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REVIEW ARTICLE

Inhibition of Bacterial Carbonic Anhydrases as a Novel Approach to Escape Drug Resistance

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Abstract: ***Background:*** Clinically used antibiotics act through one of these four mechanisms: cell wall biosynthesis inhibition, inhibition of protein biosynthesis, interference with DNA and RNA synthesis and the folate pathway.

Objective: The metalloenzymes carbonic anhydrases (CAs, EC 4.2.1.1) widespread in microorganisms and present as three genetically distinct families may be considered for the design of antiinfective agents with a different mechanism of action compared to the clinically used antibiotics. CAs are crucial for the life cycle of the pathogen, interfering with pH regulation and biosynthetic processes in which CO_2 or bicarbonate are substrates. CA inhibition was shown to lead to debilitation or growth defects of several pathogenic bacteria.

Method: CAs catalyzes the interconversion between carbon dioxide to bicarbonate, leading to the formation of protons, and thus affecting pH homeostasis. Several classes of CA inhibitors (CAIs) are known to date, among which the metal complexing anions, the unsubstituted sulfonamides, the dithiocarbamates, *etc.,* which bind to the Zn(II) ion of the enzyme either by substituting the non-protein zinc ligand or add to the metal coordination sphere.

Results: Effective inhibitors for many bacterial CAs belonging to the α-, β-, and γ-CA classes were detected, some of which inhibited bacterial growth *in vivo*. Few of the inhibitors investigated so far were also selective for the bacterial over the human CA isoforms, which may pose problems for their wide clinical applications.

Conclusion: Structure-based drug design campaigns might lead to the achievement of the desired selectivity/potency for preferentially inhibiting bacterial but not the host CAs.

Keywords: Carbonic anhydrase, Hydratase activity, Metalloenzymes, CA inhibitors, Pathogens, Selective inhibition, Antibacterial, Antiinfective, Sulfonamide, Anions, Inhibitor selectivity, Drug-design.

1. INTRODUCTION

1.1. Carbonic Anhydrases

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes that catalyze the reversible hydration of carbon dioxide with the production of bicarbonate and protons [1-4]. The CA classification uses Greek letters for the designation of the seven CA families known to date, i.e., the α, β, γ, δ, ζ η and θ-CAs [5-10]. Detailed biochemical, kinetic, phylogenetic and structural studies afforded a clear view regarding the differences between CA families. The catalytic function of CO_2 hydration is maintained for all CA classes, despite the diverse spatial reorganization of the active sites [5, 11-16]. In general the catalytic efficiency of the γ-, δ- and ζ-CAs is low compared to the β- and η-CAs, which in turn are less efficient catalysts compared to many bacterial α-CAs [6, 9, 11, 17]. Interestingly, γ-CAs use only CO_2 as substrate, while β-CAs can hydrolyze CO_2, H_2S and COS [18]. Furthermore, α-CAs not only hydrate CO_2, CS_2 but also possess esterase activity, with a range of esters of carboxylic, sulfonic or phosphate acids [6, 9, 11, 17, 19, 20]. Generally, bacterial genome encodes for three CA classes that in agreement with the CA family nomenclature are designed as α, β and γ [17]. CAs distribution pattern in bacteria is very intriguing [17]. In bacteria, CAs belonging to one class, two or three different genetic families were evidenced, whereas in few of them no CAs seem to be present, i.e., Gram-negative bacteria of the genera Buchnera and Rickettsia [17]. The evolutionary relationship of CAs from pro-

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karyotes is thus characterized by a high degree of complexity, not entirely understood at the present time.

Recently, we investigated the diverse CAs present in the genome of thermophilic and psychrophilic bacteria, whereas in mesophilic bacteria their presence was investigated for some species in the last 15 years [7-9, 12, 13, 17, 21-24]. The metal ion from the enzyme active site is coordinated by three His residues in the α-, γ- ,δ- and θ-classes, by one His, and two Cys residues in β- and ζ-CAs or by two His and one Gln residues in η-class with the fourth ligand being a water molecule/hydroxide ion acting as nucleophile in the catalytic cycle of the enzyme [9, 12, 17, 25]. The α-, β-, δ- and, probably η- and θ-CAs use Zn(II) ions at the active site, γ-CAs are Fe(II) enzymes but they are active also with bound Zn(II) or Co(II) ions, whereas ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis [6, 9, 12, 17, 26, 27]. The α-, β-, γ- and ζ-CAs have been crystallized, but not δ-, η- and θ-CAs. The three-dimensional structures of the different CA-classes, *e.g.* the the overall shape of the molecules, the protein folding patterns as well as the oligomeric state, are very different for the three CA classes present in bacteria [8, 28]. For example, α-CAs are normally monomers and rarely dimmers; β-CAs are dimers, tetramers or octamers; γ-CAs are trimers; the only ζ-CA crystallized so far has three slightly different active sites on the same polypeptide chain [8, 28]. Phylogenetic analysis has shown that the most recent form is the α-CA class, whereas the γ-class is the most ancient CA class, catalyzing only CO_2 hydration with a rather weak catalytic efficiency and is the only CA class mainly identified in *Archaea*, the most ancient microorganisms that exist on earth [6, 12, 17, 29].

1.2. CA Inhibitors

Several classes of CA inhibitors (CAIs) are known to date: (i) the metal ion binders (anion, sulfonamides and their isosteres, dithiocarbamates, xanthates, etc, Fig. **1A**); (ii) compounds which anchor to the zinc-coordinated water molecule/hydroxide ion (phenols, polyamines, thioxocoumarins, sulfocoyumarins, *etc.,* Fig. **1B**); (iii) compounds occluding the active site entrance, such as coumarins and their isosteres – Fig. **1C**, and (iv) compounds binding out of the active site, Fig. **1D** [24, 30-33].

Few such compounds apart the anions, sulfonamides and dithiocarbamates were investigated so far for the inhibition of the bacterial CAs [12].

1.2.1. Anions

Anions, such as the inorganic metal-complexing ones or more complicated species such as the carboxylates, are also known to bind to the CAs, but generally with less efficiency compared to the sulfonamides [30, 34, 35]. Anions may bind either the tetrahedral geometry of the metal ion or as trigonal–bipyramidal adducts, as shown (for the tetrahedral geometry in Fig. 1A). Anions are an important class of CAIs, which were useful for designing novel types of more complex inhibitors possessing an organic scaffold, which afforded the discovery of totally new families of such pharmacologic agents [24, 36-42].

1.2.2. Sulfonamides

The sulfonamides were the first antimicrobial drugs, discovered in 1935 by Domagk, and they paved the way for the antibiotic revolution in medicine [43]. Prontosil, the first such derivative showing effective antibacterial action was in fact a prodrug, obtained by the bioreduction of prontosil with formation sulfanilamide, 4-aminobenzenesulfonamide. After this bacteriostatic agent, a range of analogs has entered into clinical use (constituting the so-called sulfa drug class of antibacterials), and many of these compounds are still widely used (Fig. **2**) [44]. Derivative used clinically, among which acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide, bind in a tetrahedral geometry to the Zn(II) ion in deprotonated state (Fig. **1A**), with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II) and an extended network of hydrogen bonds, involving amino acid residues Thr199 and Glu106 (numbering system used for the human CA, isoform I), also participating to the anchoring of the inhibitor molecule to the metal ion [44]. The aromatic/heterocyclic part of the inhibitor interacts with hydrophilic and hydrophobic residues of the cavity.

1.2.3. Dithiocarbamates

Dithiocarbamates (DTCs) were discovered as a completely new class of CAIs only recently, after the investigation of some inorganic anions acting in this way [45-50]. Indeed, the simple inorganic compound trithiocarbonate (TTC, $CS_3$2-), [51] for which an X-ray crystal structure in complex with the isoform hCA II was available, showed that the inhibitor is bound to the Zn(II) ion from the enzyme catalytic site through one of its three equivalent sulfur atoms, whereas a second such atom interacted with the conserved Thr199 amino acid residue. As the third sulfur did not make any interactions with the enzyme, the DTCs were synthesized, in which the third sulfur is replaced with a nitrogen atom and organic scaffold. DTCs were shown to act as micromolar- low nanomolar CAIs against many CA isoforms as their organic scaffold participates in supplementary interactions with the enzyme active site. These compounds were also investigated for the inhibition of some pathogenic CAs such as those of *Mycobacterium tuberculosis, Porphyromonas gingivalis*, *etc.* [1, 47, 52, 53]. The DTCs showed effective CA inhibitory activity against many bacterial enzymes (Fig. **3**.)

2. RESULTS AND DISCUSSION

2.1. Carbonic Anhydrases and their Implication in Bacterial Virulence

At present, infectious diseases are the second-leading cause of death in the world and the emergence of antibiotic-resistant bacteria is an inevitable and widespread phenomenon, inherent to most drugs [54, 55]. The possibility to develop new antibacterial agents raised much interest recently. The main classes of antibiotics clinically used nowadays act towards the inhibition of four classical targets: a) cell wall biosynthesis; b) protein biosynthesis; c) DNA and RNA biosynthesis; d) folate biosynthesis [56]. Recently, carbonic

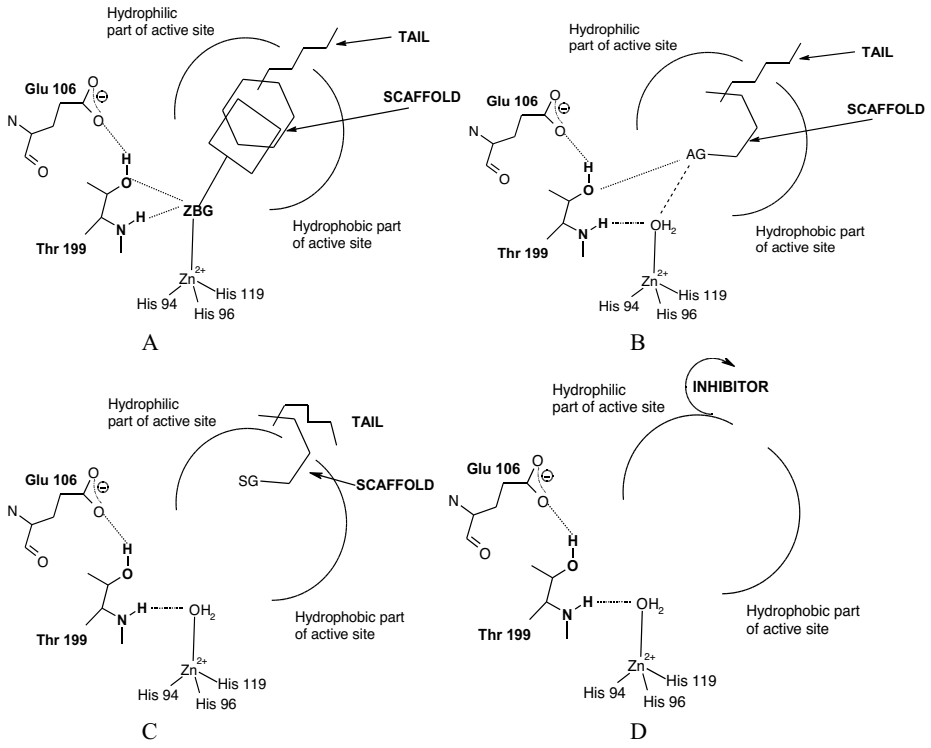

Fig. (1). CA inhibition mechanisms: A. Zinc-binding; B. Anchoring to the metal ion coordinated water; C. Occlusion of the active site entrance. D. Out of the active site binding.

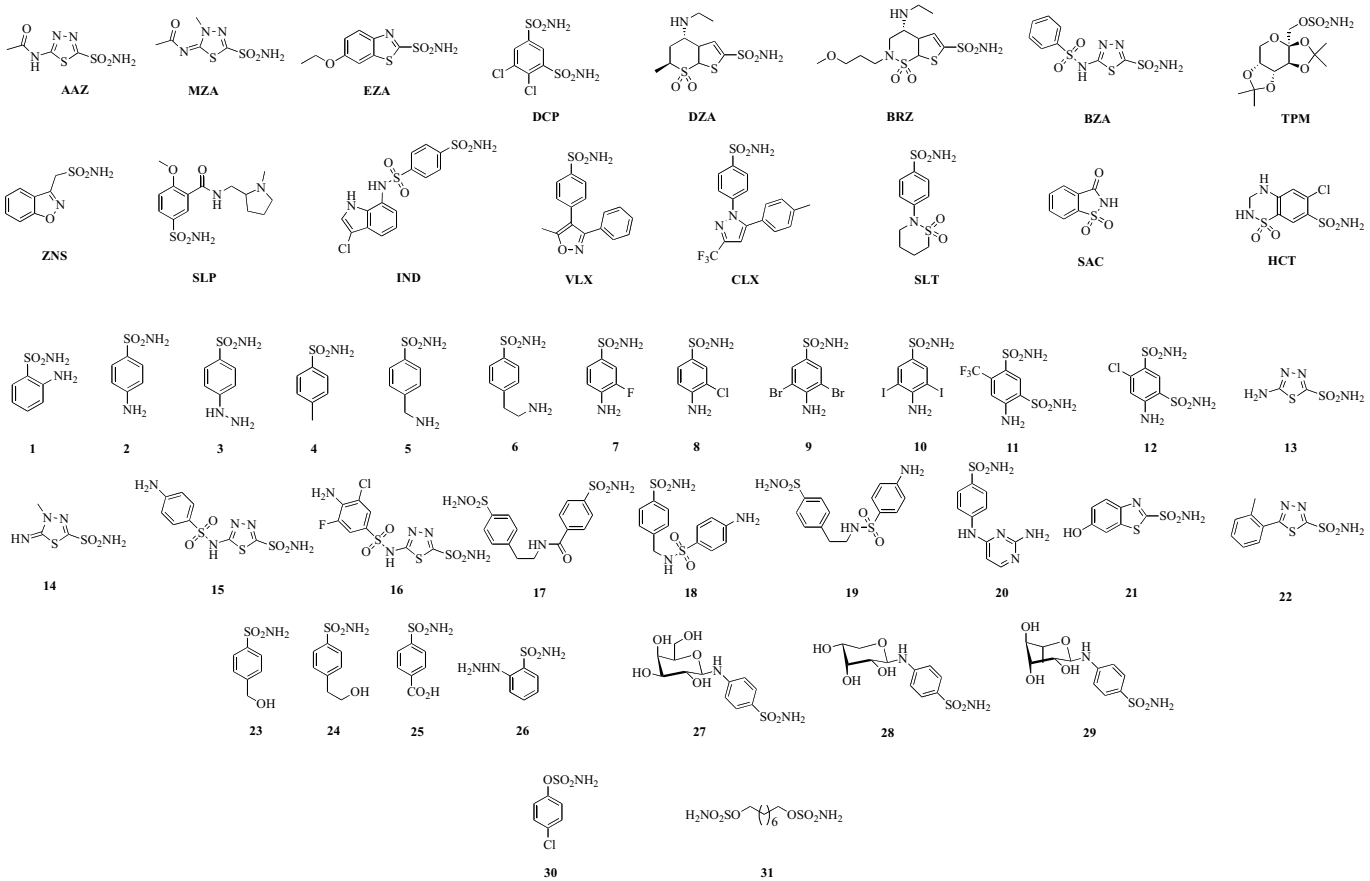

Fig. (2). Sulfonamides investigated as inhibitors of bacterial CA

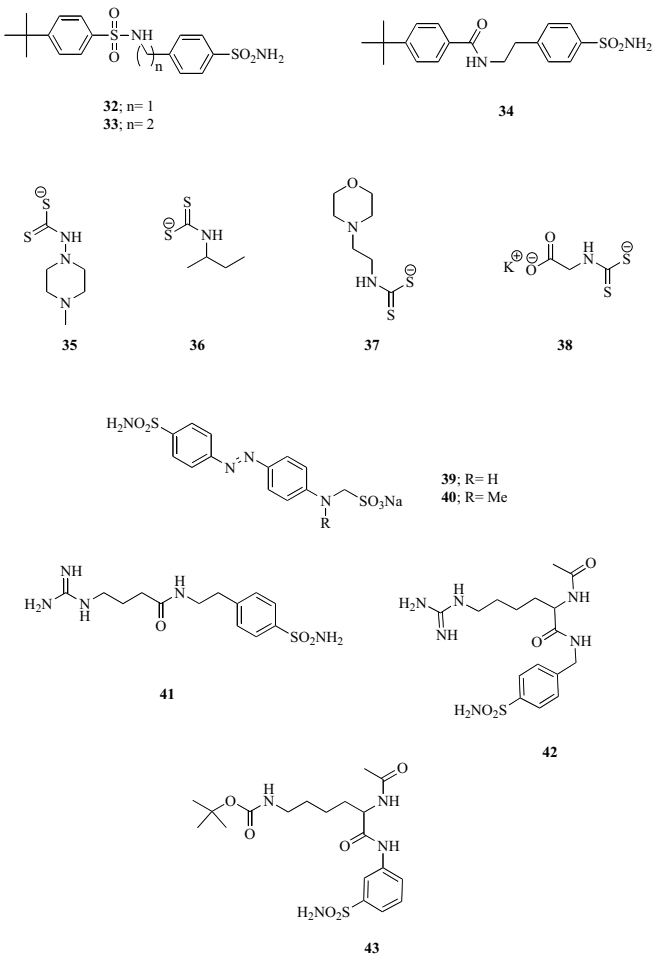

Fig. (3). Sulfonamides and dithiocarbamates **32-43** showing selective bacterial CA inhibitory properties.

anhydrases (CAs, EC 4.2.1.1) started to be investigated in detail in pathogenic bacteria, in the search for antibiotics with a novel mechanism of action, since it has been demonstrated that in many bacteria, CAs are essential for the life cycle of the microoorganism and that their inhibition leads to growth impairment or growth defects of the pathogen [24, 34, 57-59].

2.1.1. Vibrio Cholerae

The Gram-negative bacterium *Vibrio cholerae* is the causative agent of cholera [60-62]. The genome of *V. cholerae* encodes for putative CAs belonging to each bacterial class: α, β and γ. The α-CA has been cloned, purified, and characterized from our group and it was named VchCA [60-62]. Recently, our group purified the recombinant α- and β-CAs (named VchCA and VchCAβ, respectively) identified in the genome of this pathogenic bacterium. [60-63]VchCA was shown to possess a significant catalytic activity for the reaction that converts the CO_2 to bicarbonate and protons, with a k_{cat} of 8.23 x 10^5 s^{-1} and a k_{cat}/K_m of 7.0 x 10^7 M^{-1} x s^{-1} [61, 62], whereas VchCAβ had the following kinetic parameters: a k_{cat} of 3.34 x 10^5 s^{-1} and a k_{cat}/K_m of 4.1 x 10^7 M^{-1} x s^{-1}. [63]Moreover, our subsequent work allowed the crystallization of VchCAβ, showing that the zinc ion is coordinated by four amino acid residues, Cys42, Asp44, His98, Cys101, in an approximately tetrahedral geometry. VchCAβ belongs to the type II subclass β-CAs, characterized by four protein-derived ligands that coordinate the catalytic zinc ion, contrary to the type I β-CAs that have only two cysteines and one histidine residues in the zinc coordination sphere with a fourth coordination site occupied by a water molecule/hydroxide ion acting as nucleophile in the catalytic cycle [63]. The crystal structure was determined at pH 8.0, when a bicarbonate ion bound in a non-catalytic binding pocket close to the zinc ion was also observed [48]. At pH 8.3, the enzyme showed a significant catalytic activity for the physiological reaction of CO_2 hydration to bicarbonate and protons as demonstrated by the following kinetic parameters: k_{cat} of 3.34 x 10^5 s^{-1} and a k_{cat}/K_m of 4.1 x 10^7 M^{-1}x s^{-1} [63].This is in fact the situation for all type II β-CAs, which at pH values > 8 become of type I, with the asp residue being involved in a salt bridge with a neighboring conserved Arg, and an incoming water molecule/hydroxide ion replacing the Asp residue [49-54]. Recently it was reported that sodium bicarbonate induces cholera toxin (CT) expression [64, 65]. It was demonstrated that bicarbonate stimulates virulence gene expression by enhancing ToxT, a regulatory protein that directly activates transcription of the genes encoding CT activity [65]. Addition of CA inhibitors caused a significant reduction in virulence gene expression. Thus, bicarbonate was the first positive effector for ToxT activity to be identified. The bicarbonate ion is present at a high concentrations in the upper small intestine colonized by *V. cholera* suggesting that the potential of *V. cholera* CAs could be implicated in the microbial virulence [60-62].

2.1.2. Helicobacter pylori

Helicobacter pylori is a neutralophile with a bioenergetics profile suited for growing at neutral pH. This pathogenic bacterium lives in the highly acidic environment of the stomach (pH = 2-3). Its genome encodes three CAs: one α-, one β-, and one γ-CA, respectively [66-70]. The α-CA was shown to possess a periplasmic localization, the β-CA has been found in the cytoplasm, whereas no information is available on the expression/localization of the *H. pylori* γ-CA. *H. pylori* has developed exclusive adaptive mechanisms for growing in the highly acid environment of the stomach (pH between 1 and 2) [71]. These mechanisms involve the urease and the CA enzymes, which allow the bacteria to colonize the stomach by regulating the periplasmic pH and elevating the cytoplasmic pH trough the production of NH_3. Under acidic conditions, urea goes into the cytoplasm through the urea channel [3]. In the bacteria cytoplasm $2NH_3$ and CO_2 are produced by hydrolysis of urea. CO_2 in the cytoplasm is hydrated by the β-CA, while the CO_2 that diffused into the periplasm is converted to HCO_3- by the periplasmic α-CA. The produced ions, such as H^+ are used by NH_3 to form NH_4^+ in the periplasm and cytoplasm, respectively [3]. Thus, as showed in (Fig. **1**), the role of periplasmic α-CA and the cytoplasmic β-CA is to generate HCO_3- and protons, to both neutralize entering acid and buffer the periplasm and the cytoplasm [3]. The crucial role played by these CAs present in *H. pylori* is the acid acclimatization of the pathogen within the stomach [72, 73]. This role is corroborated by the *in vivo* inhibition studies carried out using inhibitors α and β-CAs in *H. pylori*. The inhibition of these two enzymes, in

fact, led to the death of the bacteria and a possible eradication of *H. pylori* from the stomach and has been used clinically for the treatment of gastric ulcers [66, 67].

2.1.3. Other Pathogenic Bacteria

CA enzymes were investigated in detail in other pathogenic bacteria (see Tables **1** and **2**). For some of them, X-ray crystal structures of the encoded CAs were also determined, and *in vitro* and *in vivo* inhibition studies with various classes of inhibitors, such as anions, sulfonamides and sulfamates have been reported [3, 32, 74-78]. Although the bacterial CA inhibition studies are in their infancy at this moment, cloning of more bacterial genomes may lead to the discovery of genes and proteins which may have interesting applications both in the biomedical and biotechnological fields [3, 32, 44, 74, 75, 78].

2.1.4. Two Pathogenic Bacteria from the Oral Cavity, P. gingivalis and S. mutans

Periodontal disease is a general term describing the inflammatory pathologic states of the supporting tissues of teeth, which can be grouped into two major categories, gingivitis and periodontitis. Gingivitis is defined as an inflammation of gingival tissues without affecting the attachment of teeth, while periodontitis involves the destruction of the connective tissue attachment to the tooth and the adjacent alveolar bone [79, 80]. Chronic adult periodontitis is the most common form of advanced periodontal disease. The microbes involved are extremely diverse and may be composed of more than 150 different species [81-83]. Among the bacteria regularly isolated from periodontal pockets, those producing such pathological states are generally gram-negative rods and include mainly *Porphyromonas gingivalis,* and other such as *Prevotella intermedia, Fusobacterium nucleatum, Actinobacillus actinomycetemcomitans, Capnocytophaga sputigena,* and *Wolinella recta* [84, 85]. As many other bacteria, the genome of *P. gingivalis* encodes for both a β- and γ-CA named with the acronyms PgiCAβ and PgiCA, respectively [8, 22, 28, 86-91].

Dental caries is a bacterial disease of the dental hard tissues (teeth); it is characterized by a localized, progressive, molecular disintegration of the tooth structure [92, 93]. Caries is associated with dental plaque of smooth coronal surfaces, pits, and fissures. Caries may also appear on root surfaces that are exposed to the oral environment as a result of gingival recession. The demineralization of teeth is caused by organic acid produced from the bacterial fermentation of carbohydrates present in the diet, which leads to a low pH (between 5.0 and 5.5) environment due to the selection of bacteria which are acid tolerating and capable of producing acid from carbohydrates [94, 95]. Among these bacteria, mutans streptococci, including *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus cricetus*, and *Streptococcus rattus*, were shown to be the most cariogenic. For example, the increased growth of *Streptococcus mutans* leads to the production of acid at a higher rate, enhancing demineralization of the tooth [94, 95]. In the genome of *S. mutans* a β-CA-encoding gene, denominated SMU_328, has been identified and the protein, indicated was indicated with the acronym SmuCA [96, 97].

Enzymes belonging to the β- and γ-CA classes in these two pathogens have been cloned, expressed, purified and characterized. PgiCA, PgiCAb and SmuCA showed significant catalytic activity for the physiologic reaction catalyzed by these enzymes, the hydration of carbon dioxide to bicarbonate and protons. Several inhibition studies with the main classes of classical CAIs, the anions and the sulfonamides, also led to the discovery of effective inhibitors belonging to both classes and directed towards all three enzymes [22, 28, 87, 91, 97]. Unfortunately, no *in vivo* inhibition of growth studies of the two pathogens in the presence of the CA inhibitors have been reported for the moment.

2.2. CA Inhibition Studies

2.2.1. VchCA and VchCAβ Sulfonamide Inhibition Studies

Sulfonamides and their bioisosteres (sulfamates and sulfamides) are the most investigated types of CA inhibitors. A library of 40 compounds, comprising 39 sulfonamides and one sulfamate (see Fig. **2**), investigated earlier as inhibitors of α, β or γ class CAs, were tested as inhibitors of the CAs here described [3, 4, 44, 98-100]. Derivatives **1–24** and **AAZ-HCT** are either simple aromatic/heterocyclic sulfonamides widely used as building blocks for obtaining new families of such pharmacological agents, or clinically used agents, among which acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** and dichlorophenamide **DCP** are the classical, systemically acting antiglaucoma CA inhibitors (CAIs). Dorzolamide **DZA** and brinzolamide **BRZ** are topically acting antiglaucoma agents, benzolamide BZA is an orphan drug belonging to this class of pharmacological agents, whereas topiramate **TPM**, zonisamide **ZNS** and sulthiame **SLT** are widely used antiepileptic drugs. Sulpiride **SLP** and indisulam **IND** were also shown by our group to belong to this class of pharmacological agents, together with the COX2 'selective' inhibitors celecoxib **CLX** and valdecoxib **VLX**. Saccharin and the diuretic hydrochlorothiazide **HCT** are also known to act as CAIs [3, 4, 44, 98-100].

2.2.1.1. VchCA

A VchCA inhibition study with sulfonamides and sulfamates led to the detection of a large number of low nanomolar inhibitors, among which were methazolamide, acetazolamide, ethoxzolamide, dorzolamide, brinzolamide, benzolamide, and indisulam (K_I values in the range 0.69−8.1 nM) [60, 62, 101, 102].

2.2.1.2. VchCAβ

A similar sulfonamide inhibition study of the other enzyme cloned so far in *V. cholerae*, VchCAβ, led to the detection of a large number of low nanomolar inhibitors, among which are methazolamide, acetazolamide, ethoxzolamide, dorzolamide, brinzolamide, benzolamide, and indisulam (KI values in the range 0.69-8.1 nM). Since ethoxzolamide was shown to inhibit this virulence *in vivo* [62], we proposed that VchCA might be a target for antibiotic development. Moreover, The best VchCAβ inhibitors were deacetylated acetazolamide and methazolamide and hydrochlorothiazide, which showed inhibition constants of 68.2 – 87.0 nM. Other compound, with medium potency against VchCAβ, (K_Is in

Table 1. CAs from pathogenic bacteria cloned, purified, characterized and subjected to extensive *in vitro* inhibition study.

Pathogen	CA class	Name	Inhibition study	
			Sulfonamides	Anions
Brucella suis	β	bsCA1	+	-
	β	bsCA2	+	-
Vibrio cholerae	α	VchCA	+	+
Helicobacter pylori	α	hpαCA	+	+
	β	hpβCA	+	+
Mycobacterium tuberculosis	β	mtCA 1	+	-
	β	mtCA 2	+	-
	β	mtCA 3	+	-
Clostridium perfringens	β	CpeCA	-	+
Neisseria gonorrhoeae	α	NgoCA	+	+
Neisseria sicca	α	NsiCA	+	-
Streptococcus pneumoniae	β	PCA	+	+
Salmonella enterica	β	stCA1	+	+
	β	stCA2	+	+
Haemophilus influenzae	β	HICA	-	+
Porphyromonas gingivalis	β	PgiCAb	-	+
	γ	PgiCA	+	+
Legionella pneumophila	β	LpCA1	+	+
	β	LpCA2	+	+

Table 2. Disease and characteristic of infectious caused by the pathogenic bacterium to its host.

Pathogen	Disease	Characteristic of infectious
Brucella suis	Brucellosis	A highly contagious zoonosis caused by ingestion of unpasteurized milk or undercooked meat
Vibrio Cholerae	Cholera	An infectious disease that causes severe watery diarrhea, which can lead to dehydration and even death if untreated.
Helicobacter pylori	Gastritis and gastric ulcers	An inflammation of the lining of the stomach.
Mycobacterium tuberculosis	Tuberculosis	An infectious that attacks the lungs, but can also affects other parts of the body.
Clostridium perfringens	Food poisoning	Depending on the type of infection, people can even die as a result of food poisoning.
Neisseria gonorrhoeae	Gonorrhea	A common human sexually transmitted infection.
Neisseria sicca	Septicemia	A potentially fatal whole-body inflammation
Streptococcus pneumoniae	Pneumonia	An inflammatory condition of the lung affecting primarily the microscopic air sacs known as alveoli.
Salmonella enterica	Salmonellosis	An infection that develop diarrhea, fever, vomiting, and abdominal cramps 12 to 72 hours after infection.
Haemophilus influenzae	Influenza	An infectious disease that can produce nausea and vomiting or gastroenteritis
Porphyromonas gingivalis	Periodontitis, rheumatoid arthritis	A set of inflammatory diseases affecting the tissues that surround and support the teeth. Rheumatoid arthritis is an infectious often leading to the destruction of articular cartilage.
Legionella pneumophila	Legionellosis	Is a form of pneumonia

the range of 275 – 463 nM), were sulfanilamide, metanilamide, sulthiame and saccharin whereas the clinically used agents such as acetazolamide, methazolamide, ethoxzolamide, dorzolamide, zonisamide and celecoxib were micromolar inhibitors (K_Is in the range of 4.51 – 8.57 μM). Identification of potent and possibly selective inhibitors of VchCA and VchCAβ may lead to pharmacological tools useful for understanding the physiological role(s) of this under-investigated enzyme [60-62].

2.2.2. VchCA Anion Inhibition Studies

Many inorganic anions and several small molecules were investigated as VchCA inhibitors. Inorganic anions such as cyanate, cyanide, hydrogen sulfide, hydrogen sulfite, and trithiocarbonate were effective VchCA inhibitors with inhibition constants in the range of 33-88μM. Other effective inhibitors were diethyldithiocarbamate, sulfamide, sulfamate, phenylboronic acid and phenylarsonic acid, with KIs of 7-43μM. Halides (bromide, iodide), bicarbonate and carbonate were much less effective VchCA inhibitors, with KIs in the range of 4.64-28.0mM. The resistance of VchCA to bicarbonate inhibition may represent an evolutionary adaptation of this enzyme to living in an environment rich in this ion, such as the gastrointestinal tract, as bicarbonate is a virulence enhancer of this bacterium.

2.2.3. hpαCA and hpβCA Sulfonamide Inhibition Studies

2.2.3.1. hpαCA

Dorzolamide and simple 4-substituted benzenesulfonamides were weak hpCA inhibitors (inhibition constants, K(I)s, in the range of 830-4310 nM). [103, 104] Sulfanilamide, orthanilamide, some of their derivatives, and indisulam showed better activity (K(I)s in the range of 310-562 nM), whereas most of the clinically used CA inhibitors, such as methazolamide, ethoxzolamide, dichlorophenamide, brinzolamide, topiramate, zonisamide, *etc.,* acted as medium potency hpCA inhibitors (K(I)s in the range of 124-287 nM) [70] .Some potent hpCA inhibitors were detected too (K(I)s in the range of 20-96 nM) such as acetazolamide, 4-amino-6-chloro-1,3-benzenedisulfonamide, 4-sulfanilyl-aminoethyl-benzenesulfonamide, and 4-(2-amino-pyrimidin-4-yl)-benzenesulfonamide [70] .

2.2.3.2. hpβCA

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.

2.2.4. hpαCA and hpβCA Anion Inhibition Studies

The gastric pathogen *Helicobacter pylori* encodes two carbonic anhydrases (CAs, EC 4.2.1.1), an α- and a β-class one, hpαCA and hpβCA, crucial for its survival in the acidic environment from the stomach. Sulfonamides, strong inhibitors of these enzymes, block the growth of the pathogen, *in vitro* and *in vivo*.

2.2.4.1. hpαCA

hpαCA was inhibited in the low micromolar range by diethyldithiocarbamate, sulfamide, sulfamic acid, phenylboronic acid, and in the submillimolar one by cyanide, cyanate, hydrogen sulfide, divanadate, tellurate, perruthenate, selenocyanide, trithiocarbonate, iminodisulfonate. [105]

2.2.4.2. hpβCA

hpβCA generally showed a stronger inhibition with most of these anions, with several low micromolar and many submillimolar inhibitors detected. [105]These inhibitors may be used as leads for developing anti-H. pylori agents with a diverse mechanism of action compared to clinically used antibiotics.

2.2.5. SmuCA, PgiCAb and PgiCA Sulfonamide Inhibition Studies

2.2.5.1. SmuCA

SmuCA was efficiently inhibited by most sulfonamides investigated so far (K_Is of 246 nM–13.5 μM) – Table **3**. The best SmuCA inhibitors were bromosulfanilamide **9**, deacetylated acetazolamide **13**, 4-hydroxybenzenesulfonamide **15**, the pyrimidine-substituted sulfanilamide derivative **19**, aminobenzolamide **20** and compounds structurally similar to it, as well as acetazolamide, methazolamide, indisulam and valdecoxib. These compounds showed inhibition constants ranging between 246 and 468 nM [96, 97] (Table **3**).

2.2.5.2. PgiCAb

Many of the clinically used sulfonamides as well as simple aromatic/heterocyclic sulfonamides were ineffective as PgiCAb inhibitors, whereas better inhibition was observed with simple derivatives such as metanilamide **1**, 1,3-Benzenedisulfonamide **3,** 4-aminoethylbenzenesulfonamide **5** (K_Is of 364–477 nM). The halogenosulfanilamides incorporating heavy halogens (compounds **8-10**), 4-hydroxy- and 4-hydroxyalkyl-benzenesulfonamides (compounds **15-17**) were ineffective PgiCAb inhibitors with K_Is in the micromolar range. The best inhibitors were **AAZ** and **EZA**, with K_Is of 214–280 nM [22] (Table **3**).

2.2.5.3. PgiCA

DCP, **TPM** and many simple aromatic/heterocyclic sulfonamides were ineffective as PgiCA inhibitors, whereas the best inhibition was observed with halogenosulfanilamides incorporating heavy halogens (compounds **9** and **10**), 4-hydroxy- and 4-hydroxyalkyl-benzenesulfonamide (compounds **15-17**), **AAZ, MZA, ZNS, IND, CLX, SAC and HCT** (K_Is in the range of 131–380 nM) [22, 28, 87]. Moreover, novel quinazoline derivatives endowed with a sulfonamide functionality at position-2 were tested for their ability to inhibit PgiCA [90]. Six such compounds were highly effective, nanomolar inhibitors of the pathogenic enzyme. Three of them were also highly effective sub-nanomolar inhibitors of the cytosolic human isoform II (hCA II). The best PgiCA inhibitor was a compound with K_I of 3.53 nM against the bacterial enzyme. Many of these new compounds showed a high selectivity for bacterial enzyme respect to the mammalian CA isoforms hCA I and hCA II [22, 28, 87] (Table **3**).

2.2.6. SmuCA, PgiCAb and PgiCA Anion Inhibition Studies

Inhibition data of the three CAs from the pathogenic bacteria of the oral cavity with a range of inorganic/organic

Table 3. Inhibition data and inhibitor selectivity of various compounds against human isoform hCA II (off-target) and bacterial CAs considered in the present paper. Inhibition data were obtained by a Stopped-Flow CO_2 hydrase assay, while the inhibitor selectivity is represented by the human K_I/bacterial K_I ratio. For acronyms see Table 1.

	K_I							Inhibitor selectivity *vers*us hCA II					
Compound	**hCAII**	**bsCA1**	**bsCA2**	**VchCA**	**hpαCA**	**hpβCA**	**PCA**	**bsCA1**	**bsCA2**	**VchCA**	**hpαCA**	**hpβCA**	**PCA**
SLP	40 nM	19 nM	-	-	-	-	-	2.10	-	-	-	-	-
20	33 nM	21 nM	-	-	-	-	-	1.57	-	-	-	-	-
14	19 nM	-	11.2 nM	-	-	-	-	-	1.69	-	-	-	-
27	25 nM	9.2 nM	10.1 nM	-	-	-	-	2.71	2.47	-	-	-	-
IND	15 nM	-	-	8.1 nM	-	-	-	-	-	1.85	-	-	-
BZA	9 nM	-	-	4.2 nM	-	-	-	-	-	2.14	-	-	-
EZA	8 nM	-	-	0.69 nM	-	-	-	-	-	11.59	-	-	-
MZA	14 nM	-	-	3.6 nM	-	-	-	-	-	3.88	-	-	-
21	30 nM	-	-	4.7 nM	-	-	-	-	-	6.38	-	-	-
32	104 nM	-	-	-	31 nM	-	-	-	-	-	3.35	-	-
33	94 nM	-	-	-	27 nM	45 nM	-	-	-	-	3.48	2.08	-
34	127 nM	-	-	-	62 nM	44 nM	-	-	-	-	2.04	2.88	-
FSO_3-	0.46 mM	-	-	-	-	-	0.060 mM	-	-	-	-	-	7.6
$V_2O_7$2-	0.57 mM	-	-	-	-	-	0.038 mM	-	-	-	-	-	15
SeO_4 2-	112 mM	-	-	-	-	-	0.044 mM	-	-	-	-	-	2545
35	33.0 nM	4.7 nM	-	2.6 nM	-	-	-	7.02	-	14.34	-	-	-
36	29.4 nM	6.0 nM	-	3.6 nM	-	-	-	4.9	-	8.16	-	-	-
37	36.3 nM	7.1 nM	-	2.8 nM	-	-	-	5.11	-	12.96	-	-	-
38	325 nM	7.8 nM	-	8.3 nM	-	-	-	41.66	-	30.15	-	-	-
39	105 nM	-	59 nM	-	-	-	-	-	1.77	-	-	-	-
40	104 nM	-	45 nM	-	-	-	-	-	2.31	-	-	-	-
Et_2NCS_2-	3.1 mM	-	-	-	0.36 mM	-	-	-	-	-	8.61	-	-
$SeO_4$2-	112 mM	-	-	-	0.47 mM	-	-	-	-	-	238.29	-	-
$H_2NSO_2NH_2$	1.13 mM	-	-	-	0.009 mM	-	-	-	-	-	125.55	-	-
$PhAsO_3H_2$	49.2 mM	-	-	-	0.007 mM	-	-	-	-	-	7028	-	-
AAZ	12 nM	-	-	-	-	59 nM	84 nM	-	-	-	-	-	-
MZA	14 nM	-	-	-	-	134 nM	68 nM	-	-	-	-	-	-
18	46 nM	-	-	-	-	91 nM	40 nM	-	-	-	-	-	1.15

(Table 3) contd….

	K_I					Inhibitor selectivity *vers*us hCA II			
Compound	**hCAII**	**PgiCAb**	**PgiCA**	**LpCA1**	**LpCA2**	**PgiCAb**	**PgiCA**	**LpCA1**	**LpCA2**
$PhAsO_3H_2$	49.2 mM	0.076 mM	-	-	-	647	-	-	-
Et_2NCS_2-	3.1 mM	-	0.004 mM	-	-	-	775 nM	-	-
$PhB(OH)_2$	23.1 mM	0.077 mM	0.0098 mM	-	-	300	2357 nM	-	-
41	847 nM	-	40.1 nM	-	-	-	21.12	-	-
42	824 nM	-	80.7 nM	-	-	-	10.21	-	-
43	5100 nM	-	54.2 nM	-	-	-	94.09	-	-
19	30 nM	-	-	40.3 nM	25.2 nM	-	-	-	1.19 nM

- no selective cause for this enzyme detected so far.

small molecule inhibitors/anions have been recently obtained by our groups and are shown in Table **2**.

2.2.6.1. SmuCA

SmuCA is inhibited by cyanate, carbonate, stannate, divannadate and diethyldithiocarbamate in the submillimolar range ($K_{Is}$of 0.30–0.64 mM) and more efficiently by sulfamide, sulfamate, phenylboronic acid and phenylarsonic acid (K_{Is} of 15–46 μM) [97] (Table **2**).

2.2.6.2. PgiCAb

PgiCAb is inhibited by cyanate and diethyldithiocarbamate in the submillimolar range (K_{Is} of 0.23–0.76 mM) and more efficiently by sulfamide, sulfamate, phenylboronic acid and phenylarsonic acid (K_{Is} of 60–78 μM) [86] (Table **2**).

2.2.6.3. PgiCA

Inorganic anions suchas thiocyanate, cyanide, azide, hydrogen sulfide, sulfamate and trithiocarbonate are effective PgiCA inhibitors with inhibition constants in the range of 41–97 μM. Other effective inhibitors are diethyldithiocarbamate, sulfamide, and phenylboronic acid, with K_{Is} of 4.0–9.8 μM [28, 91] (Table **2**).

3. SELECTIVE INHIBITORS FOR THE BACTERIAL OVER THE HUMAN CA ISOFORMS

Among the set of 47 sulfonamides/sulfamates investigated as inhibitors of many bacterial (as well as human) CAs, few compounds showed selectivity for the inhibition of the pathogen over the host enzyme (Table **3**). Indeed, most of these compounds showed better inhibitory properties against the mammalian over the bacterial enzymes. For example, against VchCA, the most selective sulfonamide inhibitors were BZA, EZA, MZA and **21**, which had selectivity ratios for inhibiting the bacterial enzyme over hCA II in the range of 2.14 – 11.6.The *Helicobacter* enzymes, hpαCA and hpβCA, also had several slightly selective inhibitors, such as **21** and**3**, which showed selectivity ratios of 2.04 – 3.48 against the α-class enzyme and of 2.08 – 2.88 against the β-class one. In the case of the two *Porphyromonas gingivalis* enzymes PgiCA and PgiCAb, some isoform selective inhibitors were discovered recently. For the γ-class enzyme the most selective compounds were diethyldithiocarbamate, phenylboronic acid and the newly reported sulfonamides **41-43**, which incorporate new scaffolds. These derivatives showed selectivity ratios of 10.2 – 2357 (very good one). On the other hand, for the β-class enzyme PgiCAb only phenylarsonic and phenylboronic acid showed a good selectivity profile over hCA II, with ratios of 300-647.

CONCLUSION

In last ten year a multitude of new CAs were detected, cloned and characterized in many pathogenic bacteria, such as *H. pylori, B. suis, V. cholerae, S. tiphimurium, L. pneumophila, S. pneumoniae, M. tuberculosis, C. perfringens, P. gingivalis*, *etc.* Enzymes belonging to the α-, β- and/or γ-CA classes were detected in these pathogens. Many of these enzymes were shown to possess a significant catalytic activity for the CO_2 hydration reaction, and furthermore, it has been demonstrated that in some of these pathogens they are crucial for the life cycle of the bacterium. *In vitro* inhibition studies were performed for many of these enzymes with inorganic anions, small molecules such as boronic acids, phenylarsonic acid, sulfamic acid, sulfamide, sulfonamides, and dithiocarbamates. In many cases effective inhibitors were detected, some of which also inhibited the bacterial growth *in vivo*. However, very few of the detected inhibitors were also selective for the bacterial over the human, such as hCA II isoform. This is in fact one of the main challenges in proposing CAIs as novel anti-infective with a new mechanism of action. Some recent work, in which highly selective *P. gingivalis* CA inhibitors (over hCA II) were detected, however demonstrates that this goal is not an impossible one. In fact, by using structure-based drug design processes we estimate that it will be possible to achieve the desired selectivity for inhibiting preferentially the bacterial but not the host CA isoforms.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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