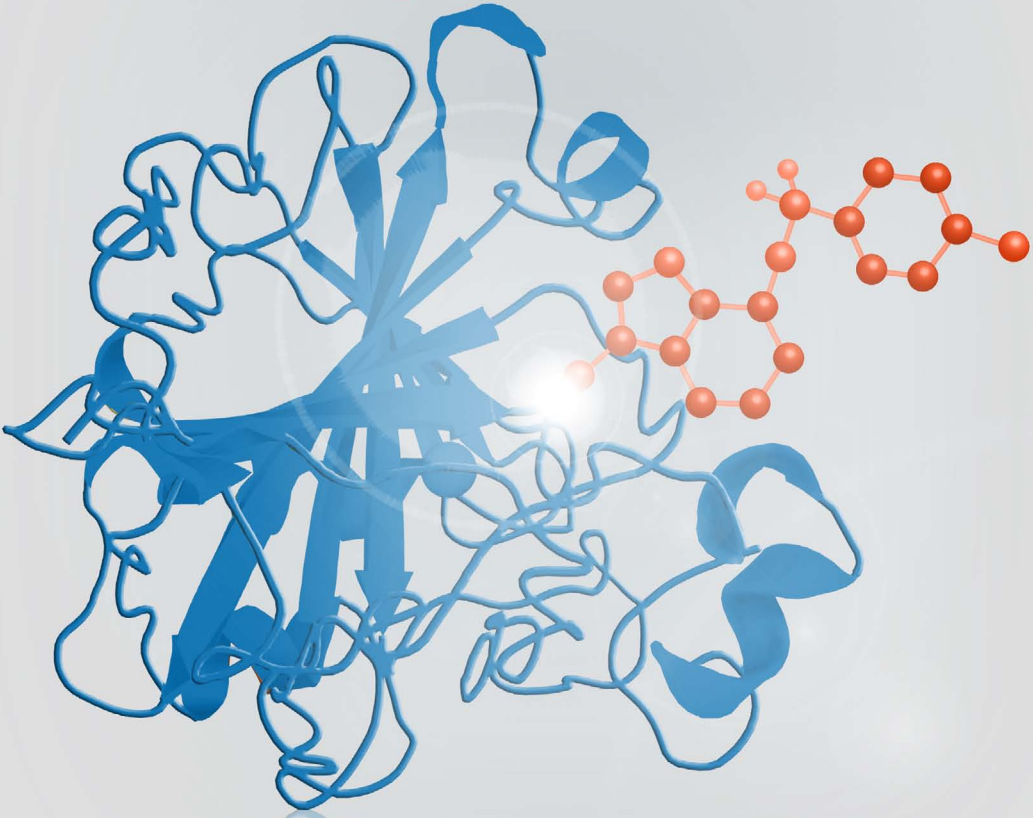
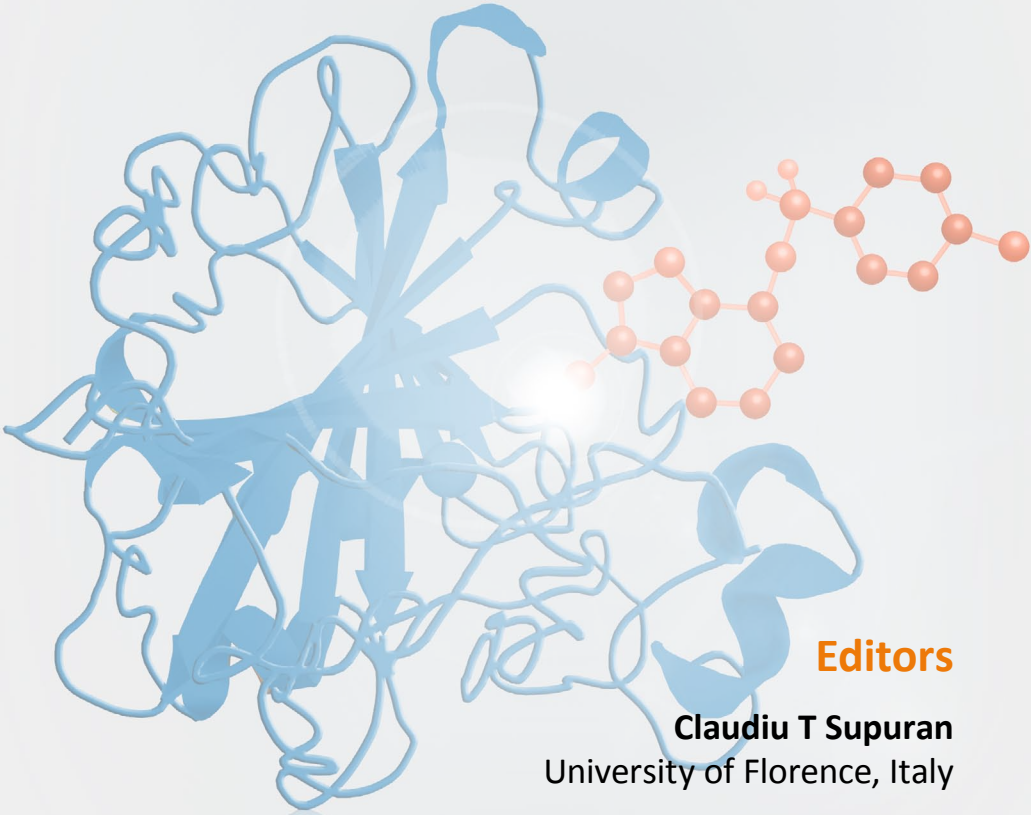
Targeting Carbonic Anhydrases



Claudiu T Supuran
& Clemente Capasso

FUTURE SCIENCE

Targeting Carbonic Anhydrases



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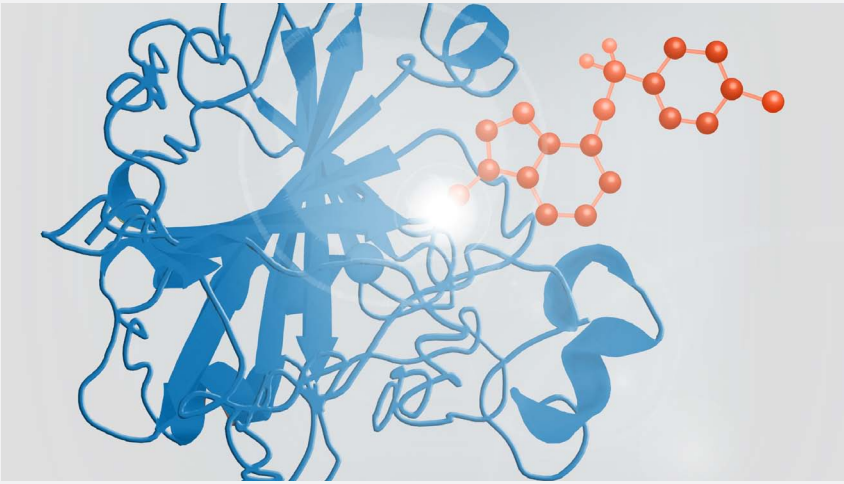

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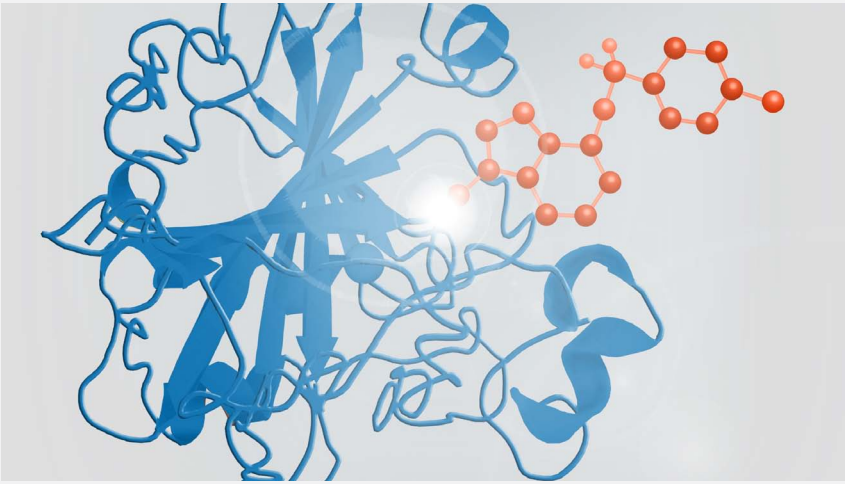

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About the Editors

Clemente Capasso

Clemente Capasso obtained his MSc in biology *cum laude* at the University of Naples 'Federico II' (Italy). His research interest at the Italian National Research Council (CNR; Rome, Italy) from 1992 to date has been the study of the structure/function relationship of both native and recombinant proteins from terrestrial and marine organisms. In particular, he studied metallothioneins and aspartic proteinases in Echinoderms and Antarctic fish. This research activity has allowed him to acquire extensive knowledge of most protein purification procedures as well as kinetic, chemical–physical and structural characterization of enzymes. He also has experience with molecular biology techniques, such as nucleic acids isolation, cloning and expression of recombinant proteins and enzymes in *Escherichia coli*. Recently, his research activity has been focused on the investigation of enzymes involved in the hydration of CO_2 (carbonic anhydrases) in extremophilic microorganisms. The preliminary results of this study are helpful in developing new methodologies for the use of enzymatic systems in biotechnologies. All of these activities have led to more than 50 publications in peer-reviewed journals. He has a long and strong scientific background, developed over 15 years of experience acquired by intense training in Italian and international laboratories. He is the Associate Editor of *Journal of Enzyme Inhibition and Medicinal Chemistry*, and is on the Editorial Board of the journal *Expert Opinion on Therapeutic Patents*.

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Foreword

Targeting carbonic anhydrases

Clemente Capasso
& Claudiu T Supuran

Carbonic anhydrases (CAs) are metalloenzymes that are intensely investigated due to the biomedical applications of their inhibitors and because they are involved in many crucial physiologic and pathologic processes. CAs equilibrate the reaction between three simple but essential chemical species: CO_2, bicarbonate and protons, which are all essential molecules/ions in important physiologic processes in all life kingdoms (Bacteria, Archaea and Eukarya), throughout the tree of life.

This book presents a collection of chapters dealing with the latest developments in CA research, which has experienced an important revival in the last 5 years, with the discovery of several classes of inhibitors and their mechanisms of action, as well as the validation of some human CA isoforms as antitumor/antimetastatic drug targets. Indeed, sulfonamide/sulfamate CA inhibitors are clinically used as diuretics, antiglaucoma, antiepileptic and antiobesity agents.

After a general overview of CAs belonging to the five genetically distinct families, the main class of inhibitors, the sulfonamides and their isosteres (sulfamates, sulfamides) are presented in several chapters. They deal both with the primary sulfonamides as well as the less investigated secondary/tertiary ones, a recently described new chemotype among these pharmacological agents.

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The totally new classes of CA inhibitors discovered in the last few years, such as the polyamines, phenols, coumarins and dithiocarbamates/xanthates are dealt with in the subsequent chapters. Bacterial, fungal and protozoan CAs and their targeting are discussed in the next chapter, while the last two chapters deal with the less investigated (up to now) CA activators, as well as the biotechnological applications of these enzymes in the CO_2 capture processes and other applications.

As this research field is a highly dynamic one, we consider that the present book offers the latest and most up-to-date findings related to CAs and their inhibitors/activators. Our hope is that the next few years will be as prolific as the last ones in the investigation of these incredible enzymes and their modulators of activity.

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About the Authors

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Chapter 1

Overview of carbonic anhydrase families/isoforms

Clemente Capasso & Claudiu T Supuran

Carbonic anhydrases (CAs; EC 4.2.1.1) are metalloenzymes that catalyze CO_2 hydration to bicarbonate and protons. These enzymes are involved in a multitude of physiologic processes in organisms all over the phylogenetic tree, with five genetically distinct CA classes known to date: the α-, β-, γ-, δ- and ζ-CAs. Their biochemical features are known in detail for at least four classes, together with their distribution and role in various organisms. Inhibition and activation studies of many such enzymes from vertebrates, protozoa, fungi and bacteria have shown that they are drug targets for obtaining pharmacological agents of the diuretic, antiglaucoma, antiobesity, antiepileptic, anticancer or anti-infective type. Many such enzymes also possess biotechnologic applications for biomimetic CO_2 capture processes. The cloning and characterization of many other such enzymes will probably lead to the discovery of other CA families as well as enzymes with potentially important technologic applications. The study of inhibitors and activators of such CAs may lead to novel types of drugs, which may be more efficient and less toxic compared with the presently used ones.

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Aa CO_2 hydration: the slow reaction between CO_2 and water at neutral pH; it leads to bicarbonate and protons, and its velocity is highly enhanced by the carbonic anhydrases. It is the most important physiologic reaction in which these enzymes are involved.

Enzyme: biologic catalyst, belonging to the protein or RNA type of macromolecule.

In this chapter, we review the various families of the metalloenzyme carbonic anhydrase (CA; EC 4.2.1.1). By catalyzing a physiologically fundamental process, the reversible hydration of CO_2 to bicarbonate and protons, five genetically distinct CA classes are known to date: the α-, β-, γ-, δ- and ζ-CAs in all organisms from the tree of life. It is not uncommon that enzymes from more than one class are present in some organisms, whereas many isoforms are also common (e.g., in humans, 15 CA isoforms belonging to the α-class have been described). CAs from four classes are characterized in detail by kinetic and x-ray crystallographic studies, whereas the δ-class is presently less well characterized. CAs are involved in crucial physiologic processes, such as pH and CO_2 homeostasis, respiration and transport of CO_2/bicarbonate, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (e.g., gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification and tumorigenicity (in vertebrates), whereas in algae, plants and some bacteria they play an important role in photosynthesis and other biosynthetic reactions. δ- and ζ-CAs play a crucial role in CO_2 fixation in photosynthetic bacteria, diatoms and algae. Some α-, β- and possibly γ-CAs are essential for the lifecycle of some pathogenic bacteria, fungi and protozoa.

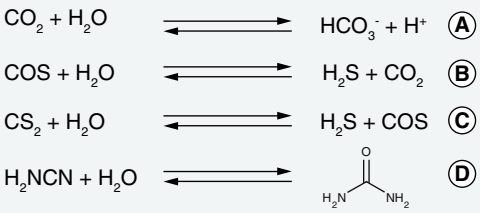
CA families

CO_2 is one of the simple molecules that was highly abundant in the primeval earth atmosphere, being a very stable form of carbon, the central element for life on this planet. This gas may react with water, leading to carbonic acid, which is unstable, being spontaneously transformed into bicarbonate and protons. However, this reaction is particularly slow at pH values in the range of <7.5. CO_2 hydration becomes, on the other hand, very effective at higher pH values, being basically instantaneous at a pH >12 [1–3]. As CO_2 is such an important molecule in all life processes, and because it is generated in high amounts [4–11], it appeared necessary to evolve catalysts for its transformation into bicarbonate. These catalysts are the CAs, (EC 4.2.1.1), metalloenzymes which catalyze not only the reversible hydration of CO_2 to bicarbonate and protons (reaction 1 in **Figure 1.1**), but also a range of other hydrolytic processes such as COS and CS_2 hydration (reactions 2 and 3 in **Figure 1.1**), cyanamide hydration (reaction 4 in **Figure 1.1**) and ester hydrolysis, among others [1–5,12–14] **(Figure 1.1)**. The way in which these processes are achieved is very simple,

as an activated 'metal hydroxide' is acting as nucleophile in all of the catalytic processes mediated by the CAs.

Organisms in all life kingdoms (Bacteria, Archaea and Eukarya) need CAs in order to manage the high amounts of CO_2 formed in metabolic reactions, so that it can be processed and hydrated to bicarbonate and protons [5–12]. By a very nice process of convergent evolution, organisms have developed at least five distinct families of such enzymes, α-, β-, γ-, δ- and ζ-CAs, to face the high concentration of CO_2 [5–12]. α-CAs are present mainly in vertebrates, fungi, protozoa, corals, algae and cytoplasm of green plants, but also in some bacteria [1,2,5–8]. β-CAs have been found in bacteria, algae and chloroplasts of both monoas well as di-cotyledons, as well as in many fungi and Archaea [1,2,4,11]. The γ-CAs have been described in Archaea, Bacteria and plants [1,9], the δ-CAs in algae and diatoms [1,2,10], whereas ζ-CAs seem to be present only in marine diatoms [10]. In such organisms, these enzymes play crucial physiological roles connected to: pH and CO_2 homeostasis (due to the fact that both protons as well as a base, bicarbonate, are involved in the catalyzed reaction); respiration and transport of CO_2/bicarbonate; electrolyte secretion in many tissues/organs; biosynthetic reactions (e.g., gluconeogenesis, lipogenesis and ureagenesis in which bicarbonate not CO_2 acts as a substrate for the carboxylation reaction); bone resorption; calcification; and tumorigenicity; all of them well studied in vertebrates [1–3,14]. In algae, plants and some bacteria, CAs play an important role in photosynthesis, by concentrating CO_2/bicarbonate nearby the RUBISCO enzyme complex, and in several other biosynthetic reactions [5,7,10]. In diatoms, δ- and ζ-CAs also play a crucial role in CO_2 fixation but probably also in the SiO_2 cycle [10].

Figure 1.1. Simple hydrolytic reactions catalyzed by members of the carbonic anhydrase superfamily.



The carbonic anhydrase superfamily of enzymes is formed by five genetically distinct classes as well as by the β-carbonic anhydrase-like enzymes that hydrolyze COS and CS_2. **(A)** CO_2 hydration, **(B)** COS hydration, **(C)** CS_2 hydration and **(D)** cyanamide hydration to urea.
Data taken from [4].

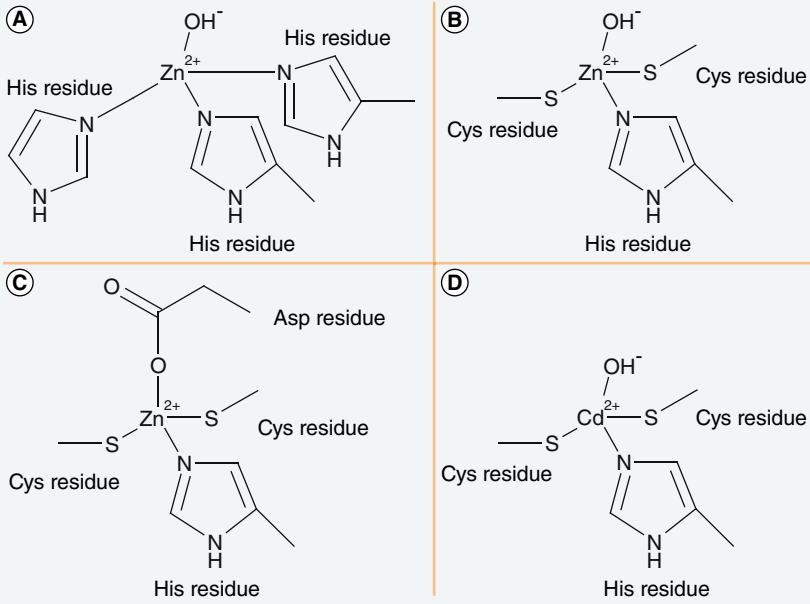
Catalytic & inhibition mechanism

All CAs are metalloenzymes and the metal ion is critical for catalysis, as the apoenzyme is devoid of activity [1,2,14–16]. The five CA families differ in their preference for metal ions used within the active site for performing the catalysis: Zn(II) ions are used by all five classes mentioned above, but the γ-CAs are probably Fe(II) enzymes (being active also with bound

Zn[II] or Co[II] ions) [1,2], whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic catalytic reaction [10]. The metal ion coordination is shown schematically in **Figure 1.2.**

In all enzyme classes, a metal hydroxide species (L_3-M^{2+}-OH^-) of the enzyme is the catalytically active species **(Figure 1.2)**, acting as a strong nucleophile (at neutral pH) on the CO_2 molecule bound in a hydrophobic pocket nearby [1,2]. This metal hydroxide species is generated from one water molecule coordinated to the metal ion, found at the bottom of the active site cavity [1,2,4]. The active center normally comprises M(II) ions in tetrahedral geometry, with three protein ligands (His or Cys residues) in addition to the water molecule/hydroxide ion. In many enzymes, generation of the metal hydroxide species from the metal-coordinated water one is the rate-determining step of the catalytic turnover, which for some α- and ζ-CAs achieve k_{cat}/K_M values >10^8 $M^{-1} \times s^{-1}$,

Figure 1.2. Metal ion coordination in the various carbonic anhydrase families.

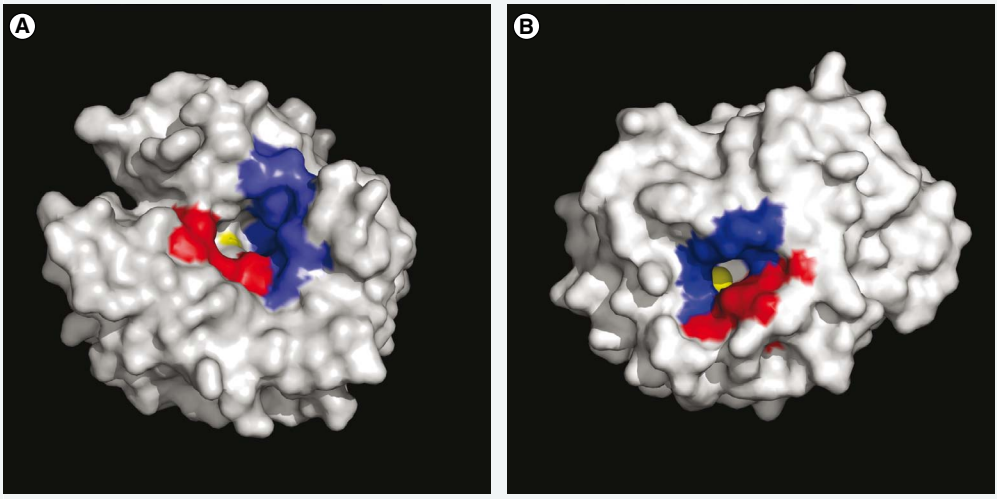


(A) By three His residues and a water molecule/hydroxide ion, in the α-, γ- and δ-CAs. **(B)** By one His and two Cys residues, together with the water molecule/hydroxide ion, in the β- and ζ-CAs (the last one when containing zinc not cadmium at the active site). **(C)** Closed active site of the β-CAs, when the Zn(II) is coordinated by two Cys, one His and one Asp residues. **(D)** ζ-CA with Cd(II) at the active site.
Data taken from [1,2,4,10,11].

making CAs among the most effective catalysts known in nature [1,2]. The metal ion ligands are three His residues in α-, γ- and δ-CAs **(Figure 1.2A)** or one His and two Cys residues in β- and ζ-CAs **(Figures 1.2B & D)** [1–12]. However, some β-class enzymes have four protein zinc ligands (i.e., one His, two Cys and one Asp coordinated to Zn(II); **Figure 1.2C**) [1,4,5,11]. For these enzymes, no water coordinated to the metal ion is present at pH values <8 [1,11]. However, at pH values >8, a conserved Arg residue in all β-CAs (belonging to a so-called 'catalytic dyad' [11]) makes a salt bridge with the Asp coordinated to Zn(II), liberating the fourth Zn(II) coordination position, which is thus occupied by an incoming water molecule/hydroxide ion [11].

A striking feature of the active site architectures of all CAs from diverse genetic families and organisms is that they are divided in two halves – one lined with hydrophobic residues (red in **Figure 1.3** for all considered enzyme families, exemplified here for an α-CA of human origin, hCA II, and a ζ-CA from the marine diatom *Thalassiosira weissflogii*), and the other lined with hydrophilic amino acid residues (blue in **Figure 1.3)** [14]. This particular 'bipolar' active site architecture had already been noticed when the first crystal structure of such an enzyme, the human isoform hCA II, was reported [1,14]. A probable explanation for this highly

Figure 1.3. The active site architecture of human isoform hCA II (α-class enzyme) and CdR1 enzyme from the marine diatom *Thalassiosira weissflogii* (ζ-class enzyme).

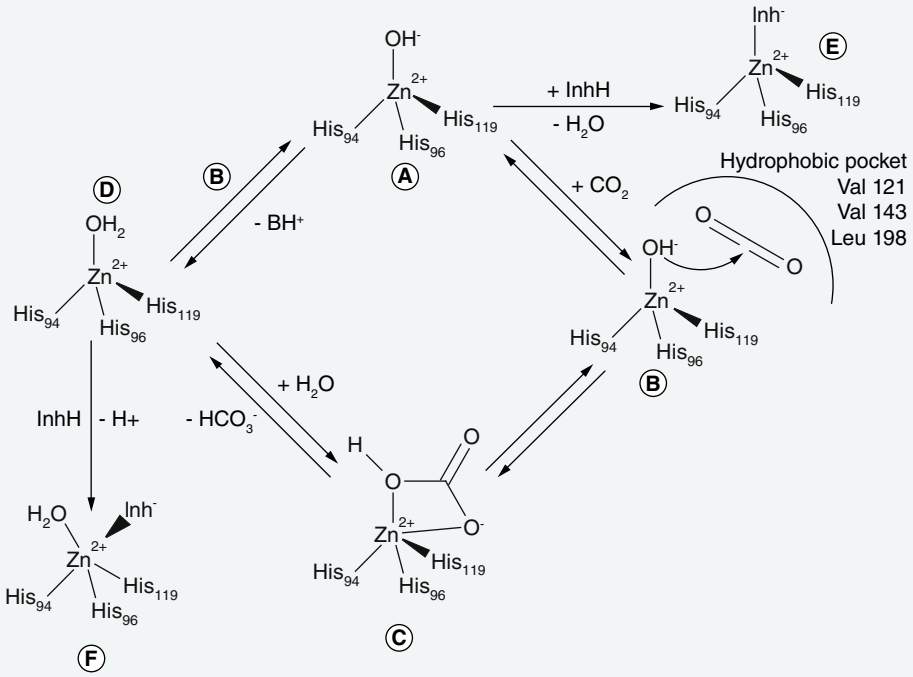


(A) hCA II. **(B)** CdR1. The metal ion is the central yellow sphere, the hydrophobic amino acid residues are shown in red, and the hydrophilic amino acid residues are shown in in blue.

particular active site architecture is that the hydrophobic part is used to entrap the CO_2 molecule (a fairly hydrophobic, not very water-soluble gas), whereas the hydrophilic half may be the part of the active site through which the polar components generated from the CO_2 hydration reaction (bicarbonate and protons) are released from the cavity into the environment [14]. At least for the protons, it is in fact well demonstrated that a relay of water molecules and polar His residues are involved in such processes, strongly facilitating the release of the protons generated by the hydration of CO_2 [1,14].

The catalytic and inhibition mechanisms of the CAs are well-understood processes and are illustrated schematically in **Figure 1.4**. The catalytically active species is the one with the metal hydroxide at the active site **(Figure 1.4A)**. The substrate CO_2 is bound in a hydrophobic pocket near the Zn(II) ion, defined among others by residues Val121, Val143 and

Figure 1.4. Catalytic and inhibition mechanisms (with zinc ion binders) of α-carbonic anhydrases (hCA I amino acid numbering of the zinc ligands).



A similar catalytic/inhibition mechanism is also valid for carbonic anhydrases from other classes (β-, γ-, δ and ζ-CAs), but either the metal ion is coordinated by other amino acid residues or a Cd(II) ion is present instead of zinc at the active site (see **Figure 1.2**). B represents a buffer present in the medium (but it may be a His residue from the enzyme active site); InhH represents an inhibitor (which in deprotonated form, Inh- binds to the metal ion).

Leu198 (in the human isoform hCA II), as represented in **Figure 1.4B**. Orientated in such a favorable position for the nucleophilic attack, CO_2 is transformed into bicarbonate, which is coordinated bidentately to the Zn(II) ion **(Figure 1.4C)**. The binding of bicarbonate to zinc is rather labile due to the trigonal bipyramidal geometry of the adduct, which is not very stable for the Zn(II) complexes and thus the intermediate **(Figure 1.4C)** is readily transformed to the structure shown in **Figure 1.4D** by reaction with water, which liberates bicarbonate into solution. In this way, the acidic form of the enzyme is generated, with water coordinated to the metal ion **(Figure 1.4D)**, which is catalytically ineffective. Generation of the nucleophilically active species of the enzyme A **(Figure 1.4A)** is achieved though a proton transfer reaction from the zinc-coordinated water (species D; **Figure 1.4D**) to the buffer (B), which is many times the rate-determining step of the entire catalytic process [1,2]. In many CA isoforms and probably in many enzyme classes (not only the α ones, which are the most investigated), this process is assisted by an active site residue able to participate in proton transfer processes. For the specific case examined in **Figure 1.4** (i.e., hCA II), it is a histidine residue placed in the middle of the active site acting as a proton shuttle residue [1,2]. For hCA II, His64 is the main proton-shuttling residue, but it may be further assisted in the proton transfer processes by a cluster of histidines extending from the middle of the cavity to its entrance and to the surface of the enzyme around the edge of the cavity (residues His4, His3, His10 and His15 [1]).

The data in **Table 1.1** also show that the CAs, irrespective of the class to which they belong, act as highly efficient catalysts for the CO_2 hydration reaction. For example, the α-class enzymes hCA II (human) or SazCA (from the extremophilic bacterium *Sulphurihydrogenibium azorense*) are among the best catalysts known in nature, possessing k_{cat} values in the range of $1.4–4.4 \times 10^6$ s^{-1} and k_{cat}/K_m values of $1.5–3.5 \times 10^8$ $M^{-1}.s^{-1}$ [1,2,8]. Enzymes from various sources (bacteria, fungi, plants and diatoms) belonging to the β-, γ-, δ and ζ-classes also possess effective catalytic activities for the physiologic reaction **(Table 1.1)**, with kinetic parameters in the range of: k_{cat} of $1.2 \times 10^5–1.5 \times 10^6$ s^{-1} and k_{cat}/K_m values of $7.5 \times 10^6–1.6 \times 10^8$ $M^{-1}.s^{-1}$. Interestingly, as far as we know, only the α-CAs possess significant esterase and phosphatase activity, whereas the β-, γ-, δ and ζ-class enzymes are devoid of such activities [13]. It may be also observed that the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) acts as an effective inhibition

Aa Enzyme inhibitor: compound interfering with the catalytic mechanism of an enzyme by blocking its activity and thus impairing its action as a catalyst. Many types of inhibitory activities/mechanisms are described.

Table 1.1. Kinetic parameters for the CO_2 hydration reaction catalyzed by various carbonic anhydrases belonging to the various families.

Isozyme	Class	Organism	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	K_i (acetazolamide) (nM)
hCA II	α	Human	1.4×10^6	1.5×10^8	12
SazCA	α	Bacterium	4.4×10^6	3.5×10^8	0.9
Can2	β	Fungus	3.9×10^5	4.3×10^7	10.5
FbiCA1	β	Plant	1.2×10^5	7.5×10^6	27
PgiCA	γ	Bacterium	4.1×10^5	5.4×10^7	324
TweCA	δ	Diatom	1.3×10^5	3.3×10^7	83
Cd(II)-R1	ζ	Diatom	1.5×10^6	1.4×10^8	82
Zn(II)-R1	ζ	Diatom	1.4×10^6	1.6×10^8	58

The α-class carbonic anhydrases (CAs) were the human cytosolic isozymes hCA II and the bacterial one SazCA (from *Sulphurihydrogenibium azorense*). The β-class includes the fungal enzyme Can2 from *Cryptococcus neoformans* and FbiCA1 from the plant *Flaveria bidentis*. The γ-class enzyme was PgiCA from the anaerobic bacterium *Porphyromonas gingivalis*, whereas the δ- and ζ-class enzymes (the last with zinc and cadmium at the active site) were from the diatom *Thalassiosira weissflogii*. Inhibition data with the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided. All data were obtained in the author's laboratories.
Data taken from [1,2,8–11].

constant in the range of 0.9–324 nM) against enzymes belonging to all five classes.

Applications of CA inhibitors & activators; biotechnologic applications of the CAs

The inhibition and activation of CAs are well-understood processes. There are many different classes of CA inhibitors (CAIs), and the most investigated ones act by binding to the metal center within the active site [1–12] in tetrahedral or trigonal bipyramidal geometries **(Figures 1.4E & 1.4F)**. The CA activators (CAAs), on the other hand, bind at the entrance of the active site cavity and participate in proton-shuttling processes between the metal ion-bound water molecule and the environment [1,2]. Recently, inhibition mechanisms other than the binding to the metal center have been reported for α-CAs, which do not directly involve the metal ion from the enzyme active site. For example phenols, polyamines and some carboxylates bind to the enzyme by anchoring to the zinc-coordinated water/hydroxide ion [4], whereas coumarins act as prodrugs and bind at the entrance of the active site cavity (in the activator binding site), rather far away from the metal ion [4,14]. Dithiocarbamates and xanthates have also been recently reported as CAIs, and they coordinate to the metal ion within the enzyme active site similar to the sulfonamides, sulfamates and sulfamides, which are the

most important classes of clinically used agents [14–20]. Indeed, sulfonamide/sulfamate CAIs are clinically used in the field of antiglaucoma, anticonvulsant, antiobesity and anticancer agents, but novel uses are emerging for designing anti-infectives (antiprotozoan, antifungal and antibacterial agents) with a novel mechanism of action [1,4,14–20]. The various classes of CAIs and CAAs are dealt with in several chapters of the present book and will be not detailed here.

Recently, due to a steep increase in the CO_2 concentration in the atmosphere and the green house effects of this gas [20], a lot of research has concentrated on the biotechnologic uses of various CAs, some of which have been isolated from not-so-common vertebrates such as extremophilic bacteria [8], with the goal of using the high catalytic activity of such enzymes for capturing atmospheric CO_2. Such a 'green', novel approach for CO_2 capture may resolve the stringent problem of global warming in an efficient and elegant manner without the pollution problems connected with the use of amines or other reagents for such processes.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Summary.

- Carbonic anhydrases (CAs) are metalloenzymes and exist as five distinct families, the α-, β-, γ-, δ- and ζ-CAs, in all organisms from the tree of life.
- They catalyze a physiologically fundamental process, the reversible hydration of CO_2 to bicarbonate and protons.
- CAs from four classes are characterized in detail by kinetic and x-ray crystallographic studies.
- CAs are involved in crucial physiologic processes (pH and CO_2 homeostasis, respiration and transport of CO_2/bicarbonate, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions, gluconeogenesis, lipogenesis and ureagenesis, bone resorption, calcification, tumorigenicity, photosynthesis and CO_2 fixation).
- Some α-, β- and possibly γ-CAs are essential for the lifecycle of some pathogenic bacteria, fungi and protozoa.
- CA inhibitors are clinically used as antiglaucoma, anticonvulsant, antiobesity and anticancer agents/diagnostic tools, but they are emerging as anti-infective (i.e., antiprotozoan, antifungal and antibacterial) agents with a novel mechanism of action.
- CAs may have biotechnologic applications in biomimetic CO_2 capture processes.

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Jean-Yves Winum received his PhD in chemistry from the University of Montpellier 2 (France) in 1998. He then worked as a postdoctoral fellow in the Department of Chemistry of Georgetown University (Washington DC, USA), and in the Department of Organic Chemistry of the University of Geneva (Switzerland). In 1999, he joined the Department of Chemistry of the University of Montpellier 2, where he is now an Associate Professor. His research interests are focused on organic/medicinal chemistry of metalloenzyme inhibitors. The results of his studies have been published in more than 110 articles. He is the editor of a more than 1000-page book on zinc enzyme inhibitors (published in 2009 at Wiley, NJ, USA) and associate editor of two journals (*Journal of Enzyme Inhibition and Medicinal Chemistry* and *Expert Opinion on Therapeutic Patents*).

Chapter 2

Classical sulfonamides and their bioisosters as carbonic anhydrase inhibitors

Jean-Yves Winum

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The α-family of the metalloenzymes carbonic anhydrase (CA; EC 4.2.1.1) found in humans is comprised of 13 different active isoforms, with diverse subcellular localization, tissue expression and susceptibility to be inhibited. Several of these isozymes are drug targets, and the design of selective inhibitors has captured the attention of researchers over the last 30 years. Numerous strategies have been developed for designing selective CA inhibitors, and modification of an essential structural element such as the zinc-binding function in the scaffold of the inhibitors has been considered as one of the most attractive and versatile tools for this goal. Important progress has been achieved in recent years, and CA inhibitors of the sulfonamide, sulfamide or sulfamate type have shown clinical applications against different pathologies such as glaucoma, epilepsy, obesity, hypertension and cancer. In this chapter, we will highlight the most significant development in the main class of such compounds, those bearing the sulfonamide zinc-binding function and its sulfamide and sulfamate bioisosters.

On the importance of zinc-binding function in the design of carbonic anhydrase inhibitors

Carbonic anhydrases (CAs; EC 4.2.1.1) belong to a superfamily of ubiquitous metalloproteins found in all kingdoms starting from Bacteria and Archaea and ending with Eukaryotes, humans included. Physiological functions of this family of metalloenzymes are essential for these organisms, as CAs catalyze a very simple fundamental physiological reaction: the reversible hydration of carbon dioxide in bicarbonate with release of a proton. This very simple reaction is involved in a plethora of biological processes such as respiration and transport of CO_2, pH regulation and homeostasis, and also several metabolic biosynthetic pathways [1].

In the α-CA family, the one found in vertebrates, including humans, 15 isoforms have now been identified, among which 12 are catalytically active. Each of these isozymes differs in their relative hydrase activity, their tissue distribution and their subcellular localization. Indeed, there are several cytosolic forms (CA I, CA II, CA III, CA VII and CA XIII), four membrane-bound isozymes (CA IV, CA IX, CA XII and CA XIV), two mitochondrial forms (CA VA and CA VB) as well as a secreted CA isozyme (CA VI) [1,2].

Numerous pathological states are linked to modifications in CA activity, some of which have been clinically exploited for the treatment or prevention of various pathologies such as glaucoma, neurological disorders and hypertension. Several of these isozymes are validated as therapeutic targets, and study in the design of selective CA inhibitors (CAIs) has always been a very dynamic research field that interests numerous research groups all over the world. During recent years, the discovery of CA isoforms implicated in cancer (CA IX and CA XII) has been made, holding great promise for therapeutic use of CAIs in oncology [2].

The catalytic mechanism of the CAs is well understood. The zinc ion is essential for the catalytic process. In the α-CA class, the metal ion is situated at the bottom of a 15 Å-deep active cleft coordinated by three histidine residues (His94, His96 and His119) and a water molecule/hydroxide ion in a tetrahedral geometry, which in turn is involved in hydrogen bonding networks with the hydroxyl moiety of Thr199 and the carboxylate group of Glu106, two conserved amino acid residues in all α-CAs [3].

From earlier studies, three major basic structural elements have emerged as crucially important to the CA recognition pharmacophore. The first one is the zinc-binding function (ZBF), which interacts with the active site metal ion and the residues Thr199 and Glu106. The

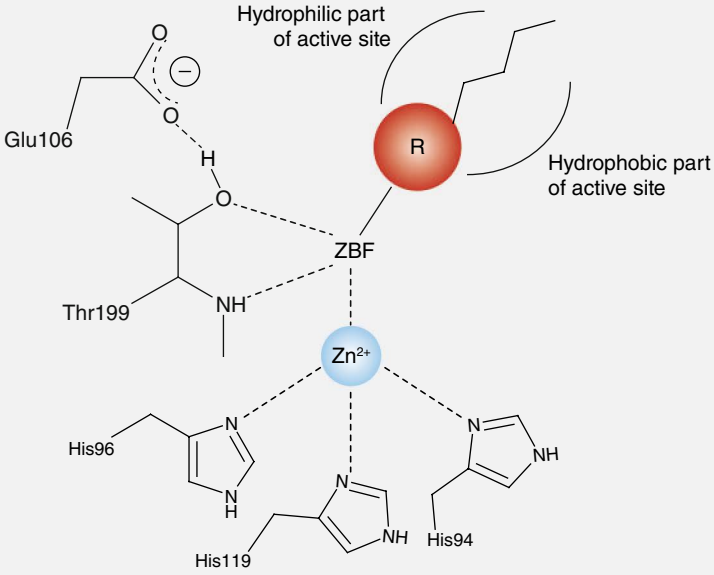
Aa Pharmacophore: ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response [27].

Aa Zinc-binding function: organic functional group able to bind Zn^{2+} cation in the active site of zinc metalloproteins.

second one is the organic scaffold, usually an aromatic or heteroaromatic moiety, which interacts both with the hydrophobic as well as the hydrophilic halves of the active site. The third one is a 'tail' attached to the organic scaffold (see **Figure 2.1**) [3].

Variations in the nature of the organic scaffold and tail moiety have been thoroughly investigated in the design of CAIs, and many studies allowed the generation of isozyme-specific lead compounds with potential biomedical applications [3]. Indeed, two main approaches have been developed by different research groups – namely the 'ring approach' and the 'tail approach' (exploring a great variety of ring systems [aromatic or heteroaromatic] or attaching different tails to the scaffolds of the inhibitors in order to modulate the physicochemical properties such as, for example, water solubility and enzyme-binding capacity, respectively). The latter was extended recently with the sugar approach, which showed that through the combination of a high degree of polyfunctionality and hydrophilicity,

Figure 2.1. General structure of an inhibitor in complex with α-carbonic anhydrase active site.



ZBF: Zinc-binding function.
Reproduced with permission from [6]. © Bentham (2006).

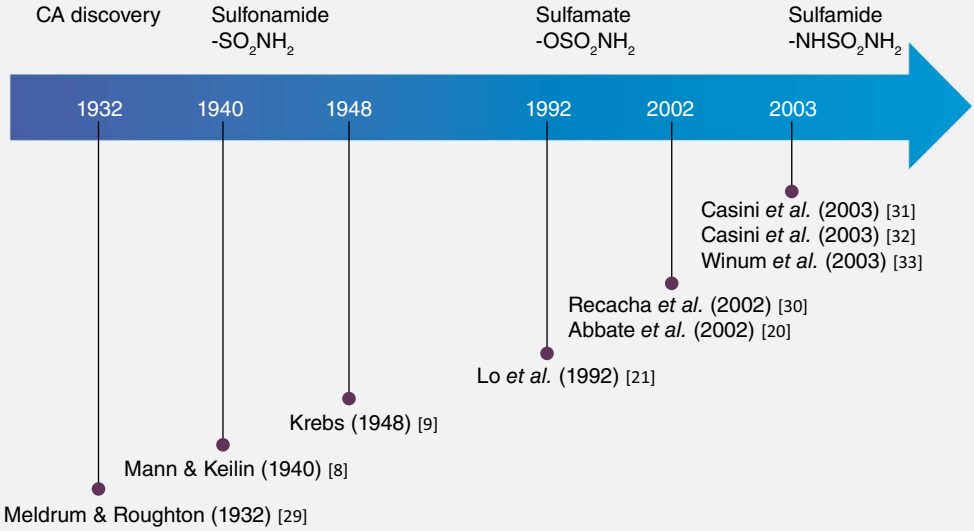
Aa Sulfonamide: important organic functional group found in the structure of carbonic anhydrase inhibitors and with the formula -SO_2NH_2. This radical can be connected to different aliphatic, aromatic or heterocyclic scaffolds.

Bioisoster: groups of atoms or molecules that have chemical and physical similarities producing broadly similar biological properties [28].

carbohydrate moieties incorporated onto aromatic sulfonamide CAs impart unique properties to this family of CAIs [4,5]. This particularly active research field has been the subject of various reviews [1–3].

The key structural element on which many studies have also focused in recent years is the ZBF [2,6,7]. Primary sulfonamide (-SO_2NH_2) has been the most common ZBF since 1940 when inhibition by sulfanilamide was reported by Keilin and Mann [8]. Even Hans Krebs contributed to early studies, with a paper in 1948 showing the relationship of 25 different sulfonamides to CA inhibition **(Figure 2.2)** [9]. This function was recognized for 40 years as the foremost example of ZBF for the design of CAIs, until the first reports in 1987 and 2003 that sulfonamide bioisosters such as sulfamate and sulfamide, respectively, can also be very good ZBF, leading to very potent and selective CAIs. Multiple libraries of this family have been reported and investigated for their interactions with all 13 catalytically active mammalian isozymes, with many low nanomolar inhibitors being detected with some of them in clinical development [1,2].

Figure 2.2. Timeline and key publications in the discovery of new zinc-binding function for the conception of carbonic anhydrase inhibitors.



CA: Carbonic anhydrase.

As it was proved by the wealth of x-ray structural data of inhibitors in complex with hCA II found in the Protein Data Bank, primary sulfonamides ($-SO_2NH_2$) as well as sulfamides ($-NHSO_2NH_2$) and the sulfamates ($-O-SO_2NH_2$) interact with the metal ion in deprotonated form similarly to the transition state of the endogeneous reaction, to form a slightly distorted tetrahedral adduct. It is worth pointing out that many anionic species such as cyanide, cyanate, thiocyanate and azide, among others, were reported to inhibit the CAs but with a mode of action different from the sulfonamides (in some cases), in which the Zn(II) has a trigonal–bipyramidal geometry [3].

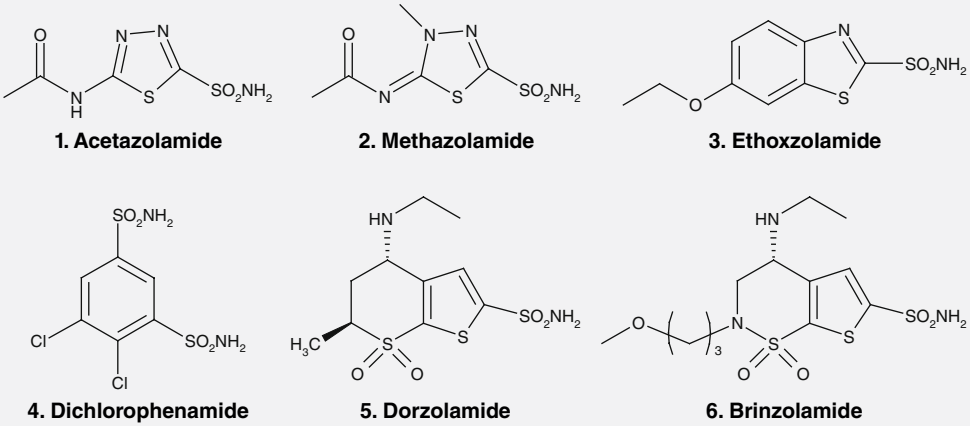
By considering the three main structural elements needed to be present in the molecule of a potent CAI – that is ZBF, an organic scaffold and one or more side chains substituting the scaffold – crucial advances have been achieved in recent years to understand the factors that govern both potency and selectivity against various CA isozymes for this class of pharmacological agents [1–3]. ZBF is critical for the design of novel classes of CAIs and exploration of new anchor functions is always of interest for the medicinal chemist in the drug design of isoform-selective compounds.

Clinically used sulfonamide CAIs

CAs have been targeted over the last 50 years for the treatment of a variety of disorders, mainly glaucoma, but also epilepsy and very recently cancer. The primary sulfonamide moiety $-SO_2NH_2$ is the main ZBF present in many clinically used drugs that have their main action as CAIs [1–3,10].

Sulfonamide CAIs such as acetazolamide (structure **1**), methazolamide (structure **2**), ethoxzolamide (structure **3**) and dichlorophenamide (structure **4**) **(Figure 2.3)** initially developed in the 1950s and 1960s are still widely used as systemic antiglaucoma drugs. These systemic inhibitors constitute an important class of drugs in the armamentarium of antiglaucoma agents and are useful in reducing elevated intraocular pressure, characteristic of this disease [1,2,11]. They represent the most efficient physiological treatment of glaucoma, acting on the ciliary process by strongly inhibiting CA II, CA IV and CA XII isoforms and thus reducing the rate of bicarbonate and aqueous humor secretion, which leads to a 25–30% decrease of intraocular pressure. These CAIs have side effects, as CA isoforms II, IV and XII are present in many other tissues/organs and their efficacy generally decreases after prolonged use. As a consequence, there are limitations of their use due to patient compliance. However, acetazolamide and dichlorophenamide are used even nowadays as components of regimens for the treatment of refractory glaucoma that does not respond to the topically acting adrenergic antagonists or prostaglandin analogs.

Figure 2.3. Structures of clinically used sulfonamides (structures 1–6).

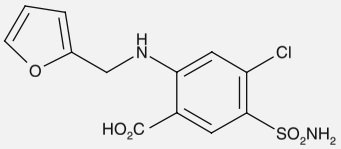


In the 1990s, water-soluble sulfonamide CAIs were developed in several laboratories as a new topical glaucoma treatment strategy, and by 1995 the first such pharmacological agent, dorzolamide (structure **5** in Figure 2.3), was launched for clinical use by Merck & Co. (NJ, USA), as 2% eye drops. A second structurally related compound, brinzolamide (structure **6** in Figure 2.3; discovered at Alcon Laboratories, Hünenberg, Switzerland), was in turn approved for the topical treatment of glaucoma in 1999. These two compounds are still the only topically acting CAIs in clinical use at the moment. Dorzolamide and brinzolamide are nanomolar CA II and CA XII inhibitors that present a good hydrophilic–lipophilic balance with good water solubility and lipophilicity sufficient to penetrate through the cornea, allowing a topical administration directly into the eye. Both drugs may be used alone, but most of the time they are used in combination with β-blockers or PGF2 receptor agonists (combination therapy, used by >60% of glaucoma patients worldwide) [1,2,11].

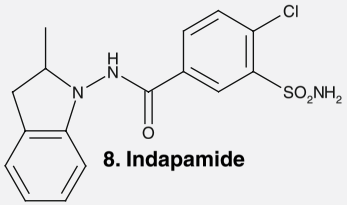
No new antiglaucoma drugs from the primary sulfonamide class have appeared in the last 15 years. Several interesting approaches have, however, been reported, and very good drug candidates such as, for example, the sulphonamide–nitric oxide donor conjugates have been claimed in recent years by many companies. Among these many interesting compounds that were evaluated in animal models of disease, none of them progressed to clinical trials, probably because the existing drugs still had patent coverage [11].

Other sulfonamide drugs such as the diuretics (furosemide [structure **7**], indapamide [structure **8**], chlorthalidone [structure **9**], bumetanide [structure **10**]

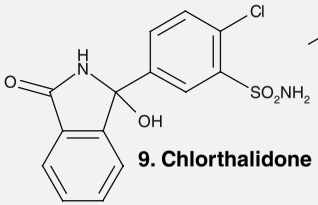
Figure 2.4. Others sulfonamide drugs used clinically and having inhibitory activity against hCA (structures 7–18).



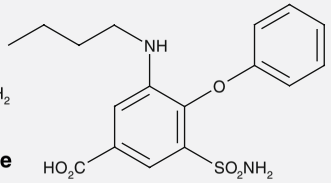
7. Furosemide



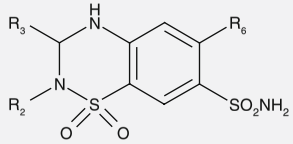
8. Indapamide



9. Chlorthalidone

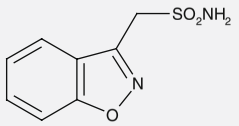


10. Bumetanide

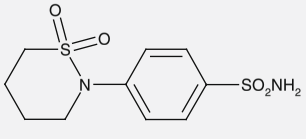


11. Benzothiadiazines

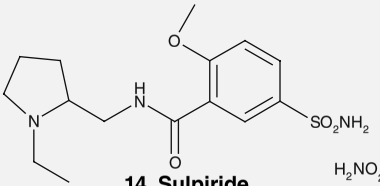
a: R_2=R_3=H R_6=Cl Hydrochlorothiazide
b: R_2=R_3=H R_6=CF_3 Hydroflumethiazide
c: R_2=H R_3=$PHCH_2$ R_6=CF_3 Bendroflumethiazide
d: R_2=H R_3=$CHCl_2$ R_6=Cl Trichloromethiazide
e: R_2=CH3 R_3=$CH_2SCH_2CF_3$ R_6=Cl Polythiazide



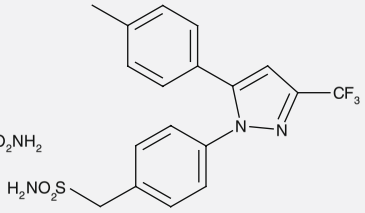
12. Zonisamide



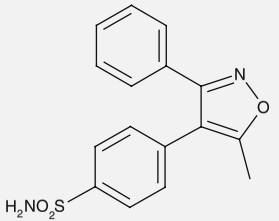
13. Sulthiame



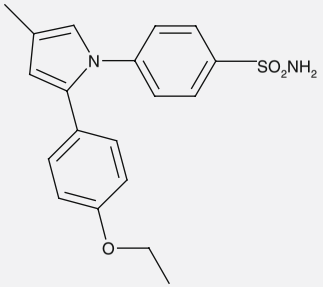
14. Sulpiride



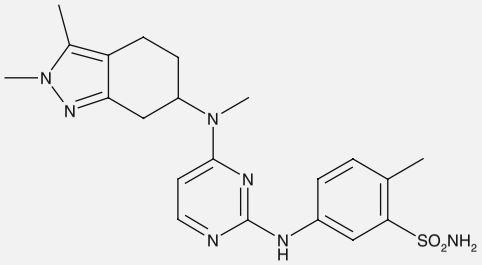
15. Celecoxib



16. Valdecoxib



17. Apricoxib



18. Pazopanib

and benzothiadiazines [structure **11**]), the antiepileptics zonisamide (structure **12**) and sulthiame (structure **13**), the antipsychotic sulpiride (structure **14**), the COX2 inhibitors celecoxib (structure **15**) and valdecoxib (structure **16**), and recently apricoxib (structure **17**) and pazopanib (structure **18**), first designed to act on targets other than CAs, were demonstrated to interfere with this family of enzymes, having nanomolar inhibitory activity against different CA isoforms. X-ray crystallographic structures are currently available for many adducts of such clinically used sulfonamide inhibitors with isozyme hCAII [1,2,10].

Most of these clinically used compounds (structures **1–18**; **Figure 2.4**) strongly inhibit many CA isozymes unselectively with affinities in the low nanomolar range [1,2]. The drug design of primary sulfonamide CAIs has been recently reviewed [10], pointing out the major problem regarding the polypharmacology of these compounds and its importance in the discovery of a new generation of isozyme-selective inhibitors for various clinical applications.

Indeed, the presence of primary sulfonamide function, for example in the molecular structure of the diuretics furosemide, indapamide, chlorthalidone, benzothiadiazines and bumetanide **(Figure 2.4)**, is responsible for the strong CA inhibitory activities of these old drugs discovered in the 1960s to 1970s, a period when only CA II was known, and which have been only recently re-evaluated against all of the mammalian active CA isoforms – that is, CA I–CAXV – showing that many of them do substantially inhibit several CA isoforms involved in crucial physiological and pathological processes. These compounds are well-known diuretics widely employed for controlling hypertension, alone or in combination with other drugs. What is important to note is the fact that the re-evaluation of these old diuretics may have relevance for the drug design of CAIs with diverse pharmacological applications [12].

Other compounds possessing primary sulfonamide function that have been detected as potent CAIs and developed for other uses are celecoxib and valdecoxib **(Figure 2.4)**, originally described as selective COX2 inhibitors and developed in the 1990s by Merck & Co. and Pharmacia–Pfizer (NY, USA) joint venture. COX2 is a member of the COX enzyme family, which catalyze the committed step in the conversion of arachidonic acid to prostaglandins and thromboxane. COX2 is considered to be mainly associated with inflammatory conditions. Side effects of this family of COX2 inhibitors, mainly due to the low nanomolar CA inhibitory activity, ultimately led to the withdrawal of valdecoxib, but with celecoxib still remaining in clinical use. Recently, another such compound, apricoxib,

has been launched successfully as an analgesic and anti-inflammatory agent, and also for its anticancer potential [1,2,10].

Another primary sulfonamide that has been approved recently for clinical use as an anticancer agent for the treatment of several types of tumor is pazopanib, a multitargeted tyrosine kinase inhibitor discovered and developed by GlaxoSmithKline (Brentford, UK). This drugs acts by binding to the VEGF receptor, PDGF receptor and several other key proteins responsible for angiogenesis, tumor growth and cell survival. The presence of the $-SO_2NH_2$ group, as in all other compounds discussed here, led as expected to low nanomolar inhibitory activity of this drug against many of the 15 human isoforms hCA I–XIV, indicating that in addition to the tyrosine kinase inhibitory action, pazopanib may also exert antitumor/antimetastatic effects, due to the potent inhibition of the tumor-associated, hypoxia-inducible enzymes CA IX and XII [13].

The development of primary sulfonamide compounds as CAIs is a very dynamic field, which has been mainly dedicated in the last year to developing selective inhibitors targeting the two isoforms CA IX and CA XII, which are involved in tumorigenesis and recently validated as anticancer drug targets [14]. Many new concepts based on the famous tail approach have been developed and patented to selectively target these isoforms, and primary sulfonamide function was one of the preferred chemotypes in the structure of these inhibitors [15].

Among these approaches, the best results were recently reported with ureido sulfonamides, which showed good CA IX/XII selective properties, inhibiting to a lower degree CA I and II, demonstrated significant inhibition of growth of primary tumors and metastases, and also produced a depletion of the cancer stem cells, all of which are important antitumor mechanisms for new generations of anticancer drugs [16–18]. Some lead compounds constitute interesting candidates for the development of conceptually novel antimetastatic drugs and are currently in clinical development by SignalChem Lifesciences Corp (BC, Canada).

Sulfamates & sulfamides as CAIs

Sulfamate and sulfamide function ($-O-SO_2NH_2$ and $-NH-SO_2NH_2$, respectively) are typically close bioisosteric variants of sulfonamide, and are very useful in medicinal chemistry, especially in the field of CAs.

The investigation of the simplest molecules (i.e., sulfamic acid [$HOSO_2NH_2$] and sulfamide [$H_2NSO_2NH_2$]) as CAIs was performed in 1996 by Supuran *et al.,* who showed these compounds to act as weak inhibitors against hCA II [19]. The x-ray crystal structure of the adducts of each of them with

hCA II was subsequently reported by the same group in 2002 [20], showing that sulfamic acid and sulfamide were able to bind to the zinc anion as dianion and monoanion species, respectively, also making a number of favorable contacts in the binding pocket of CA II [3]. These very simple lead compounds, which can be easily derivatized, have been considered as a starting point for the design of tighter-binding CAIs with potential therapeutic applications, and this has been achieved in several studies that are summarized in the following sections.

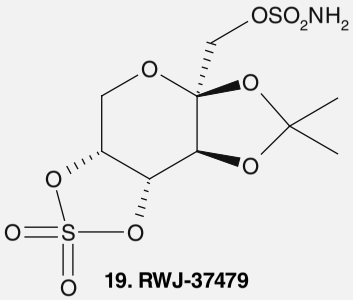
The sulfamate motif in the design of CAIs

From a historical point of view, the first CAIs in sulfamate series were reported in 1992 by Lo *et al.*, who described a small library of imidazolylphenylsulfamate and (imidazolylphenoxy)alkylsulfamate derivatives possessing nanomolar CA inhibitory properties and excellent antiglaucoma topical activity in lowering the intraocular pressure in animal model [21]. Systematic studies on the inhibition of CA isoforms by alkyl and aryl sulfamates were reported in 2003, and can be considered as the starting point for the use of this ZBF in the design of CAIs (see **Figure 2.2**) [1,2,22].

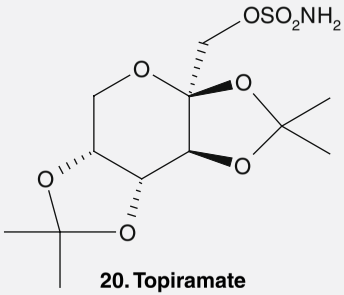
In the meantime, the x-ray crystal structure of the cyclic sulfate analog of topiramate (structure **19**) and topiramate itself (structure **20**; both in **Figure 2.5**) in complex with isozyme hCA II were reported in 2002 and 2003, respectively. Topiramate is an anticonvulsant drug developed by Johnson & Johnson (NJ, USA) first and reported in 1987. At this time, this compound was not described as a CAI, and its action was considered to derive from a multitude of biological activity, such as enhancement of GABAergic transmission, negative effects on the glutamate at the kainate/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, and negative modulation of the voltage-dependent sodium channels and the high-voltage calcium-activated channels. Last but not least, topiramate was shown to effectively inhibit several CA isoforms, especially hCA II, with an inhibitory constant of 5 nM against hCA II. These crystallographic studies revealed the classical tetrahedral coordination of the sulfamate group to the zinc ion, whereas the scaffold of the molecule (a protected fructose moiety) is entrapped into the enzymatic cleft by means of a large number of hydrogen bonding and van der Waals interactions [1,2,22].

Others sulfamates compounds such as estrone sulfamate (EMATE; structure **21**) and irosustat (structure **22**) **(Figure 2.5)**, although developed independently of their potential CAI properties, were also investigated for their CAI properties and also showed low nanomolar activity against different CA isoforms. These compounds are currently in advanced clinical trials as

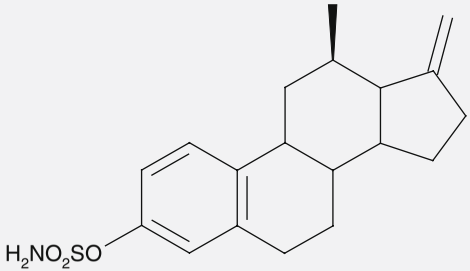
Figure 2.5. Sulfamate and sulfamide inhibitors of carbonic anhydrase (structures 19–24).



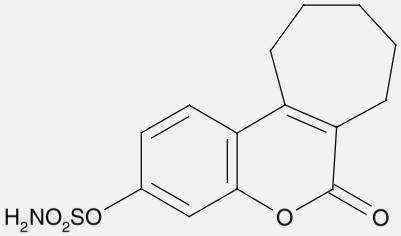
19. RWJ-37479



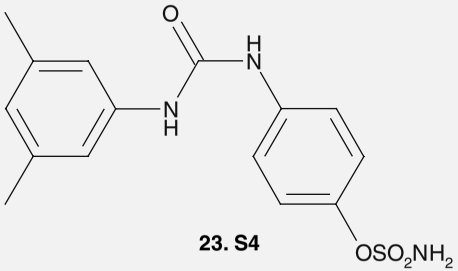
20. Topiramate



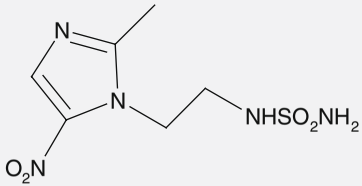
21. EMATE



22. Irosustat



23. S4



24. DH348

dual steroid sulfatase inhibitors/CAIs with anticancer effects. Irosustat is the first-in-class steroid sulfatase inhibitor to be used clinically in patients with advanced hormone-dependent cancers, but it is also a potent CAI. X-ray crystal structures of the two adducts of CA II with EMATE and irosustat were reported in 2004 and 2005, respectively, revealing the interaction of the zinc anchoring group with the Zn ion in a similar manner as sulfamate/sulfonamide, and an interaction of the scaffold with the hydrophobic half of the CA active site [1–3].

The best results that have been described in recent years on CA inhibition with sulfamate compounds are related to the inhibition of the tumor-associated membrane-bound CA IX /CA XII with a large library of ureido-substituted sulfamate compounds. This series displayed high specificity at nanomolar levels for the tumor-associated CA IX/XII isoforms and for some of them showed a positive response in *in vitro* assays for tumor cell migration and spreading under oxygen-decreased conditions as found in solid tumors. For the best compound, S4 (structure **23**; **Figure 2.5**), it was shown that low-dose maintenance therapy strongly inhibited the development of MDA-MB-231 metastases in lung with no signs of toxicity, and had no effect on the primary MDA-MB-231 tumor growth [23]. This sulfamate CAI constitutes an excellent new candidate as an antimetastatic drug in breast cancer therapy and is currently in preclinical studies.

Sulfamide motif in the design of CAIs

The sulfamide motif -NH-SO_2NH_2 moiety has been also used as a substitute for sulfonamide ZBF in the design of CAIs, starting from 2003 when the first systematic studies were published, showing that monosubstituted sulfamide incorporating aliphatic, cyclic or aromatic moieties were able to inhibit the two cytosolic isozymes hCA I and hCA II in the nanomolar range (see **Figure 2.2**). Different series of CAIs were reported in sulfamide series during the last year [1,2,24], but the most interesting results have been obtained with the 'dual-drug' approach, which consists of combining in the same molecule moieties able to inhibit CAs with moieties possessing another pharmacological action such as radiosensitizer moiety. Such an approach has been applied with success for the development of nitroimidazole derivatives and, among the series of compounds prepared, the sulfamide (structure **24**; **Figure 2.5**) was found to inhibit selectively hCA IX and to reduce hypoxic extracellular acidification, slowing down tumor growth at nontoxic doses and sensitizing tumors to irradiation all in a CAIX-dependent manner, suggesting no 'off-target' effects [25,26]. This family of compound is currently in preclinical development by the company DualTPharma (Maastricht, The Netherlands).

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Summary.

- Carbonic anhydrases (CAs) are validated targets for the treatment of glaucoma, hypertension, cancer and diseases of the CNS such as epilepsy and migraine.
- In CAs, the Zn^{2+} cation is the implied target for drug design, and almost all reported CA inhibitors (CAIs) comprise a zinc-binding function (ZBF) in their structure.
- The primary sulfonamide is the most common ZBF used in the structure of CAIs.
- The primary sulfonamide function is also responsible for the CA inhibitory activity of a lot of drugs not initially designed as CAIs.
- Sulfonamide bioisosters such as sulfamate and sulfamide represent a very good alternative ZBF to the sulfonamide. They have a similar mechanism of CA inhibition at the molecular level, but their pharmacologic properties can be rather different, leading to several interesting agents, especially in the field of CA IX/CA XII inhibitors.

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Suleyman Akocak received his BS in chemistry from the Selcuk University (Turkey). After his graduation, he was awarded a full PhD scholarship from the Ministry of National Education of Turkey. He is currently a PhD candidate in medicinal chemistry under the guidance of Marc A Ilies at Temple University School of Pharmacy (PA, USA). His research focuses on the synthesis of isozymes-selective carbonic anhydrase inhibitors useful for detection and treatment of hypoxia-related diseases.

Marc A Ilies

Marc A Ilies was born and educated in Romania, receiving his BSc and MSc degrees from the University of Bucharest in 1995 and 1996, after a research stage at ENSC Lille (France). In 1996, he joined Alexandru T Balaban's group at the Polytechnic University of Bucharest, receiving his PhD in 2001 on a thesis focused on biological applications of pyridinium compounds. From 1997, he was also a faculty in the Department of Chemistry, School of Biotechnologies, UASVM Bucharest. In 2001, he emigrated to the USA, following Alexandru T Balaban at Texas A&M University for postdoctoral studies aimed to develop new pyridinium amphiphiles for gene delivery. In 2004, he relocated to the University of Pennsylvania (USA) for postdoctoral studies towards self-assembling of amphiphilic compounds (with Virgil Percec) and their supramolecular assemblies as drug- and gene-delivery systems (with Vladimir Muzykantov and Ian Blair). Since 2007, he has been Assistant Professor in the School of Pharmacy of Temple University. His research is focused on medicinal chemistry/chemical biology of membrane interfaces using pyridinium compounds and the development of chemical solutions for the efficient and selective delivery of drugs and genes for therapeutic purposes. He has published over 55 peer-reviewed scientific papers, reviews, and book chapters, and has coauthored one US patent.

Chapter 3

Next-generation primary sulfonamide carbonic anhydrase inhibitors

Suleyman Akocak & Marc A Ilies

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This chapter describes the recent trends in the design of next-generation sulfonamide carbonic anhydrase inhibitors, emphasizing the problems and limitation of present technologies and revealing their structural and mechanistic bases. We also present the most recent synthetic solutions advanced by various research groups, together with the main biological properties of the novel inhibitors and the structure–activity and/or structure–properties relationships that were drawn in these studies.

CA isozymes: overview & inhibition

Carbonic anhydrases (CAs; E.C.4.2.1.1) are a class of ubiquitous metalloenzymes that catalyze the reversible hydration of carbon dioxide: $CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+$. Since CO_2 is the end-product of aerobic metabolism of living creatures, the CAs are found in virtually all organisms, from the most simple ones to higher vertebrates. In humans a total of 16 CA isozymes are currently known, with different tissue and organ distribution, subcellular localization and catalytic properties. Thus, one can distinguish cytosolic forms (CA I, CA II, CA III, CA VII and CA XIII), membrane-bound ones (CA IV, CA IX, CA XII, CA XIV and CA XV), mitochondrial isozymes (CA VA and CA VB) and secreted isoforms (CA VI). Some of these isozymes are very fast (CA II, CA IV, CA VII, CA IX, CA XII and CA XIII), others are slower (CA I, CA III, CA V, CA VI and CA XIV). Three CA-related proteins (CARPs) were also identified (CA VIII, CA X and CA XI) that are completely devoid of catalytic activity [1,2].

These isozymes are involved in critical physiologic and pathologic processes, including respiration, acid-base regulation, electrolyte secretion, bone resorption/calcification, gluconeogenesis, tumorigenicity and the growth and virulence of various pathogens. Some of them are overexpressed in pathological conditions such as edemas, glaucoma, obesity and cancer. Therefore CA isozymes have become important targets for pharmaceutical research [1–4].

The active site of the catalytically able CAs contains a zinc ion, which is essential for catalysis. The metal is located at the bottom of the conical active site cavity (~13 Å deep) and it is coordinated by three histidine residues (His-94, His-96 and His-119 in CA II), with the fourth ligand being a water molecule or a hydroxyl ion in the inactive/active form of the enzyme. The active site cavity is half hydrophilic and half hydrophobic, being adapted to the intrinsic polarity of the chemical species involved in the CO_2 hydration reaction [1–3].

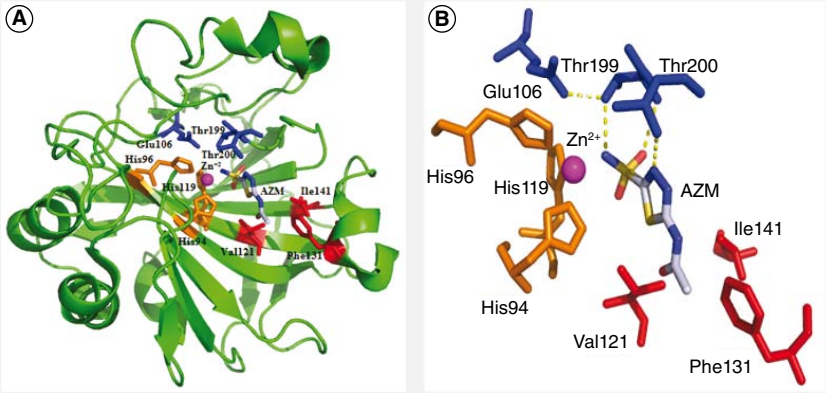
There are two main classes of CA inhibitors (CAIs), namely inorganic anions and organic ligands [1,2,5]. Within the second category we can distinguish sulfonamides, sulfamates, sulfamides and hydroxamates, as well as the recently introduced xanthates [1–4]. To date, the primary sulfonamides ($R\text{-}SO_2NH_2$) are the most investigated and used CAIs in physiological and pharmacological studies, as well as in clinical use. The sulfonamide group ionizes first and subsequently binds the zinc ion in

Aa Isoenzymes (isozymes): chemically distinct forms of a particular enzyme that differ in amino acid sequence but catalyze the same (bio) chemical reaction.

Active site (of an enzyme): a portion (region) of an enzyme where substrate binds and undergoes the chemical reaction catalyzed by the enzyme.

Enzyme inhibitor: compound that binds to an enzyme and interferes with its catalytic properties, decreasing its activity.

Figure 3.1.



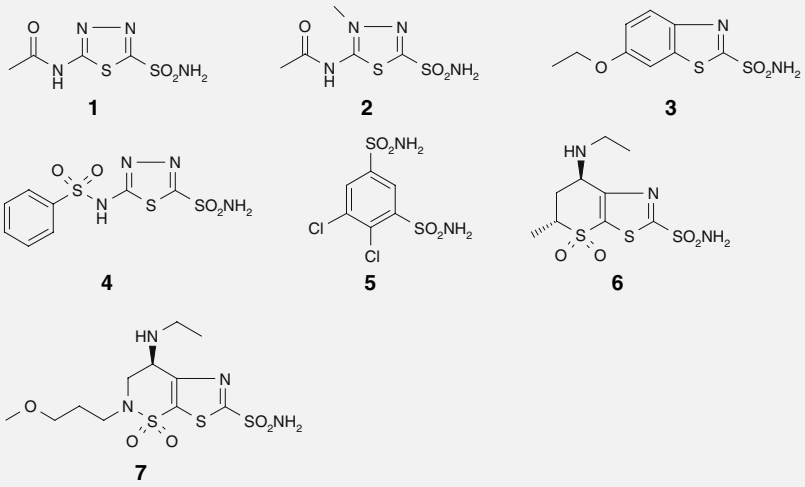
(A) Ribbon diagram of the hCA II/acetazolamide (1) adduct (pdb code 3hs4 [7]) showing interaction of the inhibitor, with key residues within the active site of the enzyme detailed in **(B)**. Helix and β-strand regions are shown in green. Zn^{2+} ion (magenta) is coordinated by three histidine residues (orange), the fourth ligand being the sulfonamide, which binds in its ionized form. Key amino acids that are stabilizing the enzyme–inhibitor complex are depicted in blue (hydrophilic ones) and red (hydrophobic ones).
Figure made using PyMol (DeLano Scientific).

deprotonated form, displacing the H_2O/OH^- ligand of Zn (II). The interaction is further stabilized by hydrogen bonding with Thr-199 and additional interactions between the backbone of the inhibitor and the two sides of the active site – one hydrophilic, the other one hydrophobic, as exemplified on the CA II – acetazolamide adduct **(Figure 3.1)** [6,7].

Thus, a large number of aromatic/heterocyclic sulfonamides were synthesized and investigated as CAIs and this research translated into the clinically used inhibitors acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, benzolamide **4**, dichlorphenamide **5**, dorzolamide **6** and brinzolamide **7** **(Figure 3.2)** [1,2,4].

Clinically used CAIs **1–5** have good tissue penetrability and inhibit efficiently most of the CA izozymes. Their rather uniform potency and lack of selectivity against different CA isozymes can be explained by the relatively high sequence homology of CA isoforms. Consequently, the systemic use of sulfonamides **1–5** is associated with serious side effects, such as fatigue, metallic taste, paresthesia and decreased libido, due to efficient inhibition of all CA isozymes in the human body [1,2]. An effective way to reduce the extent of side effects is to administer the sulfonamide CAIs topically, as in the case of dorzolamide **6** and brinzolamide **7** used in the

Figure 3.2.



Clinically used carbonic anhydrase inhibitors. The first five representatives were designed for systemic use while the dorzolamide **6** and brinzolamide **7** were designed for topical administration.

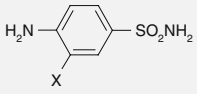
treatment of glaucoma via topical administration into the eye [1,2]. However, this strategy can be applied only for tissues amenable to topical treatment and side effects were observed even when topically active CAIs were used, although to a reduced extent compared with systemic drugs. Novel isozyme-selective CAIs delivered systemically are needed in key areas such as management of various forms of cancers in which CA IX and CA XII isozymes are overexpressed [1,2,8]. The methods available to improve the isozyme selectivity of known sulfonamide CAIs will be discussed in the following sections, emphasizing the main strategies currently pursued for next-generation sulfonamide CAIs.

Enhancing the isozyme selectivity of sulfonamide CAIs

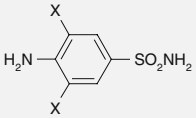
Exploiting the difference in sequence between the different isozymes

Even if CA isozymes are very closely related structurally, they are not identical. The sequence differences create small binding pockets within the active site of certain isozymes. This strategy was used to find selective inhibitors for CA IX in the first inhibition study of this isozyme with sulfonamides [5]. Two known CAI scaffolds, namely sulfanilamide and aminobenzolamide were derivatized with different halogens of increased steric bulk (F, Cl, Br, I) at selected positions of their phenyl rings **(Figure 3.3)**. One or two halogens (identical or different) were used for each scaffold, in

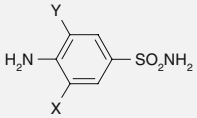
Figure 3.3.



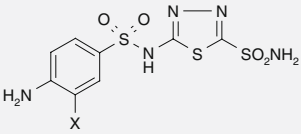
8a-d (X = F, Cl, Br, I)



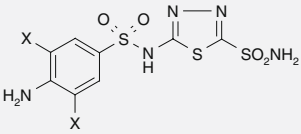
9a-c (X = Cl, Br, I)



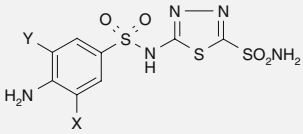
10a-f (X, Y = F, Cl; F, Br; F, I Cl, Br; Cl, I; Br, I)



11a-d (X = F, Cl, Br, I)



12a-d (X = Cl, Br, I)



13a-f (X, Y = F, Cl; F, Br; F, I Cl, Br; Cl, I; Br, I)

Halogenosulfonamides synthesized and tested as selective inhibitors against carbonic anhydrase IX.

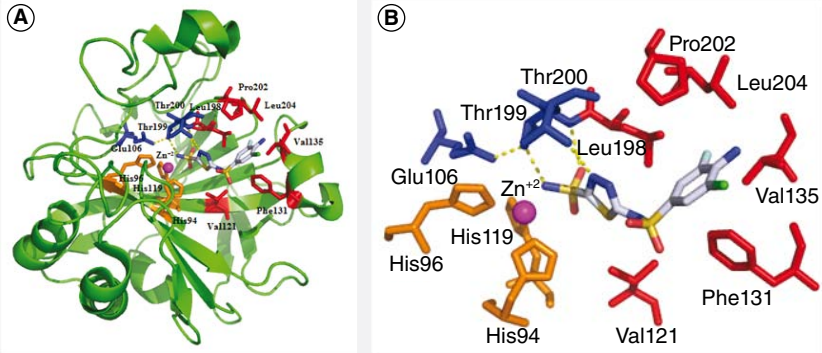
order to generate enough structural diversity for efficient sampling the active site of different CA isozymes for possible mini-pockets with atoms of appropriate size and positioning. A series of 26 compounds of types **8–13** were synthesized and tested against membrane-bound CA IX, as well as against off-target, physiologically relevant, cytosolic isozymes CA I and CA II and membrane-bound isozyme CA IV. As expected, the potency and selectivity profiles of these compounds varied significantly, with many of the novel sulfonamides surpassing the potency of clinically used inhibitors **1–7**. The most potent and selective CA IX inhibitor was 3-fluoro-5-chloro-4-aminobenzenesulfonamide **10a** ($K_{i\ hCA\ I}$ = 3800 nM, $K_{i\ hCA\ II}$ = 32 nM, $K_{i\ hCA\ IV}$ = 95 nM, $K_{i\ hCA\ IX}$ = 12 nM). In general, compounds **8–10** proved more selective against CA IX than compounds **11–13**, indicating that selectivity against CA IX can be achieved with halogeno groups placed closer to the sulfonamide group and, hence, to the zinc ion within the active site of CA isozymes. Dihalogenoderivatives **9** and **10** were more potent and selective than their mono-halogeno congeners against CA IX. Dihalogenoderivatives **9**, **10**, **12**, **13** were found to be slightly more potent than their mono-halogeno congeners **8** and **11**, proving the impact of electron-withdrawing effect of halogens on the sulfonamide moiety and consequently on the inhibitor potency.

Compounds **11–13** displayed a rather strong and uniform potency against all four isozymes, that is, **13a** ($K_{i\ hCA\ I}$ = 1.4 nM, $K_{i\ hCA\ II}$ = 0.3 nM, $K_{i\ hCA\ IV}$ = 15 nM, $K_{i\ hCA\ IX}$ = 38 nM) [5,9]. The explanation for this particular inhibition profile was provided by Supuran and DeSimone's groups via x-ray crystallography

of the adduct of 3-fluoro-5-chloroaminobenzolamide **13a** with CA II [10], which revealed a bent conformation of the inhibitor in the active site of the enzyme, with the phenyl ring of benzolamide making this time an edge-to-face interaction with Phe-131 and pointing towards an amphiphilic part of the active site delimited by this residue, Val-135, Leu-198 and Leu-204 **(Figure 3.4)**.

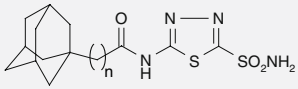
This x-ray study also revealed the difficulties in generating CA IX selective inhibitors using this strategy as the number of aminoacids from the active site that differ in CA IX from CA II is rather small [1,2]. Among them, residue 131 (Phe in CA II, Leu in CA IX) was the most promising for inducing CA IX selectivity. Since this amino acid is placed at the edge of the active site, selective inhibitors must combine structural rigidity with large steric demand (bulkiness). These desiderates were fulfilled by amido-sulfonamides **14** and **15** **(Figure 3.5)** [11] and their ureido and thioureido congeners, in which classical aromatic/heterocyclic sulfonamide-based scaffolds were decorated with adamantane moieties to generate lipophilic inhibitors of CA isozymes in the brain [12]. Among them, adamantylsulfonamide **14a** proved to strongly inhibit CA IX and CA XII ($K_{i\,hCA\,I}$ = 884 nM, $K_{i\,hCA\,II}$ = 11 nM, $K_{i\,hCA\,IX}$ = 6.4 nM, $K_{i\,hCA\,XII}$ = 2.8 nM). Its superior homolog **14b** was approximately seven-times less potent against CA IX while retaining the efficacy towards the other isozymes ($K_{i\,hCA\,I}$ = 362 nM, $K_{i\,hCA\,II}$ = 8.9 nM, $K_{i\,hCA\,IX}$ = 49.5 nM, $K_{i\,hCA\,XII}$ = 4.7 nM).

Figure 3.4.

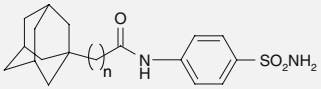


(A) Ribbon diagram of the human carbonic anhydrase II/**13a** adduct showing interactions with key amino acid residues (PDB code 2hoc [10]). **(B)** Representation of halogenosulfonamide **13a** binding within the human carbonic anhydrase II active site revealing the bent conformation of the inhibitor and the edge-to-face interaction with Phe-131.
Figure made using PyMol (DeLano Scientific).

Figure 3.5.



14a (n = 0)
14b (n = 1)

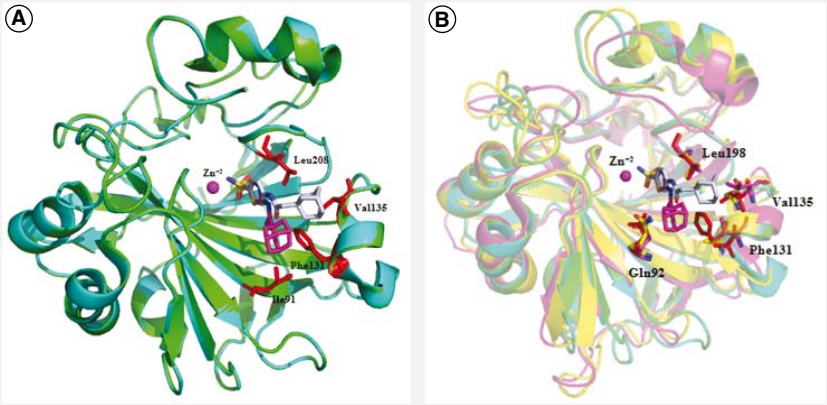


15a (n = 0)
15b (n = 1)

Adamantane sulfonamides synthesized and tested as selective inhibitors against carbonic anydrase IX.

An explanation for the different inhibitory profiles was provided by x-ray crystallography, which revealed a similar orientation of the thiadiazole moiety as in hCAII/**13a** adduct but with the adamantane tail moiety of inhibitor **14a** positioned within a different cavity, delimited by hydrophobic residues Phe-131 and Ile-91 (**Figure 3.6A**, with **14a** in pink) [9]. Interestingly, the replacement of the thiadiazole from **14b** with a phenyl ring in **15b** reorients the adamantane moiety in the pocket flanked by residues 131, 135, 198 and 204 (**Figure 3.6**, with **15b** shown in grey). Since this pocket is less hydrophobic than the first one, the result is a decrease in potency against hCA II while retaining the potency against hCA IX and hCA XII (**15a**: $K_{i\ hCA\ I}$ = 18.6 µM, $K_{i\ hCA\ II}$ = 265 nM, $K_{i\ hCA\ IX}$ = 51.3 nM, $K_{i\ hCA\ XII}$ = 7.1 nM; **15b**:

Figure 3.6.



(A) Superposition of human carbonic anhydrase (hCA) II/**14a** complex with hCA II/**15b** adduct [11], revealing the structural effect induced by the phenyl ring in **15b** that caused the reorientation of the adamantly moiety from one hydrophobic pocket to another, and **(B)** its impact on isozyme selectivity of the two inhibitors (hCA II [green/blue], CA IX [pink], CA XII [yellow]).
Figure made using PyMol (DeLano Scientific).

$K_{i\,hCA\,I}$ = 15.1 µM, $K_{i\,hCA\,II}$ = 233 nM, $K_{i\,hCA\,IX}$ = 56.5 nM, $K_{i\,hCA\,XII}$ = 9.4 nM), which have Val and Ala, respectively, instead of Phe in position 131 **(Figure 3.6B)**.

Exploiting the difference in cellular localization of CA isozymes

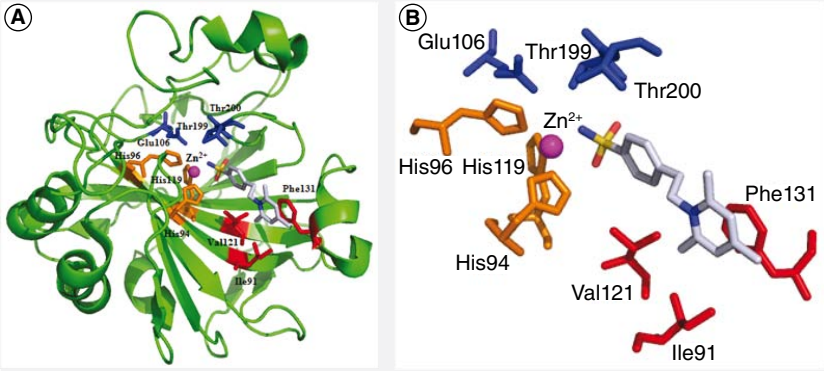
Another strategy to achieve isozyme selectivity is to restrict the permeation of the inhibitor through the membrane of target cells. A membrane-impermeant inhibitor will thus inhibit only the membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV), leaving the cytosolic isozymes unaffected. There are several ways to make an inhibitor membrane-impermeant: making the compound bulky; or making the compound very polar or charged (either positively or negatively).

The first strategy was used in the early 1980s to generate selective inhibitors for CA IV by conjugating polymers such as dextran, aminoethyldextran or polyethyleneglycol to known aromatic/heterocyclic sulfonamides. Owing to their high molecular weights, compounds such as **16**, **17** or **18** (MW in the range of 3.5–99 kDa) proved to be membrane impermeant and inhibited only membrane-bound isozyme CA IV *in vivo*. Their development was limited due to bioavailability problems and allergic reactions [4].

Another way to generate membrane-impermeant sulfonamide inhibitors is through conjugation of aromatic and heterocyclic sulfonamides with highly polar or permanently charged groups. Thus, the groups of Poulsen, Winum and Supuran prepared several series of glucoconjugate benzenesulfonamides by attaching sugar moieties to the classical aromatic sulfonamide pharmacophores [13–21]. The high polarity of various sugar moieties was used to confer poor membrane permeability to novel inhibitors. Attachment of sugars was performed either through direct conjugation [13] or through the use of triazole- (generated through 'click' chemistry [14–16,18,21]) sulfonamide- [17] or thiourea- [19,20] based linkers. Besides impairing membrane permeability, the carbohydrate moiety allows the exploitation of small differences within the active sites of different CA isozymes via their chirality and surface topology, sometimes achieved through acetylation of OH moieties, with examples such as **19**: $K_{i\,hCA\,I}$ = 2.4 µM, $K_{i\,hCA\,II}$ = 378 nM, $K_{i\,hCA\,IX}$ = 23 nM [14]; **20**: $K_{i\,hCA\,II}$ = 384 nM, $K_{i\,hCA\,IX}$ = 430 nM, $K_{i\,hCA\,XII}$ = 4.3 nM, $K_{i\,hCA\,XIV}$ = 11 nM [15]; **21**: $K_{i\,hCA\,I}$ = 560 nM, $K_{i\,hCA\,II}$ = 13 nM, $K_{i\,hCA\,IX}$ = 8.4 nM [16]; **22**: $K_{i\,hCA\,I}$ = 102 nM, $K_{i\,hCA\,II}$ = 9.1 nM, $K_{i\,hCA\,IX}$ = 95 nM, $K_{i\,hCA\,XII}$ = 8.3 nM [17]; **23**: $K_{i\,hCA\,I}$ = 4.26 µM, $K_{i\,hCA\,II}$ = 271 nM, $K_{i\,hCA\,IX}$ = 2.1 nM, $K_{i\,hCA\,XII}$ = 9.8 nM, $K_{i\,hCA\,XIV}$ = 7.8 nM; and **24**: $K_{i\,hCA\,I}$ = 112 nM, $K_{i\,hCA\,II}$ = 5.3 nM, $K_{i\,hCA\,IX}$ = 6.2 nM, $K_{i\,hCA\,XII}$ = 10 nM [21]. Notably, compound **28** and congeners proved efficient towards inhibiting the proliferation of CA IX and CA XII-expressing LS174Tr colon cancer cells [21].

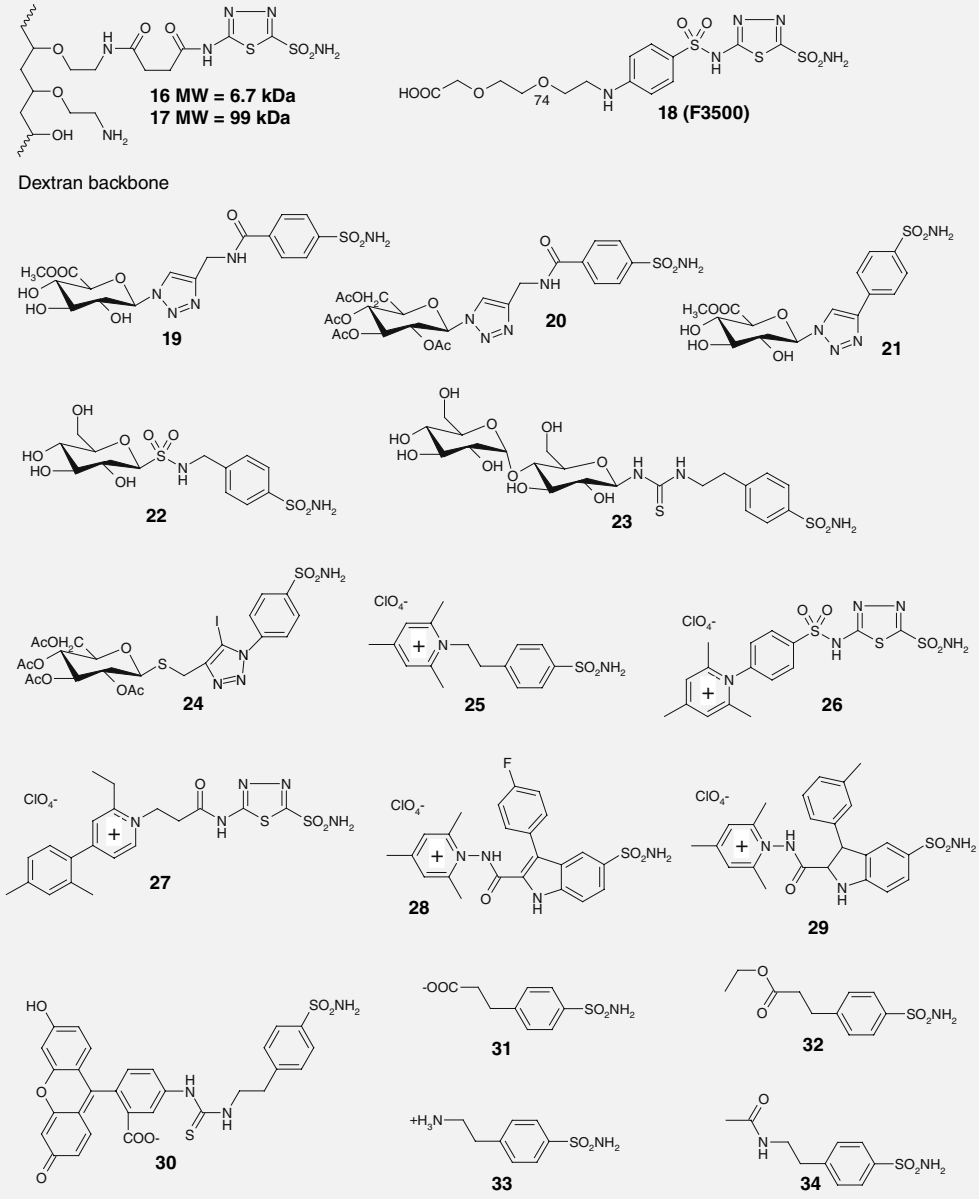
An alternative approach to membrane-impermeant CA inhibitors was proposed by Supuran, Ilies and collaborators, involving the conjugation of aromatic and heterocyclic amino-sulfonamides with pyridinium positively charged moieties, generated via reaction of pyrylium salts with primary amino groups [22–25]. Efficient sampling of the active site of CA isozymes was achieved through the use of substituents with different shapes and steric demands on the pyridinium ring, generating nanomolar potent positively charged sulphonamides, such as **25–27**, with moderate selectivity *in vitro* (**25**: $K_{i\ hCA\ I}$ = 4 µM, $K_{i\ hCA\ II}$ = 21 nM, $K_{i\ bCA\ IV}$ = 60 nM [23]; **26**: $K_{i\ hCA\ I}$ = 18 nM, $K_{i\ hCA\ II}$ = 4 nM, $K_{i\ bCA\ IV}$ = 10 nM [22]; **27**: $K_{i\ hCA\ I}$ = 300 nM, $K_{i\ hCA\ II}$ = 7 nM, $K_{i\ bCA\ IV}$ = 30 nM [24]). However, due to their salt-like nature these pyridinium sulfonamides proved to be membrane-impermeant, inhibiting only the membrane-bound isozyme CA IV while leaving unaffected the cytosolic CA I and CA II [22–25]. The high affinity of these inhibitors for CA II was proved to be related with the ∏–∏ stacking of the pyridinium moiety with Phe-131 residue within the CA II active site **(Figure 3.7)** [26]. Compounds **25** and **26** also proved efficient against tumor overexpressed isozymes CA IX and CA XII and against other CA isozymes (**25**: $K_{i\ hCA\ III}$ = 310 µM, $K_{i\ hCA\ VA}$ = 88 nM, $K_{i\ hCA\ VB}$ = 70 nM, $K_{i\ hCA\ VI}$ = 65 nM, $K_{i\ hCA\ VII}$ = 15 nM, $K_{i\ hCA\ IX}$ = 14 nM, $K_{i\ hCA\ XII}$ = 7 nM, $K_{i\ mCA\ XIII}$ = 21 nM, $K_{i\ hCA\ XIV}$ = 13 nM [1]; **26**: $K_{i\ hCA\ IX}$ = 5.8 nM, $K_{i\ hCA\ XII}$ = 48 nM [27]). The efficiency of pyridinium sulfonamides against CA IX and CA XII was reconfirmed

Figure 3.7.



(A) Ribbon diagram of the human carbonic anyhydrase II/**25** adduct showing interactions with key residues (PDB code 1ze8 [26]). **(B)** Representation of pyridinium-sulfonamide **25** binding within the human carbonic anyhydrase II active site, revealing the conformation of the inhibitor and the π-π stacking of pyridinium ring with Phe-131.
Figure made using PyMol (DeLano Scientific).

Figure 3.8.



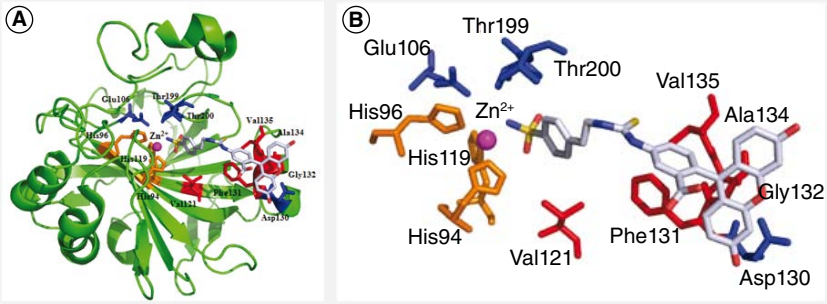
Carbonic anhydrase inhibitors designed to achieve selective membrane-bound carbonic anhydrase isozyme inhibition via restricted diffusion through biological membranes.

recently with compound **28** and congeners (**28**: $K_{i\ hCA\ I}$ = 11.3 nM, $K_{i\ hCA\ II}$ = 3380 nM, $K_{i\ CA\ IX}$ = 9.4 nM, $K_{i\ CA\ XII}$ = 9.7 nM) thus constituting, together with compounds **25–27** valuable lead compounds for further studies, which are ongoing in our laboratories. Notably, structurally related pyridinium sulfonamide **29** and several congeners were also identified as nanomolar inhibitors of β-CAs encoded by genes Rv1284 and Rv3273 from *Mycobacterium tuberculosis* while displaying good selectivity against hCA I and hCA II (**29**: $K_{i\ hCA\ I}$ = 110 nM, $K_{i\ hCA\ II}$ = 104 nM, $K_{i\ CARv1284}$ = 1.5 nM, $K_{i\ CARV3273}$ = 7.8 nM) **(Figure 3.8)** [28].

On the other hand, Pastorekova and Supuran introduced fluorescent sulfonamide **30** and congeners, synthesized via condensation of fluorescein isothiocyanate (FITC) with aminosulfonamides, as selective inhibitors of CA IX (**30**: $K_{i\ hCA\ I}$ = 1300 nM, $K_{i\ hCA\ II}$ = 45 nM, $K_{i\ CA\ IX}$ = 24 nM) [29,30]. The x-ray structure of hCA II/**30** adduct revealed the bulky fluorescein moiety extending to the rim of the active site and interacting strongly with the α-helix formed by residues Asp-130–Val 135 **(Figure 3.9)** [31]. The negatively charged carboxylate group impaired the penetrability through membranes of **30** and congeners similarly to the positively charged sulfonamides **25–29**, targeting these inhibitors to CA IX and allowing its selective detection [29,30].

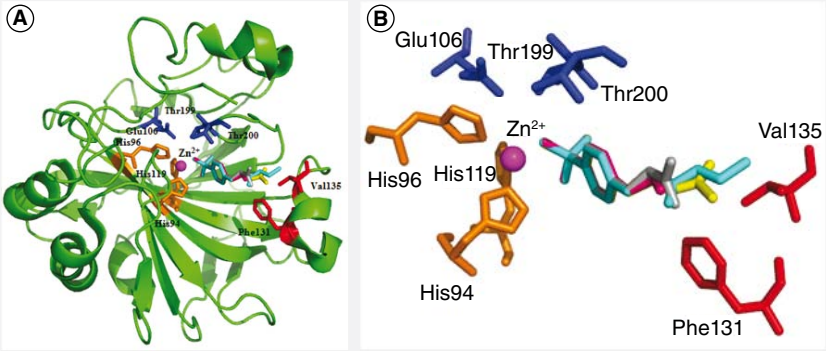
A comparison between the binding of negatively charged sulfonamide CA inhibitors in the active site of CA isozymes versus positive and neutral congeners was recently made by Srivastava, Mallik and Christianson [32]. The authors compared the x-ray crystal structure of carboxyethylbenzenesulfonamide **31** with similar structure of adducts of CA II with ester **32**, positively

Figure 3.9.



(A) Ribbon diagram and **(B)** active site binding details of the human carbonic anhydrase II adduct with negatively charged inhibitor **30** showing the conformation adopted by this bulky inhibitor within the active site of carbonic anhydrase II (PDB code 2f14 [31]). Figure made using PyMol (DeLano Scientific).

Figure 3.10.



(A) Ribbon diagram and **(B)** active site detail of the human carbonic anhydrase II depicting the superposition of its adducts with negatively charged inhibitor **31** (grey), positively charged inhibitor **33** (pink), and their neutral congeners **32** (light blue) and **34** (yellow) showing the conformation adopted by all these inhibitors within the active site of carbonic anhydrase II (PDB codes 2nno, 2nnv, 2nng, 2nns [32]).
Figure made using PyMol (DeLano Scientific).

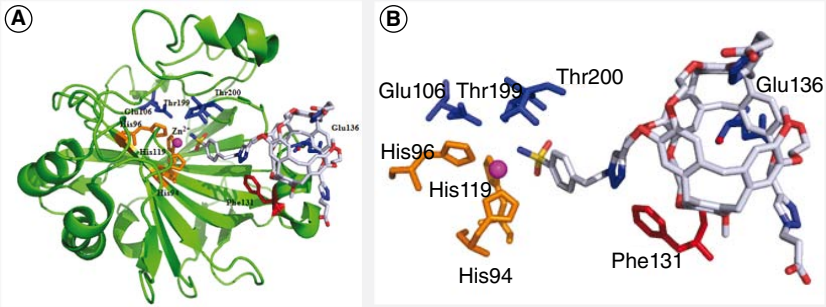
charged aminoethylbenzenesulfonamide **33** and with its acetylated congener **34**. Interestingly, no major conformational changes could be observed for adducts of hCA II with inhibitors **31–34**, despite the differences in their charged status **(Figure 3.10)** [32].

Next-generation primary sulfonamides CA inhibitors for detection & imaging of CA isozymes

The success of fluorescent sulfonamide **30** and its congeners as potent and selective inhibitors for CA IX that could be used *in vitro* [29,30] and *in vivo* [33,34] to track the presence of this isozyme overexpressed in hypoxic tumors and even inhibit the tumor growth [33] prompted the development of related primary sulfonamide markers fluorescent in near infrared such as **35–37** for the same purpose [35]. All these sulfonamides proved to possess nanomolar affinity for CA IX and CA XII, with a moderate selectivity against CA II and CA XIV. The most promising sulfonamide marker, **37** ($K_{i\,hCA\,II}$ = 248 nM, $K_{i\,hCA\,IX}$ = 7.5 nM, $K_{i\,hCA\,XII}$ = 35 nM, $K_{i\,hCA\,XIV}$ = 66 nM) showed accumulation in hypoxic tumors allowing efficient tumor mapping via noninvasive *in vivo* FMT imaging in mice [36].

Dmochowski's and Christianson's groups proposed a cryptophane–sulfonamide biosensor encapsulating ^{129}Xe as a biomolecular probe for CA isozyme imaging via ^{129}Xe- MRI. Trifunctionalized cryptophane biosensors **38a–c** were synthesized via triazole click chemistry and proved efficient

Figure 3.11.

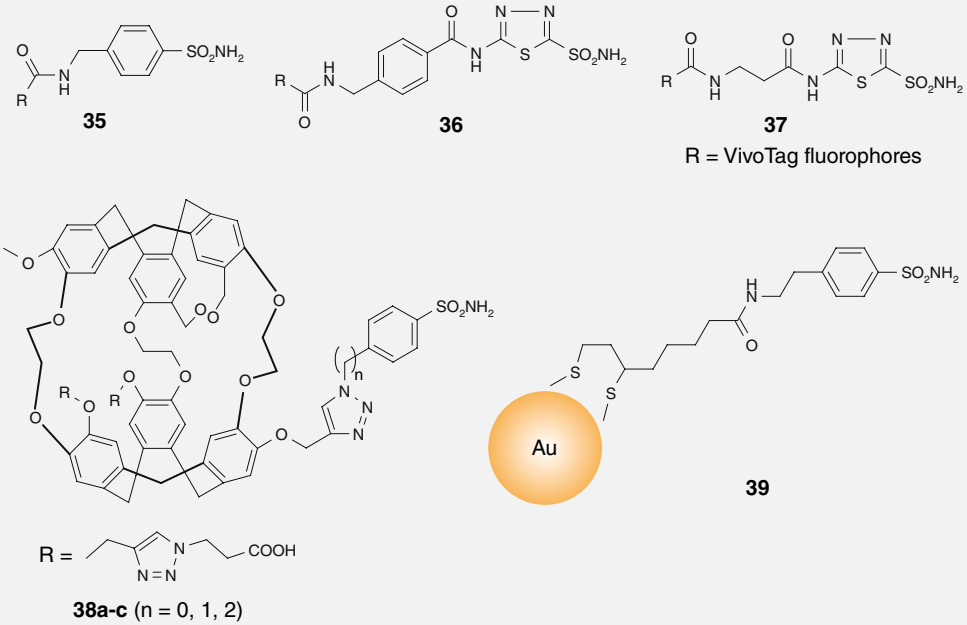


(A) Ribbon diagram and **(B)** active site detail of the human carbonic anhydrase II complexed with Xe-loaded cryptophane-sulfonamide **38c**, revealing the positioning of the inhibitor and its interaction with key amino acid residues within carbonic anhydrase active site (PDB code 3cyu [38]).
Figure made using PyMol (DeLano Scientific).

inhibitors of CA I and CA II (e.g., **38c**: $K_{D\ CA\ I}$ = 30 nM, $K_{D\ CA\ II}$ = 60 nM) [37]. An analysis of the complex between Xe-loaded **38c** and hCA II confirms the presence of Xe within the cryptophane cage that is positioned at the boundary of CA II active site, making van der Waals contacts with Gln-136 **(Figure 3.11)** [38]. This optimal positioning can explain the isozyme-specific chemical shifts obtained with biosensor **38c** and congeners, since the amino acid residues flanking the active site differ from one izozyme to another [37].

In another approach to generate membrane-impermeant CA inhibitory systems for imaging of tumors Supuran's and Winum's groups proposed gold nanoparticles coated with sulfonamide-based carbonic anhydrase inhibitors **39** **(Figure 3.12)** [39]. Energy dispersive x-ray analysis (EDX), in conjunction with transmission electron microscopy (TEM), surface plasmon spectroscopy and elemental analysis revealed a loading of approximately 140 molecules of **39** on the surface of a 3.3 nm Au nanoparticle. Biological testing showed a good affinity of this colloidal system for CA IX and a moderate selectivity against CA I and CA II ($K_{i\ hCA\ I}$ = 581 nM, $K_{i\ hCA\ II}$ = 451 nM, $K_{i\ hCA\ IX}$ = 32 nM) [39]. Considering that the system is membrane impermeant, as proved by red blood cell permeability studies, this technology constitutes another direction to be pursued for developing next-generation sulfonamide CA inhibitors and was recently improved in terms of method of preparation and stability of the nanosystem by one of the authors [40].

Figure 3.12.



Carbonic anhydrase inhibitors designed for imaging of carbonic anhydrase isozymes.

Conclusion & perspective: next-generation primary sulfonamides CAIs for cancer treatment

Besides the issue of isozyme affinity and selectivity, which remain permanent desiderates from next-generation CA inhibitors, other issues related with pharmacokinetics and pharmacodynamics of these drugs remain to be optimized in order to be used efficiently towards cancer treatment. In this direction notable progresses were made by using ureido- and thioureido-sulfonamides [33,41], which displayed good CA inhibition, isozyme selectivity and tissue penetrability, making thiourea a preferred linker used in conjunction with classical aromatic and heterocyclic sulfonamides, as well as associated with other CA active site-targeting structural motifs [1,2,8].

The aforementioned technologies involving next-generation sulfonamide CA inhibitors with improved affinity and selectivity for tumor-overexpressed isozymes, some suitable for imaging purposes, give good hope for the development of novel treatments against various hypoxic tumors, either alone or in combination with established anticancer therapies.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Summary.

- All 16 known carbonic anhydrase (CA) isozymes share a high sequence homology and metal (zinc) content that makes the design of selective inhibitors a challenging task.
- Primary aromatic and heterocyclic sulfonamides are powerful CA inhibitors that bind strongly the zinc ion in the active site of all zinc-containing isozymes, which further reduces the selectivity of this class of CA inhibitors.
- Lack of isozyme selectivity of sulfonamide CA inhibitors translates into significant side effects due to inhibition of CA isozymes with different tissue localizations.
- Main strategies for enhancing selectivity without sacrificing potency involve the exploitation of (small) differences existing between isozyme amino acid sequences and of different cellular and tissue localization of CA isozymes.
- Next-generation primary sulfonamides combine potency, isozymes selectivity with imaging capabilities and optimized pharmacokinetic and pharmacodynamic properties.

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Chapter 4

Next-generation secondary/tertiary sulfonamide carbonic anhydrase inhibitors

Sébastien Thibaudeau, Hélène Carreyre, Agnès Mingot & Claudiu T Supuran

Nowadays, cancer is becoming one of the main challenges of public health in the world. Recently, a new application of the human carbonic anhydrase inhibitors (CAIs) regards their utility in the treatment of hypoxic tumors, through the selective inhibition of human carbonic anhydrase (hCA) IX and XII, which are highly overexpressed in tumors. In the quest of selective isoforms, recent advances have been made allowing to identify novel chemotypes, acting as non-zinc-binding inhibitors. Whereas primary sulfonamides are historically known to be good hCA inhibitors (hCA IX and XII as well), their applications in this field were limited by their promiscuous inhibiting effects of many of the widespread isoforms, among which the cytosolic hCA I and II. At the same time, due to the difficulty of secondary and tertiary sulfonamides to anchor the Zn^{2+} ion within the active site of CAs, these compounds have been considered for a long time not to be good targets as inhibitors of these enzymes. The reported data in this chapter show that in

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the quest of tumor-associated hCA inhibitors, these neglected classes of compounds might find a renewed interest. Recent data are reported and emphasize that secondary and tertiary benzenesulfonamides can act as very potent and selective hCA IX inhibitors, and should be better studied in the future.

Owing to their broad biological profiles, sulfonamide-containing compounds have great importance in medicinal chemistry. Renewed interest in sulfonamides for the design of anti-tumoral, anti-bacterial and anti-inflammatory agents led to the development of innovative synthetic methods for their preparation [1]. The carbonic anhydrases (CAs; EC 4.2.1.1), ubiquitous metalloenzymes, are important targets for designing selective inhibitors with clinical applications **(Table 4.1)** [2].

Table 4.1. Human carbonic anhydrase isoforms as drug targets/off-targets in various diseases†.

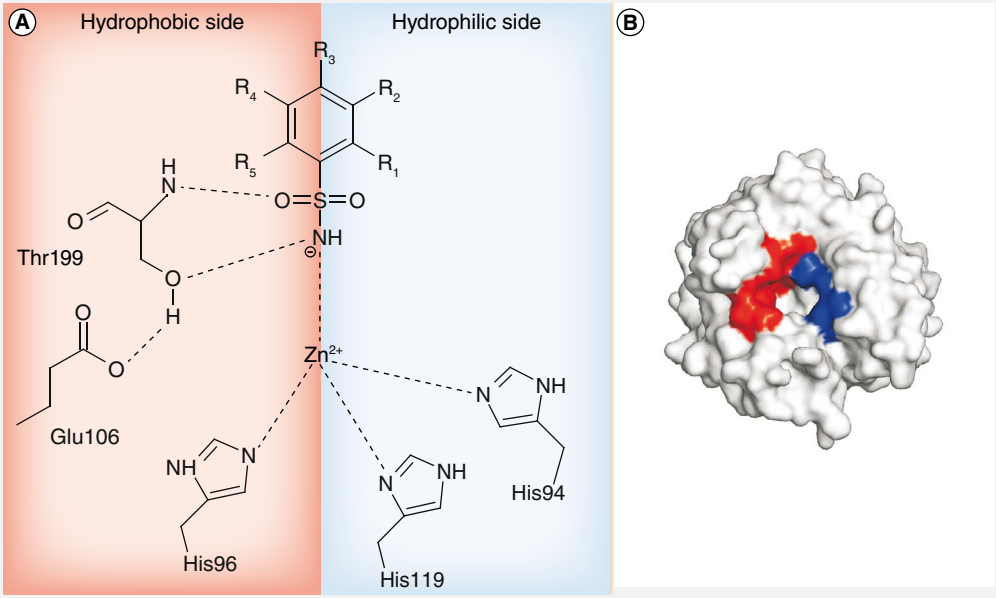
Isoform	Disease in which is involved	Possible off-targets among other hCAs
CA I	Retinal/cerebral edema	Unknown
CA II	Glaucoma Edema Epilepsy Altitude sickness	hCA I Unknown Unknown Unknown
CA III	Oxidative stress	Unknown
CAIV	Glaucoma Retinitis pigmentosa Stroke	hCA I Unknown Unknown
CA VA/VB	Obesity	hCA I, hCA II
CA VI	Cariogenesis	hCA II
CA IIV	Epilepsy	Unknown
CA VII	Neurodegeneration Cancer	Unknown Unknown
CA IX	Cancer	hCA I, hCA II
CA XII	Cancer Glaucoma	hCA I, hCA II Unknown
CA XIII	Sterility	Unknown
CA XIV	Epilepsy Retinopathy	Unknown Unknown

†No data are available in the literature on CA X and XI involvement in diseases.
hCA: Human carbonic anhydrase.

Among the various families of CAIs, to date, the sulfonamide group (RSO_2NH_2) is the most largely used function for the design of inhibitors, mainly belonging to the aromatic/heterocyclic sulfonamide classes [3]. Recently, for example, old sulfonamide diuretics, such as indapamide or furosemide, were found to considerably inhibit several CA isoforms and x-ray crystal structures of the CA II-diuretic adducts showed essential features for the design of novel classes of CAIs [4]. Crystallographic data are also available for many primary benzenesulfonamide adducts with several isozymes [5]. These studies show that primary benzenesulfonamides' binding driving force is the coordination of the deprotonated sulfonamide nitrogen to the catalytic Zn^{2+} ion, with additional hydrogen bonds between the sulfonamide moiety and Thr199 residue **(Figure 4.1A)**.

In addition, modifications of the aromatic ring induce generally a strong variation in the hCA inhibition profile (modification of the selectivity), probably due to variation of interactions between the molecule and the hydrophobic and hydrophilic half parts of the isoforms **(Figure 4.1B)**. Among CAs, the

Figure 4.1. Binding of sulfonamide inhibitors to carbonic anhydrase.



(A) Key interactions between a generic benzenesulfonamide inhibitor and the hCAII active site. **(B)** Solvent accessible surface of hCAII. Residues delimiting the hydrophobic half of the active site cleft are shown in red (Ile91, Phe131, Val121, Val135, Leu141, Val143, Leu198, Pro202,Leu 204, Val207 and Trp209) while residues delimiting the hydrophilic one are shown in blue (Asn62, His64, Asn67 and Gln92).

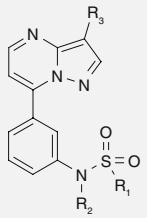
transmembrane isoforms hCA IX and XII became recently very attractive drug targets [6]. For example, hCA IX is a very peculiar isoform, as it is only expressed in a few normal tissues and overexpressed on cell surfaces of solid tumors. In addition, it was clearly demonstrated that the highly catalytically active and widespread cytosolic isoform CA II is responsible for most of the side effects of the primary sulfonamide-based CA inhibitors [7,8]. The other cytosolic isoform hCA I, also widespread in the blood and gastrointestinal tract shows a lower catalytic activity [5]. As a consequence, hCA IX and XII selective inhibitors (over the off-targets hCA I and II) became drug targets of choice in the design of new anticancer agents. As few isoform-specific inhibitors are known yet in the benzenesulfonamide series, the quest of new hCA inhibitors has given rise to the need of new chemotypes in this family [9]. To design inhibitors that would preferentially inhibit transmembrane isoforms over all other isoforms [10], elegant strategies such as structural approaches or generation of constitutional dynamic library have been recently employed [11]. When these methods focused on the fine structural variations within the isoforms catalytically active sites (close to the Zn^{2+} ion), new selective inhibitors of tumor-associated CAs have been discovered to act as non-zinc-binding inhibitors [12]. In this series, (thio)coumarins and recently sulfocoumarins were shown to be hydrolyzed by the esterase CA activity, then anchored in the enzyme without direct interactions within the Zn^{2+} ion, into a so-called coumarin-binding site [13]. These recent data clearly demonstrated that not only zinc-binding type inhibitors may exist and targeting different modes of inhibition for benzenesulfonamide inhibitors may be envisioned. When primary benzenesulfonamides were largely explored for their CA inhibition, secondary and tertiary ones were less investigated. In the meantime, the use of sulfonamide is not limited to the CAI field, and targeting secondary and tertiary benzenesulfonamides is a valid approach in medicinal chemistry investigation as shown by the recently patented benzenesulfonamides **(Figure 4.2)** [14].

Therefore, there is a growing interest in identifying new secondary and tertiary benzenesulfonamides with different mechanisms of action for hCA selective inhibition, especially hCA IX and XII selective inhibition. Recently, secondary and tertiary benzenesulfonamides acting as selective CA inhibitors were discovered and they will be reviewed in this chapter. First, a focus on novel secondary benzenesulfonamides that show selectivity toward the transmembrane hCA IX and XII isoforms versus widespread cytosolic hCA I and II, will be reported. In the second part, inhibition data of newly reported tertiary benzenesulfonamides acting as selective tumor-associated isoforms inhibitors will be detailed.

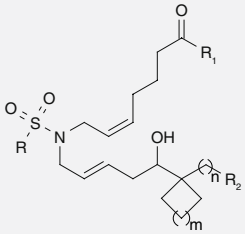
Aa Selectivity: the state or quality of being selective, a parameter essential for carbonic anhydrase inhibitor discovery.

Tumor-associated isoforms: hCA IX and XII isoforms are over-expressed in hypoxic tumor cells.

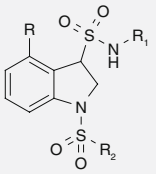
Figure 4.2. Recently patented secondary and tertiary benzenesulfonamides as promising therapeutic agents.

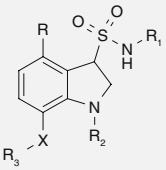


Pyrazolo-(1.5-a)-pyrimidin-7-aryl scaffolds
CNS diseases



Compounds filed as IOP lowering and neuroprotective agents
Glaucoma





Indole sulfonamides as SFRP-1 modulators
Bone remodeling

IOP: Intraocular pressure.

Secondary benzenesulfonamides acting as selective CAIs

As previously mentioned, numerous structural studies showed that benzenesulfonamides bind the zinc ion in their deprotonated form [5]. Probably due to the dominant effect of this interaction, in general primary benzenesulfonamides behave as very good CAIs but are not selective, limiting their potential. Although many SAR studies have been performed on primary benzenesulfonamides by modifying the aromatic ring substitution, so far, none of them led to the discovery of selective inhibitors. Any structural changes in the aromatic ring, aiming at modifying polar and hydrophobic interactions **(Figures 4.1A & 4.1B)**, had a rather marginal effect on the inhibitory profile. To generate selective benzenesulfonamide type inhibitors, an innovative approach was recently developed. Although showing lower affinity for the catalytic Zn^{2+} ion, secondary substituted benzenesulfonamides could be more selective toward some CA isoforms and could offer selectivity in the hCA inhibition with potent therapeutic applications. Although *N*-substituted benzenesulfonamides have been long described as inactive CAIs, one of us recently reported the evaluation of *N*-substituted secondary substituted benzenesulfonamides as CAIs, showing that

N-substituted benzenesulfonamides preserved inhibitory properties against hCA I, II, IX and XII, and thus despite the substitution of the nitrogen atom **(Figure 4.3 & Table 4.2** substrates 1–4) [15].

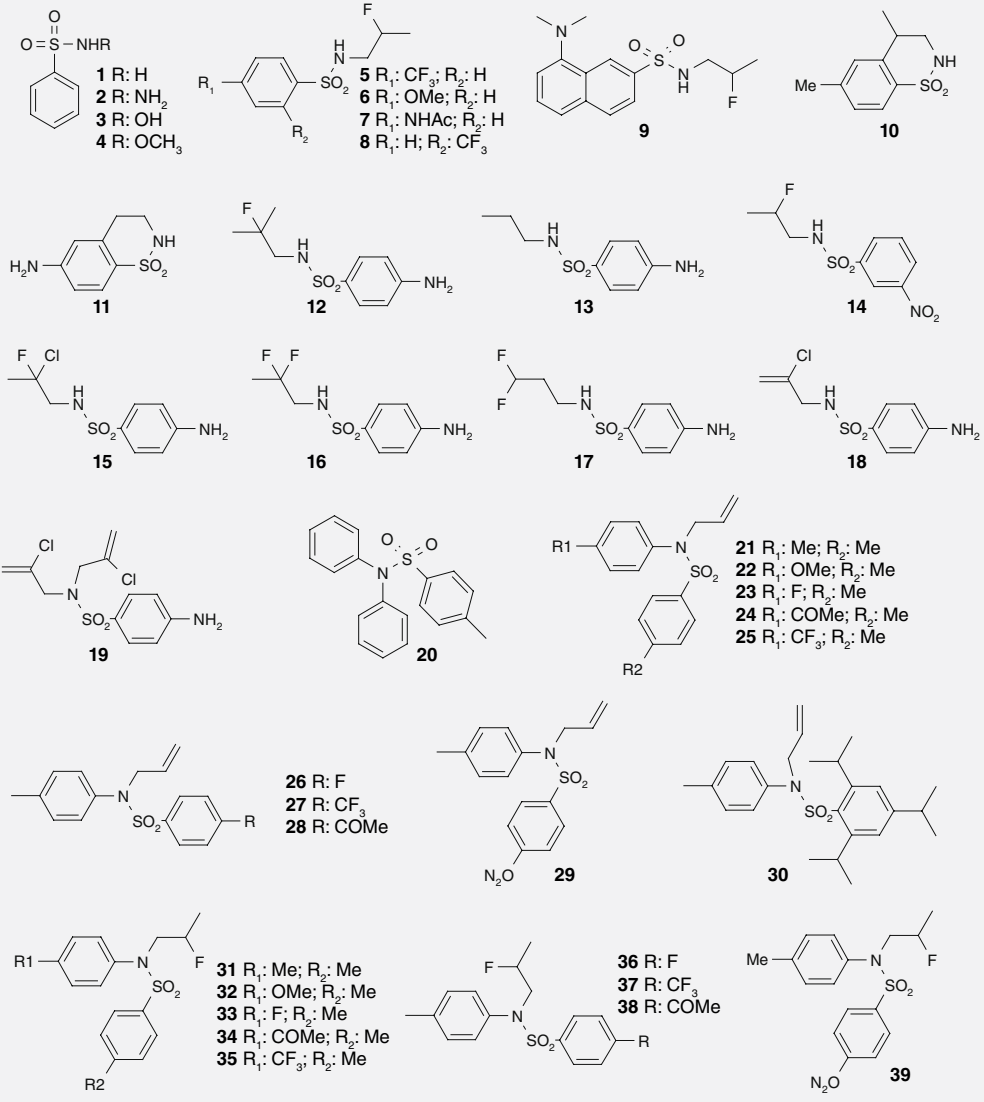
It was also interesting to note that whereas primary benzenesulfonamide **1** is active at the nanomolar (nM) level, it presents no selectivity toward hCA IX and XII. However, even if the tested *N*-substituted analogs were less efficient, the impact of the substituent on the selectivity is dramatic, as shown by the complete loss of inhibition for hCA I when the nitrogen atom is substituted with a methoxy group (compound **4**). Such a variation in the inhibition profile through a small modification of the sulfonamide function was attributed to a new binding mode for the *N*-methoxy benzenesulfonamide

Table 4.2. Inhibition data of selected secondary benzenesulfonamides against isoforms human carbonic anhydrase I, II, IX and XII.

Substrate	K_i (nM)[†]				Selectivity ratios			
	hCA I	*hCA II*	*hCA IX*	*hCA XII*	*I/IX*	*I/XII*	*II/IX*	*II/XII*
1	86	101	97	90	0.89	0.96	1.04	1.12
2	69,800	5310	51,700	2420	1.35	28.8	0.10	2.19
3	2730	5470	60,300	1530	0.04	1.78	0.09	1.08
4	[‡]	8960	64,300	8320	[§]	[§]	0.14	1.08
5	3100	4500	3300	3000	0.94	1.03	1.36	1.50
6	4600	4500	3900	3100	1.18	1.48	1.15	1.45
7	4800	4600	4400	1100	1.09	4.36	1.05	4.18
8	4700	4300	4900	1100	0.96	4.27	0.88	3.91
9	4600	2300	2700	3800	1.70	1.21	0.85	0.61
10	20,000	21,000	[§]	[§]	[§]	[§]	[§]	[§]
11	637	431	136	298	4.68	2.14	3.17	1.45
12	174	75	83	39.7	2.10	4.38	0.90	1.89
13	5142	3455	2078	1841	2.47	2.79	1.66	1.88
14	4600	2100	2900	4600	1.58	1.00	0.72	0.46
15	5179	1400	2471	1521	2.10	3.40	0.57	0.92
16	151	85.3	47.0	79.4	3.21	1.90	1.81	1.07
17	4224	4235	2123	1145	1.99	3.69	1.99	3.70
18	5626	5176	90	96	62.51	58.60	57.51	53.92

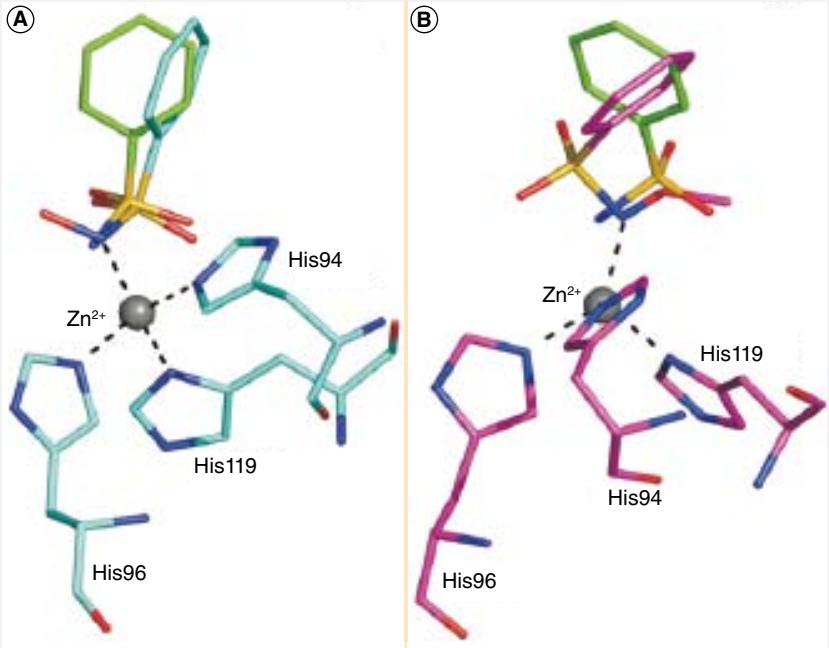
[†]Errors in the range of ± 5% of the reported data from three different assays.
[‡]Not active.
[§]Not determined.

Figure 4.3. Secondary and tertiary benzenesulfonamides acting as carbonic anhydrase inhibitors.



species **4**. This has been confirmed by analysis of the crystal structures of compounds **3** and **4** in complex with hCA II **(Figure 4.4)**. The analysis of the hCA II/**3** complex structure confirms that despite the substitution of the sulfonamido function, secondary benzenesulfonamides present a

Figure 4.4. Carbonic anhydrase II active site with sulfonamides coordinated to it.



(A) Structural superposition of benzenesulfonamide **1** (green) with **3** (cyan). **(B)** Structural superposition of benzenesulfonamide **1** (green) with **4** (magenta).
Reproduced from [15] with permission from The Royal Society of Chemistry.

binding mode to the CA active site, very close to that observed for primary benzenesulfonamide. However, the analysis of the hCA II/**4** complex revealed a completely different coordination with the sulfonamide moiety of compound **4** being rotated at almost 180° with respect to classical primary benzenesulfonamides.

This work could be considered as a proof of concept that substituting the nitrogen atom of benzenesulfonamide inhibitors is not always to the detriment of inhibition properties. This opens further potential advances in the quest for selective *N*-substituted benzenesulfonamide inhibitors interacting with regions of the active site yet unexplored.

Recently our group focused on the ability to use original superacid chemistry as a new tool to extend the molecular diversity for medicinal chemistry research. By using recently developed methodologies for benzenesulfonamides and benzofused sultams synthesis in superacid [16], a first series of secondary fluorinated and cyclic benzenesulfonamides were

synthesized and evaluated as selective hCA inhibitors [17,18].

Aa Superacid: acidic media stronger than pure sulfuric acid.

Fluorine: a nonmetallic univalent element belonging to the halogens, essential element in bio-organic and medicinal chemistry.

The introduction of fluorine atoms into biologically active organic compounds is now a common practice in SAR studies. For nitrogen-containing compounds, the modulation of the basicity of proximal functions, through a strong fluorine atom electron withdrawing effect is commonly used for improving membrane permeation and metabolic stability. In addition, α-fluorination of sulfonamide has been applied recently to develop new hCAs inhibitors, and a correlation between sulfonamide acidity enhancement and inhibition has been shown.

A similar effect could occur with the targeted fluorinated substrates. As shown by the inhibition data obtained from the representative tested secondary benzenesulfonamides, all the tested secondary benzenesulfonamides in this series were found to be potent hCA inhibitors (**Table 4.2**, substrates 5–9). They showed very close inhibition constants of 3.1–4.8 μM and 2.3–4.5 μM against the cytosolic isoforms hCA I and hCA II, respectively. The tumor-associated transmembrane isoforms hCA IX and XII were also inhibited with similar low micromolar level of inhibition. No significant effect of the aromatic modification on the selectivity or on the inhibition could have been extracted from the reported data, the tested compounds **5–9** showing a very similar inhibition profile. The inhibition profile of compound **11**, compared with the one of compound **10** clearly demonstrated that despite an increase in steric hindrance close to the Zn^{2+} active site, a simple modification of the aromatic ring allows to get good hCA inhibitors. By substitution of a methyl group of the aromatic ring by an amino group, the inhibition of hCA I and II was largely improved (from micromolar to nanomolar level of inhibition) (**Table 4.2**, substrates 10–11). The free amino group in secondary 4-aminobenzenesulfonamide was shown to be essential to get good inhibition level, a series of 'fluorinated' secondary 4-aminobenzenesulfonamide was evaluated as selective hCA inhibitors and compared with a nonfluorinated analog (**Table 4.2**, substrates 12–18).

The obtained results for compound **12** validated our approach. It demonstrated a good inhibition profile with interesting inhibition constants at the nanomolar level against tumor-associated isoforms. However, this compound could not be considered as a good selective inhibitor as it was also effective against the cytosolic isoforms hCA I and II. The impact of the insertion of the fluorine atom on the efficiency was also demonstrated, as the nonfluorinated analog **13** showed only a micromolar inhibition level.

For these β-fluorinated substrates, the impact of the second substituent geminal to the fluorine atom is dramatic. While substituting one methyl group of the substrate by a chlorine atom led to a loss of efficiency, adding a second fluorine atom did not modify the good inhibition profile of the β-fluorinated inhibitor (comparison of compounds **12**, **15** and **16**). The effect of the fluorine atom in β-position could have been attributed to a decrease of pKa correlated with an increase of efficiency. However there is a strong opposite effect of the addition of a chlorine atom compared with the addition of a second fluorine atom in the β-position. This clearly ruled out this hypothesis. Recently, O'Hagan *et al.* clearly demonstrated that through hyperconjugative interaction with a vicinal C-H bond, the C-F bond dramatically alters the conformational stabilities of substituted fluoroethanes. As a consequence, β-fluorinated nitrogen-containing compounds exhibit a preferred *gauche* conformation. For the tested substrates, the addition of a second substituent in β-position must also influence the conformational energy profile of β-fluorinated 4-aminobenzenesulfonamides, and so, the effect of the fluorinated core might be attributed to a preferential constrained conformation, essential for hCA inhibition. This hypothesis was further related by the micromolar level of inhibition for compound **17**. Increasing the distance between the zinc-chelating function and the fluorine atom had a strong negative effect on the inhibition. The strong impact of halogen substituted fragments substitution of the sulfonamide function on the inhibition profile of original secondary 4-aminobenzenesulfonamide was confirmed by testing the inhibitor activity of compound **18**. This compound was shown to be an excellent selective tumor-associated isoforms inhibitor. The low micromolar inhibition of the widespread hCA I and II isoforms, combined to a nanomolar selective inhibition of hCA IX and XII emphasized the potential of these type of inhibitors. This never yet reported inhibition profile for secondary benzenesulfonamides reinforced the potential of this strategy in the quest of selective novel benzenesulfonamide type inhibitors.

Tertiary benzenesulfonamides acting as selective CAIs

An increasing number of chemotypes other than the sulfonamides have been recently reported as CAIs [5]. All these derivatives bind to the CA active site, differently from the classical sulfonamide Zn^{2+} binding site. For example, (thio)coumarines, which show strong CA IX and XII inhibition selectivity [19] are anchored far away from the zinc ion. A recent and very popular strategy in the design of selective tumor-associated inhibitors is to conceive selective inhibitors using very specific non-zinc-binding model of inhibition. In this context, a recently designed tertiary benzenesulfonamide **19** was

evaluated as hCA inhibitor and showed an original inhibition profile for a tertiary benzenesulfonamide inhibitor (**Table 4.3**, substrate 19):

- Despite the fact that this compound cannot act in a deprotonated form, it demonstrated inhibition for both cytosolic and transmembrane isoforms;
- The micromolar level of inhibition of widespread cytosolic isoforms, combined to a nanomolar level of inhibition of tumor-associated isoforms, made this compound a very good lead for further investigations;
- This inhibition profile for a tertiary benzenesulfonamide could not come from a 'classical' zinc-binding mode of inhibition, allowing to suppose a new binding mode for tertiary benzenesulfonamides;
- Compared with the recently reported tertiary benzenesulfonamide **20** (**Table 4.3**, substrate 20) [20], the aliphatic chain impact on hCA I and II inhibition was really important emphasizing that a nonaromatic alkyl chain is essential for the inhibition efficiency of this new chemotype. It also strengthens the new binding mode hypothesis and the potential of further evaluations in this direction.

Following this innovative strategy, a series of (fluorinated) tertiary benzenesulfonamides was synthesized using a multistep synthetic strategy starting from commercially available aniline derivatives and using hydrofluorination process in superacid for the synthesis of the fluorinated analogs [21]. This method allowed to directly access a large range of 'classically' nonaccessible β-fluorinated benzenesulfonamides **(Figure 4.3)** [22]. The more significant examples are reported in **Table 4.3**. Exceptionally, all the tested tertiary benzenesulfonamides were found to be ineffective as hCA II inhibitors (or very weak ones in the case of compounds **25** and **29**) and inhibited the tumor-associated isoform IX at the nanomolar level. Such selectivity between the widespread off-target isoform II and the cancer-related isoform hCA IX was never reported before for sulfonamide-type inhibitors. What was also noticed is the strong impact of the aromatic ring substituent moieties on the efficiency of these molecules. For tosylated allylic derivatives **21–25**, when modifying the aniline core with methyl, methoxy or esters groups did not modify the inhibition efficiencies, the insertion of a fluorine atom or a trifluoromethyl group strongly impacted the inhibition profile. Fluorine substituted compound **23** was a very good hCA IX selective inhibitor only inhibiting hCA I with a higher k_i value of 238 nm (selectivity ratio I/IX: 28). When a CF_3 group was inserted, a decrease of selectivity was observed. When these modifications of the aniline core clearly impacted the selectivity of the inhibition of allylic derivatives, similar modifications on the benzenesulfonamide part had no

Table 4.3. Inhibition data of selected tertiary benzenesulfonamides against isoforms human carbonic anhydrase I, II, IX and XII.

Substrate	K_i (nM)†				Selectivity ratios			
	hCA I	*hCA II*	*hCA IX*	*hCA XII*	*I/IX*	*I/XII*	*II/IX*	*II/XII*
19	6796	4995	73.2	79.8	92.8	85.2	68.2	62.6
20	377	459	§	§				
21	75.1	‡	70.1	17.7	1.1	4.2		
22	72.5	‡	49.6	96.4	1.5	0.7		
23	238	‡	8.31	82.5	28	2.8		
24	81.7	‡	33.3	60.7	2.4	1.3		
25	58.7	366	86.9	83.6	0.7	0.7	4.2	4.4
26	154	‡	76.5	2768	2.0	0.06		
27	98	‡	61.1	3241	1.6	0.03		
28	76.1	‡	42.7	2569	1.8	0.03		
29	83.9	975	73.3	1327	1.1	0.06	13.3	0.7
30	‡	‡	451	‡				
31	73.1	‡	9.3	33.6	7.8	2.2		
32	78.6	‡	33.3	60.7	2.4	1.3		
33	9.8	‡	86.1	3511	0.1	0.003		
34	89.1	‡	9.6	83.8	9.3	1.1		
35	9.3	‡	81.2	80.4	0.1	0.1		
36	55.1	‡	35.8	1463	1.5	0.04		
37	68.3	‡	24.9	1239	2.7	0.05		
38	87.6	‡	70.6	1356	1.2	0.06		

†Errors in the range of ± 5% of the reported data from three different assays.
‡Not active.
§Not determined.

significant effect on hCA IX inhibition efficiency (Table 4.3, substrates 26–28). However, the benzenesulfonamide core modification strongly impacted hCA XII inhibition, this effect being stronger with CF_3 substituted derivatives (Table 4.3, substrates 26–28 and 36–38). Such an impact of CF_3 substituent (known to strongly modify the lipophilic character of the molecule) reinforced the hypothesis of a new binding mode for these derivatives, probably anchoring a balanced lipophilic–hydrophilic region of the active site of hCA IX isoform. The tested β-fluorinated analogs showed also a similar inhibition profile, absence of inhibition of hCA II and inhibition of hCA IX at the nanomolar level

(**Table 4.3**, substrates 31–38). The strong effect of aromatic rings modifications on the selectivity of the inhibition, and especially the impact of groups known to modify the lipophilic parameters of the molecule, led us to synthesize and evaluate the inhibitor properties of compound **30**, substituted on the benzenesulfonamide part with three isopropyl groups. Although this compound was less efficient to inhibit the targeted cancer-related isoform hCA IX (k_i = 451 nm), this compound did not inhibit hCA I, hCA II and hCA XII and can be considered as an excellent selective lead for the future quest of anticancer agent with a never yet reported inhibition profile.

Conclusion

In summary, due to the difficulty of these compounds to anchor the Zn^{2+} ion within the active site of the enzymes, for a long time, secondary and tertiary benzenesulfonamides were considered not to be good targets for generating CAIs. The reported data in this chapter show that in the quest of tumor-associated hCA inhibitors, these neglected classes of compounds might find a renewed interest. The recent data obtained in our laboratories show that novel fluorinated secondary and tertiary benzenesulfonamides act as very selective hCA IX inhibitors, making these compounds excellent leads for further development in the quest of selective anti-cancer agents. For the reported tertiary benzenesulfonamide inhibitors, the inhibition profile with excellent hCA IX selectivity confirms that a new non-zinc-binding mode has to be explored further. Furthermore, considering the large scope of potential applications for selective human CAIs (from biotechnology to medicinal chemistry), the use of secondary and tertiary benzenesulfonamides should re-emerge in this field. However, the binding mode of most of these compounds to CA is still unknown, as no x-ray structures of their adducts are yet available. We hypothesized that these compounds may bind at the entrance of the CA active site, in the coumarin-binding site, but this hypothesis needs to be verified. We estimate that clarifying the inhibition mechanism with secondary/tertiary sulfonamides may lead to interesting novel advances in generating isoform-selective CAIs.

Financial & competing interests disclosure

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Summary

- For the fluorinated secondary benzenesulfonamides, the inhibition profile is new with excellent hCA inhibition nanomolar values for secondary sulfonamides type inhibitors.
- The exceptional hCA IX selectivity of the reported tertiary benzenesulfonamides makes them good candidates in the quest of selective non-zinc-binding inhibitors.
- This long neglected type of compounds could find a rebirth in the quest of selective antitumor agents, but also for further applications as selective human carbonic anhydrase novel non-zinc-binding benzenesulfonamide chemotypes.

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Chapter 5

Next-generation polyamine human carbonic anhydrase inhibitors

Fabrizio Carta &
Andrea Scozzafava

Polyamines as small aliphatic molecules having multiple nitrogen atoms are widely present in all the organisms, including bacteria, archaea, fungi and the eukaryotic cells. Herein we review the class of polyamines as inhibitors of the catalytically active α-carbonic anhydrases (CAs; EC 4.2.1.1). The polyamines binding mode was investigated through crystallographic and kinetic experiments. The crystal structure of the spermine in adduct with the human (h) isoform hCA II is discussed in detail, as it proved a new inhibition mechanism with a novel class of CA inhibitors.

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Polyamines: role in biology

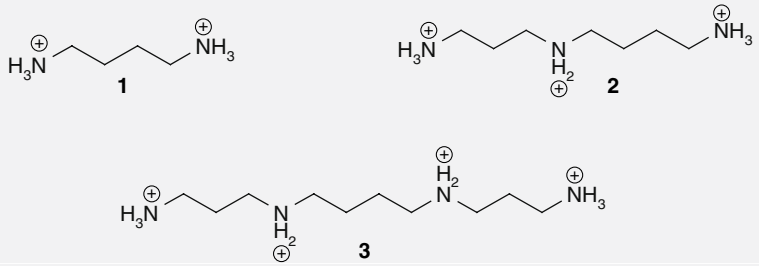
Polyamines are small aliphatic molecules bearing multiple nitrogen atoms, possessing several degrees of substitutions. Usually the inner nitrogens have two or three alkyl substituents and the terminal ones, in addition, can also be unsubstituted. Polyamines are widely present in all the organisms, including bacteria, archaea, fungi and the eukaryotic cells. Therefore, they play a fundamental role in the biological scenario [1–4].

In this chapter the authors will focus on the endogenous polyamines, such as the putrescine **1**, spermidine **2** and spermine **3** in the cells of the higher organisms **(Figure 5.1)**.

Putrescine **1**, spermidine **2** and spermine **3** represent the main pool of polyamines in the eukaryotic cells and are all derived from decarboxylation of ornithine or *S*-adenosyl-methionine operated by the enzyme ornithine decarboxylase (ODC) [5]. Polyamine biosynthesis in the eukaryotic cells is precisely regulated, as they are involved in many vital processes, such as ion channel and cell signaling modulations, stabilization of biological macromolecules and even gene regulation [2]. As polycations at physiological pHs, polyamines are reported to interact with both RNA and DNA [6]. It was demonstrated that the exposure of DNA in the form of isolated or chromatin samples, induced condensation of the genetic material [6]. In the double-strand DNA, polyamines bind both to the minor and the major grooves. Furthermore, additional electrostatic interactions can also occur with the polyphosphate DNA scaffold. The biological meaning of the polyamines interactions with the nucleic acids is not completely clear at the moment. In fact, the induced conformational changes to the nucleic acids might be considered as a temporary macromolecular stabilization or as an additional fine-tuning of the gene regulation machinery system [1].

The higher polyamines, such as the spermine **3** and spermidine **2**, get dismantled to smaller constituents through a process generally called of oxidative deamination **(Figure 5.2)** [7].

Figure 5.1. Chemical structure of the endogenous polyamines 1–3 at the physiological pH.



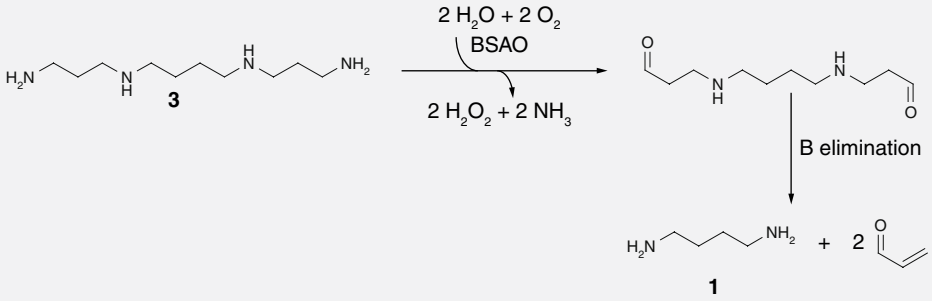
As reported in **Figure 5.2**, the starting polyamine spermine **3** gets catalytically oxidized at the terminal ends by the bovine serum amine oxidase (BSAO; EC 1.4.3.6) to generate ammonia and hydrogen peroxide. Then the dialdehyde intermediate **4** fragments to the smallest polyamine putrescine **1** and acrolein. The global result of the polyamines catabolism is the formation of highly cytotoxic metabolites, which in turn trigger cell death via apoptotic or nonapoptotic pathways [7].

In synthesis, the role of the polyamines in a biological system is to contribute in regulating cells proliferation and death mechanisms.

Thus scientists started to get attracted by the idea to consider the polyamines as an alternative way of intervention for the design of new antineoplastic drugs. Of particular importance is the development of the *N*-alkylated spermine analogs BES **5** and DENS-PM **6** **(Figure 5.3)**, which act mimicking the biogenic polyamines, but are incapable to substitute them in their biological functions [8].

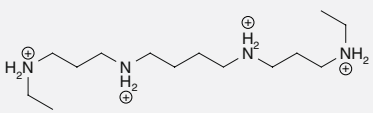
As a result, cells stop proliferating and proceed to death. Animal tumor models proved such polyamine analogs to be quite promising also because they have low toxicity profiles. Nevertheless, the use of polyamine analogs as anticancer drugs still is considered too premature as the preferential molecular targets of such molecules are far to be identified and the picture scientists have in mind is quite confusing [5,9]. Actually the cell death induced by the use polyamine analogs in cancer models is considered the outcome of a multitargeting effect, which involves genes and ion channels deregulations, macromolecules destabilization and, more importantly, disruption of the functionality of some organelles, such as the mitochondria. Although mitochondria differ from

Figure 5.2. Oxidation of spermine 3 catalyzed by the bovine serum amine oxidase.

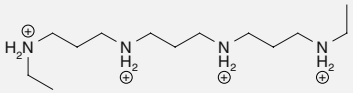


BSAO: Bovine serum amine oxidase.
Data taken from [7].

Figure 5.3. Chemical structure of the spermine derivatives BES (*N*1,*N*12-diethyl spermine tetrahydrochloride) 5 and DENS-PM (*N*1,*N*11-diethyl norspermine tetrahydrochloride) 6.



BES, 5



DENS-PM, 6

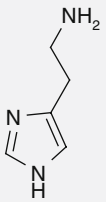
other cellular compartments as they lack of the enzymatic tools for the biosynthesis of polyamines, instead they possess efficient and specific polyamine transportation systems [10].

Polyamines as CA inhibitors

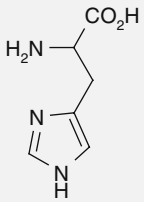
Traditionally, compounds bearing the amino functionalities are associated with CA activation [11–15]. CA activators (CAAs), such as histamine **7** or the D/L-aminoacids histidine **8** and phenylalanine **9**, and related compounds are shown in **Figure 5.4** [11–15]. A deeper discussion on CAAs is properly developed in **Chapter 10** of this book, and for the purpose of this section it is enough to mention that CAAs are able to interfere with the CA's catalytic cycle at the latest step (conversion of D to A in **Figure 5.5**), which also is the rate-determining one, at least in the hCAII. As reported in **Figure 5.5**, a CAA promotes the deprotonation of the zinc-bounded water (D) to restore the highly nucleophilic hydroxide species (A) [15].

Usually in the majority of the CAs this step is assisted by a histidine residue located at the middle of the enzymatic cavity and, acting as a proton shuttle, facilitates the extrusion of protons to the external medium. The CAAs enhance the proton extrusion by creating additional hydrogen nets through their protonable amino functionalities thus generating, as result, an enzyme catalytically more efficient towards the hydration reaction of the carbon dioxide **(Figure 5.5)** [11–14].

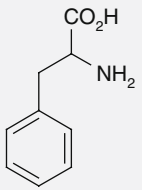
Figure 5.4. Classical carbonic anhydrase activators.



Histamine, 7

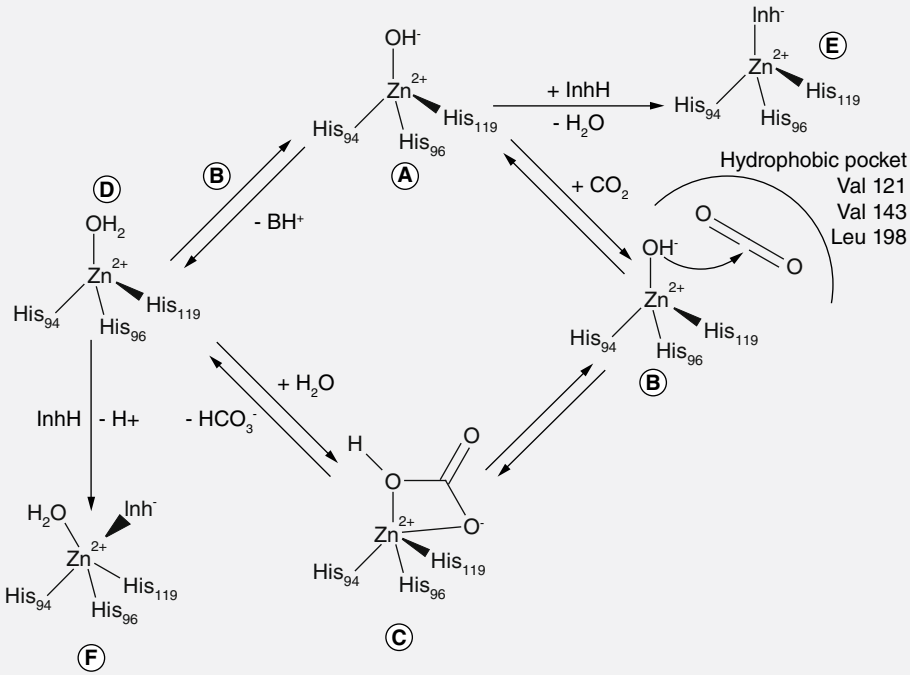


D,L-histidine, 8



D,L-phenylalanine, 9

Figure 5.5. Catalytic cycle of human carbonic anhydrases.



Data taken from [15].

From the chemical viewpoint the CAA candidate has two requirements: the molecule has to fit into the enzymatic cavity; and the structure must have at least a protonatable functional group with pKa values between 6.5 and 8.0 [11].

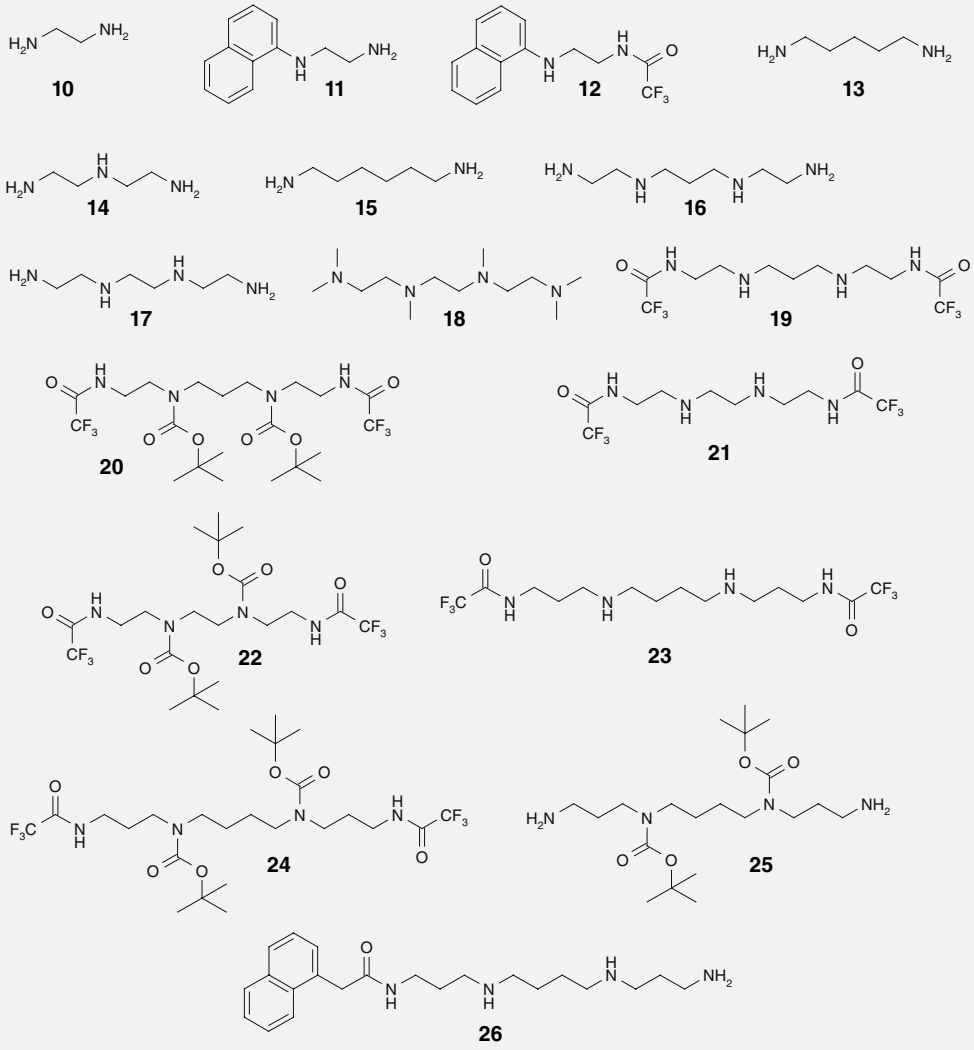
In light of such observations it was expected that the polyamines also should act as CAAs. Therefore, a series of polyamines such as the commercially available spermidine **2** and spermine **3**, as well as some synthetic derivatives depicted in **Figure 5.6**, were evaluated for their kinetic properties on the human (h) and murine (m) catalytically active CAs **(Table 5.1)** [16].

Surprisingly all the tested compounds did not show any CA activation, instead they revealed to inhibit the CA enzymes with K_i values spanning from the nano- to the milli-molar range. The only exception is ethylenediamine **10** as any appreciable enzymatic modulation was detected.

The comparison of the obtained results with the kinetic data of established CA inhibitors, such as the sulfonamide acetazolamide (AAZ) and phenol, gives a proper idea of the efficiency of the polyamines as CA inhibitor.

Among the biogenic polyamines, spermine **3** has the most relevant CA inhibitory profile. Indeed **3** is a weak inhibitor of the physiological abundant hCA isoforms (K_i 231 and 84 µM for hCA I and hCA II, respectively) and a rather good inhibitor of the mitochondrial (K_i 0.84 and 0.83 µM for hCA VA and VB), secreted (K_i 0.99 µM hCA VI) and the CNS dominant isozymes (K_i

Figure 5.6. Polyamines tested for carbonic anhydrase activity.



Data taken from [16].

Table 5.1. Human carbonic anhydrase I–XII, XIV and murine carbonic anhydrase XIII and XV inhibition data with polyamines 2 and 3, and 11–26.

No.	K_i (μM)												
	CAI	*CAII*	*CAIII*	*CAIV*	*CAVA*	*CAVB*	*CAVI*	*CAVII*	*CAIX*	*CAXII*	*CAXIII*	*CAXIV*	*CAXV*
2	1.40	1.11	11.5	0.11	1.22	1.44	1.41	1.23	1.37	44.1	11.6	1.0	10.0
3	231	84	167	0.01	0.84	0.83	0.99	0.71	13.3	27.6	22.6	0.86	74
11	>500	103	0.42	0.058	0.048	0.061	0.64	0.36	0.51	0.38	0.62	0.59	0.57
12	>500	121	128	12.3	106	107	109	1.24	12.2	21.5	127	34	110
13	13.3	11.0	0.50	0.052	0.044	0.54	0.74	0.42	0.38	0.45	0.63	0.50	0.65
14	415	118	117	116	110	11.0	11.5	12.1	10.6	11.4	11.5	10.1	105
15	12.6	34.4	0.60	0.45	0.61	0.58	0.72	0.44	0.41	0.37	0.69	0.64	0.66
16	115	75	63	44	50	59	53	58	48	68	66	36	66
17	100	64	48	35	38	49	43	45	39	57	52	12.1	59
18	>500	11.2	0.52	0.053	0.047	0.71	0.78	0.73	0.31	0.52	0.58	0.74	0.76
19	122	112	96	108	62	54	156	108	117	112	131	125	104
20	>500	>500	>500	309	416	401	>500	>500	>500	>500	>500	>500	>500
21	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
22	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
23	136	11.3	11.5	0.116	1.26	1.05	1.10	1.09	11.4	10.2	12.6	6.7	110
24	>500	107	112	104	125	103	104	107	124	175	179	85	>500
25	137	110	132	103	131	107	114	108	144	165	136	115	236
26	12.3	1.13	11.6	0.018	1.03	1.05	0.11	0.10	0.12	0.19	10.2	1.03	0.78
AAZ	0.25	0.012	200	0.074	0.063	0.054	0.011	0.0025	0.025	0.0057	0.017	0.041	0.072
Phenol	10.2	5.5	2.7	9.5	218	>500	208	>500	8.8	9.2	>500	11.5	10.5

From three different assays, errors ± 5–10 % of the reported value, CO_2 hydrase, stopped-flow assay. Data relative to ethylenediamine **10** are not shown.
Adapted with permission from [16] © American Chemical Society (2010).

0.71 and 0.86 µM for hCA VII and XIV, respectively). Interestingly spermine **3** revealed to be an exceptional inhibitor of the membrane bound hCA IV having a value of 10 nM.

Spermidine **2** still acts preferentially on the hCA IV (K_i 0.112 µM) but also strongly inhibits almost all the remaining CA isoforms (K_i 1.0–11.6 µM), except for the hCA XII (K_i 44.1 µM).

Reduction of the alkyl chain length, such as in **13–15**, resulted in a general decrease of the CA inhibitory activity, with the triamine **14** as the weakest in the series. Even so the kinetic profile for **13** and **15** is analog to the parent biogenic polyamines spermidine **2** and spermine **3**, having the hCA I and II the less inhibited among all the tested isoforms.

Direct structure–activity relationship between the CA inhibition and the chain length is also evident by comparing the kinetic data of the *N*-polysubstituted spermine derivatives **23–25** with the corresponding shorter analogs **19–22**. A remarkable dropping in the inhibitory activities is registered as the alkyl chain is reduced.

Another critical factor that modulates the CA inhibitory activity, is represented by the number of the amine functionalities and their substitution pattern. Herein all reported compounds have at least two nitrogen atoms to a maximum of four. When the chain length is kept constant and a -CH_2- is substituted by a nitrogen, such as conversion of **13** to the triamine **14**, the CA inhibition drops.

As far as it concerns the substitution at the nitrogens a good example is offered by the polyalkylation of the trien **17** to give **18**. In this case a two orders of magnitude improvement for the CA inhibitory activity is observed in all the isozymes with the exception for hCA I. In general, all the data demonstrated that polyamines functionalized at the nitrogen atoms preserve a good CA inhibitory activity when at least a free terminal amino-end is preserved. This is not the case of the spermine derivative **23** which has both the primary amino ends protected with trifluoroacetate groups. In fact the CA inhibitory activity of **23** is maintained when compared with the parent spermine **4** and even ameliorated as for hCAs I–III.

Interestingly the selective functionalization at one end enhanced the CA inhibitory activity as demonstrated by the commercially available spermine derivative **26** and even more clearly with **11**. The introduction of the naphthyl group (compound **11**) to the inactive ethylenediamine **10**, restored the CA inhibitory activity which gets attenuated when the latest amino terminal was functionalized with the trifluoroacetate group (compound **12**).

The introduction of bulky functionalities inevitably spoiled the CA inhibitory properties as clashes within the enzymatic cavity might occur (compounds **20**, **22**, **24** and **25** in **Figure 5.6**).

Crystallographic investigation

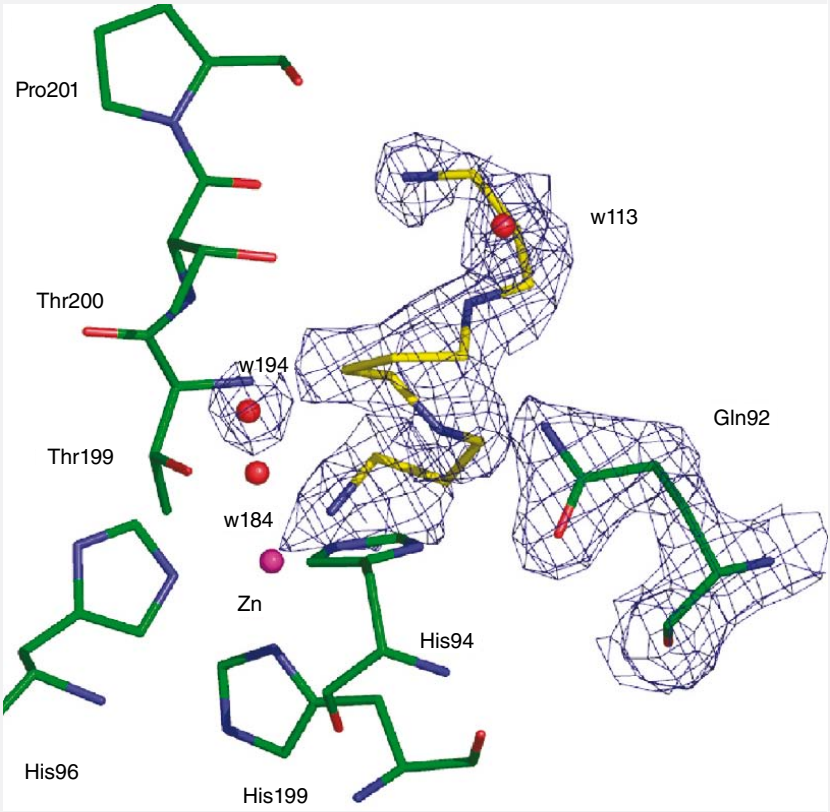
In order to reveal the detailed mode of action of the polyamines as CA inhibitors, a crystallographic investigation of the spermine **3** in adduct with the hCA II at a resolution of 2.0 Å was conducted. As for the kinetic experiments, the crystallographic investigation was performed at pH 7.4, which means the spermine **3** was fully protonated [16].

The electron density maps clearly showed the enzymatic cavity occupied by a single molecule of inhibitor, which was stabilized in a coiled conformation **(Figure 5.7)** [16].

One ammonium terminal moiety of the polyamine appeared deeply buried into the enzymatic cavity and strongly interacting with the zinc-bounded hydroxide **(Figure 5.8A)** in a fashion resembling the phenol–hCA II adduct binding mode **(Figure 5.8B)**. In particular one of the ammonium protons was connected via a hydrogen bond (2.8 Å) with the zinc-bounded hydroxide and another with the -OH (3.0 Å) of the conserved amino acid Thr199. The phenol–hCA II adduct differed at this point as the Thr199 participates to the phenol stabilization through its amidic -NH- **(Figure 5.8B)** [17]. The aliphatic carbon chain of the spermine **3** contributed to the adduct stabilization by making various Van der Waals interactions. Of particular note are the clashes the central alkyl section of the spermine **3** retained with the water molecule 113 (3.1 Å) and the amide nitrogen of Gln92 (2.9 Å). The presence of such steric clashes might explain the low inhibitory activity of the spermine **3** for hCA II (Ki 84 µM). The outer ammonium end was involved in a net of strong hydrogen bonding with Thr200 and Pro201 as shown in **Figure 5.8A**.

The terminal monoderivatized polyamines **11** and **26** possessed stronger inhibition profiles on CAs when compared with the parent precursors, ethylendiamine **10** and spermine **3**, respectively. Assuming their binding mode being similar to the spermine-CAII adduct, it is also realistic to hypothesize that the terminal derivatization at **11** and **26** offers additional binding interactions with the peripheral part of the enzymatic active site and hence reinforces the adduct interaction. Similarly the selectivity that some tested polyamines have towards the hCA IV isoform might be explained. Indeed the hCA IV differs from the other isozymes in the panel for the primary amino acidic sequence at the cavity entrance, being rich in cysteine residues, which form two disulfide bonds (Cys6-Cys11G and Cys23-Cys203) [18,19].

Figure 5.7. The electron density 2Fo-Fc map at 1σ of spermine bound within the human carbonic anhydrase II active site.



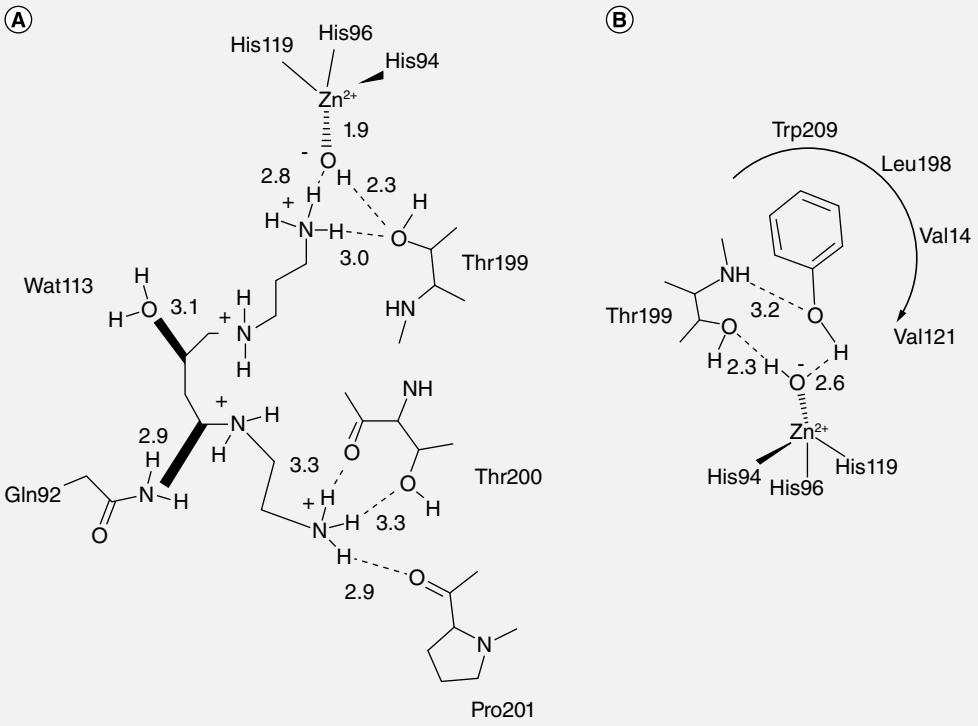
Conclusion

Polyamines revealed a completely unexpected behavior when tested for their CA activity. Indeed, they showed good CA inhibitory properties with K_i values spanning from the nano- to the milli-molar range.

Structure–activity relationship analyses revealed that many aspects account for the polyamines CA inhibitory activity such as the chain length, the number of the nitrogens atoms as well as the number of functional groups introduced and their nature.

Crystallographic experiments clearly demonstrated that polyamines interact within the CA enzymatic cavity in a very different manner when

Figure 5.8. Carbonic anhydrase inhibitors anchoring to the zinc-coordinated water molecule, spermine and phenol.



(A) Schematic representation of interactions of spermine **3** (as tetracation) with human carbonic anhydrase II active site. Figures represent distances (in Å). Hydrogen bonds are represented as dashed lines. In bold are shown two clashes [16]. **(B)** Schematic representation of the binding of phenol to human carbonic anhydrase II [17].

compared with known CA inhibitors such as the sulfonamides (or their bioisosters), coumarines or phenols. Specifically the spermine within the enzyme cavity adopts a helicoidal conformation and with the buried ammonium-end strongly interacts with the zinc-bound hydroxide ion through a network of hydrogen bonds. Additional hydrogen bonds, as well as multiple weak Van der Waals contacts, contribute to the adduct stabilization.

The discovery of the polyamines as CA inhibitors opens innovative perspectives for the development of new lead-compounds with blocking properties towards the CA enzymes.

First of all the original binding mode of the CAs with polyamines has to be taken into account when a drug-design route is started.

Additionally the selectivity of some polyamines for the hCA IV isozyme gives the opportunity to develop the polyamines as a new class of isoform-specific compounds. hCA IV is a unique enzyme which strongly differs from the other CAs as it is bounded to the plasma membranes through a glycan linkage.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Summary.

- Polyamines are small aliphatic molecules bearing multiple nitrogen atoms, possessing several degrees of substitutions. Usually the inner nitrogens have two or three alkyl substituents and the terminal ones, in addition, can also be unsubstituted.
- Polyamines biosynthesis in the eukaryotic cells is precisely regulated, as they are involved in many vital processes such as ion channel and cell signaling modulations, stabilization of biological macromolecules and even gene regulation.
- All the polyamines and their derivatives tested so far did not show any carbonic anhydrase (CA) activation, instead they revealed to inhibit the CA enzymes with K_i values spanning from the nano- to the milli-molar range.
- Spermine **3** revealed to be an exceptional inhibitor of the membrane bound hCA IV having a value of 10 nM.
- The electron density maps clearly show the enzymatic cavity occupied by a single molecule of inhibitor, which is stabilized in a coiled conformation.
- The discovery of the polyamines as CA inhibitors opens innovative perspectives for the development of new lead-compounds with modulating properties towards the CA enzymes.
- The original binding mode of the CAs with polyamines has to be taken into account when a drug-design route is started.
- Polyamines, like phenol anchor to the zinc-bound water molecule, an inhibition mode rarely observed with other classes of CA inhibitors.

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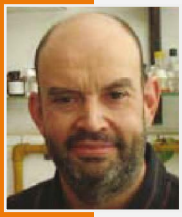
About the Author



Pedro Alfonso Colinas

Pedro Alfonso Colinas received his PhD in chemistry from the Universidad Nacional de La Plata (Argentina) in 1997. He worked as a postdoctoral fellow in the Institut für Organische Chemie, Universität Stuttgart (Germany). In 2006, he became Professor of Organic Chemistry at the Universidad Nacional de La Plata. Since 2005, he has been a member of the scientific research career of the National Scientific and Technical Research Council (CONICET; Argentina). His research interests include carbohydrate chemistry, design of enzyme inhibitors and medicinal chemistry.

Chapter 6

Next-generation phenol carbonic anhydrase inhibitors

Pedro Alfonso Colinas

This chapter will focus on the phenol-based carbonic anhydrase inhibitors. Phenol presents an unusual binding mode to the carbonic anhydrase active site. The different mechanism of inhibition of phenols compared with other inhibitors make this class of derivatives of great interest to design novel carbonic anhydrase (CA) inhibitors with selectivity and/or specificity for some of the medicinal targets belonging to this enzyme family. Natural products, synthetic compounds and their CA inhibition profile will be reviewed. Modifications of the hydroxyl group will be described in terms of their effects on CA inhibition selectivity. A new generation of effective inhibitors has been recently developed by attachment of different tails to the phenol pharmacore, and these results will be described. In addition, the 'carbohydrate approach', which led to the discovery of novel antitubercular agents, will be discussed.

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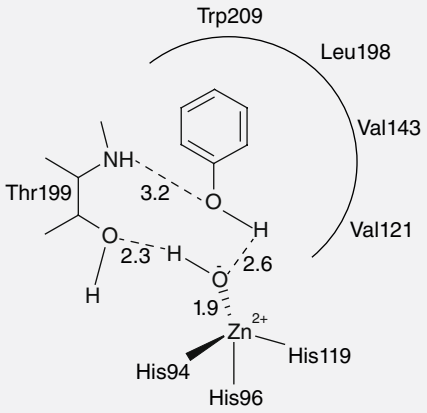
Carbonic anhydrases (CAs; EC 4.2.1.1) are the most studied members of a great family of metalloenzymes. CAs catalyze the reversible hydration of CO_2, and they are found in multiple organisms such as vertebrates, bacteria and algae, among others [1]. Five genetically distinct CA families are known to date, the α-, β-, γ-, δ- and ζ-CAs. Mammals possess only α-CAs, while many pathogenic organisms such as bacteria and fungi encode β-CAs. These enzymes contain a zinc ion (Zn^{2+}) in their active site, which is coordinated by three histidine residues and a water molecule/hydroxide ion (in the α-, γ- and δ-classes) or by two cysteine and one histidine residues (in the β- and ζ-CA classes), with the fourth ligand being a water molecule/hydroxide ion. α-CAs are involved in several physiological processes and have been exploited for the treatment or prevention of various pathologies such as glaucoma, neurological disorders, osteoporosis, obesity and cancer [1]. During the last few years, the interest in the therapeutic use of CA inhibitors (CAIs), has improved remarkably due to the validation of several CA isozymes as drug targets [2]. CAIs are classified in three main classes:

- Sulfonamides, sulfamates and sulfamides, which bind in deprotonated form, as anions, to the Zn(II) ion from the enzyme active site by replacing the zinc-bound water/hydroxide ion and leading to a tetrahedral geometry of Zn(II);
- Coumarins, which exhibit a very different binding mode with no observed interaction between the inhibitor molecule and the active site Zn(II) ion, occluding the entrance to the active site cavity;
- Polyamines and phenols, which bind by interacting with a water molecule/hydroxide ion coordinated to Zn(II) through hydrogen bonding.

Although phenol-based natural and synthetic compounds are largely known to exhibit biological activity (mainly as antioxidants), they have only recently been studied as CAIs. Even though in the early 1980s phenol was reported by Simonsson *et al.* as a CO_2-competitive CAI [3], only in 1994 did Christianson's group report the x-ray study of its adduct with the physiologically dominant human isoform CA II (hCA II) [4]. That crystallographic analysis showed that this inhibitor binds in a completely unprecedented manner: its OH moiety anchored by a hydrogen bond to the fourth zinc ligand, which is a water molecule, or a hydroxide ion, as the crystallization was performed at pH 10. There is evidence of a second hydrogen bond between the oxygen atom of phenol and the amide NH of Thr199 **(Figure 6.1)**. Moreover, the phenyl ring was found to lie in the hydrophobic part of the hCA II active site, thus explaining the behavior of phenol as a unique CO_2 competitive inhibitor.

Later, structural studies have been performed by Martin and Cohen, showing that other phenol derivatives exhibited the same binding mode [5]. It is important to point out that the presence of other functional groups in the phenyl ring of phenols could change this binding mode. The binding mode of 4-hydroxybenzoic acid in the active site of hCA II is similar to that of phenol. The molecule is anchored by a hydrogen bond with the Zn(II)-bound water molecule; however, the interaction is through the carboxylate of this compound. Poulsen's group has reported the x-ray structure of one phenol-based compound that possesses sulfonamide functionality, in adduct with hCA II [6]. As expected, the crystallographic data showed that the sulfonamide moiety interacts with the Zn(II) ion in the active site. But the most interesting result was found with a phenol derivative incorporating a methyl ester functionality. It was expected that this compound would bind to the hCA II active site by anchoring its OH moiety to the zinc-bound water molecule. Surprisingly, the crystal structure showed that the ester carbonyl interacts with the water molecule. These few examples demonstrate that the inhibition studies of phenol-based CAIs should be carefully interpreted, taking into account the presence of the different functionalities in their molecules.

Figure 6.1. Interactions of phenol with human isoform carbonic anhydrase II.



The numbers represent distances in angstroms, and hydrogen bonds are represented as dashed lines.

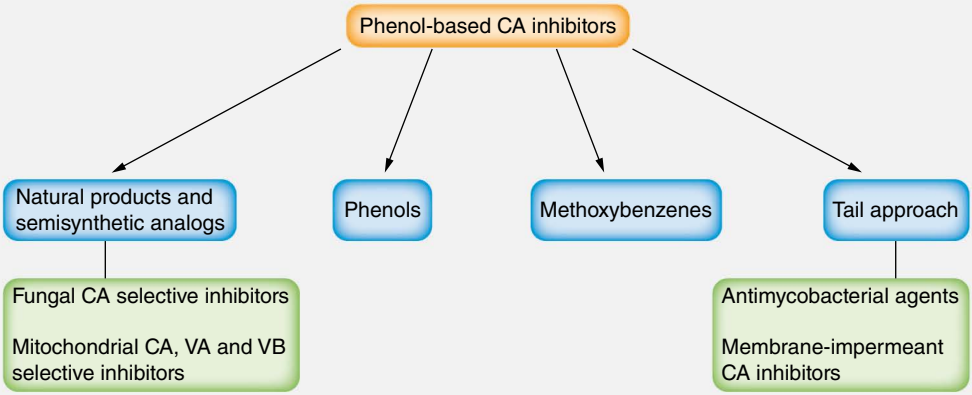
At this point, it is necessary to mention that there are two main ways of studying CA inhibition:

- Monitoring the CA-catalyzed CO_2 hydration activity [7]
- Using the esterase activity of the CA, with 4-nitrophenyl acetate as substrate [8]

Usually, the second method gives higher K_is compared with the CO_2 hydrase assay, because CAs are weak esterases. In our discussion, only the results found using the second methodology will be described. The reader interested in the phenol derivatives studied by the esterase assay is referred to the publications of Gülçin's group [8].

In the past 5 years, several phenol-based compounds have been studied as CAIs and some of them exhibit very interesting properties **(Figure 6.2)**.

Figure 6.2. Phenol-based carbonic anhydrase inhibitors.



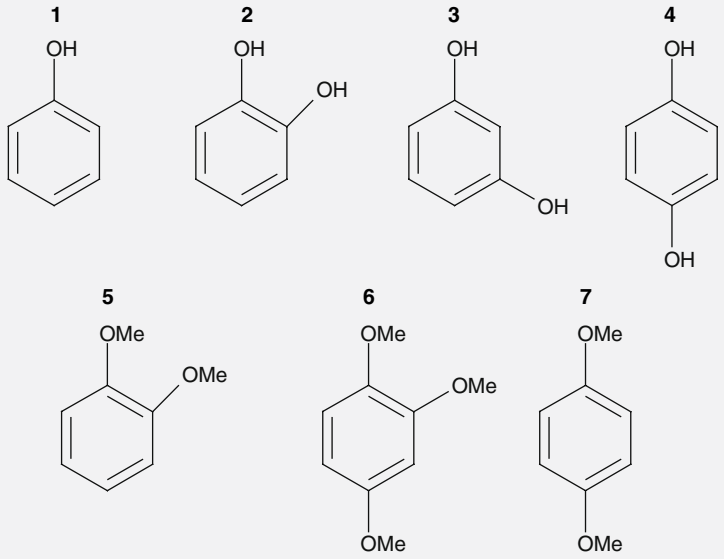
CA: Carbonic anhydrase.

Simple mono- and poly-phenols have been considered for their interaction with catalytically active mammalian CA isozymes **(Figure 6.3)** [9,10]. It is important to note that the structure–activity relationship (SAR) found in these compounds is rather complicated **(Table 6.1)** although some important features could be remarked:

- Phenol and its derivatives are efficient CAIs in the low micromolar range
- Structural changes lead to a great variation in the inhibition profile

For example, phenol (compound 1) and diphenols (compounds 2–4) showed that different substitution patterns in the aromatic ring lead to drastic changes in the CA activity and/or selectivity **(Figure 6.3)**. Against isozyme hCA I, the addition of an OH moiety in the *ortho* or *meta* position (as in pyrocathecol [compound 2] and resorcinol [compound 3], respectively) leads to a weaker inhibitor compared with the parent derivative [compound 1]). A quite different behavior is found in the inhibition profile of the ubiquitous isoform hCA II. Hydroquinone (compound 4), with the second OH moiety in *para*, is one of the best CA II inhibitors. Isozyme hCA III was inhibited in the low micromolar range by the phenols shown in compounds 1, 2 and 4 but an OH group in the *meta* position leads to a dramatic loss of inhibitory power compared with phenol. This profile was quite similar to the one found in the inhibition of the membrane-anchored isoform hCA III. The two mitochondrial isoforms, hCA VA and VB, were quite poorly inhibited by phenol but all substitution patterns in its phenyl moiety lead to much more effective inhibitors. Compounds 1–4 are quite ineffective

Figure 6.3. Phenol and methoxybenzene derivatives.



inhibitors of the isoforms hCA VI and VII. Against the tumor-associated isozyme, hCA IX, phenol (compound 1) behaves as a quite good inhibitor, whereas the other derivatives showed weaker activity. On the other hand, these substitution patterns are favorable for the inhibition of hCA XII, the second tumor-associated isozyme. A similar behavior was found with the transmembrane isozyme hCA XIV (similar to hCA IX and XII, but not tumor-associated), which was well inhibited by all the phenols. Finally, only pyrocatechol is a fairly efficient inhibitor of the cytosolic isoform hCA XIII, whereas phenol and diphenols (compounds 3 and 4 in **Figure 6.3**) behave as weaker inhibitors. It should be stressed that there are only few examples of the x-ray crystal structure of CAs with phenols, and thus there is little information on the binding mechanism of these inhibitors to the metalloenzyme active site. More such crystallographic data would be critical for better understanding the CA inhibition profile of phenols.

Methylation is one of the most common chemical modifications of the OH moiety in phenol. Only very recently, methoxyphenyl derivatives have been investigated as CAIs **(Table 6.1)** [11], because it was considered that they do not bear any moiety normally associated with CA inhibition in their molecules. Unexpectedly, di- and tri-methoxybenzenes (compounds 5–7 in **Figure 6.3**) are rather similar or better CAIs than phenol. Docking studies have been performed to explain the behavior of these compounds. It

Table 6.1. Inhibition of mammalian α-carbonic anhydrase with phenols and methoxybenzenes.

Isozyme	K_i (μM)						
	1	***2***	***3***	***4***	***5***	***6***	***7***
hCA I	10.2	4003	795	10.7	10.4	8.21	5.96
hCA II	5.5	9.9	7.7	0.090	0.50	3.35	1.43
hCA III	2.7	13.0	605	8.2	ND	ND	ND
hCA IV	9.5	10.9	570	10.8	ND	ND	ND
hCA VA	218	55.1	8.7	14.1	ND	ND	ND
hCA VB	543	4.2	7.1	12.5	ND	ND	ND
hCA VI	208	606	550	521	ND	ND	ND
hCA VII	710	714	644	883	ND	ND	ND
hCA IX	8.8	115	69.7	32.5	8.63	4.15	4.06
hCA XII	9.2	8.9	7.5	7.8	8.36	4.02	7.80
hCA XIII	697	12.2	62.6	74.3	ND	ND	ND
hCA XIV	11.5	48.9	10.7	42.0	ND	ND	ND

hCA: Human isoform carbonic anhydrase; ND: Not determined.

was found that the binding of methoxy-substituted benzene within the enzyme active site is done without interaction with the zinc ion, by means of different interactions with amino acid residues and water molecules. The compounds were located between the phenol-binding site and the coumarin-binding site, filling the middle of the enzyme cavity. Thus this completely different binding mode offers the possibility of designing CAIs with a different inhibition profile from the known inhibitors. However, only very few such derivatives have been investigated so far.

Further studies found effective CAIs within a large library of phenolic natural products and their semisynthetic derivatives **(Table 6.2)** [12]. The common feature of the compounds 8–17 is the presence of at least one phenol OH moiety and a great variety of functionalities such as carboxylic acid, amide, etc **(Figure 6.4)**. These phenolic compounds inhibited hCA I less than phenol. It is important to note that, in general, the compounds are better hCA II inhibitors but with K_i in the low micromolar range and similar to phenol. Very interesting results were found when these phenol derivatives were tested against mitochondrial isozymes VA and VB, which are involved in biosynthetic processes such as gluconeogenesis, lipogenesis and ureagenesis. These two isoforms were very weakly inhibited by phenol but, surprisingly, much more susceptible to inhibition with all the phenolic

Table 6.2. Inhibition of mammalian α-carbonic anhydrase and pathogen β-carbonic anhydrase with natural and semisynthetic phenols.

Compound	K_i (μM)							
	hCA I	*hCA II*	*hCA VA*	*hCA VB*	*mtRv3273*	*mtRv1284*	*Nce103*	*Can2*
8	309	10.3	0.101	0.105	11.4	10.8	1.02	0.9
9	309	11.2	0.092	0.081	9.12	0.85	0.91	0.84
10	265	8.6	0.100	0.118	10.8	10.3	1.08	1.12
11	237	131	0.110	0.106	11.2	10.5	1.00	0.85
12	369	107	0.109	0.125	ND	ND	ND	ND
13	201	8.4	0.093	0.103	11.4	10.5	1.06	1.12
14	374	9.2	0.094	0.102	10.9	0.99	1.01	1.08
15	368	11.7	0.091	0.069	8.92	0.82	0.73	0.77
16	354	12.1	0.098	0.079	0.89	0.80	0.70	0.95
17	307	230	0.085	0.071	9.10	0.85	0.62	0.81
Phenol	10.2	5.5	218	543	79.0	64.0	17.3	25.9

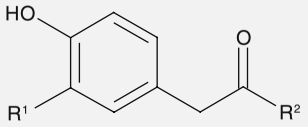
hCA: Human isoform carbonic anhydrase; ND: Not determined.

compounds analyzed. Unfortunately, it is not possible to rationalize the inhibition profile due to the little structural information available for these mitochondrial isoforms, but the high selectivity displayed by the phenolic library could be exploited in the development of antiobesity agents with a novel mechanism of action.

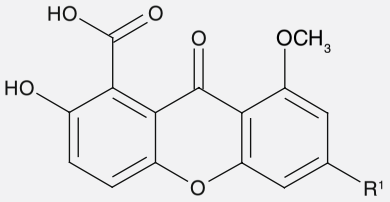
Compounds 8–17 **(Figure 6.4)** have also been assayed as inhibitors of β-CAs from pathogenic organisms [6]. The β-CAs include the enzymes Rv3273 and Rv1284 from *Mycobacterium tuberculosis*, NCE103 from *Candida albicans* and Can2 from *Cryptococcus neoformans*. Rv1284 is generally believed to be essential for survival of *Mycobacterium* and was found to be highly upregulated under starvation conditions. By contrast, there is no evidence suggesting that Rv3273 is essential for mycobacterial growth. Fungal CAs Can2 and NCE103 are essential for the survival of *C. neoformans* serotype A in its natural environment and for *C. albicans* infections in epithelial virulence models.

The mitochondrial CAs (mtCAs) were inhibited very weakly by phenol, the core scaffold of the library, while all the phenolic derivatives showed enhanced enzyme activity for these isozymes compared with phenol; however, the SAR found is very complex. A closely related situation was found with NCE103 and Can2 inhibition. Compounds 8–17 showed a relatively flat SAR with low micromolar or submicromolar activities. It

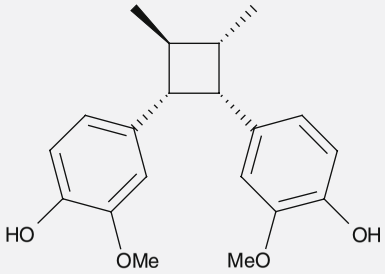
Figure 6.4. Natural phenolic compounds and semisynthetic derivatives.



8 $R^1 = H$ $R^2 = OH$
9 $R^1 = H$ $R^2 = NH_2$
10 $R^1 = Cl$ $R^2 = OH$
11 $R^1 = Cl$ $R^2 = NH_2$
12 $R^1 = Cl$ $R^2 = OCH_3$



13 $R^1 = CH_3$
14 $R^1 = CH_2OH$



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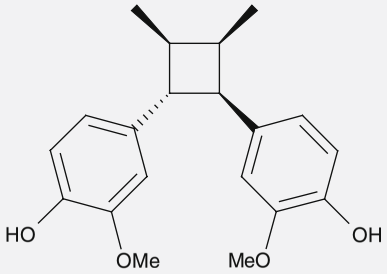

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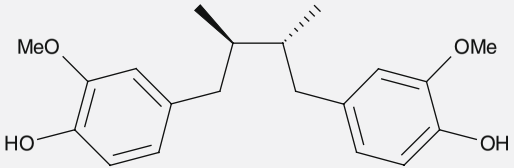

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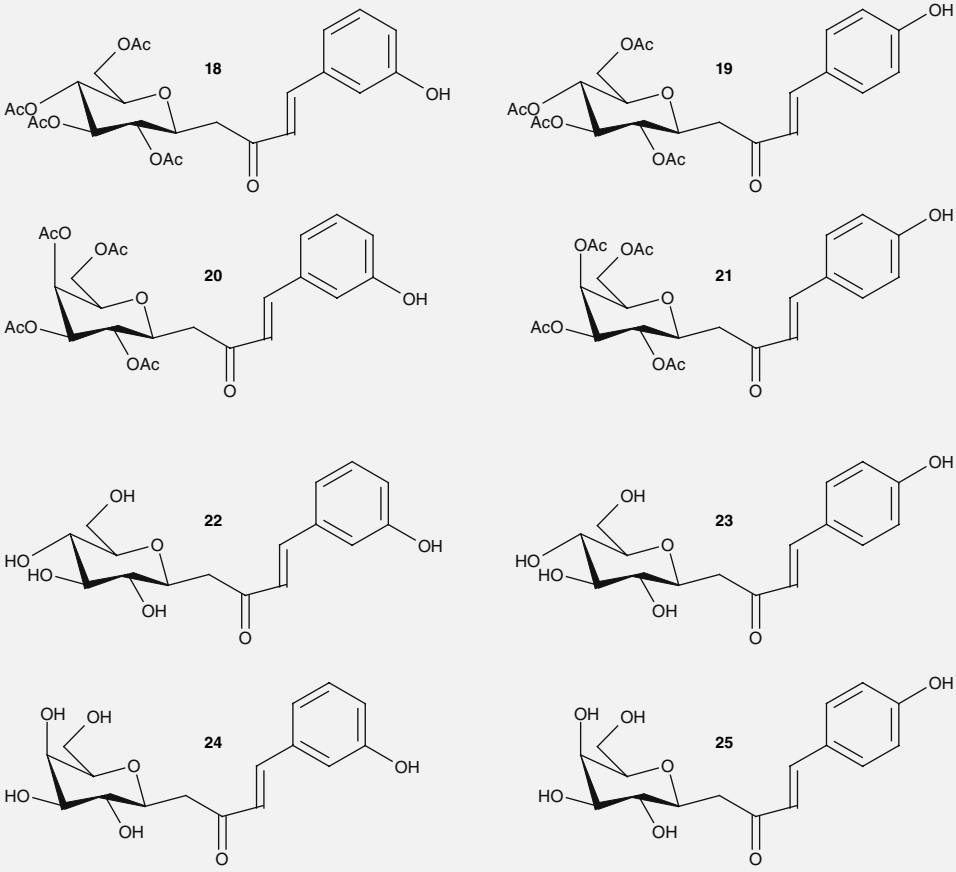
should be noted that (-)-dihydroguaiaretic acid (compound 17), a plant secondary metabolite, preferentially inhibited fungal CAs with selectivities of more than two orders of magnitude. The more flexible polyphenol (compound 17) is a weaker hCA II inhibitor compared with the more rigid cyclobutane derivatives (compounds 15–17). Indeed, changes in the scaffold led to a significant improvement in activity. Thus (-)-dihydroguaiaretic acid represents an interesting lead for the development of novel antifungal agents.

One of the most successful approaches for designing CAIs targeting all isoforms known to date was termed 'the tail approach' [13]. It was initially developed for the synthesis of sulfonamide CAIs but later extended to many other classes of such compounds. The tail approach originally consisted of attaching different tails to the scaffolds of sulfonamides to modulate the physicochemical properties of these pharmacological agents. A very good example of such 'tails' is constituted by sugars, which represent a wide range of chemotypes, thus leading to a high number of new CAIs [14]. The stereochemical diversity across the carbohydrate tails provides the opportunity for interrogation of subtle differences in active site topology of CA isozymes. Recently, our group has applied the 'sugar approach' to the preparation of *C*-glycosyl phenols, where the carbohydrate moiety is tethered to a phenol CA pharmacophore through a carbon chain **(Figure 6.5)**. In contrast to standard CAIs, the inhibition profile with human CAs for the peracetylated *C*-glycosyl compounds is flat **(Table 6.3)** [15]. Neither the stereochemistry presented by the differing carbohydrate moiety nor the position of hydroxyl in the aromatic ring altered the enzyme inhibition profile. *C*-glycosides showed better inhibitory activity against almost all α-CAs than phenol. Thus, this confirms that attaching carbohydrate moieties to phenol could improve its inhibitory activity. Later on, these compounds have been tested as inhibitors of the *M. tuberculosis* β-CAs [16].

Rv1284 and Rv3273 mtCAs were inhibited very weakly by phenol, while all *C*-glycosides exhibited enhanced enzyme inhibitory activity in the low or submicromolar range. These glycosides were also efficient inhibitors of the isoform Rv3588c found in *M. tuberculosis*. The antitubercular activity of the *C*-glycosyl phenol was investigated, allowing the identification of compound 18 as the first mtCA inhibitor with antimycobacterial activity. *C*-glycoside (compound 19) also had activity on the mycobacterial metabolism capable of restricting growth but not high enough to prevent it.

Another approach is the development of positively charged membrane-impermeant CAIs by attaching substituted pyridinium moieties to scaffolds possessing one or two phenolic OH moieties **(Figure 6.6)**. The presence of

Figure 6.5. *C*-glycosyl phenols.



the pyridinium ring would enhance the preferential inhibition of transmembrane isozymes *in vivo*. Dopamine (compound 26) and aminophenols (compounds 29 and 30) were derivatized with substituted pyrilium perchlorate to afford the pyridinium salts (compounds 27 and 28, and 31 and 32) [17].

Although dopamine is a CA activator, the derivatives 27 and 28 were effective CAIs in the micromolar or submicromolar range **(Table 6.4)**. The activating properties of dopamine are due to the proton-shuttling capacity of the amino group when the activator is bound within the enzyme cavity. In the pyridinium salts (compounds 27 and 28), the moiety responsible for this process is blocked, leading to the loss of CA activating effect. The aminophenols (compounds 29 and 30) were efficient inhibitors in the low micromolar range but

Table 6.3. Inhibition of mammalian α-carbonic anhydrase I and II; and *Mycobacterium tuberculosis* β-carbonic anhydrase with *C*-glycosyl phenols.

Compound	K_i (μM)			
	hCA II	***mt Rv1284***	***mt Rv3273***	***mt Rv3588c***
18	7.0	2.1	19.0	0.64
19	3.9	2.9	13.1	0.35
20	7.1	3.8	15.6	0.87
21	5.5	4.5	12.0	1.15
22	7.8	0.14	6.21	0.24
23	8.8	0.93	4.13	0.13
24	3.1	1.16	3.25	0.51
25	6.8	4.5	4.13	0.94
Phenol	5.5	64.0	79.0	ND

hCA: Human isoform carbonic anhydrase; ND: Not determined.

their derivatives 31 and 32 were more than one order of magnitude better inhibitors compared with their parent compounds.

Based on these findings, it is possible to hypothesize that inhibitors obtained by attaching different tails to the phenol pharmacophore could

Figure 6.6. Aminophenols and pyridinium salts.

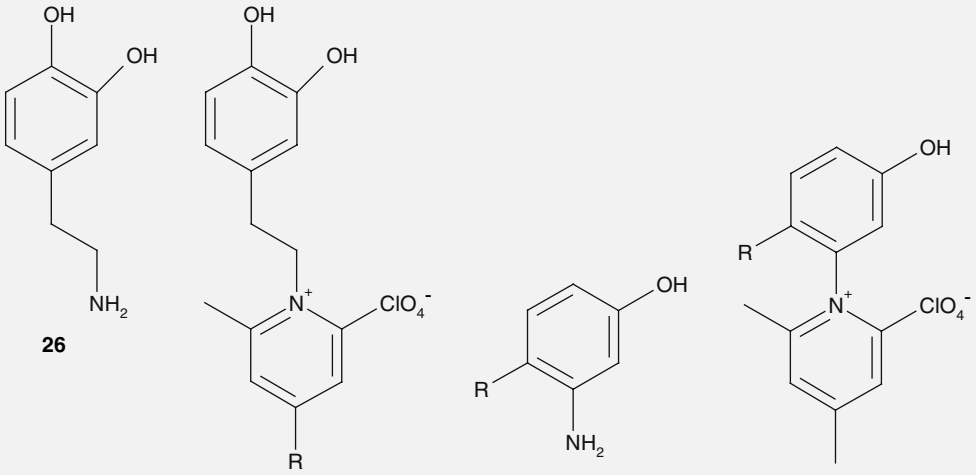


Table 6.4. Inhibition of mammalian α-carbonic anhydrase with pyridinium derivatives.

Compound	K_i (μM)					
	hCA I	*hCA II*	*hCA VII*	*hCA IX*	*hCA XII*	*hCA XIV*
27	7.4	6.8	8.7	0.83	0.76	0.81
28	5.1	4.7	7.2	0.71	0.40	0.74
29	4.9	4.7	5.9	4.9	7.7	7.2
30	6.3	4.9	6.4	4.7	7.5	6.4
31	0.39	0.32	0.72	0.68	0.54	0.70
32	0.5	0.49	0.73	0.70	0.78	0.74

hCA: Human isoform carbonic anhydrase.

improve the inhibition profile by modulating the interaction with the amino acid residues and/or water of the CA active site.

As can been seen from the above examples, phenols and their derivatives (natural and synthetic ones) constitute a class of CAIs with great promise. It should be noted that ,until recently, only phenol had been studied as a CAI. In the last few years, a wide range of phenol derivatives have been extensively studied by kinetic techniques. Unfortunately, crystallographic studies of adducts with hCA II are available only for simple derivatives such as phenol, resorcinol and hydroquinone. Lack of x-ray structures of compounds with more complicated scaffolds, bound to CAs, precludes a rationalization of SAR and a deep understanding of features associated with activity and selectivity of phenols [18]. It is desirable that, in the near future, the x-ray structures of adducts of phenols with CAs can be resolved, to increase our understanding of the binding mode of such compounds.

The 'tail approach' has been shown to be highly effective in providing phenol-based inhibitors with enhanced activity, which, in the end, may lead to better drugs belonging to this class of pharmacological agents (such as antimycobacterial ones).

Although several interesting developments have been registered in the past 5 years regarding phenol-based CAIs, this field is still young. Their inhibition mechanism exploits a different binding mode of the inhibitors to the active site, compared with the classical CAIs of the sulfonamide type. In this way, they may lead to compounds with a better inhibition profile. Thus phenol-based compounds will continue to be one of the most relevant topics in the search for pharmacologically relevant enzyme inhibitors targeting the CAs.

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Summary

- Phenols inhibit carbonic anhydrases (CAs) not by binding to the Zn of the active site but by anchoring to a zinc-bound water molecule through hydrogen bonding.
- Their inhibition profile is rather complicated and highly dependent on structural changes.
- Methoxy-substituted benzenes bind differently from all other CA inhibitors known to date.
- Several polyphenols show excellent inhibition profiles and selectivity.
- Attachment of a tail to the phenol scaffold has led to successful drug-design examples.
- More crystallographic studies are necessary for better understanding the CA inhibition profile of phenols.

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Chapter 7

Coumarins that inhibit carbonic anhydrase

Sally-Ann Poulsen

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Carbonic anhydrases (CAs) are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton [1,2]. The selective inhibition of this enzyme family has been validated as an approach towards personalized therapies against a range of human diseases, including cancer. The active site zinc cation has been the predominant focus for the development of small-molecule CA inhibitors (CAIs) and almost all reported CAIs comprise a zinc-binding group (ZBG), of which the primary sulfonamide group is the premier example. Owing to the structural similarity between the different CA isozymes it is, however, challenging to develop isozyme selective inhibitors based around the ZBG–zinc interaction and ligands with alternate mechanisms for inhibiting this class of enzymes are sought to fill this void. This chapter will highlight the recent and growing interest in the coumarin chemotype as nonclassical CAIs. The interest in coumarins as CAIs has quickly developed into a vibrant research area, triggered from the 2009 report of a natural product, coumarin, that inhibited CAs. The excitement around this finding was twofold. First, the coumarin chemotype lacks the ZBG functionality that is present in all classical CAIs, and

Zinc-binding group: inhibitors of zinc metalloproteins may contain a functional group that can bind to the zinc to block the endogenous process. These functional groups are known as zinc-binding groups, and include carboxylate, sulfonamide, hydroxamate, phosphonate, phosphate, thiol, diol and carbamate. The zinc-binding group may form monodentate and/or bidentate interactions with the zinc.

Natural products: natural products are the chemicals produced by microbes, plants and animals. They include primary metabolites that are involved in growth and metabolism (e.g., lipids, carbohydrates and proteins), and secondary metabolites that support survival functions for the specific organism that produces them (e.g., antibiotics, hormones).

second the coumarin provided uncharacteristic but promising CA isozyme selectivity. Two distinct sources of coumarins will be overviewed in this chapter: natural product coumarins; and synthetic coumarins.

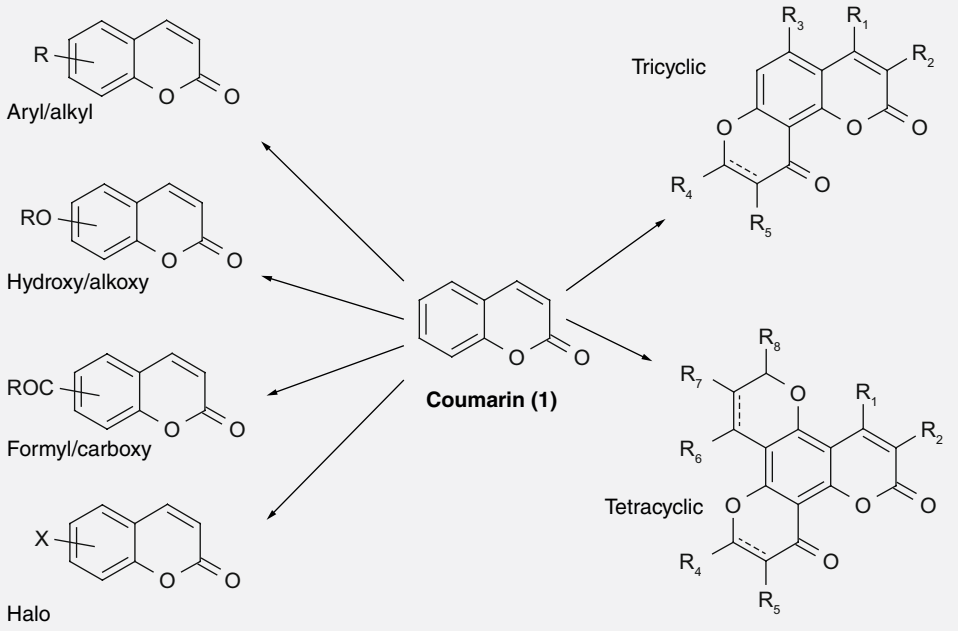
The need for selective carbonic anhydrase inhibitors

In humans, there are 12 catalytically active carbonic anhydrase (CA) isozymes (CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII and XIV) that catalyze the reversible hydration of CO_2 to bicarbonate anion and a proton: $CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$ [1,2]. CAs underpin crucial physiological and pathological roles in pH regulation, carbon metabolism and ion transport. Most human CA isozymes are recent discoveries, while the physiologically abundant 'textbook' isozymes, CA I and II, have been known since the 1930s. CAs have been characterized in an increasing number of pathogenic organisms and have been put forward as a novel target to fight infection [3]. The catalytic activity of CAs is conveyed by an active site zinc cation coordinated to three conserved histidine residues, while the fourth zinc ligand is a H_2O molecule or hydroxide anion (OH^-). Nucleophilic attack by this OH^- on a CO_2 molecule within the CA active site leads to the formation and release of HCO_3^- and H^+. Classical small-molecule CAIs are primary sulfonamide compounds ($R\text{-}SO_2NH_2$), with the deprotonated sulfonamide anion ($R\text{-}SO_2NH^-$) able to coordinate to the active site zinc cation and replace the zinc-bound H_2O/OH^- ligand to inhibit the enzymes activity. An appreciation of the therapeutic impact of inhibiting specific CA isozymes to disturb this critical biochemical equilibrium has progressed significantly in recent years. The variability in the active site between isozymes of CA is, however, subtle and this makes drug discovery targeting CAs challenging. Several selectivity analyses of CAIs have been reported to guide CA drug design, including ligand-based 3D QSAR selectivity analysis using a large dataset of structurally diverse inhibitors, and very recently, a protein data bank mining approach with CAI crystal structures [4,5]. A number of selectivity discriminating 'hot spots' across CA isozymes have been identified. The therapeutic footprint of CAIs together with the improved understanding of the physiology of CA isozymes stands to benefit from the discovery of new CAIs chemotypes with improved isozyme selectivity characteristics over classical primary sulfonamide inhibitors and the potential to underpin first-in-class next-generation therapeutics.

The coumarin chemotype

Coumarins are abundant secondary metabolites in plants where they act as phytoalexins (defense compounds produced when the plant is under threat from other organisms). The structure of coumarin **(1)** comprises a 1,2-benzopyrone (or *o*-hydroxycinnamic acid-8-lactone) core, with extensions to this core allowing extensive structural diversity across both simple and complex coumarins **(Figure 7.1)** [6]. Vast numbers of distinct coumarin structures have been isolated from nature, while variably substituted coumarins have been synthesized using a number of synthetic routes. Classical methods employ the Perkin, Pechmann or Knoevenagel reactions, while more recent approaches have employed the Wittig, Kostanecki–Robinson and Reformatsky reactions. In addition to building the heterocycle, numerous approaches that elaborate the widely available hydroxycoumarin scaffolds have also been described. These synthetic methods, including their scope and limitations, have been reviewed by Borges and colleagues [6]. Coumarins have been subjected to a broad panel of bioassays. The chemotype displays a range of biological activities, including antimicrobial, molluscicidal, acaricidal, antiviral, anticancer,

Figure 7.1. Structure of coumarin (1), simple coumarins (substituted at any of six available positions), and higher complexity coumarins.

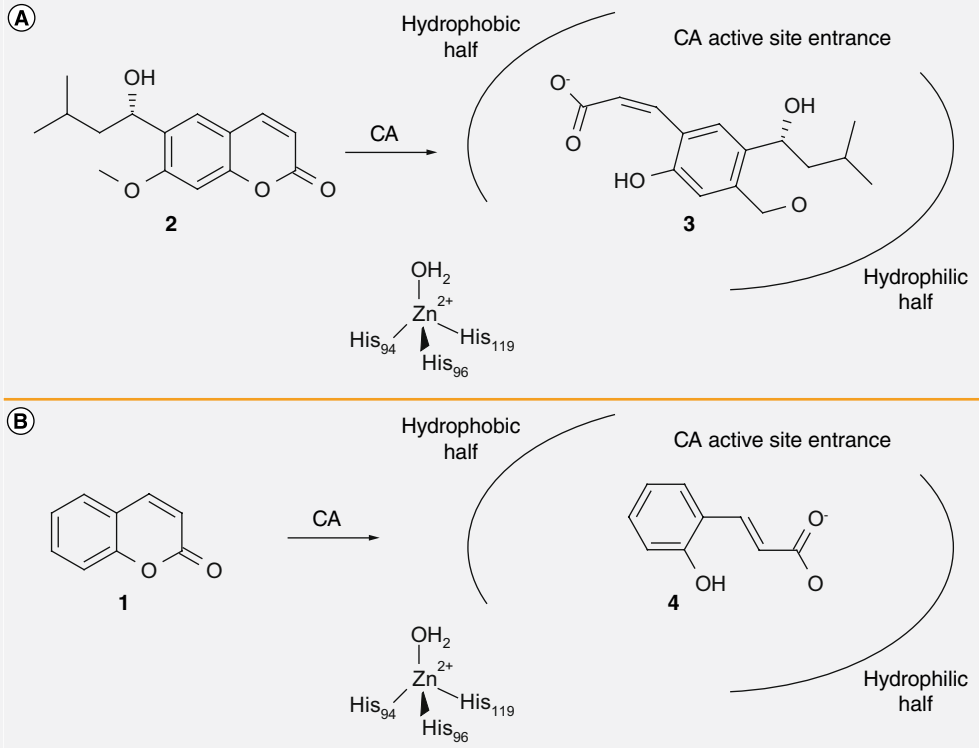


antioxidant and anti-inflammatory properties [7]. A recent review of coumarin-based drugs highlights the increasing interest in investigating the bioactivity of this chemotype for drug discovery, evidenced by approximately 42 patent publications on the biological activity of coumarins from 2008 to 2011 [7]. This review also describes some of the difficulties in advancing this compound class for drug discovery, including identification of the mechanism of action and optimizing the pharmacokinetic properties. Coumarins comprise an α,β-unsaturated ketone within the structure, this Michael acceptor motif is a common structural alert in drug discovery owing to the propensity to react with and covalently modify nucleophiles present in biomolecules. It has, however, been demonstrated that simple coumarins exhibit poor protein binding characteristics compared with other carbonyl-containing Michael acceptors. This observed lower reactivity of the coumarin double bond has been attributed to being part of a pseudoaromatic system [8]. This chapter will present the discovery, properties and development of coumarin compounds that have been shown to inhibit CAs. Coumarins isolated from natural sources as well as synthetic coumarins will be described.

Natural product coumarins

The natural product (NP) compound, 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one **(2)** was the first coumarin characterized as an inhibitor of CA enzymes **(Figure 7.2)** [9]. The compound was identified from screening a NP extract against bovine CA II using native state MS as the screening platform [10]. The mechanism of CA binding and inhibition for this compound was later explored and found to be intriguing. Under standard enzyme assay conditions (15 min incubation of test ligand and enzyme) the coumarin had only weak CA inhibition; however, following 6 h of preincubation with CA II the K_i of coumarin dropped to 60 nM (a similar reduction in K_i at other CA isozymes was also observed). The identification of this compound would have gone unnoticed using standard biochemical assay-based screening, demonstrating the importance of alternate screening platforms, such as MS, for identifying 'nonstandard' but relevant protein–ligand interactions. Using protein x-ray crystallography the crystal structure of this NP with human CA II was obtained at a resolution of 2.0 Å. The hydrolyzed coumarin, *cis*-cinnamic acid derivative **(3)**, was unexpectedly observed in the active site and not the coumarin structure of **(2)** **(Figure 7.2)**. The hydrolysis product **(3)** is bulky compared with **(2)** and was found spanning the CA active site cavity entrance rather than interacting with the catalytic zinc ion (located at the base of the active site). All previously known CAIs coordinate to the active site zinc and this dual substrate-inhibitor behavior, devoid of zinc binding, had never been observed.

Figure 7.2. Coumarins act as dual substrate-inhibitors of carbonic anhydrases.



(A) Natural product coumarin **(2)** is hydrolyzed by the CA enzyme to form to form a *cis*-cinnamic acid **(3)**.
(B) Coumarin **(1)** is hydrolyzed by the CA enzyme to form to form a *trans*-cinnamic acid **(4)**.
CA: Carbonic anhydrase.

Esterase activity is known for CAs and the observation of the cinnamic acid **(3)** rather than the coumarin **(2)** could be rationalized as a consequence of the CA II esterase activity leading to hydrolysis of the lactone of **(2)**. In a subsequent study this unusual inhibition mode behavior was also observed with coumarin **(1)**, the simplest of the coumarin structures. Similarly to the NP **(2)**, the hydrolyzed lactone product from coumarin **(1)** was observed in the crystal structure near the entrance to the CA II active site [11] this time as the *trans*-cinnamic acid isomer, compound **(4)**, and not the *cis*-cinnamic acid isomer as for compound **(3)** (Figure 7.2). It was suggested that the combined bulkiness of the two pendant substituents of **(3)** forced the thermodynamically less stable *cis*-isomer to form and stretch across the CA active site entrance.

Another notable difference between the protein–ligand structures of CA II with **(3)** and **(4)** was the reversed orientation of the carboxyethylene substituent. With the simple NP **(4)** this carboxyethylene substituent was oriented towards the hydrophilic half of the CA active site, while with the bulkier NP **(3)** it was oriented towards the hydrophobic half of the active site with the additional hydroxy-3-methylbutyl substituent of the NP oriented instead towards the hydrophilic half of the active site. Coumarin **(1)**, is not an appreciable inhibitor of most CAs with the exception of CA I and II, while coumarin **(2)** is a submicromolar inhibitor of CA I and CA II, and low-to-medium micromolar inhibitor of all other CAs (with the exception of CA III where it is a very weak inhibitor) **(Table 7.1)**. This structure–activity relationship (SAR) implies that the 6- and/or 7-substituents on coumarin **(2)** are critical to the strengthened interaction with CAs. As the entrance of the CA active site where these ligands bind is the most variable region among human CA isozymes this may account for the highly variable SAR for the NP **(2)** compared with coumarin **(1)** **(Table 7.1)**.

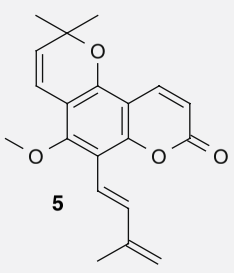
The finding of NP coumarin **(2)** has inspired a closer study of additional NP coumarins as CAIs, with a diverse selection including both simple coumarin derivatives and some substantially more complex in structure investigated, coumarins **(5–31)** **(Figures 7.3 & 7.4)** [12]. The inhibition activity data for the NP coumarins **(5–31)** following a 6 h preincubation with the CA enzyme was determined against CA I, II, IX and XII, with I and II off-target CA isozymes, and CAs IX and XI of interest to diagnose and/or treat hypoxic tumors **(Table 7.2)**. Similarly to the NP coumarin **(2)** as precedent, the SAR for these additional NP coumarins was also highly variable and substituent dependent. The SAR trends include very weak CA II inhibition, while at CA I, IX and XII many have K_is in the range of 1–10 µM. There are, however, a few outliers to this general trend and these compounds represent interesting structures owing to their CA isozyme selectivity characteristics. For

Table 7.1. Inhibition of human carbonic anhydrases by natural product coumarins following 6 h preincubation: coumarin (1) and 6-(1S-hydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one (2).

Compound	K_is (µM)[†,‡]										
	CA I	*CA II*	*CA III*	*CA IV*	*CA VA*	*CA VB*	*CA VI*	*CA VII*	*CA IX*	*CA XII*	*CA XIV*
1	3.1	9.2	>1000	62.3	>1000	578	>1000	>1000	>1000	>1000	>1000
2	0.08	0.06	>1000	3.8	96.0	17.7	35.7	27.9	54.5	48.6	7.8

[†]These inhibition data were acquired following a 6-h incubation time with enzyme using a stopped flow assay that monitors the carbonic anhydrase catalyzed hydration of CO_2.
[‡]Errors in the range of ±5% of the reported value, from three determinations.
CA: Carbonic anhydrase.

Figure 7.3. Natural product coumarins that have been evaluated as carbonic anhydrase inhibitors, compounds 5–16.



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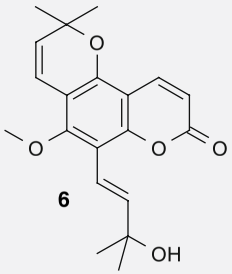

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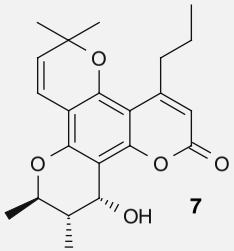

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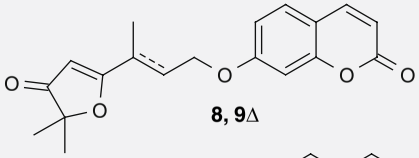

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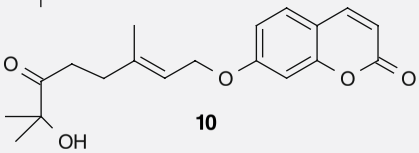

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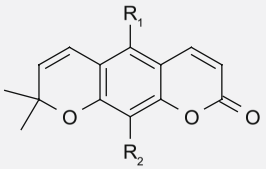
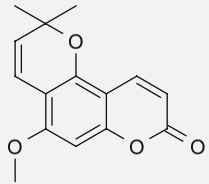


11 $R_1 = H; R_2 = H$
12 $R_1 = OCH_3; R_2 = H$
13 $R_1 = OCH_3; R_2 = OCH_3$



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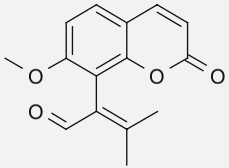

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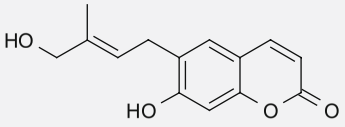
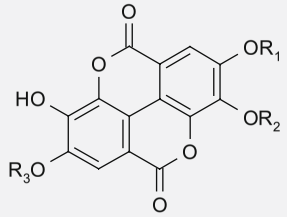


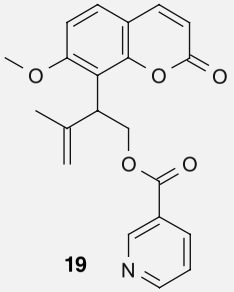
16

Figure 7.4. Natural product coumarins that have been evaluated as carbonic anhydrase inhibitors, compounds (17–31).



17 $R_1 = H; R_2 = H; R_3 = H$

18 $R_1 = CH_3; R_2 = CH_3; R_3 = CH_3$



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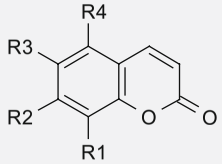


20 $R_1 = OH; R_2 = OCH_3; R_3 = OCH_3; R_4 = H$

21 $R_1 = OH; R_2 = OH; R_3 = OCH_3; R_4 = H$

22 $R_1 = H; R_2 = OH; R_3 = OCH_3; R_4 = H$

23 $R_1 = OCH_3; R_2 = OCH_3; R_3 = OCH_3; R_4 = H$

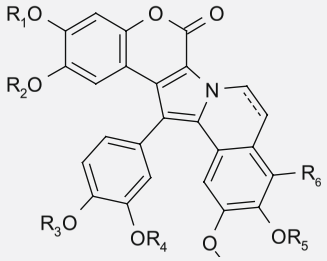
24 $R_1 = OCH_3; R_2 = OCH_3; R_3 = H; R_4 = OCH_3$

25 $R_1 = OCH_3; R_2 = OH; R_3 = H; R_4 = H$

26 $R_1 = H; R_2 = OCH_3; R_3 = OH; R_4 = H$

27 $R_1 = O\beta\text{-D-Glc}; R_2 = OH; R_3 = OCH_3; R_4 = H$

28 $R_1 = H; R_2 = O\beta\text{-D-Glc}; R_3 = OCH_3; R_4 = H$



29 $R_1 = H; R_2 = CH_3; R_3 = CH_3; R_4 = H; R_5 = CH_3; R_6 = OH$

30 $R_1 = H; R_2 = CH_3; R_3 = H; R_4 = CH_3; R_5 = CH_3; R_6 = OCH_3\Delta$

31 $R_1 = CH_3; R_2 = H; R_3 = CH_3; R_4 = H; R_5 = SO_3Na; R_6 = H$

Table 7.2. Selected inhibition data for natural product coumarins 5–31 against carbonic anhydrase isozymes I, II, IX and XII.

Compound	K_i (μM)[†,‡,§]			
	CA I	*CA II*	*CA IX*	*CA XII*
5	7.66	>100	0.62	0.79
6	8.46	>100	0.78	0.77
7	9.31	50.7	0.83	0.81
8	59.2	63.4	0.89	0.60
9	9.75	>100	0.60	0.83
10	7.81	>100	4.03	0.70
11	21.5	>100	7.51	25.7
12	7.71	>100	0.74	0.96
13	9.21	49.3	0.86	8.35
14	5.60	>100	3.50	9.10
15	9.11	>100	8.12	7.44
16	7.52	78.9	9.75	0.77
17	68.2	>100	79.8	8.15
18	44.1	>100	17.4	7.42
19	5.84	>100	0.67	7.39
20	9.89	>100	0.85	7.84
21	4.86	94.3	0.61	7.70
22	10.56	>100	0.96	4.05
23	0.0097	>100	6.58	18.2
24	4.31	9.65	0.76	0.83
25	36.4	>100	0.85	9.12
26	14.0	>100	7.37	4.14
27	5.04	>100	0.37	7.45
28	5.93	>100	8.72	0.78
29	6.45	>100	3.22	9.07
30	40.1	>100	6.33	8.51
31	6.55	>100	3.27	1.79
AZA	0.25	0.012	0.025	0.0057

The standard inhibitor acetazolamide (AZA) is included for comparison.
[†]These inhibition data were acquired following a 6 h incubation time with enzyme using a stopped flow assay that monitors the CA catalyzed hydration of CO_2. [‡]Errors in the range of ±5% of the reported value, from three determinations. [§]All proteins were recombinant.

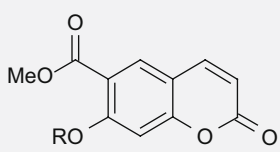
example, a subset of the NP coumarins has viable selectivity characteristics towards CA IX and XII and may warrant further studies in cell-based models of CA in cancer. This variability of the CA isozyme inhibition profile may prove a desirable attribute for drug discovery, wherein selective inhibition of specific CA isozymes is sought to deliver the desired physiological effect without side effects caused by off-target inhibition. When coupled with the complexity and diversity of NP coumarin structures, which far exceeds that described for synthetic coumarin CAIs, there remains opportunity to discover new NP coumarin CAIs with ideal characteristics for drug development.

Synthetic coumarins

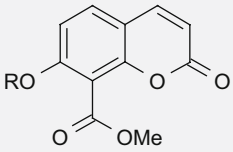
Following the report of the NP coumarin **(2)** as a CAI a number of libraries of synthetic coumarins and thiocoumarins have since been prepared and evaluated as CAIs. The first detailed investigation of synthetic coumarins described the SAR for a library of 6- or 7-monosubstituted coumarins, disubstituted coumarins (with a carboxylic acid or ester as one of the two substituents) and a thiocoumarin against 13 mammalian CA isozymes [11]. As for the NP **(2)**, the coumarins were preincubated with the target CA enzyme for 6 h prior to measuring enzyme inhibition to allow for the complete enzyme-mediated hydrolysis of the (thio)lactone to the product cinnamic acid derivative. All CAs were inhibited by the coumarins with activity in the low nanomolar to micromolar range with the substitution pattern of the (thio)coumarin dominating the inhibition profile. In a number of follow-up studies the importance of the coumarin substitution pattern was further reinforced, especially when targeting selective inhibition of cancer-associated isozymes, CAs IX and XII.

In the first of these studies the CA inhibition profile for two series of coumarins, either 6,7- or 7,8-disubstituted coumarins, was reported [13]. These compounds were synthesized by elaboration of the 7-hydroxycoumarin (umbelliferone) scaffold. CA inhibition data for selected compounds from this study, coumarins **32–39**, are given in **Figure 7.5 & Table 7.3**. The 6,7-disubstituted series, coumarins **32–35**, showed very weak or nil CA inhibition activity, while the 7,8-disubstituted series, coumarins **36–39** (structural isomers of the 6,7-disubstituted series) were also ineffective inhibitors of CA I and II however much better inhibitors of the tumor-associated isozymes, CA IX and XII **(Table 7.3)**. The combined size of the 7- and 8-substituents played a role in optimal inhibition, with the 7-*n*-propyl substituent of compound **(38)** providing a subnanomolar CA XII inhibitor (K_i = 0.98 nM), while shorter and longer/bulkier 7-alkyl substituents caused inhibition to drop off. This SAR suggests that the hydrolysis product of these

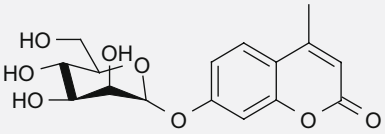
Figure 7.5. Synthetic coumarins that have been evaluated as carbonic anhydrase inhibitors (compounds 32–39) and in preclincal models of cancer (GC-204, GC-205 and ^{18}F-FEC).



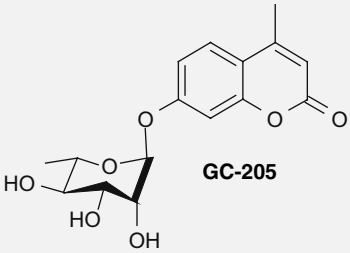
32 R = Me
33 R = Et
34 R = *n*-Pr
35 R = *n*-Bu



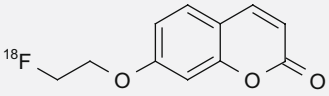
36 R = Me
37 R = Et
38 R = *n*-Pr
39 R = *n*-Bu



GC-204



GC-205



^{18}F-FEC

coumarins may span the active site entrance similarly to the NP **(2)**, with an optimum span length approached by the substituents of **38**. Additional studies on coumarins incorporating hydroxy-, chloro- and/or chloromethyl-moieties in positions 3-, 4-, 6- and 7- [14] and coumarins and thiocoumarins incorporating *tert*-butyl-dimethylsilyloxy- or allyoxy- moieties in positions 4-, 6- or 7-, synthesized from 6-, 7- or 4-hydroxycoumarins, respectively [15], have also displayed this promising CA activity and selectivity profiles.

The CA isozyme selectivity profile displayed by some of the synthetic coumarins is beneficial for therapeutic applications of CAIs and this has fuelled further interest in exploring the coumarin compound class in preclinical tumor models. Glycosyl coumarins **GC-204** and **GC-205** were synthesized from 4-methyl-7-hydroxycoumarin (4-methylumbelliferone), then evaluated in a highly metastatic and aggressive 4T1 mouse mammary tumor model that overexpresses CA IX together with a bioluminescent reporter [16]. Mice were treated daily for 6 days with **GC-204** or **GC-205** (15–40 mg/kg) by intraperitoneal injection prior to imaging of tumors. Compounds were well tolerated and compound treatment resulted in significant inhibition of both tumor growth and metastasis formation in the model, with **GC-205** being particularly efficacious at 15 mg/kg without effects on CA IX negative tumors. These results provide proof-of-principle for therapeutic intervention with CA IX inhibitors to limit breast tumor growth and metastasis. In a related

Table 7.3. Carbonic anhydrase inhibition data for synthetic coumarins 32–39 and coumarins assessed in preclinical models (GC-204, GC-205 and ^{18}F-FEC) against carbonic anhydrase isozymes I, II, IX and XII.

Compound	K_I (μM)[†,‡,§]			
	CA I	*CA II*	*CA IX*	*CA XII*
32	>100	>100	7,800	6,540
33	>100	>100	7,400	>100,000
34	>100	>100	7,580	>100,000
35	>100	>100	>100,000	>100,000
36	>100	>100	78.3	60.9
37	>100	>100	70.8	1.0
38	>100	>100	56.7	0.98
39	>100	>100	61.2	8.8
GC-204	>100,000	>100,000	9.2	43
GC-205	>100,000	>100,000	201	184
[^{18}F]FEC	4,622	>100,000	70	88

[†]These inhibition data were acquired following a 6 h incubation time with enzyme using a stopped flow assay that monitors the CA catalyzed hydration of CO_2. [‡]Errors in the range of ±5% of the reported value, from three determinations. [§]All proteins were recombinant.
CA: Carbonic anhydrase.

study **GC-204** was administered by intravenous injection at 30 mg/kg to mice with the same 4T1 mammary tumor model. A more modest but significant reduction in tumor volume (relative to intraperitoneal injection described above) was observed with the compound.

Small molecules targeting CA IX may one day play an important role in diagnosing hypoxic tumors, planning treatment strategies, and monitoring response to therapies. CA IX comprises several features that make it a potentially good option for preclinical and diagnostic imaging [17–19]. First, the active site is extracellular and so can be targeted by probes that do not have to cross the cell membrane, and second, it is expressed in few healthy tissues but upregulated to high concentrations in many hypoxic tumors. Based on this premise, together with the promising preclinical data from **GC-204** and **GC-205**, the coumarin [^{18}F]-7-(2-fluoroethoxy)coumarin (**^{18}F-FEC**) containing the PET radionuclide ^{18}F was synthesized. **^{18}F-FEC** is very selective for cancer-related CAs **(Table 7.3)**, while the compound is stable in mouse plasma with >99% of the tracer remaining intact after 2 h incubation. This compound was evaluated in HT-29 colorectal tumor

bearing mice as the CA IX expressing tumor model. Unfortunately the tumors were not visualized from the PET images obtained, instead very high liver and nasal cavity uptake was observed. An explanation for this high nontumor uptake was proposed as the action of a cytochrome P450 enzyme (CYP1A2) that is able to metabolize the structurally similar 7-ethoxycoumarin, converting it into 7-hydroxycoumarin and acetaldehyde. CYP1A2 is expressed both in liver and the nasal cavity and it was proposed that CYP1A2 may metabolize **^{18}F-FEC** into 7-hydroxycoumarin and [^{18}F]-2-fluoroacetaldehyde. This radioactive metabolite may be further metabolized into [^{18}F]-2-fluoroacetate that in turn forms [^{18}F]-2-fluoroacetyl CoA and becomes trapped within the cell. More studies are needed to assess this hypothesis and refine the pharmacokinetics of the coumarin chemotype for diagnostic applications.

Aa PET: a clinically used molecular imaging technique that enables whole body imaging of molecular targets or processes with high sensitivity within a living subject using a specific imaging agent. The images obtained using PET reflect the distribution of the PET imaging agent in the subject. In the clinic PET is mainly used to image cancer through the use of imaging agent comprising the positron-emitting radionuclide ^{18}F, such as in [^{18}F]-2-fluoro-2-deoxy-glucose ([^{18}F]FDG).

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Summary.

- The structure of coumarins comprise a 1,2-benzopyrone (or *O*-hydroxycinnamic acid-8-lactone) core.
- A vast collection of variably substituted coumarins are known from natural sources or chemical synthesis.
- Coumarins are dual acting, as both substrates and inhibitors of carbonic anhydrase (CA) enzymes.
- Coumarins inhibit CAs via an alternate and unprecedented mechanism compared with classical sulfonamides.
- Several natural and synthetic coumarins derivatives have shown promising CA inhibition profiles towards cancer-associated CAs.
- Coumarins have been evaluated in preclinical cancer models and results suggest a potential new avenue for drug development compared with the zinc-binding groups of classical CA inhibitors.

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Chapter 8

Next-generation dithiocarbamate carbonic anhydrase inhibitors

Fabrizio Carta, Murat Bozdag, Muhammet Tanc & Claudiu T Supuran

Dithiocarbamates (DTCs) are chemically referred to as functional groups analogs to the carbamates, containing sulfur instead of oxygen atoms. Herein we report the kinetic results of a series of DTC-containing compounds tested on α and β-carbonic anhydrases (EC 4.2.1.1). The DTCs binding mode was also investigated by means of crystallographic experiments of the selected DTC **10**, **11** and **14** in adduct with human carbonic anhydrase II.

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Dithiocarbamates (DTCs) are chemically referred to as functional group analogs to the carbamates, containing two sulfur atoms instead of oxygen atoms. DTCs are stable in the form of both inorganic and organic salts or esters, and are easily obtained by coupling reactions of primary/secondary amines (aliphatic or aromatic) with carbon disulfide in the presence of an appropriate base. The introduction of an alkyl halide in the reaction medium affords the corresponding esters [1]. The use of DTC-bearing compounds in large amounts began in the early 20th century, with their industrial application as accelerators in the rubber vulcanization process [1]. This method proved to be much more efficient than the primitive, sulfur-based procedures introduced by Goodyear and Hancock approximately 60 years earlier [1]. Actually many DTCs are widely used in different fields such as in the analytical, macromolecular and organic chemistry, medicine and biology [1]. Undoubtedly the plant disease management is the area in which DTCs are predominantly employed [1]. The role DTCs play nowadays is heading for strengthening in the near future, due to the global growth population estimations (~9 billion in 2050) and the necessity to control plant diseases by means of chemical agents [1,2].

DTCs & the carbonic anhydrases

The main feature of DTCs is the ability to form stable complexes with metal ions, and plenty of reports accounting for the characterization of such derivatives are available in the literature [1]. However, the first study dealing with DTCs and metalloenzymes only appeared in 1983 with the investigation of the interaction between *N*,*N*-diethyldithiocarbamate **1** and various metal-containing bovine carbonic anhydrases (bCAs) [3]. In particular, the study reported the enzymatic inhibition of **1** with the native zinc-containing bCA, the ability of **1** to extrude the catalytic metal ion from the enzymatic site, and the authors proposed a trigonal bipyramidal geometry for the **1**-Co(II)-bCA adduct [3]. However the scientific community ignored the topic until the kinetic profiles of a series of unexplored inorganic anions on the human (h) and murine (m) CAs (EC 4.2.1.1) were recently reported by our group [4,5]. Such results demonstrated that most common inorganic anions explored [6], possess inhibition constants (K_is) between the millimolar to the sub-millimolar range. It is worth mentioning that the cyanide, azide and hydrogen sulfide were the only known exceptions [6]. Among the newly investigated inorganic species, trithiocarbonate **2** showed the highest affinities (Kis 8.7, 9.9,

Aa Dithiocarbamates (DTCs): chemically referred as functional groups analogs to the carbamates, containing two sulfur instead of oxygen atoms.

Carbonic anhydrase inhibitors (CAIs): in general referred as chemical species able to inhibit the activity of the carbonic anhydrase enzymes. Classical CAIs include the sulfonamides and their bioisosters the sulfamates and the sulfamides.

9.7 and 120 μM) for various CA isoforms, such as hCA I, II, IX and XII, respectively **(Table 8.1)** [4,5]. These studies validated trithiocarbonate **2** as a new inorganic CA inhibitor (CAI) and its binding mode was then explored by means of x-ray crystallography on the **2**-hCA II adduct [7]. Along with trithiocarbonate **2** the kinetic studies were also extended to the organic, structurally related compound *N,N*-diethyldithiocarbamate **1**. The rationale of this investigation was to explore whether organic compounds incorporating the trithiocarbonate moiety merged into organic scaffolds, such as the DTCs, might act as CAIs. The known metal chelating properties of the DTCs and the preliminary investigations by Morpurgo *et al.* previously discussed, indeed represented an interesting starting point to validate such a hypothesis. Indeed, we definitely proved that *N,N*-diethyldithiocarbamate **1** not only efficiently inhibits hCAs but it is even a better inhibitor when compared with its inorganic lead trithiocarbonate **2**, having K_i values as low as 24.6-times those for the hCA VII [4,5]. Afterwards, we reported a series of new DTC derivatives **3–28**, which have been prepared and screened for their inhibition activities on several α- and β-CAs. The biological implications as well as structure–activity relationship (SAR) investigations will be developed later in this chapter, now it is important to focus on the DTCs binding mode, which was explored by means of x-ray crystallography on adducts of several such DTCs, that is, **10**, **11** and **14,** bound to hCA II **(Figure 8.1)** [8,9].

The DTCs were found deeply buried into the enzyme active site. The CS_2^- groups upon displacement of the zinc-bound water/hydroxide, directly interacted through a sulfur atom with the catalytic zinc ion at a distance Zn-S of 2.3 Å. The angles defined by the zinc-coordinating histidines (His94, His96 and His119), the metal ion and the DTCs sulfur atom closely associated, account for a distorted tetrahedral geometry, which resembles the binding mode of the classical CAIs, such as the sulfonamides and their bioisosters sulfamates and sulfamides **(Figure 8.1A–8.1C)** [8,9]. The major differences in the interaction modes of **10**, **11** and **14** with the hCA occur at the organic scaffold level. The benzyl ring of compound **14** engages a T-shaped π-staking interaction with the imidazole ring of the His64, which, as a result, was frozen in the 'in' conformation. Furthermore, **14** is stabilized by the weaker van der Waals interactions occurring with the side chains of Asn62, Glu92, His94, Val121, Phe131, Leu198, Thr200 and Pro202 **(Figure 8.1C)** [8,9]. On the other hand, compound **10** positioned its phenyl ring onto a hydrophobic pocket located at the opposite site of the enzymatic tunnel bearing the His64 residue **(Figure 8.1A)** [8,9]. The pendant His64 imidazole ring was at a distance of 5 Å from **10**, being thus not involved in any valuable interaction with the inhibitor and is allowed to flip

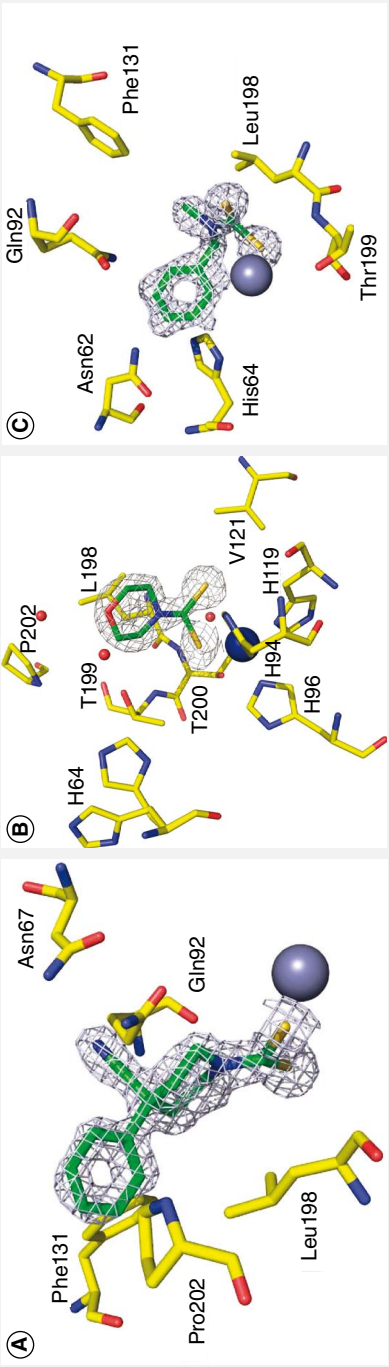

Figure 8.1. Electron densities for dithiocarbamates (A) 10, (B) 11 and (C) 14 in adduct with human carbonic anhydrase II.

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adopting both the 'in' and 'out' conformations. The phenyl ring of **10** headed towards the rim of the catalytic cleft, interacting with Phe131 and Pro202, whereas the cyano group hydrogen bonds with Asn67 and Gln 92 and a water molecule locate nearby **(Figure 8.1A)** [8,9]. Compound **11** was the deepest buried among all, and, as for compound **10**, no interactions with the His64 side chain occurred. The only contacts detected for the compound **11** scaffold in adduct with hCA II were with two water molecules, Wat393 and 540 **(Figure 8.1B)** [8,9].

The inhibition profiles of the DTCs **1** and **3–28** on hCA I, II, IX and XII are reported in **Table 8.1** and compared with the acetazolamide (**AAZ**) as CAI reference compound [8].

Compounds **1** and **3** are less effective as CAIs against all the screened isoforms. The elongation of both the aliphatic chains, as for **4–6**, ensures a progressive amelioration in the affinities toward all hCA isozymes tested, with K_is in the range of 5.8–1838 nM. A slight selectivity of **4** and **5** for the tumor-associated isoform hCA XII is observed (Kis 7.0 and 5.8 nM for **4** and **5,** respectively). The introduction of terminal hydroxide groups, as in **7**, results into an overall improvement of the CA inhibitions (Ki 4.0–9.2 nM) and further enhancements are gained when the linear side chains are substituted with branched analogs as in **8**. The K_i values for the *i*-Bu **8** were lower of one or two order of magnitude compared with the parent linear compound **5** (Kis 0.97, 0.95, 4.5 and 0.99 nM for **8** and K_is 43.1, 50.9, 50.3 and 5.8 nM for **5** on hCA I, II, IX and XII, respectively). The idea to join the linear alkyl

Table 8.1. Human carbonic anhydrase I, II, IX and XII inhibition data with dithiocarbamates 1, 3–28 and trithiocarbonate 2.

Compound	R^1	R^2	K_I (nM)			
			hCA I	*hCA II*	*hCA IX*	*hCA XII*
1	Et	Et	790	3100	1413	11
2†	CS_3^-		8.7×10^3	9.9×10^3	9.7×10^3	120×10^3
3	Me	Me	699	6910	714	798
4	*n*-Pr	*n*-Pr	1838	55.5	53.8	7.0
5	*n*-Bu	*n*-Bu	43.1	50.9	50.3	5.8
6	*n*-Hex	*n*-Hex	48.0	51.3	27.4	16.1
7	$HOCH_2CH_2$	$HOCH_2CH_2$	9.2	4.0	4.3	4.2
8	*i*-Bu	*i*-Bu	0.97	0.95	4.5	0.99
9	$(CH_2)_5$		0.96	27.5	70.4	46.1
10	$(NC)(Ph)C(CH_2CH_2)_2$		48.4	40.8	757	169
11	$O[(CH_2CH_2)]_2$		0.88	0.95	6.2	3.4
12	Et	*n*-Bu	157	27.8	25.9	7.5
13	Me	Ph	39.6	21.5	28.2	7.7
14	Me	$PhCH_2$	69.9	25.4	53.0	3.0
15	H	*sec*-butyl	21.1	29.4	4.6	31.7
16	H	Ph	4.8	4.5	4.2	4.3
17	H	2-thiazolyl	3.9	4.6	12.6	22.0
18	H	$PhCH_2$	4.1	0.7	19.2	11.5
19	H	4-PyridylCH_2	3.5	16.6	26.0	24.1
20	H	Imidazol-1-yl-$(CH_2)_3$	8.6	24.7	4.3	6.5
21	H	$O[(CH_2CH_2)]_2N$	4.8	3.6	29.1	9.2
22	H	$O[(CH_2CH_2)]_2N(CH_2)_2$	31.8	36.3	4.5	4.2
23	H	$[(CH_2)_5N]CH_2CH_2$	4.5	20.3	3.6	20.5
24	H	$MeN[(CH_2CH_2)]_2N$	33.5	33.0	22.1	17.5
25	$NaS(S=C)N[(CH_2CH_2)]_2$		12.6	0.92	37.5	0.78
26	H	$KOOCCH_2$	13.1	325	57.1	6.7
27	(*S*)-$[CH_2CH_2CH_2CH(COONa)]$		2.5	17.3	4.1	4.0
28	H	$N[(CH_2CH_2)N]_3$	31.9	13.5	27.4	9.3
AAZ			250	12	25	5.7

†hCA III, VII and XIII not shown.
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chains by means of a -CH_2- (compound **9**), ends in affinity values similar to the best alkyl derivative **8** only for the cytosolic hCA I, whereas the other isoforms keep their K_i closer to the linear DTCs **4–6** (Kis 0.96, 27.5, 70.4 and 46.1 nM for **9** on hCA I, II, IX and XII, respectively) **(Table 8.1)**. The substitution of the joint -CH_2-protons with bulkier groups (compound **10**) causes a sensible drop in the inhibitory activities (Kis 48.4, 40.8, 757 and 169 nM for **10** on hCA I, II, IX and XII, respectively). On the other end, replacement of the -CH_2- with a sp^3-hybridized oxygen, as in **11**, greatly enhances the enzymatic affinities (Kis 0.88, 0.95, 6.2 and 3.4 nM on hCA I, II, IX and XII, respectively). The asymmetric *N,N*-disubstituted DTCs **12**, **13** and **14** preferentially inhibit the tumor-associated hCA XII isoform (Kis 7.5, 7.7 and 3.0 nM for **12**, **13** and **14,** respectively, on hCA XII). The monosubstituted *sec*-butyl derivative **15** has inhibition values comprised between the parent linear *N,N*-dialkylated **5** and the branched **8** (Kis 21.1, 29.4, 4.6 and 31.7 nM for **15** on hCA I, II, IX and XII, respectively). When an aromatic moiety is introduced, such as the phenyl ring in **16** or the heterocyclic thiazolyl group in **17,** the K_i values get flattened to the low nanomolar levels, and only a minor selectivity for the hCA I and hCA II is observed for **17** **(Table 8.1)**. The insertion of a spacer between the aromatic/heteroaromatic tail and the DTC nitrogen (compounds **18**, **19** and **20**) did not improve the inhibition profiles **(Table 8.1)**. Interestingly, the morpholine compound **21** showed preferential inhibition for the cytosolic hCA I and II, whereas the introduction of an ethylenic spacer (compound **22**) shifted the selectivity toward the tumor-associated isoforms hCA IX and XII. The replacement of the morpholinic oxygen in **22** with a -CH_2- , to give **23**, lowers the hCA I and IX K_i values of 4.5- and 5.7-fold compared with the corresponding cytosolic hCA II and tumor-associated hCA XII isoforms. The bis DTC **25** had a complementary kinetic profile to **23**, as shows stronger affinity for the hCA II and XII over the corresponding hCA I and IX **(Table 8.1)**. The aminoacidic derivatives glycine **26** and *S*-proline **27** were screened for their inhibitory activities. In particular **26** badly interacts with the hCA II (Ki of 325 nM). Despite the *S*-proline **27** reported K_i values at the low nanomolar level for all the tested hCAs, in analogy to **26**, keeps the inhibition for hCA II weaker **(Table 8.1)**. In conclusion the SAR of compounds **1** and **3–28** validated the DTCs as new class of CAIs, having inhibition constants spanning from the micro- to the nano-molar range.

Aa Glaucoma: consists of a group of progressive optic neuropathies characterized by a slow and progressive degeneration of retinal ganglion cells and their axons. It shows a broad spectrum of clinical presentation and etiologies, which lead to a permanent loss of visual function due to the damage of the optical nerve. Most types of glaucoma are characterized by an elevated intraocular pressure.

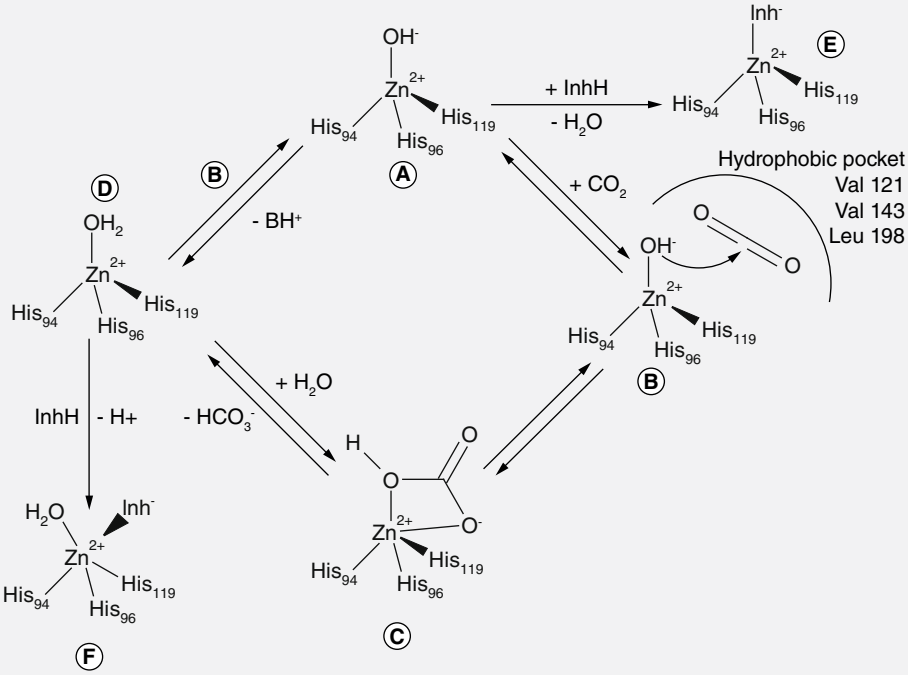
In light of such considerations DTCs **11** and **25** were investigated for their intraocular pressure (IOP) inhibition properties on the carbomer-induced glaucoma model on

rabbits, and compared with the standard clinically used dorzolamide (**DRZ**) [8]. Glaucoma is estimated to affect more than 70 million people worldwide and is clinically characterized by a series of neuropathic diseases, which progressively affect the retinal systems toward irreversible visual impairment and blindness [6,10,11]. The majority of glaucomas are characterized by elevated IOPs as result of unbalance between flow-out and production of the aqueous humor [6,10,11]. The metalloenzyme CAs plays a crucial role in the production of the aqueous humor, which is particularly rich in the bicarbonate ions [6,10], and the pioneering report of Becker in 1955 created the base for the use of CAIs as antiglaucoma agents [12].

As previously discussed DTCs take part in the CA catalytic cycle in the same manner as the classical CAIs such as sulfonamides and their bioisosters, to afford the intermediate **E** in **Figure 8.2** [13]

The result is the interruption of the CAs catalytic cycle with a diminished production of bicarbonate and protons as in agreement with $CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+$. The biological consequences are a lower production of the

Figure 8.2. Catalytic cycle of human carbonic anhydrases.



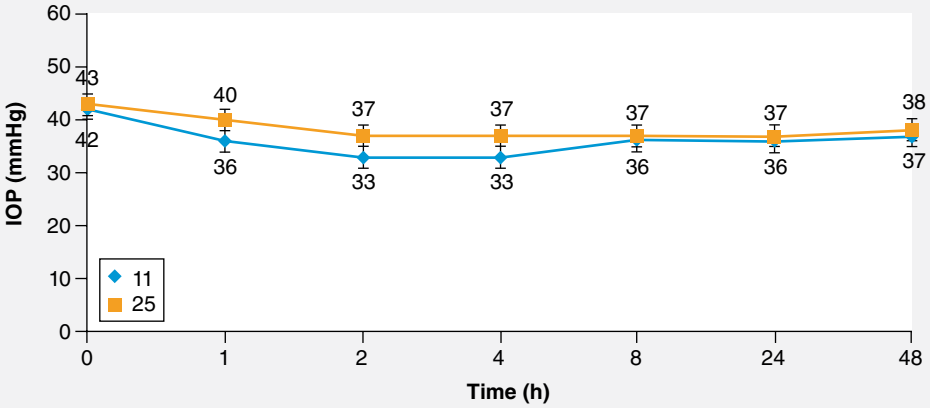
Data taken from [13].

humor liquid and IOP lowering as demonstrated by the **Figure 8.3** referred to **11** and **25** used at 2% concentration [8]. Both compounds show a persistent IOP lowering effect up to 8 h after local administration with **11** been slightly the more potent. In any case both DTCs **11** and **25** proved to be superior in terms of potency and lasting to the clinically used **DRZ** at the same concentration [8].

DTCs & α-CAs from *Drosphila melanogaster*

The arthropod *Drosophila melanogaster*, also known as fruit fly, became of particular attention as it represented the first animal, along with the nematode *Caenorhabditis elegans*, in which the presence of a β-class CA (DmBCA) was reported [14,15]. CA enzymes are classified into five unrelated gene families (α-, β-, γ-, δ- and ζ) and usually it is assumed that each CA-class is expressed only within specific species [6]. Despite the new scientific findings, little is known about the properties of the α-CAs abundantly expressed in *D. melanogaster.* A study of this kind recently appeared [16], and it reported the expression, purification and characterization of the *D. melanogaster* CAH1 and CAH2 along with their tissue distributions. Both enzymes were also considered for their carbon dioxide hydration reaction activities by means of stopped-flow measurements. Interestingly CAH1 and CAH2 revealed high catalytic turnovers with values similar to the highly active hCA II, but slightly lower when compared with the DmBCA (6.4×10^6 for CAH1,

Figure 8.3. 48 h intraocular pressure monitoring of compounds 11 and 25 at 2% concentration in aqueous solution.



Vehicle treatment not shown.
IOP: Intraocular pressure.
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6.0×10^6 for CAH1, 9.5×10^5 for DmBCA and 1.4×10^6 for hCA II) [14,16]. CAH1 and CAH2 are efficiently inhibited by the CAI acetazolamide (**AAZ**), even if with K_is higher than the hCA II and DmBCA values (Kis 106, 78, 49 and 12 nM for **AAZ** on CAH1, CAH1, DmBCA and hCA II, respectively) [14,16].

The DTCs **1** and **3–28** previous reported were also screened for their inhibition profiles on both CAH1 and CAH2, and their data were compared with the hCA II K_i values obtained in the same conditions **(Table 8.2)** [16]. A general overview reveals that all DTCs tested have good inhibition properties, with the only exceptions of **1** and **3** been the less active in the series **(Table 8.2)**.

Elongation of the aliphatic chains as in **4–6** drives the selectivity toward CAH2 over CAH1 and hCA II **(Table 8.2)**. Similarly **10**, the *N*-monosubstituted DTC **15**, and the tris-DTC **28** show analogous inhibition profiles. On the other hand the heterocyclic thiazolyl derivative **17**, the methyl piperazine **24** and *S*-proline **27** have preferential inhibition for the CAH1 over the other tested isozymes. The kinetic investigation reported is of particular importance as subcellular localization predictions account for the cytoplasmic and extracellular membrane-bounded expression of CAH1 and CAH2, respectively [16]. Moreover, in support of the localization predictions, the phylogenetic tree reveals CAH1 sharing a common ancestor with the cytosolic hCA I and hCA II, whereas CAH2 been related to the extracellular human isoforms [16]. Thus compounds, as the DTCs, which show selectivity for specific arthropod CAs, are of particular relevance for the management of parasites or organisms acting as vectors for potential diseases.

DTCs as new β-CAIs

The β-CAs, in agreement with their primordial origins, are largely encoded in archaea, bacteria, algae and fungi. In this contest is important to consider the problems associated to the management of diseases directly provoked of transmitted by such organisms [6,17]. Moreover the appearance of drug-resistant (DR) and multiple drug-resistant (MDR) bacteria are the principal causes of failure in the treatment of infections. Nevertheless, the US FDA registered a drop in the approval of new drugs as antibacterials up to 56% in the last 20 years [17]. Therefore, the selective targeting of the β-CAs represents a very promising approach for the development of new anti-infectives, and the validation of the DTCs as new CAIs indeed might provide such opportunity.

The *Mycobacterium tuberculosis* genome encodes for three β-CAs (mtCA1, mtCA2 and mtCA3) [18,19]. The bacterial enzymes have been cloned, characterized and screened for their kinetic profiles with

Aa Anti-infectives: molecules of natural or synthetic origin able to suppress microorganisms dangerous for human health.

Table 8.2. hCA II, CAH1 and CAH2 inhibition data with dithiocarbamates 1 and 3–28.

Compound	R^1	R^2	K_i (nM)		
			hCA II	*CAH1*	*CAH2*
1	Et	Et	3100	2355	654
3	Me	Me	6910	3461	769
4	*n*-Pr	*n*-Pr	55.5	23.4	3.9
5	*n*-Bu	*n*-Bu	50.9	17.1	0.5
6	*n*-Hex	*n*-Hex	51.3	1.4	3.8
7	$HOCH_2CH_2$	$HOCH_2CH_2$	4.0	3.2	134
8	*i*-Bu	*i*-Bu	0.95	20.2	0.9
9	$(CH_2)_5$		27.5	135	4.7
10	$(NC)(Ph)C(CH_2CH_2)_2$		40.8	12.8	3.7
11	$O[(CH_2CH_2)]_2$		0.95	13.8	0.8
12	Et	*n*-Bu	27.8	10.5	21.9
13	Me	Ph	21.5	67.1	5.9
14	Me	$PhCH_2$	25.4	50.2	12.4
15	H	*sec*-butyl	29.4	325	0.9
16	H	Ph	4.5	21.4	33.9
17	H	2-thiazolyl	4.6	0.5	3.9
18	H	$PhCH_2$	0.7	0.8	213
19	H	4-PyridylCH_2	16.6	35.1	58.8
20	H	Imidazol-1-yl-$(CH_2)_3$	24.7	0.9	24.1
21	H	$O[(CH_2CH_2)]_2N$	3.6	13.1	20.7
22	H	$O[(CH_2CH_2)]_2N(CH_2)_2$	36.3	12.1	10.4
23	H	$[(CH_2)_5N]CH_2CH_2$	20.3	57.1	26.7
24	H	$MeN[(CH_2CH_2)]_2N$	33.0	0.9	13.8
25	$NaS(S=C)N[(CH_2CH_2)]_2$		0.92	0.9	21.6
26	H	$KOOCCH_2$	325	12.3	76.5
27	(*S*)-$[CH_2CH_2CH_2CH(COONa)]$		17.3	1.4	13.6
28	H	$N[(CH_2CH_2)N]_3$	13.5	134	1.5
AAZ			12	106	78

classical CAIs as the sulfonamides [19–21]. DTCs **1** and **3–27** were investigated for their ability to inhibit mtCA1 and mtCA3 and the results are reported below in comparison with the cytosolic human hCA I and hCA II **(Table 8.3)** [22].

Table 8.3 shows that almost all tested DTCs have good affinities for both the mtCA1 and mtCA3, with K_is spanning from 0.9 to 893 nM. Similarly to the human cytosolic isozymes hCAI and hCA II, compounds **1** and **3** were the less active (Kis 431–893 nM). Chain elongation, as for **4–6**, accounts for a slightly preferential inhibition of the mtCA3 isoform over the mtCA1 **(Table 8.3)**. Such effect becomes more evident when the branched *i*-Bu substituents are present (Kis 86.2, 43.0 nM for **8** on mtCA1 and mtCA3, respectively). The introduction of the hydroxyl functionalities, as for compound **7**, results in an overall enhancement of the mtCAs inhibitory properties (Kis 7.5, 6.0 nM for **7** on mtCA1 and mtCA3, respectively). Inclusion of the DTC nitrogen into a cyclohexane ring, as in **9**, shifts the inhibition toward the mtCA3 isozyme over the mtCA1 (Kis 90.5, 4.1 nM for **9** on mtCA1 and mtCA3, respectively). Interestingly the substitution in **9** of the distal -CH_2- with an oxygen (compound **11**) lowers both the mtCAs K_i values, and it completely abolishes any preferential inhibition. Also the asymmetric *N,N*-disubstituted DTCs **12–14** show good inhibition with K_is comprised between 25.2 and 91.6 nM. The *N*-monosubstituted DTCs **15**, **16**, **19**, **20–24** reveal better profiles among all the series having K_is in the low nanomolar range and comprised between 2.4 and 9.1 nM. Interestingly, **17** and **18** are 9.4- and 12.3-fold more selective toward the mtCA3 and mtCA1, respectively **(Table 8.3)**. The bis-DTC **25** offers better selectivity when compared with **17**, as possesses a K_i for the mtCA3 48-times lower to the corresponding for mtCA1. Finally the amino acidic derivatives **26**, **27** and the tris-DTC **28** are good mtCAs inhibitors with K_is between 4.0 and 8.0 nM

As for the *M. tuberculosis*, the β-CAs from the pathogenic fungi *Cryptococcus neoformans*, *Candida albicans* and *Candida glabrata* (Can2, CaNce103 and CgNce103) were investigated for their inhibition profile with DTCs **1**, **3–28** **(Table 8.4)** [23].

In general, all tested compounds show good inhibition profiles towards all three fungal CAs, with the smaller derivatives **1** and **3** being the less active in the series (K_is 962–802 nM). Again the chain elongation, as for compounds **4–6**, or the introduction of the branched *i*-Bu substituents (as for **8**) lowered the enzymatic K_is up to low nanomolar values **(Table 8.4)**. In particular the *n*-hexyl-disubstituted DTC derivative **6** had the lowest K_i ever reported for Can2 (K_i 0.75 nM). Among the remaining *N,N*-disubstituted derivatives **7** and **9–14**, compounds **10** and **14** have the better affinities (K_is

Table 8.3. hCAI, II, mtCA 1 and mtCA 3 inhibition data with dithiocarbamates 1, 3–28.

Compound	R^1	R^2	K_i (nM)			
			hCA I	*hCA II*	*mtCA 1*	*mtCA 3*
1	Et	Et	790	3100	615	431
3	Me	Me	699	6910	893	659
4	*n*-Pr	*n*-Pr	1838	55.5	74.8	80
5	*n*-Bu	*n*-Bu	43.1	50.9	81.7	72.8
6	*n*-Hex	*n*-Hex	48.0	51.3	95.4	51.7
7	$HOCH_2CH_2$	$HOCH_2CH_2$	9.2	4.0	7.5	6.0
8	*i*-Bu	*i*-Bu	0.97	0.95	86.2	43.0
9	$(CH_2)_5$		0.96	27.5	90.5	4.1
10	$(NC)(Ph)C(CH_2CH_2)_2$		48.4	40.8	93	61.2
11	$O[(CH_2CH_2)]_2$		0.88	0.95	0.94	0.91
12	Et	*n*-Bu	157	27.8	91.6	63.5
13	Me	Ph	39.6	21.5	25.2	46.8
14	Me	$PhCH_2$	69.9	25.4	72	62.5
15	H	*sec*-butyl	21.1	29.4	6.0	3.6
16	H	Ph	4.8	4.5	5.6	2.5
17	H	2-thiazolyl	3.9	4.6	89.4	9.5
18	H	$PhCH_2$	4.1	0.7	7.1	87.3
19	H	4-PyridylCH_2	3.5	16.6	5.4	5.7
20	H	Imidazol-1-yl-$(CH_2)_3$	8.6	24.7	5.3	8.7
21	H	$O[(CH_2CH_2)]_2N$	4.8	3.6	6.1	2.4
22	H	$O[(CH_2CH_2)]_2N(CH_2)_2$	31.8	36.3	7.1	2.8
23	H	$[(CH_2)_5N]CH_2CH_2$	4.5	20.3	9.1	8.8
24	H	$MeN[(CH_2CH_2)]_2N$	33.5	33.0	4.7	2.6
25	$NaS(S=C)N[(CH_2CH_2)]_2$		12.6	0.92	37.5	0.78
26	H	$KOOCCH_2$	13.1	325	7.7	8.0
27	(*S*)-$[CH_2CH_2CH_2CH(COONa)]$		2.5	17.3	7.1	6.4
28	H	$N[(CH_2CH_2)N]_3$	31.9	13.5	4.2	4.0
AAZ			250	12	481	104

Table 8.4. hCA II, Can2, CaNce103 and CgNce103 inhibition data with dithiocarbamates 1, 3–28.

Compound	R^1	R^2	K_i (nM)			
			hCA II	*Can2*	*CaNce103*	*CgNce103*
1	Et	Et	3100	802	950	874
3	Me	Me	6910	876	962	913
4	*n*-Pr	*n*-Pr	55.5	88.1	69.4	76.2
5	*n*-Bu	*n*-Bu	50.9	75.8	67.0	73.2
6	*n*-Hex	*n*-Hex	51.3	0.75	6.3	3.9
7	$HOCH_2CH_2$	$HOCH_2CH_2$	4.0	65.5	69.0	70.3
8	*i*-Bu	*i*-Bu	0.95	6.2	6.0	5.7
9	$(CH_2)_5$		0.96	64.5	62.1	72.9
10	$(NC)(Ph)C(CH_2CH_2)_2$		48.4	7.3	7.2	7.6
11	$O[(CH_2CH_2)]_2$		0.88	5.4	4.2	7.8
12	Et	*n*-Bu	27.8	7.6	5.7	7.1
13	Me	Ph	21.5	15.2	16.8	14.9
14	Me	$PhCH_2$	25.4	8.3	6.5	7.4
15	H	*sec*-butyl	29.4	71.3	50.8	88.2
16	H	Ph	4.5	4.8	6.4	7.8
17	H	2-thiazolyl	4.6	64.4	58.5	72.3
18	H	$PhCH_2$	0.7	21.7	37.3	34.5
19	H	4-PyridylCH_2	16.6	5.7	5.3	8.7
20	H	Imidazol-1-yl-$(CH_2)_3$	24.7	6.7	5.3	7.2
21	H	$O[(CH_2CH_2)]_2N$	3.6	5.6	6.6	9.2
22	H	$O[(CH_2CH_2)]_2N(CH_2)_2$	36.3	6.6	6.0	8.4
23	H	$[(CH_2)_5N]CH_2CH_2$	20.3	5.1	7.5	6.0
24	H	$MeN[(CH_2CH_2)]_2N$	33.0	7.1	7.5	8.4
25	$NaS(S=C)N[(CH_2CH_2)]_2$		12.6	6.3	7.4	7.7
26	H	$KOOCCH_2$	325	50.8	61.3	47.0
27	(*S*)-$[CH_2CH_2CH_2CH(COONa)]$		2.5	6.4	6.2	7.2
28	H	$N[(CH_2CH_2)N]_3$	13.5	60.5	466	85.9
AAZ			12	10.5	132	11

4.2–8.3 nM). Also the monosubstituted DTCs **16** and **19–24** possess very good inhibitory properties for all the fungal CAs (K_is 4.8–9.2 nM) **(Table 8.4)**. The only exceptions are derivatives **15**, **17** and **18** (K_is 21.7–88.2 nM). The bis derivative **25** reports good inhibition profile too (K_i 6.3–7.7 nM). Among the amino acid derivatives **26** and **27** it is interestingly to note they differ for an order of magnitude in inhibiting all the fungal CAs, with the latter been the more active **(Table 8.4)**. Finally the tris DTC **28** showed a slightly preference for the inhibition of the Can2 and CgNce103 isozymes over the CaNce103 with a selectivity of 7.7 and 5.4, respectively.

Conclusion

The DTCs are indeed a new potent class of CAIs. Their salt character provides them good solubility in aqueous media, thus allowing for a vast array of biomedical applications such as anti-glaucoma and anti-infectives (antibacterial, antifungal agents) or in agriculture.

The crystallographic experiments of several DTCs in adduct with hCA II revealed a new binding mode of these compounds to the enzyme, which although similar to that of the classical CAIs, the sulfonamides, is quite distinct. The DTCs are monodentate ligands of the Zn(II) ion from the enzyme active site, binding to it by means of a sulfur atom at a distance Zn-S of 2.3 Å.

In the series of approximately 30 DTCs investigated so far as CAIs, it has been observed that small structural modifications in the inhibitor scaffold lead to different location of the molecules within the enzyme active site, leading also to quite variable SAR for the various isoforms or enzyme classes (α- and β-CAs of mammalian, insect, fungal or bacterial origin). Such observations are in agreement with the reported kinetic profiles as small modifications in the DTC scaffolds account for sensible changes in the inhibition potency and selectivity over the CA isozymes tested so far. We estimate that in the future more classes and variants of DTCs will be investigated as CAIs and that all enzyme classes (apart from the α- and β-ones) will be included in such studies.

Financial & competing interests disclosure

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Summary.

- Dithiocarbamates (DTCs) are chemically referred as functional groups analogs to the carbamates, containing sulfur instead of oxygen atoms.
- The main feature of DTCs is the ability to form stable complexes with metal and alkali-metal ions.
- The rationale of this investigation was to explore whether organic compounds incorporating the trithiocarbonate moiety merged into organic scaffolds, such as the DTCs, might act as carbonic anhydrase inhibitors (CAIs).
- In any case both DTCs tested proved to be superior in terms of potency and lasting to the clinically used DRZ at the same concentration.
- The selective targeting of the β-CAs represents a very promising approach for the development of new anti-infectives and the validation of the DTCs as new CAIs indeed might provide such opportunity.
- Their salt character provides them good solubility in aqueous media, thus allowing for a vast array of biomedical applications such as glaucoma and anti-infectives or agriculture.
- The crystallographic experiments of DTC **10**, **11** and **14** in adduct with hCA II reveal a binding mode similar to the classical CAIs, as the sulfonamides, and also show that small structural modifications in the inhibitor determine dissimilar allocation of the molecule portions within the enzymatic cleft.
- Such observations are in agreement with the reported kinetic profiles as small modifications in the DTC organic scaffolds account for sensible changes in the inhibition potency and selectivity over the CA isozymes tested.

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Clemente Capasso obtained his MSc in biology *cum laude* at the University of Naples 'Federico II' (Italy). His research interest at the Italian National Research Council (CNR; Rome, Italy) from 1992 to date has been the study of the structure/function relationship of both native and recombinant proteins from terrestrial and marine organisms. In particular, he studied metallothioneins and aspartic proteinases in Echinoderms and Antarctic fish. This research activity has allowed him to acquire extensive knowledge of most protein purification procedures as well as kinetic, chemical–physical and structural characterization of enzymes. He also has experience with molecular biology techniques, such as nucleic acids isolation, cloning and expression of recombinant proteins and enzymes in *Escherichia coli*. Recently, his research activity has been focused on the investigation of enzymes involved in the hydration of CO_2 (carbonic anhydrases) in extremophilic microorganisms. The preliminary results of this study are helpful in developing new methodologies for the use of enzymatic systems in biotechnologies. All of these activities have led to more than 50 publications in peer-reviewed journals. He has a long and strong scientific background, developed over 15 years of experience acquired by intense training in Italian and international laboratories. He is the Associate Editor of *Journal of Enzyme Inhibition and Medicinal Chemistry*, and is on the Editorial Board of the journal *Expert Opinion on Therapeutic Patents*.

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Chapter 9

Protozoan, fungal and bacterial carbonic anhydrases targeting for obtaining anti-infectives

Clemente Capasso & Claudiu T Supuran

Carbonic anhydrases (CAs) play an important role for the life cycle of the microorganisms and the investigations of such enzymes in protozoa, fungi and bacteria domains certainly may reveal novel aspects of microbial virulence. The search for CA inhibitors with a novel mechanism of action is of pivotal importance for reducing or stopping microorganism virulence. The mechanism of inhibition of CA is well characterized and it is, therefore, easy to screen inhibitors. In this chapter we will review the current state of the art for inhibiting microbial CAs with the goal of developing antiprotozoal, antifungal or antibacterial agents exploiting a mechanism of action different from that of the clinically used drugs to which a considerable degree of drug resistance has been reported. Over the last several years, effective *in vivo* inhibition of parasite growth was evidenced in several organisms, such as *Trypanosoma cruzi, Plasmodium falciparum, Helicobacter pylori, Mycobacterium tuberculosis* and *Brucella suis*. As this field is still in its infancy, we predict that in the future many interesting anti-infectives will be designed considering parasite CAs as drug targets.

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Microorganism virulence is referred to as the degree of pathogenicity of a microbe that enables it to cause a disease through its ability to enter a host, evade host defenses, grow in the host environment, counteract host immune responses, assimilate iron or other nutrients from the host or sense environmental change [1–3]. As described in the Overview of carbonic anhydrase families/isoforms (**Chapter 1**) of the present book, carbonic anhydrases (CAs) are enzymes present in several pathogenic microorganisms belonging to protozoa (e.g., *Leishmania donovani chagasi*, *Plasmodium* spp. and *Trypanosoma cruzi*), fungi (e.g., *Malassezia globosa*, *Candida albicans*, *Candida glabrata and Cryptococcus neoformans*) and bacteria (e.g., *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Brucella suis*, *Vibrio cholerae* and *Porphyromonas gingivalis* among others). They catalyze a physiologically fundamental process, the reversible hydration of CO_2 to bicarbonate and protons.

CAs are metalloenzymes and exist as five distinct families, the α-, β-, γ-, δ- and ζ-CAs, in all organisms from the tree of life [1–3]. CAs are involved in crucial physiologic processes, (pH and CO_2 homeostasis, respiration and transport of CO_2/bicarbonate, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions, gluconeogenesis, lipogenesis and ureagenesis, bone resorption, calcification, tumorigenicity, photosynthesis, CO_2 fixation) [1–3]. The search of inhibitors with a novel mechanism of action is of pivotal importance for reducing or stopping microbial virulence, since it has been demonstrated that in many of them CAs are essential for their life cycle. The mechanism of inhibition of CA is well characterized and it is therefore easy to screen inhibitors and to examine designed inhibitors. Known α-CA inhibitors (CAIs) include various anions, imidazole, phenols, hydroxyurea, carboxylates, organic phosphates and phosphonates, and various sulfonamide derivatives ($R\text{-}SO_2NH_2$), which represent the main class of clinically used CAIs. Many of the mammalian enzymes are drug targets for antiglaucoma, antiepileptic or anticancer drugs, as well as diuretics. Some sulfonamides (and one sulfamate) have been in clinical use for a long time, mainly as antiglaucoma, antiepileptic and diuretic agents [1,3]. CAIs seem to have important applications, which virtually can

Aa Virulence: the degree of pathogenicity of a parasite that enables it to cause a disease through its ability to invade the host.

Protozoa: a subkingdom of the kingdom Protista, although in the classical system they were placed in the kingdom Animalia. Protozoa are single-celled organisms. They are found in most habitats and hunt other microbes as food. All higher animals are infected with one or more species of protozoa.

Fungus: these organisms are classified as a kingdom, Fungi, which is separate from plants, animals, protists and bacteria. Fungi include microorganisms such as yeasts and molds, as well as the more familiar mushrooms. Fungal cells have cell walls that contain chitin, while the cell walls of plants and some protists contain cellulose.

Bacteria: prokaryotes characterized by having the genetic material, or DNA, which is organized in a single circular chromosome located in the cytoplasm and not enclosed in a cellular compartment called the nucleus.

lead to new antibacterials, antifungals or antiprotozoal agents. Intriguingly, *V. cholerae* utilizes the CA system to accumulate bicarbonate, which is a potential inducer of virulence gene expression [4]. Taking in account these data, the investigations of CA in protozoa, fungi and bacteria domains certainly may reveal novel aspects of microbial virulence. In this chapter we will review the current state of the art for inhibiting CAs belonging to the protozoan, fungal and bacterial domains with the goal of developing antiprotozoal, antifungal or antibacterial agents possessing a different mechanism of action compared with the clinically used drugs to which a considerable degree of drug resistance has been reported.

Protozoan CAs

The malaria-provoking organism *Plasmodium falciparum*, encode for an α-CA named PfaCA. It was the first parasite CA that was shown to be a druggable target. *P. falciparum*, as demonstrated by Krungkrai's and our laboratories, encodes for several a-class CAs [5]. These enzymes show significant hydratase and esterase catalytic activities. All identified CAs are inhibited by primary sulfonamides, the main class of CAIs. Some benzenesulfonamide derivatives were effective *in vitro* as *P. falciparum* CAIs and also inhibited the *ex vivo* growth of the parasite efficiently [5]. CAIs show antimalarial activity because they inhibit the first step of pyrimidine nucleotide biosynthesis in the protozoan parasite, that is, the CA-mediated carbamoylphosphate biosynthetic pathway.

Trypanosoma cruzi, the causative agent of Chagas disease, encodes for an α-CA (TcCA) possessing high catalytic activity [6]. Aromatic/heterocyclic sulfonamides incorporating halogeno/methoxyphenacetamido tails inhibited TcCA with K_is in the range of 0.5–12.5 nM, being less effective against the human off-target isoforms hCA I and II. The aromatic sulfonamides were weak inhibitors (K_is of 192 nM–84 μM), whereas some heterocyclic compounds inhibited the enzyme with K_is in the range of 61.6–93.6 nM [6]. The thiols were the most potent *in vitro* inhibitors (K_is of 21.1–79.0 nM) and some of them also inhibited the epimastigotes growth of two *T. cruzi* strains *in vivo*.

On the other hand, a β-carbonic anhydrase was cloned and characterized from the protozoan, *L. donovani chagasi*, which causes visceral leishmaniasis [7]. The characterized CA, denominated LdcCA, had an effective catalytic activity for the CO_2 hydration reaction, with a k_{cat} of 9.35×10^5 s^{-1} and k_{cat}/K_M of 5.9×10^7 M^{-1} s^{-1} [7]. A large number of aromatic/heterocyclic sulfonamides and 5-mercapto-1,3,4-thiadiazoles were investigated as LdcCA inhibitors. The sulfonamides were medium potency weak inhibitors (K_is of 50.2 nM–9.25 μM), whereas some heterocyclic thiols inhibited the

enzyme with K_is in the range of 13.4–152 nM. Some of the investigated thiols efficiently inhibited the *in vitro* growth of *L. chagasi* and *L. amazonensis* promastigotes, impairing the flagellar pocket and movement of the parasites, and inducing autophagic vacuoles, which cause intracellular damages and parasite death. Thus, protozoan CA inhibition may be a valid strategy to control infection with protozoans provoking serious diseases, such as malaria and Chagas disease [7].

Fungal CAs

Fungal CAs play an important role in the CO_2-sensing of the fungal pathogens and in the regulation of sexual development. Physiological concentrations of CO_2/HCO_3^- induce filamentation in *C. albicans* and capsule biosynthesis in *C. glabrata* or *C. neoformans* through direct activation of the fungal adenylyl cyclase [8,9]. The link between cAMP signaling and CO_2/HCO_3^- sensing is conserved in fungi and revealed CO_2 sensing to be an important mediator of fungal metabolism and pathogenesis. Fungal CAs belong to β-CA class. The CAs named CaNce103 (*C. albicans* CA) or the analogous CgNce103 (*C. glabrata* CA) are essential for pathogenesis of these fungi in niches where the available CO_2 is limited (e.g., the skin), or essential for the growth of *C. neoformans* (CA called Can2) in its natural environment [8,9]. A β-carbonic anhydrase from the fungal pathogen *M. globosa* has been cloned, characterized and studied for its inhibition with sulfonamides [10]. This enzyme, designated with the name MgCA, has significant catalytic activity in the CO_2 hydration reaction and was inhibited by sulfonamides, sulfamates and sulfamides with K_is in the nanomolar to micromolar range [10]. Several sulfonamides have also been investigated for the inhibition of growth of *M. globosa*, *Malassezia dermatis*, *Malassezia pachydermatic* and *Malassezia furfur* in cultures [10]. A mouse model of dandruff showed that treatment with sulfonamides led to fragmented fungal hyphae, as for the treatment with ketoconazole, a clinically used antifungal agent. These data prompted the inventors to propose MgCA as a new antidandruff drug target.

The β-CAs from *Saccharomyces cerevisiae* (ScCA), *C. albicans* (CaNce103), *Candida glabrata* (CgNce103) and *Cryptococcus neoformans* (Can2) which are all encoded by the *Nce103* gene, were shown to be effective catalysts for CO_2 hydration to bicarbonate and protons [8,9,11]. For example, ScCA has a k_{cat} of 9.4×10^5 s^{-1}, and k_{cat}/K_M of 9.8×10^7 $M^{-1}s^{-1}$ [11]. Bromide, iodide and sulfamide, were the best anion inhibitors, with K_is of 8.7–10.8 μM. Benzenesulfonamides substituted in 2-, 4- and 3,4-positions with amino, alkyl, halogeno and hydroxyalkyl moieties had K_is in the range of 0.976–18.45 μM. Better inhibition (K_is in the range of 154–654 nM) was observed for

benzenesulfonamides incorporating aminoalkyl/carboxyalkyl moieties or halogenosulfanilamides [11]. All these enzymes have been investigated for their inhibition profiles with anions, sulfonamides, sulfamates and carboxylates, and many efficient inhibitors (and in some cases also selective ones) have been detected.

Bacterial CAs

CAs started to be investigated in detail recently 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 organism [12–14]. CAs are involved in the transport of CO_2 or HCO_3^-, in supplying CO_2 or HCO_3^- for the biosynthetic reactions (and thus metabolisms), in pH regulation and also in cyanate degradation (e.g., *E. coli*), as well as in the survival of intracellular pathogens within their host. In bacteria, genes encoding CAs from three classes, α-, β- and γ-CAs, were identified [12–15].

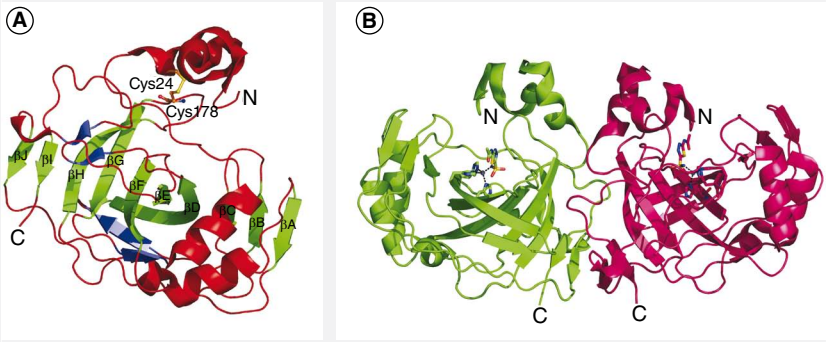
Enzymes from *Neisseria gonorrhoeae*, *Neisseria sicca*, *H. pylori* and *V. cholerae* encode CAs belonging to the α-class [4,16]. These enzymes, rather homologous to mammalian CAs, showed high CO_2 hydratase activity as well as esterase activity for the hydrolysis of p-NpA. *Vibrio* CA, for example, has a significant catalytic activity, and a large number of low nanomolar inhibitors, among which are methazolamide, acetazolamide, ethoxzolamide, dorzolamide, brinzolamide, benzolamide and indisulam (K_is values in the range 0.69–8.1 nM) [4]. Many inorganic anions and several small molecules were investigated as VchCA inhibitors, too. Inorganic anions such as cyanate, cyanide, hydrogen sulfide, hydrogen sulfite and trithiocarbonate were effective VchCA inhibitors with inhibition constants in the range of 33–88 µM. Other effective inhibitors were diethyldithiocarbamate, sulfamide, sulfamate, phenylboronic acid and phenylarsonic acid, with K_i of 7–43 µM. Halides (bromide, iodide), bicarbonate and carbonate were much less effective VchCA inhibitors, with K_is in the range of 4.64–28.0 mM. 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 GI tract, as bicarbonate is a virulence enhancer of this bacterium. As bicarbonate is a virulence factor of this bacterium and since ethoxzolamide was shown to inhibit the *in vivo* virulence, we proposed that VchCA may be a target for antibiotic development, exploiting a mechanism of action rarely considered until now [4]. Our laboratories cloned and purified novel bacterial α-CAs from thermophilic bacteria, belonging to the genus *Sulfurihydrogenibium*, living in hot springs all over the world, at temperatures up to 110°C [17,18]. The CAs of these bacteria (*Sulfurihydrogenibium yellowstonense* and

Sulfurihydrogenibium azorense) are probably involved in the CO_2 fixation and biosynthetic processes as for other bacteria, algae and plants. Such enzymes show greater stability at higher temperatures, and some of them are also highly active for the hydration of CO_2 to bicarbonate and protons. One of the discovered α-CA identified in the bacterial species *S. yellowstonense* and denominated SspCA retained its high catalytic activity for the CO_2 hydration reaction even after being heated at 80°C for several hours. [17,18]. The other new α-CA, named SazCA and identified by translated genome inspection in *S. azorense*, resulted to be the most catalytically effective CA ever investigated, and as far as we know, the second fastest enzyme after human SOD. SazCA, was a 2.33-times better catalyst for the CO_2 hydration reaction compared with hCA II, and is considered up to now the best catalyst in this family of enzymes, possessing a k_{cat} of 4.40×10^6 s^{-1} and a k_{cat}/K_M of 3.5×10^8 M^{-1} s^{-1} [18]. SazCA was less effective as an esterase with 4-nitrophenyl acetate as substrate, but was highly thermostable, surviving to incubation at 90–100°C for several hours, without any loss of the catalytic activity. SspCA and SazCA showed an inhibition profile very similar to that of the predominant human cytosolic isoform hCA II. Some clinically used drugs, such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, topiramate, celecoxib and sulthiame were low nanomolar SspCA/SazCA/hCA II inhibitors (K_is in the range of 4.5–12.3 nM) whereas simple aromatic/heterocyclic sulfonamides were less effective, micromolar inhibitors [17,18]. As this highly catalytically active and thermostable enzyme may show biotechnological applications, its inhibition studies may be relevant for designing on/off systems to control enzyme activity and for new drug design.

X-ray crystal structures of some of these CAs were also determined. For example, one of the last bacterial CAs crystallized by our group is the thermostable SspCA. As described above, this enzyme is an efficient catalyst for the hydration of CO_2 and presents exceptional thermostability. Indeed, SspCA retains a high catalytic activity even after being heated to 80°C for several hours. The crystallographic structure of this α-carbonic anhydrase suggests that increased structural compactness, together with an increased number of charged residues on the protein surface and a greater number of ionic networks, seem to be the key factors involved in the higher thermostability of this enzyme with respect to its mesophilic homologues. These findings are of extreme importance, since they provide a structural basis for the understanding of the mechanisms responsible for thermal stability in the α-CA family for the first time **(Figure 9.1)**.

CAs belonging to the β-class were cloned, purified and characterized from *E. coli*, *Salmonella enterica*, *H. pylori*, *Haemophilus influenzae* and from other

Figure 9.1. View of the folding of the extremo-carbonic anhydrase from *Sulfurihydrogenibium yellowstonense*.



(A) 3D fold of the a-carbonic anhydrase from *Sulfurihydrogenibium yellowstonense* (SspCA) as determined by x-ray crystallography. **(B)** SspCA is a homodimer (one dimer is shown in green, the second one in magenta) [17].

pathogens. The β-class displayed excellent activity for the CO_2 hydration but lack esterase activity, similar to the β-class enzymes isolated from plants. A β-CA from the thermophilic bacterium *Methanobacterium thermoautotrophicum* has also been identified. Interestingly, the enzyme from *M. thermoautotrophicum* was stable at temperatures up to 75°C. The bacterial pathogen *B. suis* encodes two CAs belonging to the β-class, bsCA1 and bsCA2, which are crucial for its life cycle [19]. Sulfonamides, strong inhibitors of these enzymes, were shown to block the growth of the pathogen *in vitro*. The enzyme bsCA1 was inhibited in the low micromolar range by sulfamide, sulfamic acid, phenylboronic/arsonic acid, and in the submillimolar range by diethyldithiocarbamate. Isoform bsCA2 generally showed a stronger inhibition with most of these anions, with several low micromolar and many submillimolar inhibitors detected. Micromolar inhibition against bsCA2 was observed for sulfamide and sulfamic acid, whereas diethyldithiocarbamate, perruthenate, pyrovanadate, tellurate and phenylarsonic acid showed inhibition constants in the range of 0.29–1.52 mM. These inhibitors may be used as leads for developing anti-*Brucella* agents with a diverse mechanism of action compared with clinically used antibiotics.

CA belonging to the γ-class are present in archaea, bacteria and plants but, except the *Methanosarcina thermophila* enzymes, CAM and CAMH, they have been poorly characterized so far. Our laboratories, cloned, purified and characterized a γ-CA, named (PgiCA), from the oral cavity pathogenic bacterium *P. gingivalis*, the main causative agent of periodontitis [20]. PgiCA showed a good catalytic activity for the CO_2 hydration reaction, comparable

with that of the human (h) isoform hCA I. Inorganic anions such as thiocyanate, cyanide, azide, hydrogen sulfide, sulfamate and trithiocarbonate were effective PgiCA inhibitors with inhibition constants in the range of 41–97 μM [20]. Other effective inhibitors were diethyldithiocarbamate, sulfamide, and phenylboronic acid, with K_is of 4.0–9.8 μM [20]. The role of this enzyme as a possible virulence factor of *P. gingivalis* is poorly understood at the moment but its good catalytic activity and the possibility to be inhibited by a large number of compounds may lead to interesting developments in the field.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Summary.

- Microorganism virulence is referred as to the degree of pathogenicity of a microbe that enables it to cause a disease through its ability to enter a host, evade host defenses, grow in the host environment, counteract host immune responses, assimilate iron or other nutrients from the host or sense environmental change.
- Carbonic anhydrases (CAs) are involved in crucial physiologic processes and some α-, β- and possibly γ-CAs are essential for the life cycle of some pathogenic bacteria, fungi and protozoa.
- The search for inhibitors with a novel mechanism of action is of pivotal importance for reducing or stopping the microorganism virulence.
- CA inhibitors are clinically used as antiglaucoma, anticonvulsant, antiobesity, and anticancer agents/diagnostic tools, but they are emerging as anti-infectives, that is, antiprotozoan, antifungal and antibacterial agents with a novel mechanism of action.

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Giuseppina De Simone graduated in chemistry at the University of Naples Federico II (Italy) in 1993. She worked from August 1994 to January 1995 at the Drug Design Group 'Sandoz Pharma AG' in Basel, Switzerland, and from September 1995 to February 1996 at the University of Pavia. From November 1996 to January 1997, she was visiting scientist at the Max Plank Institute, Hamburg, Germany. In 1998, she received her PhD at the University of Naples. Since 2003, she has been Senior Researcher at the Institute of Biostructures and Bioimaging (IBB) of the Italian National Research Council. Her main research interests include x-ray crystallography of macromolecules, structure-based drug design, carbonic anhydrases, serine proteases, esterases/lipases, peptide nucleic acids. She has published more than 80 papers in these fields.

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Chapter 10

Amine, amino acid and oligopeptide carbonic anhydrase activators

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The rate determining step in the carbonic anhydrase (CA; EC 4.2.1.1) catalytic cycle is a proton transfer reaction from the water molecule coordinated to the metal ion to the reaction medium, with formation of the catalytically active, metal hydroxide species of the enzyme. This process is assisted by amino acid residues from the active site, which act as proton shuttling residues. A His is the most common such residue (His64, hCA I numbering; h = human isoform) in the α-CAs, whereas for other enzyme classes the proton shuttling residue is less clear. Compounds able to participate in proton transfer processes, such as amines, amino acids and oligopeptides, may assist this proton transfer, enhance the activity of the enzyme and are denominated CA activators (CAAs). X-ray crystal structures are available for several such compounds in adduct with hCA I and II (the main human isoforms), but all the mammalian CAs have been investigated for their activation with amines and amino acids, together with several β- and γ-CAs from different organisms (corals,

fungi, bacteria). CAAs, similar to the CA inhibitors (CAIs) may have pharmacologic applications for the management of aging or Alzheimer's disease, conditions in which a reduction of the CA activity in the brain has been reported.

CA activation mechanism

The catalytic mechanism of the carbonic anhydrases (CAs) was discussed in Chapter 1 of this book and will be not detailed here. According to it, the physiological CO_2 hydration reaction involves the nucleophilic attack of a metal-bound hydroxide species of the enzyme on the substrate CO_2 optimally positioned and orientated in a hydrophobic binding pocket within the active site cavity, not far away from the metal ion [1–4]. The next step of the cycle involves the replacement of the formed bicarbonate by a water molecule, with generation of the acidic, catalytically inactive form of the enzyme $EM^{2+}–OH_2$ **(Equation 10.1)**, where M can be Zn, Cd or Fe(II) [4–6]. For regenerating the catalytically active form with hydroxide coordinated to the metal ion, a proton transfer reaction occurs from the water bound to M(II) within the enzyme active site, to the external medium [7]. In many α-CAs (the most investigated enzymes belonging to this superfamily) this step **(Equation 10.2)** is assisted by an active site amino acid residue (e.g., His 64), placed in the middle of the cavity or at the entrance of the active site [1,6,7]. The imidazole moiety of this His residue acts as an 'internal' buffer, being able to be protonated/deprotonated. This step is also rate-determining for the whole catalytic cycle in many CAs and the shuttling effects of His64 also explains the very high efficiency of some CA isoforms (e.g., hCA II, hCA IX) as catalysts, with maximal turnover numbers of approximately 1.6×10^6 s^{-1} [2,6,7].

$$EM^{2+}–OH^- + CO_2 \Leftrightarrow EM^{2+}–HCO_3^- \overset{H_2O}{\Leftrightarrow} EM^{2+}–OH_2 + HCO_3^- \quad \textbf{(Equation 10.1)}$$

$$EM^{2+}–OH_2 \Leftrightarrow EM^{2+}–HO^- + H^+ \quad \textbf{(Equation 10.2)}$$

In the presence of activators, we proposed and later demonstrated by means of kinetic and x-ray crystallographic studies [7–15], that an enzyme–activator complex is formed. In such a complex, the activator participates in proton transfer processes through moieties able to accept or donate H^+ ions [7–9]. The enhanced catalytic rate is thus due to the fact that intramolecular reactions are more rapid than intermolecular ones. Thus, **Equation 10.2** becomes **Equation 10.3** in the presence of activators (A):

Aa CO_2 hydration: the slow reaction between CO_2 and water, at neutral pH, leads to bicarbonate and protons, and its velocity is highly enhanced by the carbonic anhydrases. It is the physiologic reaction in which these enzymes are involved.

Enzyme activator: compound interfering with the catalytic mechanism of an enzyme, by enhancing its activity and favoring a crucial step of the catalytic cycle.

$$EZn^{2+}–OH_2 + A \Leftrightarrow [EZn^{2+}–OH_2 \text{ - } A] \Leftrightarrow [EZn^{2+}–HO^- \text{- } AH^+] \Leftrightarrow EZn^{2+}–OH^- + AH^+ \quad \textbf{(10.3)}$$

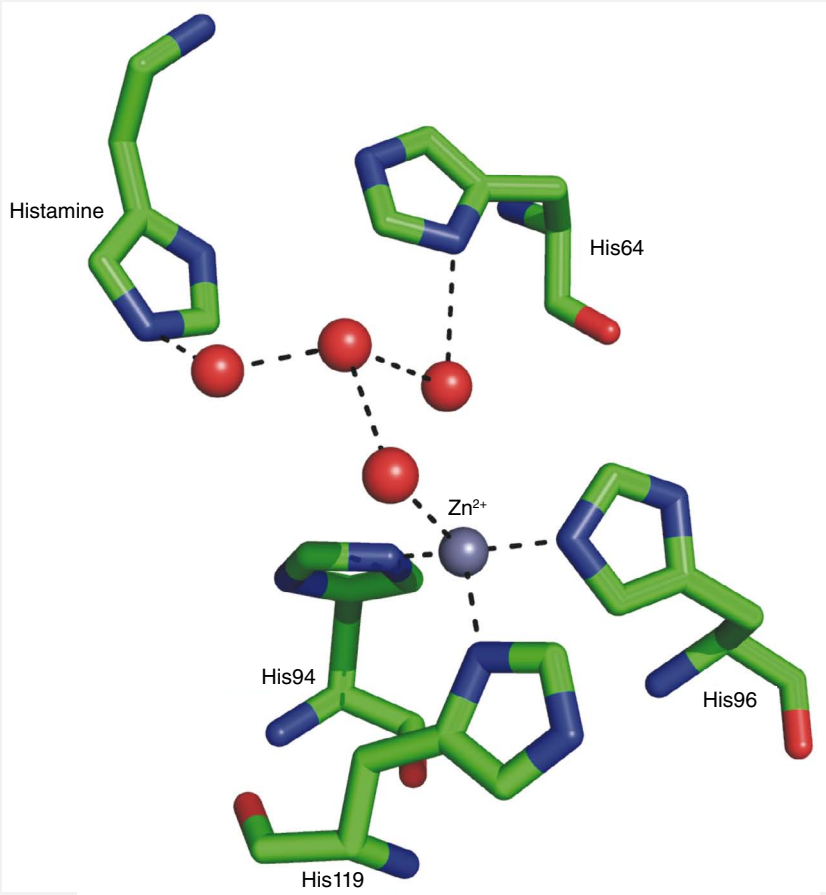
Enzyme–activator complexes

The above activation mechanism is probably valid for all CA classes, as diverse metal hydroxide species (L_3-M^{2+}-OH^-) of the enzyme are the catalytically active nucleophiles in all of them, irrespective of the nature of the metal ion. As mentioned above, Zn(II), Cd(II), Co(II) or Fe(II) ions were found within the active site of different CAs belonging to the five genetic families, the α-, β-, γ-, δ- and ζ-CAs [1–6]. It has also been demonstrated that a relay of water molecules and polar His residues are involved in the proton transfer processes (at least for the α-CAs), strongly facilitating the release of the protons generated by the hydration of CO_2 and thus enhancing the rate-determining step of the catalytic cycle [7–15]. For hCA II, one of the most investigated such enzymes, His64 is the main proton-shuttling residue, but it is further assisted in the proton transfer processes by a cluster of histidine residues prolonging from the middle of the cavity to its entrance and till the surface of the enzyme around the edge of the cavity (His4, His3, His10 and His15 [7]).

Amine activators of the human CA isoforms

The first adduct of a CA activator studied by this technique was the histamine–hCA II complex, characterized at a resolution of 1.95 Å and reported by our group in 1997 [7], the structure being available in the RCSB Protein Data Bank (file code 1AVN). The histamine molecule binds at the entrance of the active site cavity, being anchored to several amino acid side-chains and water molecules by means of hydrogen bonds **(Figure 10.1)**. The evidenced hydrogen bonds involved only the nitrogen atoms of the imidazole moiety, as the aliphatic NH_2 group did not experience contacts with the enzyme. The aminoethyl fragment of histamine was found orientated towards the exit of the active site cavity, towards the solvent accessible areas [7]. The two imidazolic nitrogen atoms were observed to be engaged in hydrogen bonds with the side-chains of Asn62 and Gln92, as well as to a water molecule, Wat152. The binding of histamine to hCA II displaced three water molecules from the active site cavity, being accompanied by a substantial rearrangement of the water structure in the enzyme active site. The orientation of the His 64 side chain, which was well defined and orientated towards the inside of the cavity (the 'in' conformation), pointing towards the metal site, was different with respect to its orientation in the noncomplexed (i.e., activated) enzyme. A network of hydrogen bonds linking the zinc coordinated-water to histamine, through two waters present from the active site cavity, numbered as Wat129 and Wat152, has been evidenced in the hCA II–histamine adduct. This network contributes to the release of

Figure 10.1. The human carbonic anhydrase II–histamine adduct.



The Zn(II) ion, its ligands, His64 and the water molecules (red spheres) involved in the carbonic anhydrase activation mechanism are shown [7].

protons from the active site to the environment. A second alternative pathway exists through Wat129 and Wat130, reaching then the residue His64, which, as mentioned earlier, is the natural proton shuttle residue of the enzyme. The activator basically allows a supplementary proton shuttling from the zinc-bound water molecule to the reaction medium, favoring thus the formation of the nucleophilic species of the enzyme [7]. All activators investigated in detail by means of x-ray crystallography showed the same pattern of binding and probably share a common activation mechanism with histamine [7,12–15].

Considering the aforementioned x-ray crystal structure, which evidenced that the imidazole ring of histamine **1** is mainly responsible for the shuttling of protons in the CA activation cycle, and that the primary amine group of **1** is orientated towards the exit of the active site cavity, considerable efforts to obtain other classes of CA activators (CAAs) starting from histamine concentrated on derivatization of this amine moiety. Indeed, as shown in **Figure 10.2**, the primary amine group of histamine was converted to sulfonamide (**2**), arylsulfonylureido (**3**), carboxamide (**4**), urea, thiourea or guanidine (**5**) moieties, or derivatized by using polyamino–polycarboxylate moieties (such as the EDTA in **6** or DTPA in 7 derivatives), with bifunctional such compounds being obtained **(Figure 10.2)** [8–11]. Many of these derivatives were highly effective, low nanomolar activators of the human (h) or bovine (b) isoforms hCA I, II and bCA IV, having a higher affinity for these enzymes compared with the lead compound histamine [8–11].

Again considering histamine **1** as lead compound, other amine CAAs were obtained by replacing the imidazole ring of histamine by other five- or six-membered ring systems, such as pyridinium (**8**), 1,3,4-thiadiazole (**9** and **10**), as well as bis-/tris-azoles (imidazoles or pyrazoles variously substituted) of types **11–13** **(Figure 10.2)** [8–11,15]. These compounds were investigated so far only as hCA I and II activators, showing generally a good activity. As histamine, many of these amines were better hCA I than hCA II activators, probably due to the fact that His64 has not a very precise role as proton-shuttling residue in hCA I, whereas in hCA II its role for the proton transfer is very well defined [7,12–15].

In **Figure 10.4**, some other amines structurally related (in some cases) to histamine **1**, which have been investigated as CAAs of all 15 mammalian isoforms hCA I – mCA XV (m = murine isoform) are shown [8–11,15].

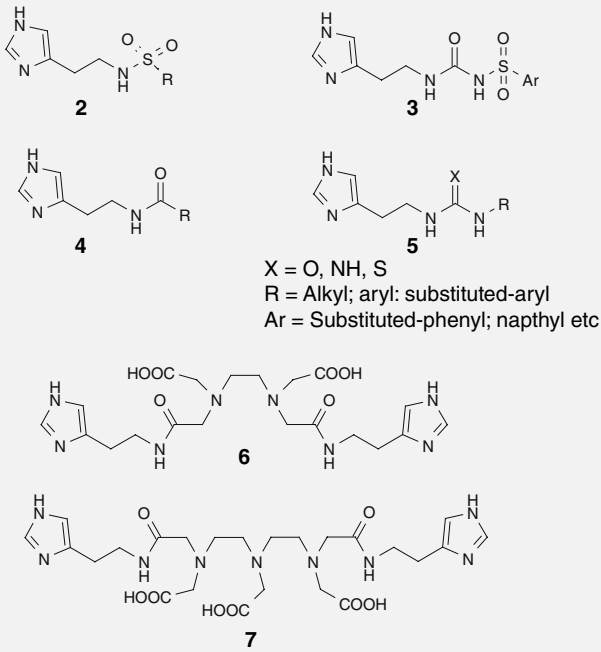
They include dopamine **14**, serotonin **15**, the pyridyl-alkyl amines **16** and **17**, the heterocyclic compounds **18** and **19**, as well as L-adrenaline **20**. Many of them play important physiological functions as autacoids [8–11,15].

Amino acid activators of the human CA isoforms

The amino acids that have been investigated in detail as CAAs of all mammalian isoforms hCA I–mCA XV, are shown in **Figure 10.5**, and they include most of the common, aromatic/heterocyclic amino acids, such as His, Phe, DOPA, Trp, Tyr and some of their derivatives, such as 4-amino-phenylalanine (**31**). Both the L- and D-enantioners of most of them have been included in such studies [12–20].

It should be noted that each different isoform investigated so far had a diverse affinity for the various amine or amino acid activator. Furthermore,

Figure 10.2. Histamine derivatives synthesized as carbonic anhydrase activators by derivatization of the primary amine moiety.



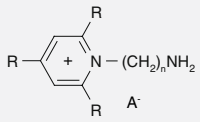
Data taken from [8–10].

there were always notable differences of activity between structurally related derivatives and even enantiomers of the same compound. For example, CA XV, the last mammalian isofom discovered, was strongly activated by some amino acids, such as D-Phe, L-/D-DOPA, D-Trp, L-Tyr, and amines, such as dopamine, serotonin, L-adrenaline and 4-(2-aminoethyl)-morpholine), which showed activation constants in the range of 4.0–9.5 µM. Other compounds, such as L-/D-His, L-Phe, histamine and several other heterocyclic amines showed less efficient activation of CA XV, with activation constants K_As in the range of 11.6–33.4 µM [15].

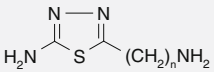
These diverse profiles of activation may in fact easily be explained by considering the x-ray crystal structures of L-/D-Phe or L-/D-His bound to hCA II or hCA I, reported by our group **(Figure 10.6)** [12,13,15].

The x-ray crystallographic structure have also been reported for hCA II complexed to L- and D-His as amino acid activators. The activators were found at the entrance of the active-site cavity **(Figure 10.6)**, participating

Figure 10.3. Amine carbonic anhydrase activators 8–13 obtained by replacing the imidazole ring of histamine 1 by other five- or six-membered ring systems.

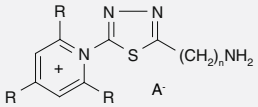


8

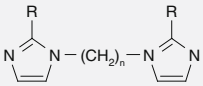


9

R = alkyl, aryl; n = 2, 3



10



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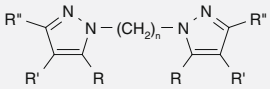
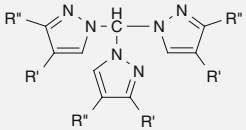


12



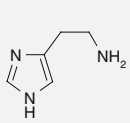
13

n = 1, 2; R, R', R" = H, Me, Et

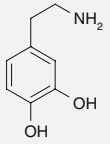
Data taken from [8–11,15].

in an intricate network of interactions with amino acid residues and water molecules, as for the histamine adduct discussed above. This may explain their different potency (against hCA II) as well as the diverse interaction patterns with various other CA isozymes of these two amino acid activators [12,15]. For the hCA II–L-His adduct, His64, Asn67, and Gln92 were the residues interacting with the imidazole moiety of the activator. Three water molecules connected the activator to the zinc-bound water in this complex. For the D-His adduct, the residues involved in recognition of the activator

Figure 10.4. Amines 1 and 14–20 investigated as carbonic anhydrase activators of all mammalian isoforms.



1



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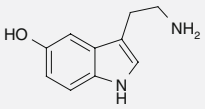
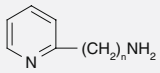
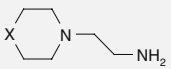


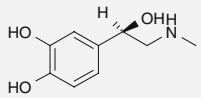
15



16: n = 1
17: n = 2



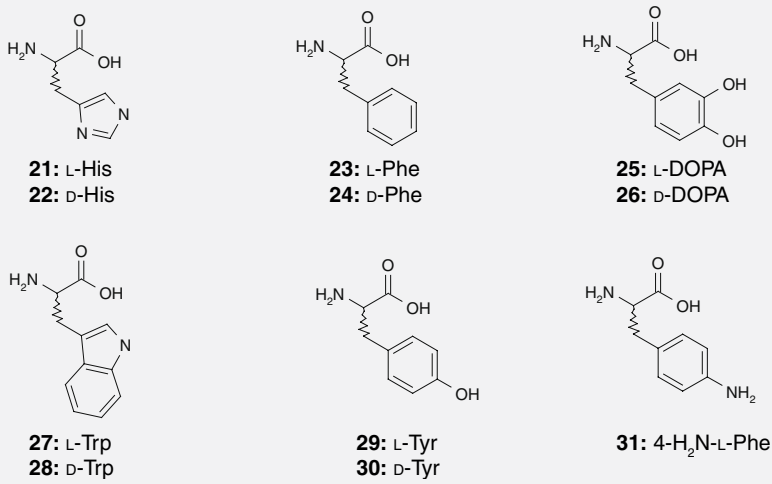
18: X = NH
19: X = O



20

Data taken from [8–11,15].

Figure 10.5. Amino acids investigated as carbonic anhydrase activators against all mammalian carbonic anhydrase isoforms.



Data taken from [12–20].

were Trp5, His64, and Pro201, whereas two water molecules connected the zinc-bound water to the activator. For both complexes, an extended network of hydrogen bonds contributed to the release of the proton from the zinc-coordinated water to the reaction medium as discussed above for the histamine adduct [5,12,13].

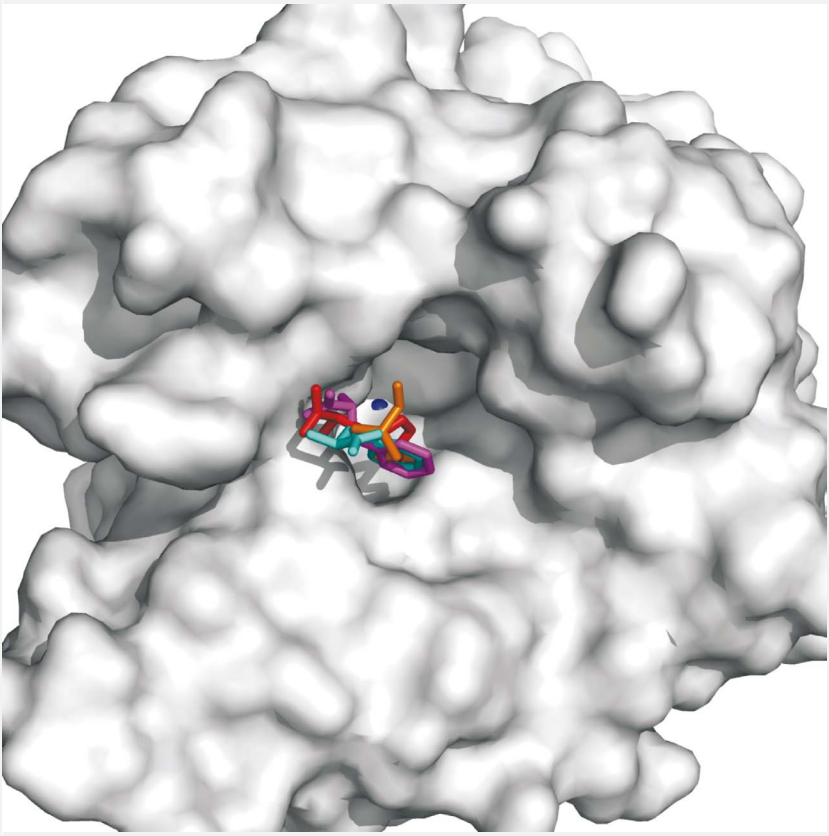
The x-ray crystal structures were also reported for the adducts of hCA II with L- and D-Phe [13]. It was observed, as for the L-/D-His adducts mentioned earlier, that the two stereoisomeric amino acids acting as activators bind differently to the enzyme. In the hCA II-L-Phe and hCA II-D-Phe complexes, the pattern of hydrogen bonds and hydrophobic contacts with amino acid residues, present within the activator binding site, were completely different compared with those present in the hCA II – histamine, L-His or D-His adducts**.** Thus, for the hCA II-L-Phe adduct, the activator molecule was anchored by its amino group by means of two hydrogen bonds with the indole nitrogen of Trp5, and with one imidazole nitrogens of His64 [13]. Some of these residues are part of the activator binding pocket, and they were shown to be involved in the binding of the other activators investigated earlier. For the hCA II-D-Phe adduct, the same four amino acid residues interacted with the activator but in a different manner compared with the hCA II-L-Phe adduct. The NH_2 group of the activator participated in two hydrogen bonds with the carbonyl oxygen of Pro201

and the OH moiety of Thr200, whereas the carboxylate moiety of D-Phe made a hydrogen bond with the indole NH of Trp5 and a contact with a nitrogen atom of His64 **(Figure 10.6)** [13].

Oligopeptide activators of the human CA isoforms

Carnosine (β-Ala-His) was among the first oligopepetides to be investigated as CAAs [10]. This molecule incoporates the imidazole moiety from a His residue, which is known to be able to participate in the proton shuttling processes and thus possess CA activation effects. We reported a novel class of tight binding CAAs, which were designed by using carnosine as the

Figure 10.6. Structural superposition of L-Phe (cyan), D-Phe (magenta), L-His (orange), D-His (red) when bound to human carbonic anhydrase II.

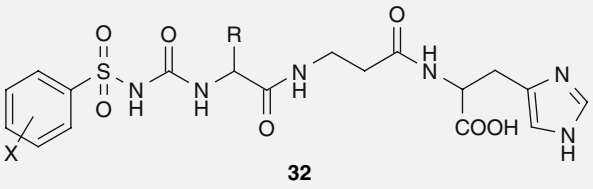


The enzyme is shown as surface representation (in gray). The catalytic zinc ion is shown as a blue sphere, at the bottom of the active site cavity.

starting material. Many such derivatives were synthesized by reaction of appropriately derivatized b-alanines with imidazole/carboxy-protected histidine. Coupling of 4-fluorophenylsulfonylureido amino acids (fpu-AA) or 2-toluene-sulfonylureido amino acids (ots-AA) with β-Ala leading to compounds of type **32** **(Figure 10.7)** lead to the derivatized β-alanines. Structurally related dipeptides with the general formula fpu/ots-AA1-AA2 (AA, AA1 and AA2 represent amino acyl moieties) were also prepared by a similar strategy, being used thereafter for obtaining activators incorporating tetrapeptide scaffolds. Many such tri-/tetra-peptides proved to be efficient *in vitro* activators of three CA isozymes, hCA I, hCA II and bCA IV. Affinities in the 1–20 nM range were observed against hCA I and bCA IV, and of 10–40 nM against hCA II, respectively. Some of these activators strongly enhanced cytosolic red cell CA activity after incubation with human erythrocytes, offering thus the possibility to develop drugs/diagnostic tools for the management of CA deficiency syndromes, for the pharmacological enhancement of synaptic efficacy, spatial learning and memory. This may constitute a novel approach for the management of Alzheimer's disease and other conditions in need of achieving memory therapy.

In another study, di-/tri- and tetra-peptides incorporating the sequence DADD, which is present in the C-terminal region of the bicarbonate/chloride anion exchanger AE1, were prepared by an original strategy and investigated as hCA I, II and IV activators [11]. It was in this way that demonstrated that these oligopeptides strongly activate isozyme hCA II, whereas they acted as more inefficient activators of isozymes hCA I and IV. This discovery suggested that in the proposed metabolon between hCA II and the anion exchanger AE1, a fragment of the exchanger AE1 has a role as a CA activator, in addition to being involved in the bicarbonate transporter process. The explanation why these acidic oligopeptides (incorporating several aspartic acid moieties) efficiently bind to hCA II but not to other isoforms,

Figure 10.7. Dipeptidyl carbonic anhydrase activator 32 obtained from carnosine (β-Ala-His) as lead molecule.



32

Data taken from [10].

such as CA I and IV, was explained based on the fact that the amino terminal part of hCA II (which presumably interacts with the anion exchanger), is rich in His residues (a His cluster of 5 such residues was observed, as discussed above), which may interact with the carboxylate moieties from the DADD tetrapeptide [11].

Activation studies of α-CAs other than the vertebrate ones & of β- & γ-CAs

Several other CAs have been investigated for their activation. Among them, two α-CAs from the coral *Stylophora pistillata*, named STPCA1 and STPCA2 [16]. STPCA, for example, is a secreted isoform and, owing to its specific secretion by the calicoblastic calcifying ectoderm, it was proposed to play a direct role in biomineralization processes. Many amine and amino acids were shown to efficiently activate it. For example D-DOPA was found to be the best coral enzyme activator, with a K_A of 0.18 μM [16].

Apart the α-CAs, activation of other classes of CAs has been recently investigated, mainly in our laboratories [17]. The archaeal β-class (Cab) and γ-class (Cam) CAs from two thermophilic Archaea (Cab from *Methanobacterium thermoautotrophicum*, and Cam from *Methanosarcina thermophila*) were investigated with a series of natural and non-natural amino acids as well as aromatic/heterocyclic amines [17]. The three enzymes had an activation profile with natural, L- and D- amino acids very different of those of other CAs, such as for example hCA I, II and III. D-Phe and L-Tyr were effective as Cab activators, with activation constants in the range of 10.3–10.5 μM. 2-Pyridylmethylamine and 1-(2-aminoethyl)-piperazine effectively activated Zn-Cam (K_As of 10.1–11.4 μM), whereas serotonin, L-adrenaline and 2-pyridylmethylamine were the best Co-Cam activators, possessing activation constants in the range of 0.97–8.9 μM against the archaeal enzyme. It seems that the activation mechanisms of the α-, β-, and γ-class CAs are similar. However, the activation profiles with amines/amino acids of different CAs differed dramatically between these diverse enzymes [17].

The β-CAs encoded by the Nce103 genes of *Candida albicans* and *Cryptococcus neoformans* play crucial roles in the life cycle of these pathogens, among which CO_2 sensing, capsule biosynthesis regulation, filamentation and adaptation of the fungus/yeast to various pH and CO_2 conditions in niches where they fungi [18,19]. Activation studies of these enzymes, CaNce103 and Can2, with amines and amino acids were recently reported. Amino acids such as L-/D-His, L-D-Trp, L-Tyr effectively activated CaNce103, the *C. albicans* enzyme, with activation constants in the range of 19.5–46 μM. Some amines, including histamine, dopamine, 2-aminoethyl-piperazine and

L-adrenaline, were even better CaNce103 activators, with K_As in the range of 13.2–18.5 μM. The best activators of this enzyme were L- and D-DOPA. The enzyme present in the second fungal pathogen *C. neoformans*, Can2, showed lower sensibility activators belonging to the amino acid and amine classes, with activation constants in the range of 28.7–47.2 μM. The best Can2 activator was L-Trp [18,19]. The same series of activators was also investigated for their interaction with the similar enzyme from another fungal pathogen, *Candida glabrata* [18].

A final mention should be made regarding the design of CAAs of the nanoparticle type [20]. New types of CAAs were obtained, which incorporated lipoic acid moieties. They were attached to amines or amino acids of the types discussed here, by formation of carboxamide functionalities. The lipoic acid conjugates of histamine, L-histidine methyl ester and L-carnosine methyl ester were obtained. Subsequently these activators were attached to gold nanoparticles (NPs), by reaction with gold(III) salts in the presence of sodium borohydride. The CAA coated NPs were highly effective, nanomolar activators of CA I, II, IV, VA, VII and XIV, with activation constants in the range of 1–9 nM. The NPs were also effective CA activators *ex vivo*, in whole blood experiments, leading to an increase of 200–280% of the enzyme activity. NPs loaded with CAAs may lead to biomedical applications for conditions in which the CA activity is diminished, as shown above, that is, for the management of aging, Alzheimer's disease or the CA deficiency syndrome [20].

Summary

- Carbonic anhydrases (CAs) are metalloenzymes and exist as five distinct families, the α-, β-, γ-, δ- and ζ-CAs, in all organisms from the tree of life.
- They catalyze a physiologically fundamental process, the reversible hydration of CO_2 to bicarbonate and protons with high efficacy, and the rate-determining step is a proton transfer reaction.
- Compounds able to participate in proton-shuttling between the active site of the enzyme and the reaction medium are termed CA activators (CAAs).
- The mechanism of action of the CAAs is understood in detail, at the molecular level, through kinetic and x-ray crystallographic experiments. All CAAs facilitate the proton-shuttling, rate-determining step of the CA catalytic cycle.
- The most important classes of CAAs are the amines, amino acids and oligopeptides.
- The structure-activity relationship of most classes of CAAs are understood in detail for all the mammalian CA isoforms (CA I–XV) and many other such enzymes, belonging to the α-class (e.g., from corals), the β-, and γ-CA families, from bacteria, Archaea or fungal pathogens.
- CAAs may have pharmacologic applications in the treatment of ageing or Alzheimer's diseases, conditions in which a diminished CA activity in the brain was observed.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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About the Authors

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Clemente Capasso obtained his MSc in biology *cum laude* at the University of Naples 'Federico II' (Italy). His research interest at the Italian National Research Council (CNR; Rome, Italy) from 1992 to date has been the study of the structure/function relationship of both native and recombinant proteins from terrestrial and marine organisms. In particular, he studied metallothioneins and aspartic proteinases in Echinoderms and Antarctic fish. This research activity has allowed him to acquire extensive knowledge of most protein purification procedures as well as kinetic, chemical–physical and structural characterization of enzymes. He also has experience with molecular biology techniques, such as nucleic acids isolation, cloning and expression of recombinant proteins and enzymes in *Escherichia coli*. Recently, his research activity has been focused on the investigation of enzymes involved in the hydration of CO_2 (carbonic anhydrases) in extremophilic microorganisms. The preliminary results of this study are helpful in developing new methodologies for the use of enzymatic systems in biotechnologies. All of these activities have led to more than 50 publications in peer-reviewed journals. He has a long and strong scientific background, developed over 15 years of experience acquired by intense training in Italian and international laboratories. He is the Associate Editor of *Journal of Enzyme Inhibition and Medicinal Chemistry*, and is on the Editorial Board of the journal *Expert Opinion on Therapeutic Patents*.

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Claudiu T Supuran received his BSc in chemistry from the Polytechnic University of Bucharest (Romania) in 1987, and PhD in chemistry from the same university in 1991. In 1990, he became Assistant and then Associate Professor of Chemistry at the University of Bucharest. In 1995, he moved to the University of Florence (Italy), where he is currently a Professor. His main research interests include medicinal chemistry, design of enzyme inhibitors and activators, heterocyclic chemistry, chemistry of sulfonamides, sulfamates and sulfamides, biologically active organoelement derivatives, QSAR studies, x-ray crystallography of metalloenzymes, metal complexes with biologically active ligands (metal-based drugs), carbonic anhydrases, cyclooxygenases, serine proteases, matrix metalloproteinases, bacterial proteases and amino acid derivatives, among others. He has published two books and more than 820 papers in these fields. He is the Editor-in-Chief of *Journal of Enzyme Inhibition and Medicinal Chemistry*, and of *Expert Opinion on Therapeutic Patents*.

Chapter 11

Targeting carbonic anhydrases in biotechnology

Clemente Capasso & Claudiu T Supuran

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes involved in many physiologic processes, such as respiration and CO_2 transport photosynthesis, as well as metabolism of xenobiotics (e.g., cyanate in *Escherichia coli*). CAs catalyze a simple but physiologically crucial reaction in all life kingdoms, CO_2 hydration to bicarbonate and protons: $CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+$. Here, we will discuss the current state of the art for utilizing CAs from different sources in biomedical and biotechnological applications, such as in the achievement of an artificial respiration system, selective biosensors for metal ions, and in the carbon capture and sequestration process (CCS).

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Carbonic anhydrase features

Carbonic anhydrases (CAs; EC 4.2.1.1) are metalloenzymes that catalyze a simple but physiologically crucial reaction in all life kingdoms, CO_2 hydration to bicarbonate and protons: $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$ [1–3]. Various classes (α, β, γ, δ and ζ) of CA were described, all of them being metalloenzymes that contain Zn(II), Cd(II) or Fe(II) ions at their active sites. Bacteria encode for enzymes belonging to the α-, β-, and γ-classes, fungi α- and β-CAs, whereas in protozoa only α-type CAs were reported so far. Mammalian and bacterial α-CAs are among the most active enzymes ever described with a turnover higher than 10^6 molecules of CO_2 per second [4–7]. They contain zinc ion (Zn^{2+}) in their active site, coordinated by three histidine residues and a water molecule/hydroxide ion (in the α-, γ- and δ-classes) or by two Cys and one His residues (in the β- and ζ-CA classes), with the fourth ligand being a water molecule/hydroxide ion. Some of the catalytically active α-CAs can also catalyze the hydrolysis of esters, for example 4-nitrophenyl acetate (4-NpA; and other hydrolytic reactions as well). These enzymes are thus involved in many physiologic processes, such as photosynthesis, respiration, CO_2 transport, as well as metabolism of xenobiotics (e.g., cyanate in *Escherichia coli*) [8,9].

Known α-CA inhibitors (CAIs) include various anions, imidazole, phenols, hydroxyurea, carboxylates, organic phosphates and phosphonates, and sulfonamides ($R\text{-}SO_2NH_2$) and their derivatives (e.g., sulfamates, sulfamides), which represent the main class of clinically used CAIs [10]. This chapter will discuss the current state of the art for utilizing CAs from different sources in biomedical and biotechnological applications, such as in the achievement of an artificial respiration system, selective biosensors for metal ions, and in the carbon capture and sequestration process (CCS). In this regard, bacterial CAs from extremophile organisms are particularly attractive candidates for such applications because of their high stability and catalytic activity, as well as possibility to be expressed in large quantities from *E. coli* [6,11].

Aa Biosensor: an analytical device that combines a biological component with a physicochemical detector for revealing a substance or chemical constituent that is of interest in an analytical procedure.

CO_2 capture: the term 'CO_2 capture' describes a set of technologies aimed at capturing CO_2 emitted from industrial and energy-related sources before it enters the atmosphere.

Artificial respiration systems

The micro-porous hollow fiber membranes (HFMs), formed by poly(methylpentene) and poly(propylene) materials, have been used to develop artificial respiration systems, named here 'artificial lungs'. HFMs are routinely employed to both oxygenate blood and remove carbon dioxide in an extraluminal blood flow models, but their use in

large blood-containing surfaces is very challenging. Indeed, it would require massive and continuous anticoagulant assistance with new and more efficient devices than the existing ones [12]. A lot of research is underway to increase the artificial lung biocompatibility, one of the ideas being to increase the gas exchange rate of HFM-based devices by reducing their surface. Another approach could be one described in the literature about the ability of CAs covalently immobilized to the surface of a conventional HFM, which was shown to facilitate the diffusion of CO_2 toward the fiber membranes, by mimicking the function of the enzymes on lung capillary membranes. Such results showed that CO_2 removal was increased by 75% in the model device using the immobilized CA [13]. Another possibility to increase the CO_2 transfer across the HFM of artificial lungs is the use of impeller devices that increase the rate of blood mixing [13]. Until now, this method has not been combined with that of the immobilized CA because it requires a more stable form of enzyme that should not be denatured by the shear forces. Nowadays, with the discovery of robust and stable CAs by Capasso *et al.* from thermophilic microorganisms (see the 'Novel thermostable and fastest CAs to be used in the CO_2 capture process' section) may allow the possibility to realize a combined device with smaller dimensions and increased efficacy/stability.

Biosensors

The fluorescence based biosensors exploit the ability of CAs to bind metal ions with high affinity, to determine the presence of free metals in solution. In these biosensors, the mammalian CAs have been used in the apoprotein form to detect Cu^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} and Ni^{2+} at concentrations lower than the picomolar range by following the change in the fluorescence emission and excitation wavelength ratios, lifetimes and anisotropy [14]. Moreover, changing the primary structure of the protein by site-directed mutagenesis, it has been possible to change the sensitivity, the selectivity, the metal binding, the kinetics and stability of the biosensors. These studies led to the development of a versatile way to measure metal ions using highly selective and sensitive fluorescence-based biosensors mostly for Zn^{2+} and Cu^{2+}, with some important applications. In fact, the CA-based Cu^{2+} biosensor has been used to obtain real-time measurement of free Cu(II) at picomolar concentrations in seawater, while the CA-base Zn^{2+} biosensor has been used for measurement of free Zn ion at picomolar levels in cultured cells [14].

The carbon cycle in nature

The carbon cycle consists of the movement of carbon between land, ocean and atmosphere, which is made possible by the processes of photosynthesis and respiration.

Most life on earth depends on the production of sugars from sunlight and CO_2 (photosynthesis) and the metabolic breakdown (respiration) of those sugars to produce CO_2 and the energy needed for movement, growth and reproduction. CO_2 and its hydration product bicarbonate (HCO_3^-) are substrates and products of various metabolic reactions in the cells of different living organisms [9]. The interconversion of CO_2 and HCO_3^- is spontaneously balanced to maintain the equilibrium between dissolved inorganic CO_2, carbonic acid and carbonate of which HCO_3^- is physiologically the most important one as it is a substrate for several carboxylating enzymes involved in biosynthetic pathways, such as biosynthesis of fatty acids, amino acids and nucleotides [9,15]. The uncatalyzed hydration–dehydration of CO_2–HCO_3^- is slow at physiological pH and, thus in biological systems, the reaction must be accelerated by an enzymatic catalyst, which is the CA. The increase of gases with greenhouse effect in the atmosphere, including CO_2, coming from the combustion of fossil materials, represents one of the leading factors of high impact on environmental stress and it is considered a major cause of climate change [16]. The production of 'eco-compatible' combustible materials and/or the reduction of CO_2 accumulation in the atmosphere represent the highest priority for a better quality of human life. A number of CO_2 sequestration methods have been proposed in order to capture and concentrate CO_2 from combustion gases, for its transit and storage. Among them the most used ones are: the sequestration of CO_2 as a salt, which is interesting since the carbonate minerals constitute the largest reserve of CO_2 on earth; and the chemical absorption of CO_2 by alkanolamines, to produce a gas that can be easily transported to and disposed of, after stripping with steam, at a geologic or marine sequestration site.

The last few years have seen the development of a biomimetic approach as an interesting strategy in the CO_2 capture process [16]. This method offers important advantages if compared with the other methods for CO_2 capturing since it is specific for this gas, is an eco-compatible process allowing the specific concentration of CO_2 from other gases, its solubilization as ions and its further use for the growth of algae or other microorganisms. The enzymatic capture technique is classified as a 'postcombustion method' withdrawing CO_2 from the industrial fumes produced by the combustion of hydrocarbons. The biomimetic approach uses microorganisms capable of fixing CO_2 through metabolic pathways or via the use of CA. The use of novel thermostable bacterial CAs, either free or

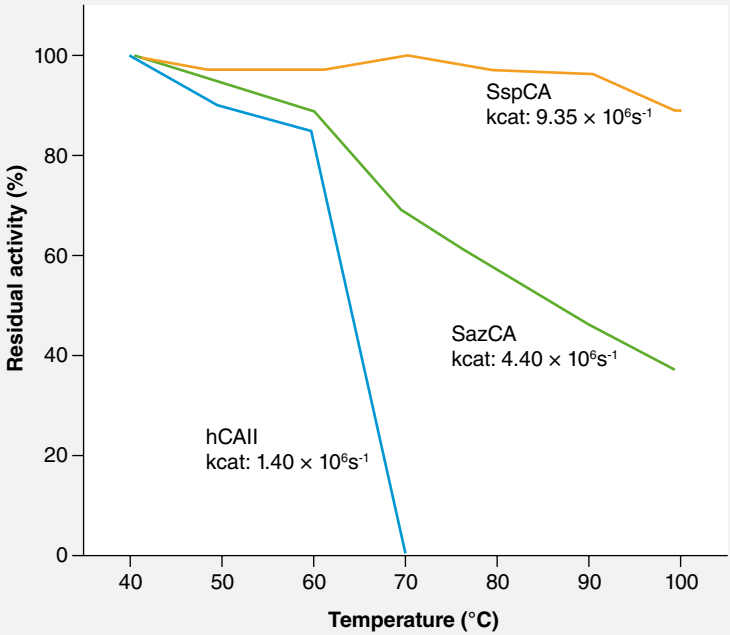
Aa Biomimetic: refers to human-made processes, substances, devices, or systems that imitate nature. It is of special interest to researchers in nanotechnology, robotics, artificial intelligence (AI), the medical industry and the military.

immobilized, has been reported in several technical approaches for capturing CO_2 from combustion or from gas mixtures, and its subsequent utilization or disposal.

Novel thermostable & highly efficient CAs

The temperature of combustion gasses or liquids into which the CO_2 are dissolved may easily exceed the optimal temperature for the enzyme used in the capture process. One of the drawbacks of using enzyme-based approaches is that an extensive cooling is necessary before adding the enzyme to the gas or liquid containing CO_2, and cooling is an energy-consuming process. There is, however, a search for CAs, which are more stable and more active in the operative conditions and, in addition, enzymes that could be produced in large amounts at low purification costs. With the technologies available on the market it is possible to produce large amounts of enzymes at moderate costs as happens, for example, for the enzymes utilized in detergents plants. Generally, CAs isolated from mammals or prokaryotes are active at physiological temperatures and are, as with many enzymes, quite unstable under extreme conditions, such as high temperature and high concentrations of salts. Taking into account the biodiversity, studies of enzymes from thermophiles, that is, microorganisms living at temperatures ranging from 70°C to 110°C, have demonstrated that such enzymes are thermostable, thermoactive and generally better supporting common enzyme denaturants. Recently, Capasso *et al.* cloned and purified novel bacterial α-CAs from thermophilic bacteria, belonging to the genus *Sulfurihydrogenibium* living in hot springs worldwide, at temperatures up to 110°C [11,16–19]. The α-CAs from *Sulfurihydrogenibium yellowstonense* and *Sulfurihydrogenibium azorense*, denominated SspCA and SazCA, respectively, are probably involved in the CO_2 fixation and biosynthetic processes, as for other bacteria, algae and plants. The aforementioned enzymes are thermostable enzymes showing an excellent catalytic activity after incubation for more than 3 h at 100°C **(Figure 11.1)**, and they are also highly effective catalysts for the CO_2 hydration reaction. SazCA is the fastest CA known to date, and the second most efficient enzyme (after superoxide dismutase), with a kcat value of 4.40×10^6 and a kcat/K_M value of 3.5×10^8 M^{-1} s^{-1}. SspCA also showed a good catalytic activity for the same reaction, with a kcat value of 9.35×10^5 s^{-1} and a kcat/K_M value of 1.1×10^8 M^{-1} s^{-1}, proving that the 'extremo-α-CAs' are indeed among the most effective CAs known to date. These findings strongly support the utilization of 'thermostable-α- CAs' in the CO_2 capture process. The biotechnological applications, in fact, require robust and catalytically active enzymes able to function in the harsh conditions typical of industrial processes.

Figure 11.1. Effect of temperature on the stability of SspCA and SazCA with respect to the mesophilic counterpart, represented by the hCAII.



In order to compare the stability at different temperatures of the α-CAs, enzymes at the concentration of 3 µg/ml in 10 mM Tris/HCl, pH 8.3 were incubated at different temperature (40, 50, 60, 70, 80, 90 and 100°C) for 30 min performing the reaction in triplicate. The percentage residual activity has been calculated with reference to the enzyme activity at 25°C. hCAII: Human carbonic anhydrase II; SspCA: *Sulfurihydrogenibium yellowstonense* carbonic anhydrase; SazCA: *Sulfurihydrogenibium azorense* carbonic anhydrase.

Carbon capture assisted by CAs

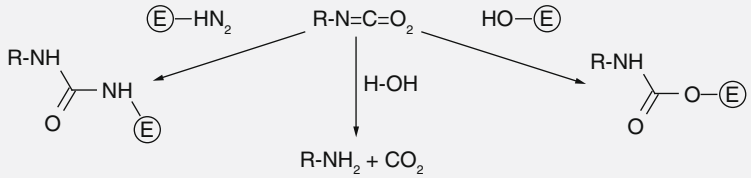
A number of CO_2 sequestration methods have been proposed in order to capture CO_2 using different types of CA enzymatic bioreactors. These comprised membrane contactors using free CA solutions for releasing gaseous CO_2 as well as to precipitate it as calcium carbonate, contactors using immobilized CA, namely counter-current and cross-concurrent packed columns, and contactors using either free or particle-immobilized CA [20]. Overall, three enzymatic CO_2 capture techniques are being industrially developed. In a first process the enzyme is immobilized on a solid support, itself packed in a bed reactor. An aqueous solution is sprayed through a nozzle at the

Aa Bioreactor: in this context, the term bioreactor is referring to a container in which a chemical process is carried out using microorganisms or biochemically active molecules.

top of the reactor. It washes a counter flow of the gas containing the CO_2 to be captured, itself injected at the lower end of the reactor. Capture of the CO_2 occurs when the opposite flows of aqueous solution and gas percolate through a supported enzyme bed. A second reactor in which the former aqueous CO_2 solution is sprayed, for instance in a carrier gas or in a partial vacuum, makes it possible to recover the CO_2 gas. A third type of deposited reactor according to which the liquid layer flows as droplets on the enzyme immobilized on elongated supports, in such a way that the CO_2 hydration reaction occurs within the flowing liquid. In a variant of such a process, CA was immobilized by covalent grafting on silica coated porous steel and water was sprayed down through the flue gas. For example, a process has been developed formed by a membrane sandwich to which the CO_2 is captured through thin aqueous films in which CA is dissolved or a network of hollow microporous fibers in which the flue gas and the release gases could flow immobilizing the enzyme directly on the external faces of the microfibers. All these methods utilize mesophilic CAs, but there are some disadvantages related to it. Indeed, the stability of such enzymes is low even if they are immobilized on appropriate supports. Immobilizing the enzyme may eliminate some of these disadvantages, but not the thermal instability of mesophilic CAs. Entrapment in matrices, adsorption on the solid surfaces, covalent bonding, and cross-linking within polymeric networks are some example of enzyme immobilization techniques. CA (usually bovine or human isoforms) has been immobilized on some solid and polymeric supports, such as chitosan-alganite beads by encapsulation, silicamonoliths by the sol-gelmethod, poly(acrylic acidco-acryamide) hydrogel by entrapment and hydrophobic adsorbent of Sepharose 4B by adsorption.

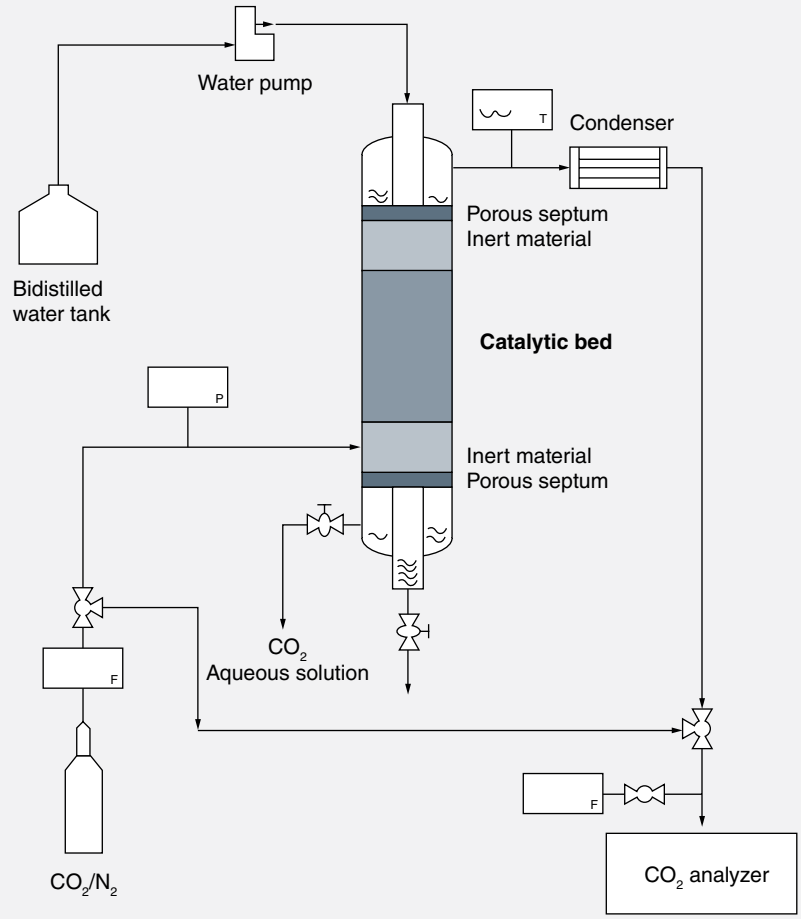
Apart from these strategies, our groups have immobilized the highly thermostable SspCA in a polyurethane foam (PU) [16]. PUs are widely used as supports for immobilization of enzymes and cells. A few steps are involves in the immobilization process **(Figure 11.2)**.

Figure 11.2. Enzyme immobilization using the prepolymer HYPOL (R in the figure).



The letter E is the enzyme.

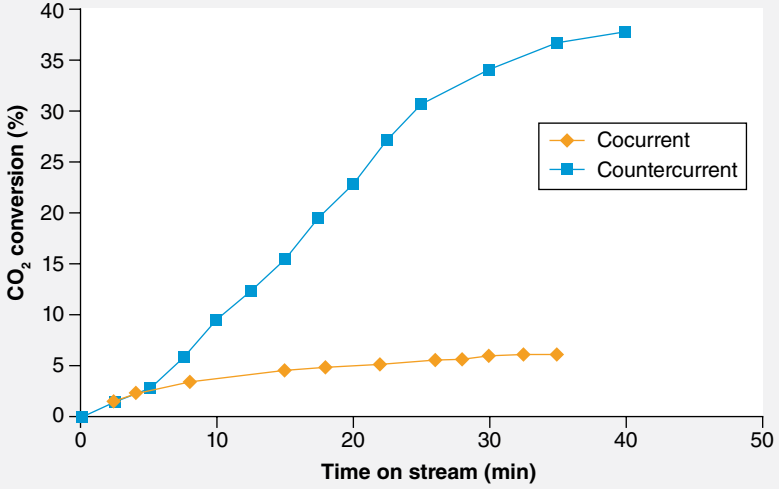
Figure 11.3. Scheme of the bioreactor used for CO_2 biomimetic absorption.



First, the aqueous enzyme or cell solution is mixed with a prepolymer (called HYPOL). HYPOL is a prepolymer of poly(ethylene glycol) capped with isocyanate-terminated end groups. The immobilization of the enzymes within the PU foam and the polymerization of HYPOL in an aqueous solution is due to isocynate chemistry. The polymerization is initiated by a nucleophilic attack by an OH- at the carbonyl group (NCO) following a protonation and deprotonation and a release of CO_2 from an unstable carbamic acid intermediate, thus converting one isocyanate group into an amine group (NH_2). The produced -NH_2 groups react immediately with a

neighboring isocyanate group, leading to an urea functionality and resulting in a crosslinking between two prepolymer chains. This process continues until all of the isocyanate groups are consumed. The released CO_2 makes a porous and sponge-like polymeric matrix of the PU foam. Because the amine and/or hydroxyl groups are readily available on the surface of the enzyme, the enzyme itself is preferentially crosslinked by the isocyanates of the prepolymer. Consequently, a covalently immobilized enzyme containing biocatalytic material can be obtained in a crosslinked form. The advantages of this procedure are that the process is faster and higher activity retention could be obtained. Capasso *et al.* realized a three-phase reactor filled with shredded foam composed of PU-SspCA or PU [16], in order to evaluate the absorption capability in presence and absence of the catalytic effect. In the bioreactor, a gas phase, containing CO_2, was put in contact with a liquid phase under conditions where the CO_2 contained in the gas phase is absorbed by the liquid phase and converted into bicarbonate by carbonic anhydrase **(Figure 11.3)**. A gaseous mixture of CO_2 in N_2 was fed to the reactor in both cocurrent downflow and countercurrent flow with bi-distilled water, the CO_2 concentration was fixed at 20% by volume since this value corresponds to the average concentration present at the exhaust of power plants.

Figure 11.4. CO_2 biomimetic absorption by three-phase trickle-bed reactor.



Effect of reactor on CO_2 conversion efficiency as function of time on stream (for details see [16]).

Under the experimental condition described by Migliardini *et al.*, the CO_2 conversion in countercurrent modality resulted significantly higher with respect to the cocurrent operation (38 and 6%, respectively). **Figure 11.4** shown the results obtained by Migliardini *et al.* [16].

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Summary

- Carbonic anhydrases (CAs; EC 4.2.1.1) are ubiquitous metalloenzymes involved in many physiologic processes, such as respiration, CO_2 transport , photosynthesis, as well as metabolism of xenobiotics.
- CAs facilitate the diffusion of CO_2 toward the fiber membranes, by mimicking the function of the enzymes on lung capillary membranes.
- CAs have been used in the apoprotein form to detect Cu^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} and Ni^{2+} at concentrations lower than the picomolar range by following the change in the fluorescence emission and excitation wavelength ratios, lifetimes and anisotropy.
- The use of novel thermostable bacterial CAs, either free or immobilized, has been reported in several technical approaches for capturing CO_2 from combustion or from gas mixtures, and its subsequent utilization or disposal.
- A three-phase reactor filled with shredded foam composed of polyurethane-*Sulfurihydrogenibium yellowstonense* CA in order to evaluate the CO_2 absorption capability.

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