM (Table 2). Most of these compounds (except **17** and **MZA** which inhibited stCA 2 with $K_I$s < 100 nM) showed a similar behavior against stCA 2, with $K_I$s in the range of 338–860 nM. It can be observed that structurally they are rather heterogeneous, but most of them are either heterocyclic sulfonamides (**13, 14, 21, 22, MZA, EZA, DZA, BRZ**) or possess a rather elongated molecule incorporating the benzenesulfonamide zinc-binding function (e.g., **17, 20**). Topiramate, the only sulfamate included in our study, is also in this group of compounds.

(iii) Derivatives **15, 16, 18, 19, AAZ, DCP** and **BZA**, were the best stCA 1 inhibitors, with $K_I$s in the range of 51–91 nM. The same compounds, together with **17** and **MZA**, constituted the best stCA 2 inhibitors, with $K_I$s in the range of 31–96 nM nM. Thus, the best inhibitors are characterized by the presence of benzolamide-like structures (**15, 16** and **BZA** itself), aromatic benzolamide-like structures (i.e., elongated sulfonylated aminosulfonamides, such as **18** and **19**) as well as the simple **AAZ, MZA** and **DCP** scaffolds. Sar is thus quite well defined for the inhibition of stCA1 and stCA 2 with sulfonamides/sulfamates. It is noteworthy that highly effective CAIs targeting these bacterial enzymes have already been detected. For example **15, 16, 18** and **19** were the best stCA2 inhibitors with inhibition constants of 31–44 nM. Compounds **16** and **AAZ** were on the other hand the best stCA 1 inhibitors, with inhibition constants of 51–59 nM.

(iv) The inhibition profile of the two bacterial CAs investigated here are very different from those of the human isoforms hCA I and II, with this class of inhibitors. Most of the time the α-CAs were

better inhibited than the β-class enzyme by most of these sulfonamides/sulfamate investigated here (Table 2). However, considering that the 3D structure of many of the α-CAs and of stCA 1 are reported, it is possible to start rational drug design campains thaty may eventualy lead to bacterial CAs-selective inhibitors that should spare the human enzymes.

But what is the significance of these *in vitro* inhibition data for the in vivo situation ? A hypothesis can be made, considering the fact that Valdivia and Falkow[37] reported that mig-5 (this is how they nominated a protein whose function was unknown in 1997 when their work was published, before the sequencing of the *Salmonella* genome),[4] which is now known to encode for stCA 1, is 24-fold increased in macrophages upon infection with the bacterium. In the same work it has been noted that mig-5 (i.e., stCA 1) maps to the virulence plasmid of *S. typhimurium*, being located around 2 kb upstream of the *spv* virulence operon, which is required by the pathogen to cause systemic disease.[37] These data pinpoint to the fact that very probable, at least stCA 1 is a virulence factor for *Salmonella*, and that the protein is being accumulated notably in macrophages upon infection. Further work is warranted to understand whether inhibition of this β-CA (and eventually also of the second one, stCA 2) has consequences for the infectivity and dissemination of *Salmonella* infections.

## 4. Conclusions

Two β-carbonic anhydrases from the bacterial pathogen *S enterica*, serovar Typhimurium, stCA 1 and stCA 2, possess appreciable activity as catalysts for the hydration of $CO_2$ to bicarbonate, with a $k_{cat}$ of $(0.79–1.0) \times 10^6$ $s^{-1}$, and $k_{cat}/K_m$ of $(5.2–8.3) \times 10^7$ $M^{-1}$ $s^{-1}$. A large number of sulfonamides and one sulfamate have been investigated for inhibition of these enzymes. All types of inhibitors have been detected, with $K_I$s in the range of 31 nM–5.87 μM. The best stCA 1 inhibitors were acetazolamide and benzolamide-based compounds, whereas the best stCA 2 inhibitors were sulfonylated benzenesulfonamides and amino-benzolamide derivatives ($K_I$s in the range of 31–90 nM). As many strains of *S. enterica* show extensive resistance to classical antibiotics, inhibition of the β-CAs investigated here may be useful for developing novel antibacterials.

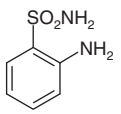
## 5. Experimental

### 5.1. Chemistry

Compounds **1**–**26** and **AAZ**–**HCT** are either commercially available (Sigma–Aldrich, Milan, Italy) or were prepared as described earlier.[31,32]

**Table 2**
Human (h) hCA I, II, and *Salmonella* enzyme (stCA 1 and stCA 2) inhibition data with compounds **1–26** and the clinically used derivatives **AAZ**–**HCT**

| Inhibitor | $K_I$* | | | |
|---|---|---|---|---|
| | hCA I[a] (μM) | hCA II[a] (μM) | stCA 1[b] (μM) | stCA 2[b] (μM) |
| **1** | 45,400 | 295 | 5.58 | 7.24 |
| **2** | 25,000 | 240 | 6.51 | 8.80 |
| **3** | 28,000 | 300 | 6.14 | 6.98 |
| **4** | 78,500 | 320 | 9.38 | 6.44 |
| **5** | 25,000 | 170 | 6.45 | 7.75 |
| **6** | 21,000 | 160 | 5.93 | 6.76 |
| **7** | 8300 | 60 | 6.55 | 4.22 |
| **8** | 9800 | 110 | 9.55 | 9.60 |
| **9** | 9650 | 73 | 7.06 | 6.81 |
| **10** | 14,000 | 124 | 6.91 | 7.00 |
| **11** | 5800 | 63 | 6.79 | 8.16 |
| **12** | 8400 | 75 | 7.30 | 7.03 |
| **13** | 8600 | 60 | 0.770 | 0.765 |
| **14** | 9300 | 19 | 0.671 | 0.703 |
| **15** | 6 | 2 | 0.068 | 0.044 |
| **16** | 1.4 | 0.3 | 0.051 | 0.038 |
| **17** | 40 | 5 | 0.124 | 0.096 |
| **18** | 164 | 46 | 0.091 | 0.040 |
| **19** | 185 | 50 | 0.072 | 0.031 |
| **20** | 109 | 33 | 0.246 | 0.631 |
| **21** | 95 | 30 | 0.642 | 0.860 |
| **22** | 690 | 12 | 0.424 | 0.338 |
| **23** | 55 | 80 | 6.82 | 7.54 |
| **24** | 21,000 | 125 | 5.75 | 6.09 |
| **25** | 23,000 | 133 | 6.01 | 8.19 |
| **26** | 24,000 | 125 | 12.1 | 12.9 |
| **AAZ** | 250 | 12 | 0.059 | 0.084 |
| **MZA** | 50 | 14 | 0.134 | 0.068 |
| **EZA** | 25 | 8 | 0.528 | 0.721 |
| **DCP** | 1200 | 38 | 0.090 | 0.095 |
| **DZA** | 50,000 | 9 | 0.445 | 0.607 |
| **BRZ** | 45,000 | 3 | 0.687 | 0.412 |
| **BZA** | 15 | 9 | 0.085 | 0.098 |
| **TPM** | 250 | 10 | 0.624 | 0.697 |
| **ZNS** | 56 | 35 | 5.43 | 5.70 |
| **SLP** | 1200 | 40 | 5.64 | 8.73 |
| **IND** | 31 | 15 | 6.86 | 6.90 |
| **CLX** | 50,000 | 21 | 5.83 | 6.11 |
| **VLX** | 54,000 | 43 | 6.85 | 6.58 |
| **SLT** | 374 | 9 | 6.80 | 4.67 |
| **SAC** | 18,540 | 5950 | 26.7 | 14.8 |
| **HCT** | 328 | 290 | 6.81 | 6.87 |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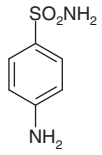
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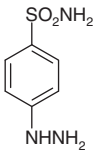
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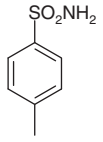
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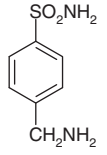
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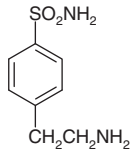
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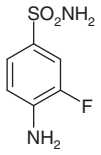
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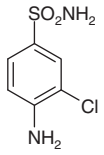
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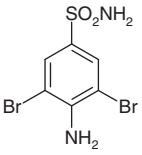
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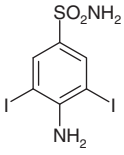
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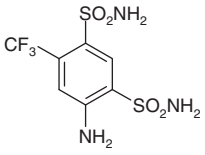
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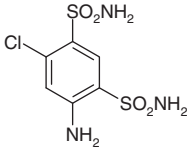
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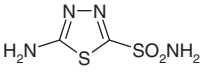
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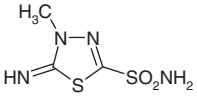
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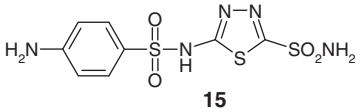
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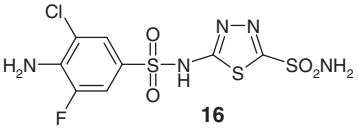
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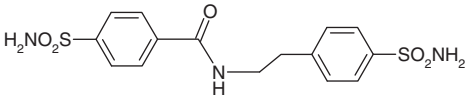
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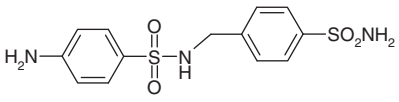
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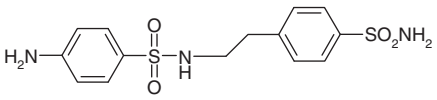
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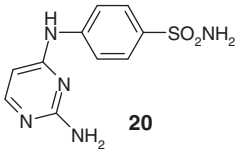
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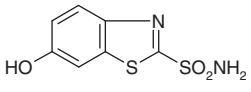
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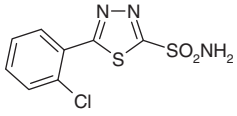
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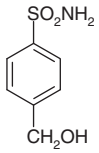
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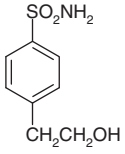
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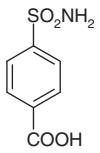
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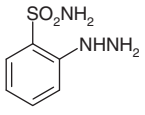
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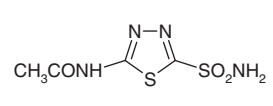


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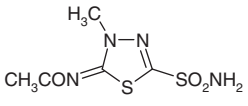


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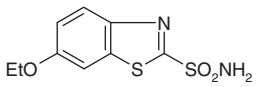


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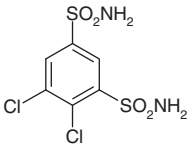


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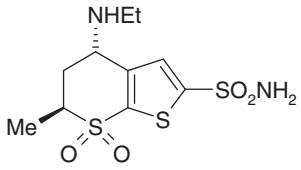


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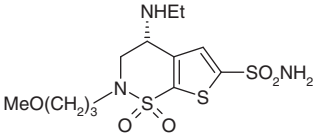


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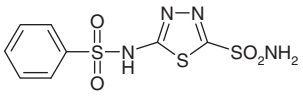


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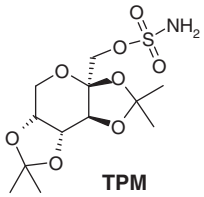


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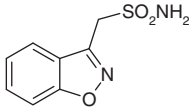


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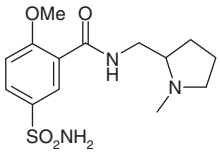
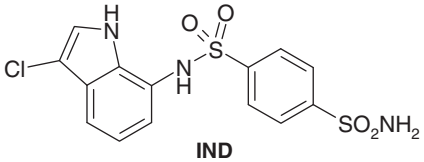
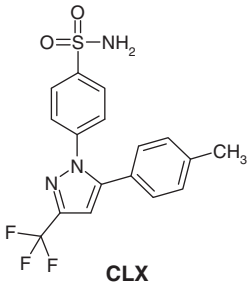


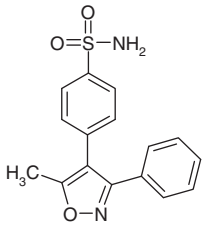

**SLP**



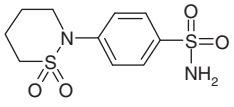

**IND**



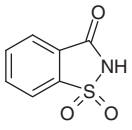

**CLX**



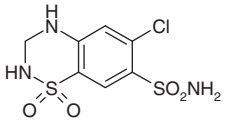

**VLX**




**SLT**




**SAC**




**HCT**

[*] 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, from Ref. 11,13c
[b] Recombinant stCA 1 and 2, stopped flow $CO_2$ hydrase assay method, this work.

### 5.2. Cloning, protein expression and purification of stCA 1 and 2

Preliminary data have been presented in ref.[24] Here we present all details regarding the cloning and purification of these enzymes. We purchased the genomic DNA, which was isolated from *Salmonella enterica* subspecies *enterica* serovar Typhimurium strain LT2 from American Type Culture Collection (ATCC 700720D-5™, Manassas, VA, USA). This serovar used to be one of the major

causes of food poisoning (gastroenteritis) and has been the principal laboratory strain for many important biochemical and genetic experiments, including classical phage transduction studies.[1–9] The sequences of the adopter primer pairs were as follows (*Eco*RI and *Sal*I recognition sequences were underlined and the extended Kozac sequence was double-underlined): 5′-CGGGAATTCCCAT GAAAGACATAGATACAC-3′ and 5′-CGGTCGACTTATTGGTGGGGA ATGTATTTC-3′ for stCA 1 and 5′-CGGGAATTCCCGCCGCCA CCATGG AACAAAACCAACCAG-3′ and 5′-CGGTCGACTTATACTTCGAA AAACT-CAACC-3′ for stCA 2.

It should be noted that we initially set a 5′-primer based on the wild-type sequence but failed to obtain the soluble protein product (data not shown). Accordingly, we added the consensus sequence for translation initiation (the extended Kozac sequence) to the 5′-end, which was shown by a survey of 699 vertebrate mRNAs (Protocol Online; http://www.protocol-online.org) and successfully obtained the protein product. The PCR reaction was hot-started with incubation for 2 min at 94 °C, consisted of 35 cycles of 15 s at 94 °C, 30 s at 58 °C and 1 min at 68 °C, followed by incubation for 5 min at 68°C. Proper DNA sequences of the PCR products were reconfirmed by direct sequencing using an ABI PRISM Dye Termination Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA) and an ABI 370A DNA sequencer (ABI, Foster City, CA, USA). The PCR products were cleaved with *Eco*RI and *Sal*I, and then ligated in-frame into the pGEX-4T2 vector (Amersham, Tokyo, Japan). The subcloned plasmid vectors were transfected into *E. coli* strain BL21 for production of the glutathione *S*-transferase (GST)-CA fusion protein as previously reported.[13,14] Following induction of the proteins expression by adding 1 mM isopropyl-γ-D-thiogalactopyranoside (IPTG) at 20 °C for stCA 1 and at 30 °C for stCA 2, the bacteria were harvested and sonicated in phosphate buffer. The sonicated cell extracts were further homogenized twice with a Polytron (Brin$K_m$ann) for 30 s each at 4 °C. Centrifugation at 30 Kg for 30 min afforded the supernatant containing the soluble proteins. The obtained supernatants were then applied to pre-packed Glutathione Sepharose 4B columns (Amersham, Milan, Italy). 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 (Sigma–Aldrich, Milan, Italy), as described earlier.[13,14] The obtained stCA recombinant proteins were further purified by sulfonamide affinity chromatography.[13,14]

### 5.3. CA catalytic activity and inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed $CO_2$ hydration activity.[30] 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 $NaClO_4$–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 non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver-Burk plots, as reported earlier,[13,14] and represent the mean from at least three different determinations.

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