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REVIEW ARTICLE

Exploring Natural Product Derivatives having Carbonic Anhydrase Inhibitory Activity

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Abstract

Carbonic anhydrase is an enzyme that plays an active role in many biological functions of the human body. It is omnipresent in all biological organisms with eight different genetic families. Its primary role is to catalyze the reversible hydration of Carbon dioxide to bicarbonate and protons. Though it is beneficial in many aspects it is also equally important for the cancer cells because of its pH-regulatory nature. For tumor cells to survive and metastasize the regulation of pH and the creation of a hypoxia condition is very much needed, as Carbonic anhydrase is an extended family of sixteen isozymes, some of which are very essential for tumor cells. Much research is going on to inhibit the selective enzyme overexpressed in the tumor cells. Carbonic anhydrase IX and XII are the most important enzymes overexpressed in the tumor cells. Sulfonamides and their bioisosteres sulfonamides and sulfamates have been employed for decades in treating conditions like glaucoma, epilepsy, and diuretics. Notably, this class of compounds has been extensively studied for its role as carbonic anhydrase inhibitors, showcasing their significant use and exploration in various therapeutic applications. In recent years, there has been a notable shift in the study of Carbonic anhydrase inhibitors toward natural products in addition to synthetic derivatives. Traditional drug design methods have historically yielded synthetic CAIs, but the exploration of Natural product derivatives has significantly advanced the field. Natural products, such as Psammaplin C and Altemicidin, containing primary sulfonamide or sulfamate groups, are gaining attention. The chemical diversity, binding specificity, and interaction tendencies of natural product derivatives make them appealing for molecular probes in research.

Keywords: Cancer, Carbonic Anhydrase, Natural Product Derivatives, Selective Inhibition

Abbreviations: CO_2 - Carbon dioxide; CARPs - Carbonic Anhydrase Related Proteins; hCAs - human Carbonic anhydrases; CAIs - Carbonic anhydrase inhibitors; H_2CO_3 - Carbonic acid; PPARg2 - Peroxisome Proliferator-Activated Receptor-g2; Pgp - P-glycoprotein; ZBG - Zinc Binding Group; NPs - Natural products; AG - Anchoring Group; SG - Sticky group

1. Introduction

Carbonic anhydrases (EC 4.2.1.1) are ubiquitous zinc metalloenzymes found in all living species. They are encoded by eight distinct genetic groups that are evolutionary unrelated, including α, β, η, δ, ζ, η, θ, and ι, which are known as CAs[1]. Protozoa, vertebrates, algae, cytoplasm of green plants, and several Gram-negative bacteria have α-CAs[2]. The β-CAs are extensively found in numerous fungi, algae, mono- and dicotyledon chloroplasts, and both positive and negative gram negative and positive bacteria. Archaea, cyanobacteria, and most species of bacteria include γ-Cas[3]. Click or tap here to enter text. On the other hand, marine diatoms appear to contain exclusively δ-, ζ-, and θ-CAs, whereas protozoa such as *Plasmodium falciparum* contain η-Cas[4]. Recently, it was found that ι-CAs are found in bacteria as well as marine phytoplankton[5,6].

The human carbonic anhydrase (hCA) family comprises sixteen distinct isoforms falling under the

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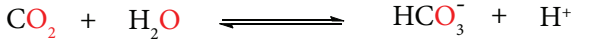
category of α-carbonic anhydrases (α-CAs). These isoforms exhibit notable diversity concerning their tissue distribution, cellular localization, expression levels, and responses to different classes of inhibitors. The classification of hCAs encompasses various subsets, including membrane-bound isoenzymes such as CA IV, CA IX, CA XII, and another CA IV variant. Additionally, there are mitochondrial isoforms, specifically CA VA and CA VB, as well as secreted isoforms exemplified by CA VI, predominantly found in saliva. Cytosolic isozymes, namely CA I, CA II, CA III, CA VII, and CA XIII, contribute to the comprehensive array of hCAs. Remarkably, among the sixteen hCA isoforms, only twelve (I–IV, VA, VB, VI, VII, IX, XII-XIV) display catalytic activity. These enzymatically active isoforms feature active sites characterized by the presence of three histidine residues in triple coordination with a zinc ion, underscoring the pivotal role of zinc in their catalytic function. In contrast, the remaining three isoforms, namely CA VIII, CA X, and CA XI lack a Zn^{2+} ion and consequently exhibit no catalytic activity. These non-catalytic isoforms are designated as CA-related proteins (CARPs), emphasizing their distinction from their enzymatically active counterparts. Furthermore, it is noteworthy that CA-XV is a variant found in mice, denoted as mCA XV, adding a layer of species-specific diversity to the carbonic anhydrase family. The nuanced differences in catalytic activity, zinc coordination, and cellular localization among these isoforms underscore the intricacies of the hCA family, highlighting the diverse functional roles played by different members in various physiological contexts[7-11].

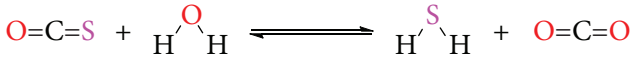
Carbonic acid (H_2CO_3) is produced when a neutral CO_2 molecule reacts with water[12]. It spontaneously separates into bicarbonate ion and proton. At high pH, this reversible reaction occurs spontaneously, whereas, at physiological pH, it proceeds slowly [turnover number (k_{cat}) of 10^{-1} s^{-1}][13,14]. Carbonic anhydrases can catalyze this process because they have evolved to withstand the enormous levels of CO_2 that most organisms produce, which makes their substrates readily available to cells. The hydrogen and bicarbonate ions that are produced have buffering properties that are crucial for pH regulation.

Carbonic anhydrases (CAs) catalyze the following reaction:

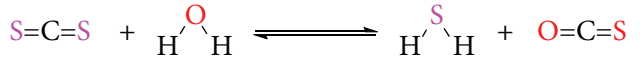


(Equation 1)

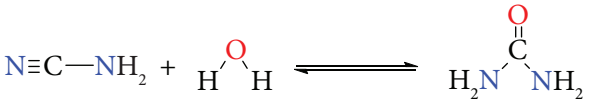
While the primary function of this class of metalloenzymes is to catalyze the reversible hydration of CO_2 to bicarbonate and proton (all CAs), some members of the entire Carbonic anhydrases family also catalyze various other hydrolytic processes, such as the hydration of COS and CS_2 (β CAs) (Equation 2 and 3), the hydration of cyanamide to urea (Equation 4), and the hydrolysis of various esters (α CAs) (Equation 5)[15-18].



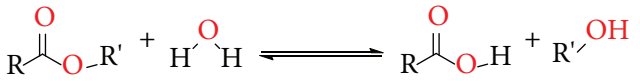
(Equation 2)



(Equation 3)



(Equation 4)



(Equation 5)

The orchestration of numerous physiological processes hinges significantly on the regulatory influence exerted by Carbonic Anhydrases (CAs). These enzymes play pivotal roles in processes such as the intricate exchange of CO_2 and bicarbonate between the lungs and metabolizing tissues, respiratory functions, cellular homeostasis, electrolyte secretion across various tissues and organs, gastric acid secretion, and the orchestration of biosynthetic reactions encompassing gluconeogenesis, lipogenesis, ureagenesis, bone resorption, calcification, renal acidification, cellular stress adaptation, and tumorigenicity, among others[19,20].

The far-reaching impact of CAs is underscored by their involvement in the pathophysiology of

numerous diseases. Conditions such as edema, obesity, mental disorders, glaucoma, cancer, and certain disorders of the central nervous system, including neuropathic pain, idiopathic intracranial hypertension, epilepsy, and osteoporosis, are intricately linked to the regulatory functions of CAs in these processes. The critical roles played by CAs in disease-associated pathways have positioned them as promising targets for therapeutic interventions. The development of selective human Carbonic Anhydrase Inhibitors (hCAIs) has emerged as a focal point in the quest for effective treatments for the aforementioned diseases. These inhibitors aim to modulate the activity of CAs, offering a targeted approach to mitigating the underlying dysregulation contributing to the pathogenesis of these diverse medical conditions. The multifaceted involvement of CAs in physiological and pathological processes emphasizes their significance as potential therapeutic targets, highlighting the intricate interplay between enzyme function and human health[21].

1.1 Isoforms of A – Carbonic Anhydrases

While novel generation compounds are receiving clinical development as anti-obesity and anticancer drugs/diagnostic tools, CA Inhibitors (CAIs) were first utilized as diuretics[22], antiglaucoma medicines[23], antiepileptics[24,25], and in the management of altitude sickness[26]. Conversely, the CA Activators (CAAs) possess pharmacological applications in memory therapy[27], and emotional memory modulation, suggesting their potential utility in the treatment of disorders such as phobias, obsessive-compulsive disorders, post-traumatic stress disorder, and generalized anxiety, for which there are currently limited treatment alternatives[28-30].

CA I and CA II are found in erythrocytes at high concentrations, and since CA I is implicated in cerebral and retinal edema, inhibiting it may be a useful treatment for these disorders[31]. Among human CAs, CA II is the most active isoenzyme and is mostly located in the kidney, inner ear, Central Nervous System (CNS), and eye. Clinically utilized medications such as diuretics, antiglaucoma, and anticonvulsants target this enzyme[32,33]. Because of its special capacity to regulate the production of peroxisome proliferator-activated receptor-g2 (PPARg2)[34], CA III is expressed in tissues such as skeletal muscles and adipose tissues, and it is directly associated with adipogenesis. Furthermore, it was shown that CAIII was downregulated in several illnesses, including systemic lupus erythematosus and rheumatoid arthritis that were connected to exhaustion and muscular soreness[35]. A unique glycosylphosphatidylinositol anchor holds CA IV, the most common membrane-associated CA isoform, to the membrane's outer surface. The human body has large amounts of CA IV in many different parts, such as the kidneys, lungs, colon, pancreatic cell plasma membranes, eyes, heart muscle, etc. Possible involvement of the mutant version of the CA IV isoform in the development of retinitis pigmentosa, stroke, and glaucoma has been discovered[36,37].

Two Mitochondrial isoforms, CA VA, which exist in the liver aid in ureagenesis by supplying bicarbonate ions for carbamoyl phosphate and CA VB enhances lipogenesis by promoting carboxylase activity[38]. Due to their dual effects on gluconeogenesis and lipogenesis, these enzymes can serve as valuable therapeutic targets in the management of insulin resistance and obesity[39]. After being initially identified in saliva, CA VI has now been demonstrated to be expressed in pulmonary airways, tears, milk, and nasal secretions, as well as enamel organ cells[40,41]. Inhibition of the CA VI isoform results in loss of taste or, rarely, anomalies in taste perception[42].

The CA VII isoform is expressed in the liver, brain, skeletal muscles, and colon. There are two versions of CA VII: one has the whole amino acid sequence, while the other has an N-terminal truncation of 56 residues. Like CA III, CA VII has two glutathionylated cysteine residues on its surface that protect cells from oxidative damage[43]. Because it reduces neuronal excitation, the inhibition of CA VII is considered a promising target for the treatment of neuropathic pain and seizures[44].

The CA VIII, X, and XI are categorized as carbonic anhydrase-related proteins (CA-RPs). They are non-catalytic isoforms and are mostly located in the brain. Their physiological role and functions remain unclear as of yet. Ataxia, mild mental retardation,

and quadrupedal gait have all been associated with the CA 8 gene, suggesting that CA VIII has a role in neurodegenerative diseases[45]. CA XIII is expressed in organs including the kidney, thymus, submandibular glands, small intestine, and reproductive organs. According to several theories, CA XIII is essential for controlling the pH of reproductive activities, such as sperm motility.CA XIV is one of the transmembrane isoforms and is expressed in the bladder, kidney, colon, small intestine, brain, and other organs. It shares a considerable amount of sequence similarity with CA XII.

CA IX and XII are well-known targets for anticancer drug and their expression in normal conditions is limited to epithelium lining the gastrointestinal tract. The over-expression of these enzymes primarily aids in the growth, metastasis, angiogenesis, and proliferation of tumors[46]. The hypoxic tumor condition, in which the tumor mass's vasculature system is unable to keep up with the oxygen demand of rapidly proliferating cancer cells, results in areas with insufficient oxygen supply. This leads to a decrease in ATP production due to reduced oxidative phosphorylation of glucose[47]. Tumor cell survival, proliferation, and gene expression undergo significant alterations as a result of this hypoxic state. The ability of proliferating cancer cells to meet their energy needs through an alternate glycolytic route is contingent upon the metabolic shift brought on by hypoxia. However, this change also results in a rise in lactic acid production as a metabolic byproduct, acidifying the extracellular environment acidic[48,49].

The ability of cancer cells to adapt to the pH shift in their microenvironment caused by the buildup of CO_2 and lactic acid is crucial to their survival. So, the overexpression of CA IX genes is the most notable adaptation under these hypoxic conditions. In hypoxic tumors, overexpression of hCA IX lowers the extracellular matrix's pH, which promotes cancer cells' survival and advancement[50]. As a result of strong transcriptional activation produced by Hypoxia Inducible Factor-1[51], it is now well established that CA IX is overexpressed in a variety of tumor types, including brain, breast, kidney, and colorectal cancers[52]. Expression of CA IX in non-cancerous tissues is uncommon and typically limited to the intestine, pancreas, stomach, and gallbladder epithelia[53,54]. It has also been discovered that tumor hypoxia makes cancer cells more resistant to weakly alkaline anticancer medications. Therefore, in the last ten years, researchers have focused a lot of attention on CA IX in an effort to create novel anticancer drugs[55].

The fact that hypoxia also controls the second tumor-associated isozyme, CA XII, is most likely not a coincidence. CA XII performs vital roles in normal physiological conditions and is found in numerous normal tissues and organs, including the endometrium, prostate, colon, kidney, and eye. The expression of this gene has been found in a variety of tumor types, such as renal cell, breast, ovarian, pancreatic colorectal, and gastrointestinal carcinoma[56]. These tumor forms are typically linked with less aggressive and well-differentiated phenotypes.

Many malignancies eventually acquire drug resistance after initially responding to chemotherapy; at this point, treatment is no longer effective, and the disease returns and worsens. When compared to drug-sensitive cancer cells, the subgroup of cancer cells that resist medication therapy exhibits inherent morphological differences. The upregulation of drug efflux transporters, such as P-glycoprotein (Pgp), often referred to as multidrug resistance protein 1, is one of the main and prevalent mechanisms underlying drug resistance. If a cancer starts from a cell type that has high basal Pgp expression, then overexpression of Pgp in malignancies may develop intrinsically. After it was found that the CA XII, which counteracts extracellular acidosis in hypoxic tumors, indirectly lowers Pgp activity in resistant cancer cells, a novel idea of using CA XII inhibition to target the pH microenvironment to overcome Pgp-mediated drug resistance, emerged. A novel approach to specifically target Pgp in cancer cells exclusively is made possible by the close correlation between the co-expression of CA XII and Pgp and a drug-resistant phenotype[57,58]. Notably, this relationship does not exist in healthy cells.

As a result, hCA XII is another excellent biomarker for the suppression of different hypoxic tumors in both the initial and metastatic stages (Table 1).

Table 1. CO_2 hydration catalytic activity and off-targets of hCA in various diseases[59]

hCA isoforms	CO_2 hydration Catalytic activity	Diseases in which involved	Possible off-targets
hCA I	Low	Cerebral/ retinal edema Hemolytic anemia	-
hCA II	High	Glaucoma Edema Epilepsy	hCA I
hCA III	Very Low	Oxidative stress Muscular soreness	-
hCA IV	Medium	Glaucoma Stroke Retinitis pigmentosa	hCA I
hCA VA	Low	Obesity and	hCA I and hCA II
hCA VB	High	Insulin resistance	
hCA VI	Low	Cariogenesis	hCA II
hCA VII	Low	Epilepsy	-
hCA VIII	No activity	Neurodegenerative diseases	-
hCA IX	High	Cancer	hCA I and hCA II
hCA X	No activity	-	-
hCA XI	No activity	-	-
hCA XII	Low	Cancer	hCA I and hCA II
hCA XIII	Low	Sterility	-
hCA XIV	Low	Retinopathy	-

2. Carbonic Anhydrase Catalytic Mechanism

The earliest evidence of CAs was found in 1933 when it was found that erythrocytes had stoichiometric zinc levels and an abundant protein known as carbonic anhydrase, which was later shown to be necessary for the enzymatic activity of CO_2 hydration[60].

Metalloenzymes, a fascinating class of proteins, exhibit catalytic efficiency contingent upon the presence of a metal ion nestled within the confines of the active site cavity. Intriguingly, the apoenzymes, devoid of this essential metal cofactor, remain catalytically inert. Within the diverse spectrum of CAs, the α-, β-, and δ-CAs feature active sites adorned with Zn(II) ions, essential for their optimal functionality. The γ-CAs, on the other hand, display versatility by accommodating either Zn(II) or Co(II) ions, although they are often speculated to primarily associate with Fe(II) ions. Remarkably, the metal ion in ζ-CAs is typically replaced by cadmium, highlighting the intricate variations in metal coordination among different CA isoforms[61,62].

One captivating facet is the revelation that certain isoforms of carbonic anhydrases, known as ι-CAs, showcase catalytic activity even in the absence of a metal ion cofactor, defying the conventional paradigm[63]. The structural intricacies of the active sites provide further insight into the diversity of metal coordination. In α-, γ-, and δ-CAs, the Zn metal ions intricately coordinate with three histidine amino acid residues (His94, His96, and His119), along with a water molecule or hydroxide ion, forming the catalytic core. This coordination occurs at the base of a 15Å deep active-site cleft, emphasizing the precision required for their enzymatic function[25,64].

Conversely, β- and ζ-CAs exhibit distinct amino acid ligands within their active sites, featuring one histidine and two cysteine residues for metal coordination[65]. Intriguingly, in silico studies shed light on the coordination sphere of η-CAs, suggesting a unique arrangement with two histidine residues and one glutamine residue participating in the coordination of the presumed Zn(II) ion, alongside the requisite water molecule or hydroxide ion. This diversity in metal coordination among various CA isoforms underscores the intricate interplay between structural nuances and catalytic function, contributing to the versatile roles these enzymes play in physiological processes. The reverse hydration of CO_2 by CAs follows a two-step catalytic mechanism:

$$E\_\_M^{2+}\_\_OH^- \rightleftharpoons E\_\_M^{2+}\_\_HCO_3^- \rightleftharpoons E\_\_M^{2+}\_\_H_2O + HCO_3^-$$

(Equation 6)

$$B + E\_\_M^{2+}\_\_H_2O \rightleftharpoons B\_\_H^+ + E\_\_M^{2+}\_\_OH^-$$

(Equation 7)

The catalytically active species that powers the catalytic activity is the metal hydroxide species of the enzyme (E-M^{2+}-OH). There is a designated pocket for CO_2 in the active site of CA. This active species shows strong nucleophilicity (at neutral pH) towards the CO_2 molecule trapped in a nearby hydrophobic pocket during the initial phase of the reaction. This results in the production of HCO_3^-, which a water molecule then removes from the active site (Equation 6). The regeneration of the metal hydroxide species through a proton transfer reaction from the M^{2+} bound water to either an external proton acceptor (B in Equation 7) or an active site residue[27] is the second stage in the catalysis process, and it is the step that limits the pace. Several of the α- and ζ-CAs are among the most potent natural catalysts because of their catalytic turnover k_{cat}/KM values, which are above 108 M^{-1} x s^{-1}. The Cl/HCO_3^-exchanger and the Na/HCO_3^- exchanger, among many others, are two examples of transporters or gateway channels in which the displaced HCO_3^- ion is involved. These channels have different roles in various physiological processes.

The active sites of all families of CAs have a unique architecture that allows for their partition into two very distinct environments: one-half of the active site is lined by hydrophilic residues, while the other is bordered by hydrophobic residues. This architecture is what allows for such efficient catalysis. A plausible theory states that the hydrophobic component functions by capturing the lipophilic CO_2 molecule, while the hydrophilic component is responsible for the escape of the polar species generated by the CO_2 hydration reaction into the environment. The latter procedure, which helps the protons reach the cavity's exterior with the help of a network of water molecules and Histidine residues, has at least been demonstrated for the protons.

2.1 New Inhibition Mechanisms and New CAI Classes

Before our studies in the field of CA, the only inhibitors known to exist were metal-complexing anions and sulfonamides, some of which are currently being used in clinical settings. Sulfonamides are still a highly significant family of CAIs with numerous established drugs, as was previously indicated; nonetheless, these two ZBGs are isosteres of the sulfonamide moiety. The sulfamate topiramate was initially deemed not to act as a CAI by those who discovered it in 1987.

Christianson's group used X-ray crystallography in 1994 to show that phenol binds in a very different way than sulfonamides: it anchors using the OH moiety to the zinc-coordinated water molecule. When Lindskog's group reported in 1982 that phenol functioned as a CAI, it was considered a kind of curiosity. The discovery of novel chemotypes acting as CAIs, such as coumarins, polyamines, dithiocarbamates, xanthates and monothiocarbamates, hydroxamates, selenols, etc., witnessed a "revival" in 2008.

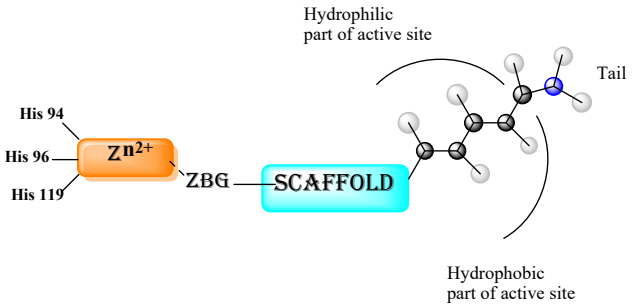


Figure 1. Schematic representation of the general structure of zinc-binding CAI in the CA active site.

The intriguing aspect is that these several chemotypes attach in four different ways, each of which has a unique inhibitory mechanism[61,62,66].

2.1.1 Zinc Binding Inhibitors

A general method of CA suppression by Zinc binders, like sulfonamides, sulfamides, and sulfamates requires the direct interaction of the Zinc Binding Group (ZBG) with the zinc metal ion (Figure 1). These inhibitors after deprotonation bind as anions to the metal ion, which is in tetrahedral geometry with the ZBG. In addition, two conserved residues in all α -CAs engage with the ZBG, serving as "gatekeepers." These residues are Thr199, which is involved in a hydrogen bonding with the zinc in the uninhibited enzyme via its OH group, and Glu106, which forms a hydrogen bonding with Thr199 in the enzyme-inhibitor adducts by its carboxylate group[67,68].

2.1.2 Inhibitors Anchoring to the Zinc Coordinated Water Molecule

Compounds employing this specific mechanism to inhibit Carbonic Anhydrases (CAs) stand out due

to the incorporation of an Anchoring Group (AG) linked to a scaffold, potentially featuring appended tails capable of engaging with both sides of the active site, akin to the binding characteristics observed in zinc binders. The uniqueness of this inhibition lies in the strategic combination of the anchoring group with a scaffold, forming a molecular structure that facilitates effective interaction with the target enzyme. The potential inclusion of tails further enhances the versatility of these compounds, allowing them to establish interactions not only with the active site itself but also with adjacent regions, thereby optimizing their inhibitory efficacy. This nuanced approach highlights the intricate design principles employed in developing inhibitors that operate through this distinctive mechanism, emphasizing the significance of the anchoring group and its synergy with the scaffold for targeted and efficient CA inhibition. When compared to the zinc binders, these inhibitors do not make direct contact with the metal ion. With the anchoring group, these CAIs can establish two hydrogen bonds, one between the inhibitor's hydrogen atom with the metal ion-coordinated water molecule/ hydroxide ion, and the other hydrogen bond involves the participation of NH of Thr 199 with the inhibitor. This mechanism of inhibition was first found for phenol and has since been found for polyamines, thioxocoumarins, catechols, sulfocoumarins (which bind as sulfonates when hydrolyzed), and polyamines (using X-ray crystallography and other biophysical measures). Consequently, OH, NH_2, or SO_3H moieties are frequently found in AG[69-71].

2.1.3 Inhibitors that Occlude the Entrance of the Active Site

In contrast to Zinc binders or compounds that attach to the zinc-coordinated water molecule, these inhibitors establish binding at a considerable distance from the metal ion, avoiding any direct interaction with it. Their inhibitory action involves obstructing the entrance of the active site cavity, a region characterized by high variability among various isoforms, while also maintaining relative homology with the 16 mammalian counterparts. The molecules that work via this inhibitory mechanism are attached to a scaffold by an aromatic, heterocyclic, or aliphatic Sticky Group (SG). Because these compounds attach in a somewhat exterior region of the cavity, they may also have a tail that extends out from the active site. The natural substance coumarin was the first instance of such an inhibitor that was identified. However, further investigation demonstrated that all coumarins and structurally similar mono- and bicyclic 5 and 6-membered lactones possess this type of action. It triggers the lactone ring to hydrolyze, resulting in the formation of hydroxy-acids for 5- and 6-membered lactones and hydroxy-cinnamic acids for coumarins. Coumarins and their derivatives serve as suicide (or prodrug) inhibitors, which sets them apart from other groups of CAIs due to their binding location near the opening of the active site cleft[72,73]. It is explained by the fact that they bind amongst the different isoforms in the changeable portion of the active site, which is why they produced highly isoform-specific CAIs.

2.1.4 Inhibitors Binding out of Active Site

The inhibitors in question exhibit a unique binding pattern, occupying an adjacent pocket near the entrance, distinct from the active site cavity itself. This unconventional binding behavior, highlighted through crystallography and kinetic studies involving a benzoic acid derivative featuring an ortho-benzyl sulfoxide moiety, is particularly evident in the hydrogen bonding interactions with His64, a crucial proton shuttle residue in α-carbonic anhydrases (α-CAs). The catalytic cycle disruption ensues as a consequence of this binding, marking a departure from the conventional inhibitory mechanisms observed with other inhibitors. To date, only 2-benzoyl sulfonyl-benzoic acid has been identified as functioning through this atypical inhibitory pathway. When 2-(benzylsulfonyl)-benzoic acid was subjected to co-crystallization with hCA II, the inhibitor's electronic density manifested in a binding pocket adjacent to the active site, positioned at a considerable distance. This interference occurs precisely at the step crucial for determining the rate of the entire catalytic cycle – the proton transfer from the zinc-coordinated water molecule to the environment and the subsequent regeneration of the enzyme-zinc hydroxide complex. A pivotal aspect of this inhibitory mechanism involves the flexibility of the amino acid His64, which exists in two primary conformations: an "in" conformation, closer to the metal ion, and an "out" conformation, directed towards the exit of the

active site cavity. In its "in" conformation, the imidazole moiety of His64 becomes protonated by extracting a proton from the zinc-coordinated water, while in the "out" conformation, the same proton may be released into the environment. The inhibitors disrupt this critical process, impeding the enzyme's function by preventing the proton shuttle from traversing a network of hydrogen bonds. Consequently, the complete catalytic cycle is hindered, underscoring the distinctive inhibitory mechanism employed by these inhibitors[74].

For decades, several sulfonamides, as well as their bioisosteres, sulfamides, and sulfamates, have been purportedly used as diuretics and to cure glaucoma and epilepsy. Of all the chemicals in this class, carbonic anhydrase inhibitors (CAIs) are the ones that are being studied the most. Acetazolamide (AAZ) and dorzolamide, for instance, are categorized as first- and second-generation medicines used as CAIs and are primary sulfonamides (Figure 2). These drugs usually have significant CA activity, but their selectivity for a specific isoform of an enzyme linked to disease is usually insufficient, and their off-target inhibition causes important adverse effects. In actuality, the field of CA inhibition has a recurring issue with the discovery of isoform-specific inhibitors[75,76].

The catalytic binding region across all 16 α-Carbonic Anhydrase (CA) isoforms manifests impressive structural conservation, posing a formidable challenge in the development of isoform-specific inhibitors. Despite this structural uniformity, comparative analyses have unearthed the existence of a specialized pocket near the zinc ion, situated at the periphery of the binding site, which exhibits selectivity towards α-CAs. Crafting an effective Carbonic Anhydrase Inhibitor (CAI) necessitates the incorporation of three indispensable structural components: a hydrophobic moiety, a Zinc-Binding Group (ZBG), and a "tail". Critical residues Thr199 and Glu106, conserved within the active sites of all α-CAs, along with the catalytic zinc ion, play pivotal roles in binding with the ZBG group. The hydrophobic moiety, crucial for the inhibitory action, features a tail intricately linked to an organic scaffold, often characterized by an aromatic or heteroaromatic ring. The unique structural arrangement allows the tail to interact directly with either the hydrophobic or hydrophilic half of the binding site, providing versatility in targeting both polar and lipophilic groups. This strategic design consideration underscores the intricate balance required for successful CAI development, navigating the complex structural landscape of

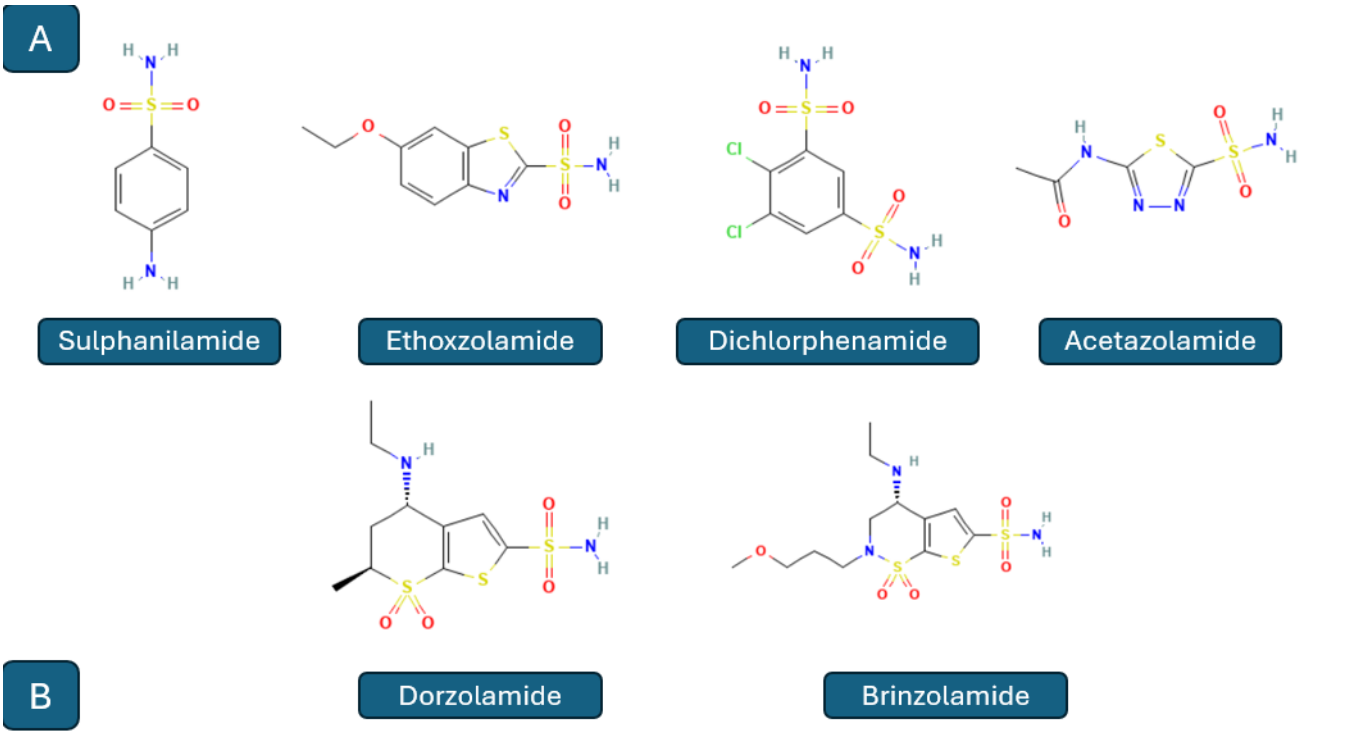


Figure 2. Molecular structures of A) First and B) Second generation CAIs.

α-carbonic anhydrases while leveraging the selective pocket near the zinc ion for isoform specificity[77].

The development of Carbonic Anhydrase Inhibitors (CAIs) commonly employs the benzene sulfonamide scaffold[78], a prominent Zinc Binding Group (ZBG) crucial for forming coordinating bonds with the zinc central metal atom of Carbonic Anhydrase (CA), thereby inhibiting the enzyme. A notable example is 4-(4-fluorophenylureido) benzene sulfonamide, denoted as SLC-0111, currently undergoing phase II clinical trials for treating solid hypoxic metastatic tumors, demonstrating its potency as a CA IX inhibitor[79,80].

Traditionally, the majority of pharmacologically effective CAIs, whether in clinical use or under development, have been synthetic derivatives resulting from conventional drug design methods applied to synthetic lead compounds. However, a paradigm shift has occurred in the last decade, with increased attention directed towards Natural Products (NPs) as potential CA inhibitors[81]. This exploration of NPs has significantly advanced the field, providing an alternative avenue for the discovery of new chemotypes with biological activity and serving as a source of novel lead compounds[82]. The appeal of natural products lies in their chemical diversity, efficacy, specificity of binding, and proclivity for interactions with biological targets, rendering them attractive options for molecular probes in the hands of researchers. This evolving trend underscores the potential synergy between traditional drug design and the exploration of natural products, paving the way for innovative approaches in the development of carbonic anhydrase inhibitors.

A small number of naturally occurring substances are known to include either a primary sulfamate or primary sulfonamide group. Mujumdar and Poulsen[83] reported on the identification, isolation, bioactivity, and synthesis of five NP primary sulfamate compounds, including nucleotide, 5'-O-sulfamoyl adenosine, 5'-O-sulfamoyl 2-chloroadenosine, 5'-O-sulfamoyl 2-bromoadenosine, and 5'-O-sulfamoyl tubercidin, as well as two natural products primary sulfonamides, psammaplin C (1), and Altemicidin (2).

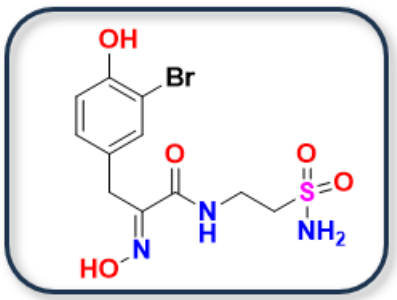
3. Psammaplin C

In 1991, the discovery of Psammaplin C marked a significant advancement in the field of natural products. Isolated from the sponge *Pseudoceratina purpurea*, this primary sulfonamide natural product exhibited a unique structural composition. Psammaplin C features a bromotyrosine functionalized moiety, with an amide linking the bromophenol fragment to an ethylene sulfonamide group, thereby incorporating the primary sulfonamide group. The confirmation of its structure was achieved through a comprehensive spectroscopic investigation, revealing a distinctive Zinc-binding sulfonamide functional group absent in other psammaplin family members[84]. The research conducted by Mujumdar and Supuran's group[85] delved into the Carbonic Anhydrase (CA) inhibition properties of Psammaplin C across a panel of 10 hCA isoforms. The binding pose within the hCA active site was meticulously evaluated, uncovering remarkable findings.

Psammaplin C exhibited exceptional inhibition for CA XII, with a Ki value of 0.79 nM. Notably, its inhibition constants (Ki) spanned five orders of magnitude (0.79 nM – 10630 nM) across various CA isoforms, showcasing significant variability compared to the more closely clustered CA inhibition of AZA (Ki range 2.5 – 250 nM). The sulfonamide proved to be less effective against hCA VI and XIII, the two isoforms displaying lower sensitivity to this inhibitory compound (Figure 3).

Moreover, the research extended to the modification of the hCA II protein to mimic the active regions of hCA IX and hCA XII. The X-ray crystal structure analysis provided insights into the diverse interactions of compound (1) bound to the active site. Consistent with previous CA-sulfonamide adducts, the deprotonated sulfonamide moiety of Psammaplin C bound with the zinc ion and formed interactions with 'gatekeepers' Thr 199 and Glu 106[85].

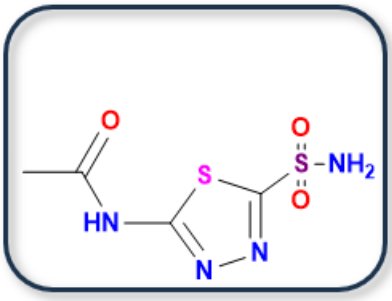
Beyond its inhibitory effects, Psammaplin C exhibited no toxicity and demonstrated efficacy only in combination with chemotherapy. To explore its potential further, a series of 45 Psammaplin C derivatives were synthesized, reported in a subsequent study[86]. This initiative aimed to establish Structure-Activity Relationships (SARs) around compound 1, focusing on its action against aggressive glioblastoma xenografts with an unconventional mechanism of action capable of reversing multidrug resistance. All the synthesized derivatives retained the crucial feature of an



1

Ki (nM)

hCA I - 48.1	hCA VII - 1.7
hCA II - 88.0	hCA IX - 12.3
hCA IV - 75.3	hCA XII - 0.79
hCA VA - 154	hCA XIII - 10630
hCA VI - 9680	hCA XIV - 379



2

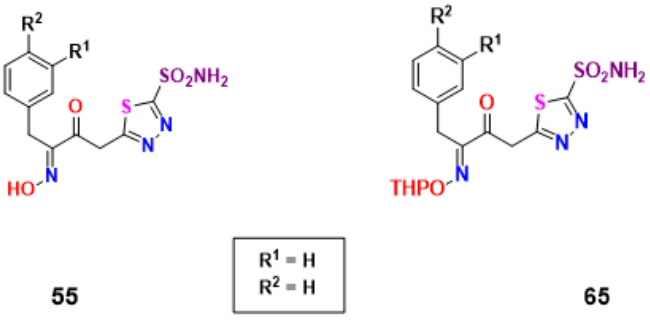
Ki (nM)

hCA I - 250	hCA VII - 2.5
hCA II - 12	hCA IX - 25.0
hCA IV - 74.2	hCA XII - 5.7
hCA VA - 63.1	hCA XIII - 16.4
hCA VI - 11.0	hCA XIV - 41.3

Figure 3. CA inhibition profile of Compound (1) and standard reference CAI Acetazolamide.

unhindered primary sulfonamide group, a prerequisite for effective CA inhibition. Structural modifications, including changes in the 3-bromo, 4-hydroxy benzyl moiety, the oxime group, and the ethylene-sulfonamide group, resulted in highly effective CAIs for hCA I, II, IX, and XII isoforms.

Noteworthy among the derivatives were Compounds 55 and 65, derivatives of acetazolamide-type Psammaplin C with a thiadiazoyl sulfonamide moiety in place of the ethylene sulphonamide group (Figure 4). These compounds exhibited potent inhibition of hCA IX in low-nanomolar concentrations and hCA XII in sub-nanomolar concentrations. Further studies, both *in vitro* and *in vivo* using samples from glioblastoma patients, demonstrated enhanced activity when combined with the clinically used agent temozolomide. This comprehensive investigation



55 65

Figure 4. CAIs for Compounds 55 and 65.

exemplifies how marine nanoparticles, exemplified by Psammaplin C, can pave the way for the development of novel compounds with potent anticancer activity.

4. Altemicidin

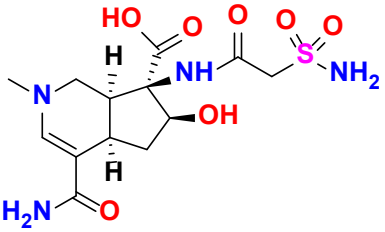


Figure 5. Chemical structure of Altemicidin.

Alkaloids are a class of basic, naturally occurring organic compounds that contain at least one nitrogen atom. In 1989, the discovery of Altemicidin (2) (Figure 5) marked a significant milestone in natural product research. This unique sulfonamide compound was isolated from a Japanese sea mud sample originating from the actinomycete strain *Streptomyces sioyaensis* SA-1758. Despite its identification more than three decades ago, Altemicidin remains the sole primary sulfonamide natural product. Notably, its isolation from a marine source added to its novelty, setting it apart from other compounds of its kind. Altemicidin (2) displayed antitumor properties, yet its administration in mice

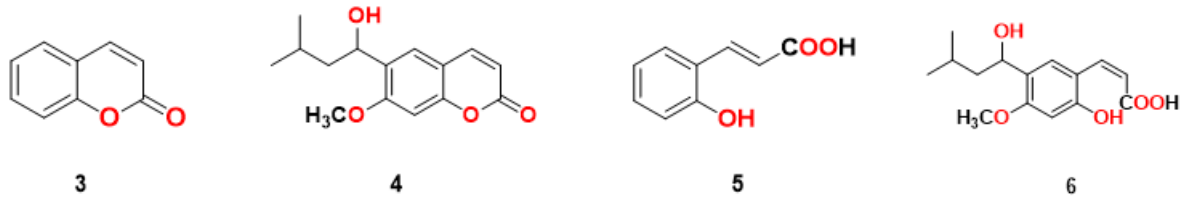
revealed a noteworthy drawback—acute toxicity[87]. The compound boasts a distinctive chemical structure, characterized by the presence of a carboxamide-sulfamoyl group connected to a bicyclic heterocyclic ring system. This unique configuration suggests a potential for strong CA inhibitory effects, a hypothesis supported by the compound's notable growth inhibition of two tumor cell lines: IMC carcinoma and murine L1210 lymphoid leukemia. The values for these inhibitory concentrations were measured at 2.12 µM and 0.8 µg/mL, respectively[83]. Despite its promising medicinal potential, the exploration of Altemicidin's CA inhibitory effects remains limited. The primary hindrance to further investigation lies in the compound's complex synthesis process, which involves 27 steps. Additionally, the compound's rarity and the challenges associated with sourcing chemical reagents contribute to its limited accessibility among researchers and suppliers[88]. The realm of marine-derived alkaloids has witnessed numerous discoveries from plants and animals, showcasing a diverse array of biological activities, including potent cytotoxicity. However, the occurrence of cytotoxic alkaloids produced by actinomycetes is an uncommon phenomenon. Altemicidin stands out as a pioneering example, being the first reported alkaloid of marine origin derived from an actinomycete with cytotoxic properties[89]. This groundbreaking revelation adds a new dimension to our understanding of the potential bioactive compounds originating from marine environments.

5. Coumarins

Coumarins are oxygenated heterocyclic polyphenolic compounds widely distributed in plant families and essential oils, and also present in smaller amounts in microbes and animal sources, and have been employed for several purposes. Coumarins can be classified into the following types based on the core benzopyrone ring as: a) simple coumarins, b) Coumarinolignans, c) pyranocoumarins, d) bis- and tris-coumarins, and e) Furanocoumarins (Figure 6).

Given that coumarins do not contain any zinc-binding scaffolds as other known CAIs, it seemed highly relevant to interpret the mechanism of CA inhibition of this new derivative and investigate its inhibition profile concerning the mammalian catalytically active isoforms[90]. Based on comprehensive kinetic, mass spectrometric, and crystallographic investigations, Maresca *et al.,* established that coumarins do have a distinct CA inhibitory mechanism. X-ray crystallographic investigations using hCA II, a simple coumarin derivative (3), and a coumarin derivative obtained from the Australian plant *Leionema ellipticum* support the fact that coumarins, unlike other CAIs, serve as suicide inhibitors (4)[91]. These observations allowed scientists to conclude that 2-hydroxy cinnamic acids (5) and (6) are formed when the coumarin ring is hydrolyzed by CA esterase activity. The esterase activity of human carbonic anhydrases (hCAs), as elucidated by Supuran's groups[92], has been demonstrated to catalyze the hydrolysis of the lactone ring in coumarins. Subsequently, the hydrolyzed products exhibit binding in the vicinity of the active site cavity opening, situated approximately 8-10 Å away from the zinc ion. Remarkably, within the diverse landscape of 15 distinct human CA isoforms, this region stands out for possessing the most varied amino acid sequence[11], Compounds capable of interacting with and binding to the amino acid residues in this specific area of the active site, in contrast to those binding deeper within the active site where a substantial fraction of amino acid residues is shared among multiple CA isoforms, often demonstrate heightened isoform selectivity. This strategic insight into the differential binding preferences within the active site sheds light on a potential avenue for achieving enhanced selectivity in the design of carbonic anhydrase inhibitors.

From the plant *Magydaris pastinacea*, fifteen coumarins were isolated and reported by Fois *et al*[93].



3 4 5 6

Figure 6. Naturally occurring coumarin types.

These coumarins showed strong inhibition towards the tumor-related isoforms hCA IX and XII (Ki > 10,000 nM), but their activity against the cytosolic isoforms hCA I and II was found to be relatively low, which is a good thing because it is believed that these isoforms are what cause the side effects of CAIs. A tricyclic ring structure that complements the furan heterocycle is present in some of these compounds (7-21), which are variably substituted furocoumarins (psoralens) and are found in numerous other plants. Polyprenylated, Isoprenyl-, or hydrated isoprenyl moieties are included in the remaining derivatives, which are likewise common for many NPs. Remarkably, all coumarins/furocoumarins (7-21) displayed an excellent, and substantial inhibitory action towards the tumor-related isoforms hCA IX and XII at nanomolar concentration, however, they were inactive as hCA I and II inhibitors (Figure 7).

Melis *et al.*,[68] synthesized and reported a library of psoralen derivatives including carboxylic acid or ester moieties and their benzene sulfonamide derivatives and examined their activities against hCA I, II, IX, and XII isoforms. Because of the photochemical characteristics of the psoralen ring system, this class of compounds has garnered ongoing research for its possible use as antifungal agents or photo-activatable anticancer medications. The *Magydaris pastinacea* derivatives that were studied for their coumarins

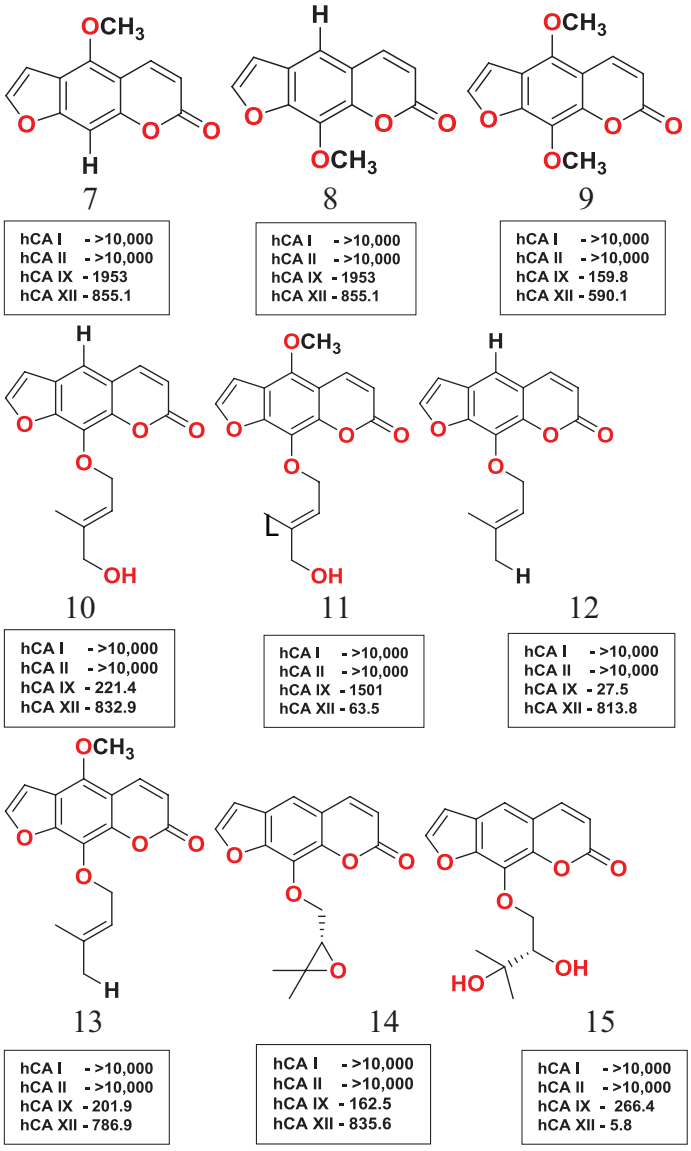


Figure 7. Inhibition profile of Natural Product Coumarin 7-21 against hCA I, II, IX, and XII.

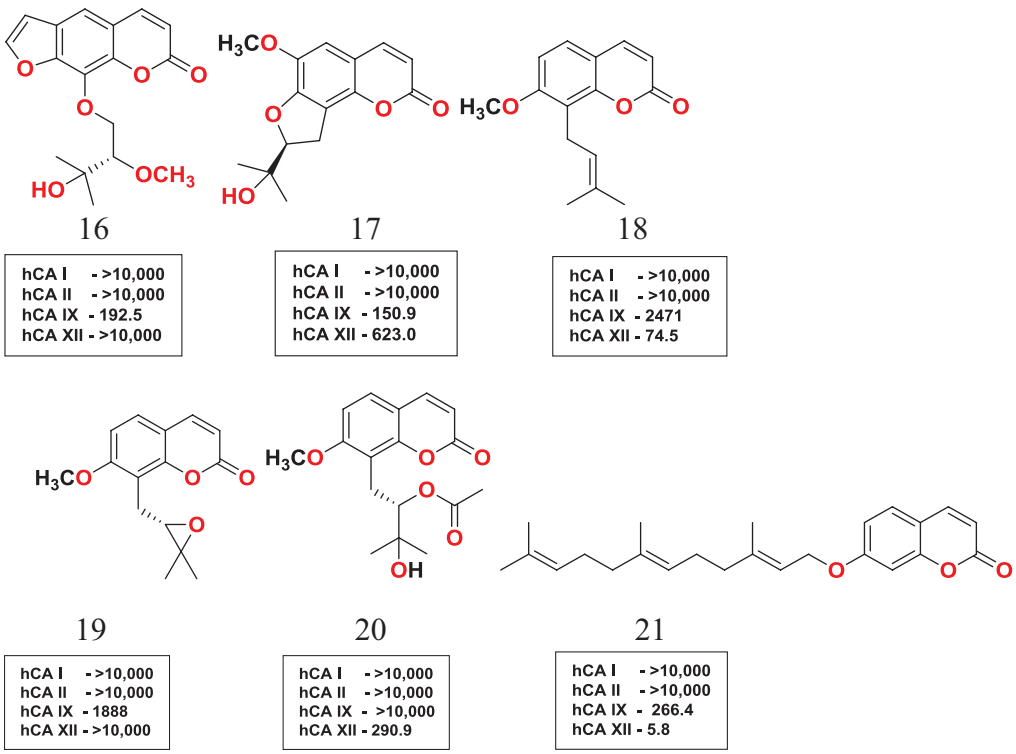


Figure 7. Continued.

Table 2. Inhibition profile Ki(nM)against hCA I,II,IX, and XII of compounds 22-25, 26-29, and 30-33

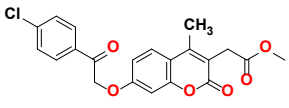
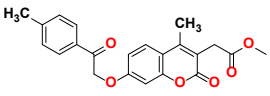
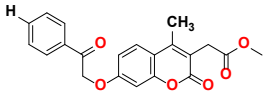
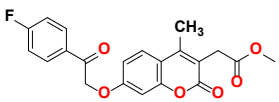
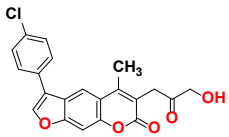
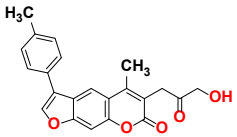
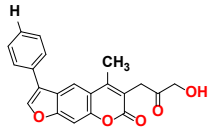
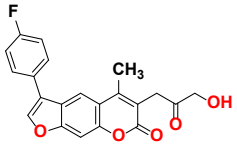
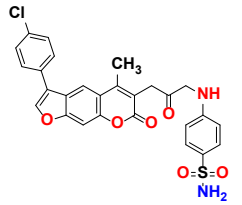
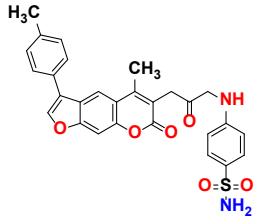
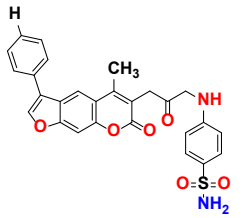
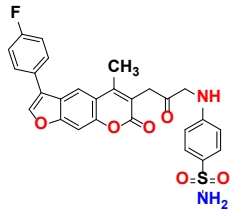
		Ki (nM)		
Compound	**hCA I**	**hCA II**	**hCA IX**	**hCA XII**
 22	>10,000	>10,000	23.6	446.6
 23	>10,000	>10,000	122.8	56.6
 24	>10,000	>10,000	89.7	72.5
 25	>10,000	>10,000	84.7	250.0
 26	>10,000	>10,000	94.7	9.3

Table 2. Continued

		Ki (nM)		
Compound	**hCA I**	**hCA II**	**hCA IX**	**hCA XII**
 27	>10,000	>10,000	23.0	9.1
 28	>10,000	>10,000	17.5	9.4
 29	>10,000	>10,000	17.7	7.4
 30	6829.7	55.1	17.8	2.4
 31	7069.0	560.0	91.6	3.4
 32	7016.1	46.6	16.5	3.6
 33	7148.1	79.5	108.4	49.9

and psoralens exhibited weak inhibition against the ubiquitous hCA I and II isoforms. However, they effectively inhibited tumor-associated, hCA IX and XII isoforms at low nanomolar concentrations, which makes them relevant for anticancer research. These findings suggested that both psoralen and hybrid compounds may potentially make good scaffolds for the development of hCAs IX and XII isoforms selective inhibitors. But the derivatives of coumarin (22-25) and psoralen (26-29) consistently showed extremely high selectivity, the hybrid compounds (30-33) than the hybrid compounds. The details of structure and activity data are presented in Table 2. Presumably, this is due to the existence of the Zinc binding group – benzene sulfonamide, which can produce exceedingly potent molecules but influence selectivity[94-96].

6. Conclusion

In conclusion, the intricate role of Carbonic Anhydrase (CA) in various biological functions has spurred extensive research, particularly due to its significance in cancer cells. The enzyme, with its eight genetic families, catalyzes the reversible hydration of CO2 to bicarbonate and protons, influencing pH regulation crucial for tumor survival and metastasis. Notably, CA IX and XII, among the sixteen isozymes, emerge as key players overexpressed in tumor cells. The quest for selective inhibition of these enzymes has led to the exploration of sulfonamides and their derivatives, historically employed in treating conditions like glaucoma and epilepsy. The evolving landscape in CA inhibitor research has witnessed a noteworthy shift toward natural products, complementing synthetic derivatives. Psammaplin C and Altemicidin, featuring primary sulfonamide or sulfamate groups, exemplify this trend. The chemical diversity, binding specificity, and interaction tendencies of these natural product derivatives make them intriguing candidates for molecular probes in research. This paradigm shift from traditional drug design methods to a focus on natural products highlights the potential for novel therapeutic applications. As investigations progress, these natural compounds may pave the way for innovative and effective strategies in cancer treatment, showcasing the dynamic and promising future of CA inhibition research.

7. Acknowledgement

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8. References

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