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Bacterial carbonic anhydrases as drug targets: toward novel antibiotics?

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Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes which catalyze the hydration of carbon dioxide to bicarbonate and protons. Many pathogenic bacteria encode such enzymes belonging to the α-, β-, and/or γ-CA families. In the last decade, the α-CAs from *Neisseria* spp. and *Helicobacter pylori* as well as the β-class enzymes from *Escherichia coli, H. pylori, Mycobacterium tuberculosis*, *Brucella* spp., *Streptococcus pneumoniae*, *Salmonella enterica*, and *Haemophilus influenzae* have been cloned and characterized in detail. For some of these enzymes the X-ray crystal structures were determined, and *in vitro* and *in vivo* inhibition studies with various classes of inhibitors, such as anions, sulfonamides and sulfamates reported. Although efficient inhibitors have been reported for many such enzymes, only for *Neisseria* spp., *H. pylori, B. suis*, and *S. pneumoniae* enzymes it has been possible to evidence inhibition of bacterial growth *in vivo*. Thus, bacterial CAs represent promising targets for obtaining antibacterials devoid of the resistance problems of the clinically used such agents but further studies are needed to validate these and other less investigated enzymes as novel drug targets.

Keywords: carbonic anhydrase, alpha-class, beta-class, bacterial enzyme, sulfonamide, antibacterials, overcome resistance

INTRODUCTION

Resistance to antibiotics belonging to several different classes is escalating and represents a worldwide problem (Ginsberg, 2008; Dye, 2009; Furtado and Nicolau, 2010), as both Gram-negative and Gram-positive bacteria (such as among others *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Brucella suis, Streptococcus pneumoniae*, etc.,) no longer respond to many such drugs (Cloeckaert and Schwarz, 2001; Nickerson and Schurr, 2006; Bush and Macielag, 2010). Cloning of the genomes of many bacterial pathogens offers however the possibility to explore alternative pathways for inhibiting virulence factors or proteins essential for their life cycle (Suerbaum and Michetti, 2002; Payne et al., 2007; Showalter and Denny, 2008; Tsolis et al., 2008). Among the many such new possible drug targets explored recently, are a class of enzymes catalyzing a simple but physiologically relevant process, carbon dioxide hydration to bicarbonate and protons (Smith et al., 1999; Supuran, 2008, 2010a,b). These enzymes are denominated carbonic anhydrases (CAs, EC 4.2.1.1), and they are all metalloenzymes. Five different genetically distinct CA families are known to date, the α-, β-, γ-, δ-, and ζ-CAs (Pastorekova et al., 2004; Supuran, 2008, 2010a). Whereas α-, β-, and δ-CAs use Zn(II) ions at the active site, 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 (Supuran, 2008, 2010a,b). The 3D fold of the five enzyme classes are very different from each other (a nice example of convergent evolution – Supuran, 2010b), as it is their oligomerization state: α-CAs are normally monomers and rarely dimmers; 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 (Supuran, 2008, 2010a). Many representatives of all these enzyme classes have been crystallized and characterized in detail, except the δ-CAs (Supuran, 2008, 2010a,b). The mammalian CAs and their inhibition/activation have been recently reviewed (Supuran, 2008, 2010a,b) and no detailed discussion of these enzymes are presented in this review.

The α-CAs are present in vertebrates, protozoa, algae, and cytoplasm of green plants and in some *Bacteria*; the β-CAs are predominantly found in *Bacteria*, algae, and chloroplasts of both mono- as well as dicotyledons, but also in many fungi and some *Archaea* (Smith et al., 1999). In bacteria and fungi they are homodimers, as shown in **Figure 1** for one of the enzymes from *Salmonella enterica*, stCA 1 (Brunzelle et al., submitted; Vullo et al., 2011). The γ-CAs were found in *Archaea* and some *Bacteria*, whereas the δ- and ζ-CAs seem to be present only in marine diatoms (Smith et al., 1999; Supuran, 2008, 2010a,b). In most organisms these enzymes are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes (thoroughly studied in vertebrates), whereas in algae, plants and some bacteria they play an important role in photosynthesis and biosynthetic reactions. In diatoms δ- and ζ-CAs play a crucial role in carbon dioxide fixation (Smith et al., 1999; Zimmerman et al., 2007; Supuran, 2008, 2010a,b).

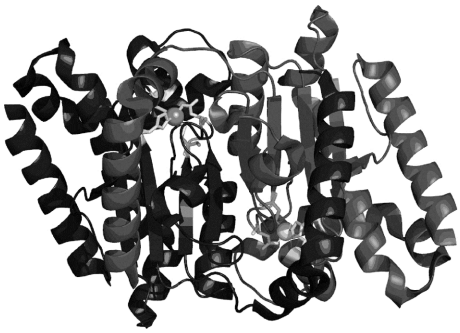


FIGURE 1 | View of the dimeric stCA 1 as obtained by X-ray crystallography (PDB file 3QY1). The polypeptide chains are represented as ribbons. The Zn(II) ions (gray spheres) and their ligands (Cys42, Asp44, His98, and Cys101) are shown as stick representation. The two active sites are identical and consist of a long channel at the bottom of which is found the Zn(II) ion in a tetrahedral geometry.

The classical CA inhibitors (CAIs) are the primary sulfonamides, RSO_2NH_2, which are in clinical use for more than 50 years as diuretics and systemically acting antiglaucoma drugs (Supuran et al., 2003; Supuran, 2008, 2010a,b). In fact there are around 30 clinically used drugs (or agents in clinical development) belonging to the sulfonamide or sulfamate class, which show significant CAI inhibitory activity (Supuran, 2008). However, it has emerged in the last years that sulfonamide/sulfamate CAIs have potential as anticonvulsant, antiobesity, anticancer, antipain, and antiinfective drugs (Supuran et al., 2003; Supuran, 2008, 2010a,b). All these drugs target in fact mammalian CAs, of which 16 different isoforms are known so far (Supuran et al., 2003; Supuran, 2008, 2010a,b).

Except vertebrates in which they have been extensively studied for decades as shown above, CAs are present in many human pathogens such as the malaria provoking protozoa *Plasmodium falciparum* (Krungkrai and Supuran, 2008; Krungkrai et al., 2008), bacteria such as *Escherichia coli* (Cronk et al., 2001), *H. pylori* (Nishimori et al., 2006, 2007, 2008), *M. tuberculosis* (Suarez Covarrubias et al., 2005, 2006; Carta et al., 2009; Güzel et al., 2009; Minakuchi et al., 2009; Nishimori et al., 2009, 2010; Davis et al., 2011), *Brucella* spp. (Joseph et al., 2010, 2011; Vullo et al., 2010; Winum et al., 2010), *S. pneumoniae* (Burghout et al., 2011), *S. enterica* (Vullo et al., 2011), and *Haemophilus influenzae* (Cronk et al., 2006; Hoffmann et al., 2011) as well as pathogenic fungi (Schlicker et al., 2009). Inhibition of these enzymes started to be investigated with sulfonamide/sulfamate inhibitors, but several other chemotypes were also explored, such as phenols, boronic acids, metal complexing anions, and other similar small molecules. As bacteria predominantly encode for β-class CAs, which are not present in vertebrates (Smith et al., 1999; Supuran, 2008), these enzymes started to be considered as possible drug targets for obtaining antibacterials devoid of the resistance problems mentioned above, which affect most classes of antibiotics in clinical use (Nishimori et al., 2010; Supuran, 2010a,b; Winum et al., 2010).

Here we review the current state-of-the art regarding the bacterial CAs cloned and characterized so far, as well as the *in vitro* and *in vivo* inhibition studies of these enzymes, which may reply to this stringent question: are the bacterial CAs future drug targets for obtaining conceptually novel antibiotics?

BACTERIAL α-CARBONIC ANHYDRASES AND THEIR INHIBITION

Table 1 shows the α-CAs cloned and characterized so far from pathogenic bacteria. The first one is an enzyme from *Neisseria gonorrhoeae* (Chirică et al., 1997; Elleby et al., 2001), although older report mention a similar CA in *N. sicca* and related species (which have not been cloned so far; Sanders, 1967; Adler et al., 1972). The *N. gonorrhoeae* CA contains 252 amino acid residues and has a molecular mass of 28 kDa, being quite homologous to mammalian CAs (Chirică et al., 1997). A comparison with the amino acid sequences of human isoforms hCA I and II suggested that the secondary structures are essentially identical in the bacterial enzyme but several loops are much shorter than in the human isoforms (Chirică et al., 1997). This has been confirmed thereafter by resolving the X-ray crystal structure of this enzyme (Elleby et al., 2001). Most of the active-site residues are indeed identical to those found in hCA II, the crucial Zn(II) ion being coordinated by three His residues and a water molecule/hydroxide ion, being placed at a bottom of a rather deep and large active site. The bacterial enzyme showed a high CO_2 hydrase activity, with a k_{cat} of 1.1×10^6 s^{-1} and Km of 20 mM (at pH 9 and 25°C; Chirică et al., 1997). The enzyme also showed esterase activity for the hydrolysis of 4-nitrophenyl acetate, similarly to the mammalian isoforms hCA I and II.

Several studies showed in fact much earlier that the activity and the growth of *N. sicca* and related species (*N. meningitides*, *N. gonorrhoeae*, and *N. lactamica* among others) were inhibited by the sulfonamide CAIs used clinically acetazolamide and ethoxzolamide (MacLeod and DeVoe, 1981; Vaneechoutte et al., 1988; Nafi et al., 1990). Such inhibition was completely overcome by the addition of exogenous bicarbonate, proving that the process was indeed mediated by the bacterial CA. Nafi et al. (1990) also observed that a number of bacterial strains including members of the genera *Pseudomonas, Staphylococcus, Streptococcus, Serratia*, and *Proteus* also strongly expressed gene products immunologically related to the *N. sicca* CA, but these enzymes were not characterized at that time (and except the *S. pneumoniae* one, see later in the text, even today.

But the best studied bacterial α-CA is the one from the gastric pathogen provoking ulcer and gastric cancer, *H. pylori*, hpαCA (Chirică et al., 2002; Marcus et al., 2005; Shahidzadeh et al., 2005; Nishimori et al., 2006, 2007) – see **Table 1**. The genome project of *H. pylori* identified in fact two different classes of CAs, with different subcellular localization: a periplasmic α-class CA (hpαCA) and a cytoplasmic β-class CA (hpβCA; Nishimori et al., 2008). These two CAs were shown to be catalytically efficient with almost identical activity to that of the human isoform hCA I, for the CO_2 hydration reaction, and highly inhibited by many sulfonamides/sulfamates, including acetazolamide, ethoxzolamide, topiramate, and sulpiride, all clinically used drugs (Nishimori et al., 2008). Furthermore, certain CAIs, such as acetazolamide and methazolamide, were shown to inhibit the bacterial growth in cell cultures (Nishimori et al., 2008). Since the efficacy of *H. pylori* eradication therapies currently employed has been decreasing due

Table 1 | CAs from pathogenic bacteria cloned and characterized so far, and their inhibition studies.

Bacterium	Family	Name	Inhibition study		Reference
			In vitro	*In vivo*	
Neisseria gonorrhoeae	α	–	Sulfonamides, anions	Sulfonamides	Chirică et al. (1997), Elleby et al. (2001)
Neisseria sicca	α	–	Sulfonamides	Sulfonamides	Adler et al. (1972), Sanders (1967)
Helicobacter pylori	α	hpαCA	Sulfonamides, anions	Sulfonamides	Chirică et al. (2002), Nishimori et al. (2006, 2007), Marcus et al. (2005), Shahidzadeh et al. (2005)
H. Pylori	β	hpβCA	Sulfonamides, anions	Sulfonamides	Chirică et al. (2002), Nishimori et al. (2006, 2007)
Escherichia coli	β	–	NI	NI	Cronk et al. (2006)
Haemophilus influenzae	β	HICA	Bicarbonate	NI	Cronk et al. (2006), Hoffmann et al. (2011)
Mycobacterium tuberculosis	β	mtCA 1	Sulfonamides	NA	Suarez Covarrubias et al. (2005, 2006)
	β	mtCA 2	Sulfonamides	NA	Minakuchi et al. (2009), Nishimori et al., 2009, 2010
	β	mtCA 3	Sulfonamides	NA	Güzel et al. (2009), Carta et al. (2009), Davis et al. (2011), Winum et al. (2010)
Brucella suis	β	bsCA 1	Sulfonamides	Sulfonamides	Joseph et al. (2010), Vullo et al. (2010)
	β	bsCA 2	Sulfonamides	Sulfonamides	Joseph et al. (2011), Winum et al. (2010)
Streptococcus pneumoniae	β	PCA	Sulfonamides, anions	NI	Burghout et al. (2010, 2011)
Salmonella enterica	β	stCA 1	Sulfonamides, anions	NI	Vullo et al. (2011)
	β	stCA 2	Sulfonamides, anions	NI	Vullo et al. (2011)
Vibrio cholerae	Unknown	–	Sulfonamide	Sulfonamide	Kovacikova et al. (2010), Abuaita and Withey (2009)

– Means not named; NA, no activity in vivo (presumably due to penetration problems); NI, not investigated.

to drug resistance and side effects of the commonly used drugs, the dual inhibition of α- and/or β-CAs of *H. pylori* could be applied as an alternative therapy in patients with *H. pylori* infection or for the prevention of gastroduodenal diseases provoked by this widespread pathogen (Nishimori et al., 2008). In fact, in a pilot study Shahidzadeh et al. (2005) showed the efficacy of acetazolamide in the treatment of gastric ulcer. This compound (as well as ethoxzolamide) were in fact widely used as antiulcer agents in the 70- and 80-s, although their mechanism of action was not properly understood at that time (Puscas, 1984).

BACTERIAL β-CARBONIC ANHYDRASES AND THEIR INHIBITION

As mentioned above, the β-CA class is the most widespread in bacteria (Smith et al., 1999; Supuran, 2008, 2010a,b). The proof-of-concept study that such an enzyme may be a drug target has been published recently by Nishimori et al. (2007) who cloned and purified the *H. pylori* enzyme (hpβCA), showing that it is highly susceptible to be inhibited by sulfonamides and sulfamates (see Discussion above for the *in vivo* data). Afterward, a rather large number of other β-CAs were cloned, purified, and characterized from other pathogens (**Table 1**). The X-ray crystal structures are also available for the *E. coli* (Cronk et al., 2001), *H. influenzae* (Cronk et al., 2006), two of the three *M. tuberculosis* enzymes (Suarez Covarrubias et al., 2005, 2006), and one *S. enterica* (stCA 1) β-CA (Brunzelle et al., submitted). The 3D folds of these enzymes are rather conserved and similar to the stCA 1 shown in **Figure 1**. The two active sites are identical, being rather long channels at the bottom of which is found the catalytic zinc ion, tetrahedrally coordinated by Cys42, Asp44, His98, and Cys101 (in stCA 1). This is the so called "closed active site," since these enzymes are not catalytically active (at pH values < 8.3; Suarez Covarrubias et al., 2005, 2006). However, at pH values > 8.3, the "closed active site" is converted to the "open active site" (with gain of catalytic activity), this being associated with a movement of the Asp residue from the catalytic Zn(II) ion, with the concomitant coordination of an incoming water molecule approaching the metal ion. This water molecule (as hydroxide ion) is in fact responsible for the catalytic activity, as for the α-CAs investigated in much greater detail (Suarez Covarrubias et al., 2005, 2006). It should be also mentioned that some β-CAs possess and open active site at all pH values (Schlicker et al., 2009).

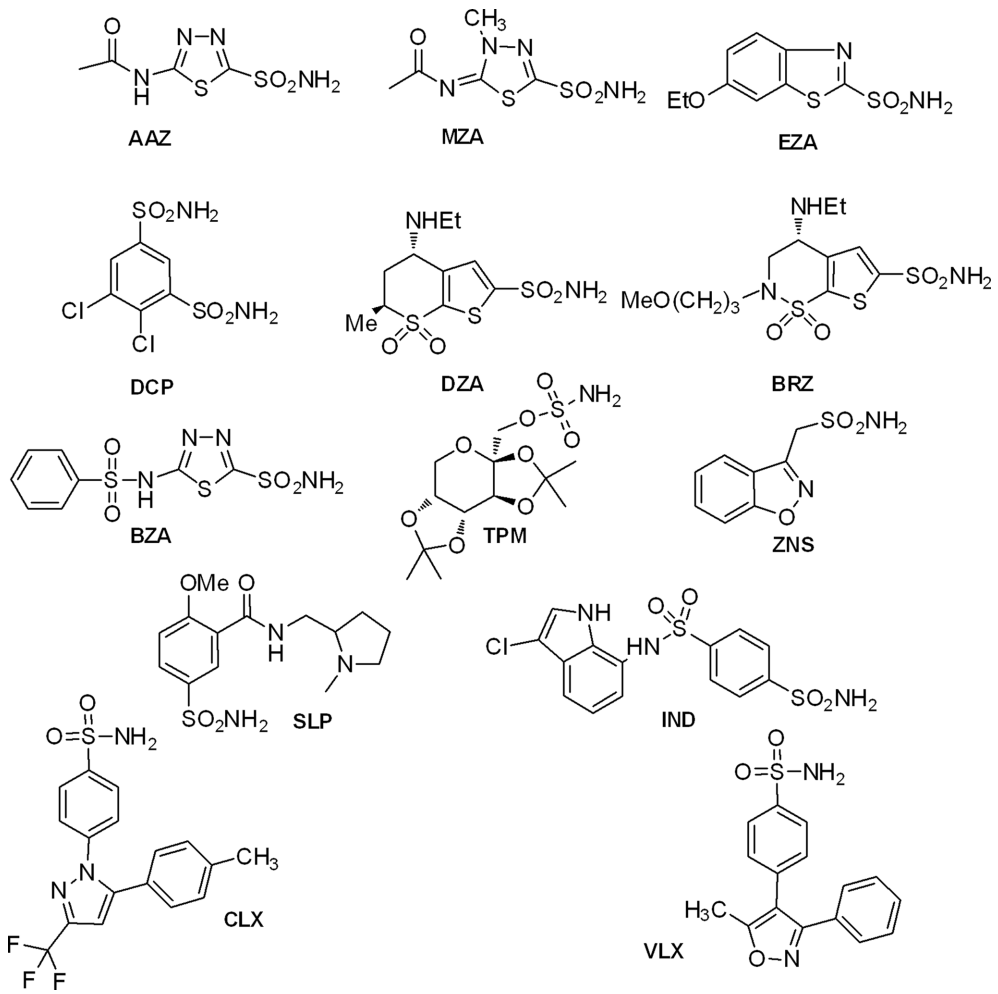
Many of these enzymes displayed excellent activity for the physiologic CO_2 hydration reaction and were inhibited (sometimes in the low nanomolar range) by sulfonamides and sulfamates (Nishimori et al., 2010; Supuran, 2010a,b; Winum et al., 2010). However, *in vivo*, it has been possible to observe inhibition of the bacterial growth only for *H. pylori*, *S. penumoniae*, and *B. suis* (Nishimori et al., 2008; Burghout et al., 2010; Winum et al., 2010). Especially in the case of *M. tuberculosis*, although nanomolar and sub-nanomolar *in vitro* inhibitors were detected (Güzel et al., 2009), no *in vivo* inhibition of growth has been observed, probably because the highly polar sulfonamides have difficulties to penetrate through the bacterial wall of these pathogens (Nishimori et al., 2010). Thus, much work is warranted in order to detect potent *in vitro* CAIs that also work *in vivo*, in order to validate these β-CAs as drug targets.

Table 2 shows the *in vitro* inhibition data of several of these enzymes with sulfonamide/sulfamates, which represent one of the main classes of CAIs (Supuran, 2008, 2010b). Such compounds are clinically used drugs, e.g., AAZ, acetazolamide; MZA, methazolamide; EZA, ethoxzolamide; DCP, dichorophenamide; DZA, dorzolamide; BRZ, brinzolamide; BZA, benzolamide; TPM, topiramate; ZNS, zonisamide; SLP, sulpiride; IND, indisulam; CLX, celecoxib; VLX, valdecoxib; as diuretics, antiepileptics, antiglaucoma, and antiinflammatory agents (Supuran, 2008, 2010b). It may be

Table 2 | ***In vitro*** **inhibition data of bacterial CAs with sulfonamides and sulfamates, some of which are clinically used drugs (only the enzymes for which these data were reported in the literature are included).**

Compound	Ki (μM)								
	hpαCA	**hpβCA**	**mtCA 1**	**mtCA 2**	**mtCA 3**	**bsCA 1**	**bsCA 2**	**stCA1**	**stCA2**
AAZ	0.021	0.040	0.481	0.009	0.104	0.063	0.303	0.059	0.084
MZA	0.225	0.176	0.781	0.660	0.562	0.054	0.642	0.134	0.068
EZA	0.193	0.033	1.03	0.027	0.594	0.017	0.420	0.528	0.721
DCP	0.378	0.105	0.872	2.01	0.611	0.058	0.112	0.090	0.095
DZA	4.36	0.073	0.744	0.099	0.137	0.021	0.923	0.445	0.607
BRZ	0.210	0.128	0.839	0.127	0.201	0.026	0.625	0.687	0.412
BZA	0.315	0.054	0.810	0.467	0.338	0.075	0.117	0.085	0.098
TPM	0.172	0.032	0.612	0.474	3.02	0.057	0.099	0.624	0.697
ZNS	0.231	0.254	28.68	0.876	0.208	1.85	0.406	5.43	5.70
SLP	0.204	0.035	2.30	0.266	7.92	0.019	0.084	5.64	8.73
IND	0.413	0.143	0.097	0.717	7.84	0.050	0.130	8.86	6.90
CLX	nt	nt	10.35	0.713	7.76	0.018	0.128	5.83	6.11
VLX	nt	nt	12.97	0.682	7.81	0.019	0.612	6.85	6.58

Nt, not tested.



observed that most CAs from bacterial pathogenic organisms are inhibited in the micro – nanomolar range by many such sulfonamide/sulfamate drugs. It should be mentioned that no rational drug design campaigns have been done to detect better CAIs targeting bacterial CAs so far, but the preliminary screening results summarized in **Table 2** are indeed promising, since a lot of effective led compounds have been detected. It is envisageable that more research in this area may lead to highly effective and bacterial CA selective compounds which may validate these enzymes as antibacterial drug targets.

As mentioned above, it is very probable that many β-CAs (or enzymes belonging to other CA families) are present in other bacterial pathogens. For example, in *Vibrio cholerae* it has been recently shown that sodium bicarbonate induces cholera toxin (CT) expression (Abuaita and Withey, 2009). Although the mechanism for bicarbonate-mediated CT induction has not been defined in detail, it has been demonstrated that bicarbonate stimulates virulence gene expression by enhancing ToxT (a regulatory protein that directly activates transcription of the genes encoding CT) activity (Abuaita and Withey, 2009). The sulfonamide CAI ethoxzolamide, inhibited bicarbonate-mediated virulence induction, suggesting that conversion of CO_2 into bicarbonate by a CA plays a role in virulence induction in *V. cholerae*. Thus, bicarbonate was the first positive effector for ToxT activity to be identified. Given that bicarbonate is present at high concentration in the upper small intestine where *V. cholerae* colonizes, bicarbonate is likely an important chemical stimulus that *V. cholerae* senses and that induces virulence during the natural course of infection (Abuaita and Withey, 2009; Kovacikova et al., 2010). However, the CA involved in these processes was not yet cloned and characterized, and as a consequence, their inhibition not properly understood.

CONCLUSION

By catalyzing the simple but highly important hydration of carbon dioxide to bicarbonate and protons, bacterial CAs are probably involved in critical steps of the bacterial life cycle, some of which are important for survival, invasion, and pathogenicity. Bacteria encode such enzymes belonging to the α-, β-, and/or γ-CA families, but up to now only the first two classes have been investigated in some detail in different species. Indeed, the α-CAs from *Neisseria* spp. and *H. pylori* as well as the β-class enzymes from *E. coli, H. pylori, M. tuberculosis*, *Brucella* spp., *S. pneumoniae*, *S. enterica*, and *H. influenzae* have been cloned and characterized. For some of these enzymes the X-ray crystal structures were determined at rather high resolution, allowing for a good understanding of the catalytic/inhibition mechanisms. However no adducts with inhibitors of these enzymes have been characterized so far, although *in vitro* and *in vivo* inhibition studies with various classes of inhibitors, such as anions, sulfonamides, and sulfamates have been reported. Efficient *in vitro* inhibitors have been reported for many such enzymes, but only for *Neisseria* spp., *H. pylori, B. suis*, and *S. pneumoniae* CAs it has been possible to evidence inhibition of bacterial growth *in vivo*. Thus, bacterial CAs represent at this moment very promising targets for obtaining antibacterials devoid of the resistance problems of the clinically used such agents but further studies are needed to validate these and other less investigated enzymes as novel drug targets.

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