Multi- and poly-pharmacology of carbonic anhydrase inhibitors

Claudiu T. Supuran

Neurofarba Department, University of Florence, Section of Pharmaceutical Sciences, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Florence, Italy.

Phone/Fax: +39-055-4573729, E-mail: claudiu.supuran@unifi.it

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b) Corresponding author: Claudiu T. Supuran, Neurofarba Department, University of Florence,

Section of Pharmaceutical Sciences, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Florence, Italy.

Phone/Fax: +39-055-4573729, E-mail: claudiu.supuran@unifi.it

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d) Non standard abbreviations

ACC – acetyl-coenzyme A carboxylase

AD – Alzheimer's disease

Aβ – amyloid β

CA – carbonic anhydrase

CAA – CA activator

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CAI – CA inhibitor

DNL – *de novo* lipogenesis

GIT – gastrointestinal tract

hCA – human CA

IOP – intraocular pressure

PD – Parkinson's disease

pMCAO – permanent middle cerebral artery occlusion

PC – pyruvate carboxylase

VRE – vancomycin-resistant enterococci

ZBG – zinc-binding group

Abstract. Eight genetically distinct families of the enzyme carbonic anhydrase (CA, EC 4.2.1.1) were described in organisms allover the phylogenetic tree. They catalyze the hydration of CO_2 to bicarbonate and protons, and are involved in pH regulation, chemosensing and metabolism. The 15 α-CA isoforms present in humans are pharmacological drug targets known for decades, their inhibitors being used as diuretics, antiglaucoma, antiepileptic or antiobesity drugs, as well as for the management of acute mountain sickness, idiopathic intracranial hypertension and recently, as antitumor theragnostic agents. Other potential applications include the use of CA inhibitors (CAIs) in inflammatory conditions, cerebral ischemia, neuropathic pain, or for Alzheimer's/Parkinson's disease management. CAs from pathogenic bacteria, fungi, protozoans and nematodes started to be considered as drug targets in recent years, with notable advances registered ultimately. CAIs have a complex multipharmacology probably unique to this enzyme, which has been exploited intensely but may lead to other relevant applications in the future, due to the emergence of drug design approaches which afforded highly isoform-selective compounds for most α-CAs known to date. They belong to a multitude of chemical classes (sulfonamides and isosteres, (iso)coumarins and related compounds, mono- and dithiocarbamates, selenols, ninhydrines, boronic acids, benzoxaboroles, etc). The polypharmacology of CAIs will also be discussed since drugs originally discovered for the treatment of non-CA related conditions (topiramate, zonisamide, celecoxib, pazopanib, thiazide and high-ceiling diuretics) show efective inhibition against many CAs, which led to their repurposing for diverse pharmacological applications.

Significance Statement

Carbonic anhydrase inhibitors have multiple pharmacologic applications as diuretics, antiglaucoma, antiepileptic, antiobesity, anti-acute mountain sickness, anti-idiopathic intracranial hypertension and as antitumor drugs. Their use in inflammatory conditions, cerebral ischemia, neuropathic pain, or neurodegenerations started to be investigated recently. Parasite carbonic anhydrases are also drug targets for antiinfectives with novel mechanisms of action which can bypass drug resistance to commonly used such agents. Drugs discovered for the management of other conditions that effectively inhibit these enzymes exert interesting polypharmacologic effects.

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1. Introduction

CO_2 is one of the simplest molecules involved in crucial physiological processes and also a very stable and abundant form of carbon, the central element connected with life processes on earth. This gas reacts slowly with water, generating carbonic acid, which is unstable and spontaneously dissociates to bicarbonate and a proton (equation *1* in Fig. 1A) (Maren, 1967; Supuran, 2008; Supuran, 2023a,b). In this way, from two neutral molecules, CO_2 and water, a weak base (bicarbonate) and a strong acid (H^+ ions) are generated. In order to use this system as a biological buffer, the reaction must occur much more efficiently than the uncatalyzed one mentioned above, which has a k_{cat} of 0.15 s^{-1} at pH 7.4 (Supuran, 2023a), and for this reason, catalysts acting on CO_2 hydration evolved, which are enzymes called carbonic anhydrases (CAs, EC 4.2.1.1), discovered already in 1933 (Meldrum and Roughton, 1933). This process, the interconversion between CO_2 and bicarbonate catalyzed by CA, which may occur with huge k_{cat} values (in the range of 10^4-10^6 s^{-1} for different such enzymes) (Lindskog, 1997; Mishra et al., 2020; Supuran, 2016a), was thereafter shown to be physiologically relevant for many systems in organisms throughout the phylogenetic tree, being connected not only with pH regulation (in most cells, tissues and organs), but also with CO_2/bicarbonate sensing as well as several metabolic pathways (Arechederra et al., 2013; Capasso and Supuran, 2015; Maren, 1967; Neri and Supuran, 2011; Santi et al, 2013; 2021; 2022; 2023c,d). The extremely high catalytic efficiency of the CAs (an increase of the conversion rate of CO_2 to bicarbonate of $10^5 - 10^7$ times over the non-catalytic process) probably explains why these enzymes are present in most living organisms, with the exception of few bacteria and one archeon, which do non express them (Smith et al., 1999; Ueda et al., 2012). Presently, eight genetically different CA families are known, which represent an interesting and unique case of convergent evolution at the molecular level (Aspatwar et al., 2022b; Supuran, 2023a,b). Indeed, the CA enzyme families described so far are the α-, β-, γ-, δ-, ζ-, η-, θ- and ι-CAs (Alber and Ferry, 1994; Alterio et al., 2021; Cox et al., 2000; Del Prete et al., 2014; Di Fiore et al., 2022; Hirakawa et al., 2021; Jin et al.,

2020; Kikutani et al., 2016; Nocentini et al., 2021b; Xu et al., 2008), with a new class being discovered each 2-3 years over the past decade (Supuran, 2023a).

Fig. 1 here

The first seven CA families are metalloenzymes and they use a metal hydroxide mechanism to achieve the very efficient catalytic process of CO_2 hydration to bicarbonate (and obviously also the reverse reaction), as shown schematically in equations *2* and *3* in Fig. 1A. The metal hydroxide species of the enzyme is situated at a bottom of a rather spacious active site, as illustrated for the human isoform hCA II in Fig. 2A. This metal hydroxide species has a strong nucleophilic character and acts on the CO_2 bound in a hydrophobic pocket nearby (Fig. 2B), as demonstrated by X-ray crystallography (Domsic et al., 2008; Sjöblom et al., 2009), leading to the formation of bicarbonate bound to the metal ion (Fig. 2B), which is thereafter replaced by an incoming water molecule, leading to the formation of the "acidic" species of the enzyme, EM^{2+}——OH_2 (Lindskog and Coleman, 1973; Lindskog and Malmstrom, 1962). This is a catalytically ineffective species, and in order to form the nucleophilic species, the acidic form EM^{2+}——OH_2 transfers one proton from the metal coordinated water molecule to the environment (eq. *3* in Fig. 1A), usually with the assistance of active site residues (His in α-CAs, and presumably a Glu or Asp in β- and γ-CAs) (Steiner et al., 1975; Tu et al., 1989; Smith et al., 2002; Tripp and Ferry, 2000), regenerating thus the catalytically effective metal hydroxide species. In most CAs this is also the rate-determining step of the entire catalytic cycle (Steiner et al., 1975; Lindskog, 1997). This structural feature is shown in Fig. 2A for one of the most studied such enzyme, the human (h) isoform hCA II, by evidencing residue His64, the proton shuttle in this and many other hCAs, and the His cluster (comprising residues His4, 3, 10, 15 and 17) which all participate to proton transfer processes for generating the nucleophilic, zinc hydroxide species of the enzyme (Briganti et al., 1997).

Fig. 2 here

In the seven CA families which use metal ions in their active site, the nature of the cation is variable (Fig 1A), but most CAs are active with Zn(II) bound at the active site (Lindskog and Malmstrom, 1962; Supuran, 2023a). The γ-CAs might contain Fe(II) in their natural (reducing) environment (Macauley et al, 2009) although they are active also with Zn(II) or Co(II) bound (Ferry, 2010). The ζ-CAs contain Cd(II) which may be substituted with Zn(II), both enzymes showing similar activity (Alterio et al., 2012; Xu et al., 2008). The other classes (δ-, η- and θ-) seem to be zinc enzymes (Del Prete et al., 2014; Kikutani et al., 2016) but no detailed studies regarding their metal ion preferences are available so far. The metal ion ligands are three His residues in α-, γ- and δ-CAs, two Cys and one His residue in β-, ζ- and θ-CAs, and two His and a Gln in η-CAs, whereas the geometry of the cation is usually tetrahedral, distorted tetrahedral and rarely trigonal bipyramidal (Supuran, 2016a,b).

The most recently described class, the ι-CA was investigated in two different organisms, the cyanobacterium *Anabaena* sp. PCC7120, and the chlorarachniophyte alga *Bigelowiella natans* (Hirakawa et al. 2021). Surprisingly, it has been demonstrated by using X-ray crystallography that no metal ions are present in these enzymes. However, similar to the remaining seven CA genetic families discussed above, the CO_2 hydration catalyzed by ι-CAs is achieved by an active site activated water molecule (Fig. 1B). But for this class, the water activation for the nucleophilic attack is performed by three amino acid residues conserved in all ι-CAs investigated so far: Thr106, Ser199 and Tyr124 without the need of metal ions (Nocentini et al., 2021b), although, initially these enzymes were reported to contain Mn(II) at their active site (Jensen et al., 2019).

Thus, the ubiquity of CO_2 which is generated in most metabolic processes in all types of organisms, its gaseous nature and the need to be transformed in water soluble products, reaction which in turn is connected with pH changes, led to the emergence of CAs as widespread, versatile and highly effective catalysts (second only to superoxide dismutase as efficiency, when considering k_{cat}/K_M values of all enzymes) (Supuran, 2008; Hekmat et al., 2024).

The presence of CAs in vertebrates, humans included, is exploited pharmacologically for almost seven decades nowadays (Maren, 1967; Supuran 2008,2021). However, one of the most intriguing aspects related to CAs is the fact that a large number of isoforms is present in humans (15), with a varied localization and functions in many cellls, tissues and organs, which leads to a multiplicity of pharmacological and clinical applications of their inhibitors, and to a lower extent also of their activators (Supuran, 2018a). Thus, the multi-pharmacology of CA inhibitors (CAIs) will be discussed in this review in the context of the recent developments in the field of both classical applications of these drugs (diuretics, antiglaucoma, antiepileptic, antiobesity, anti-acute mountain sickness and antitumor agents) but also newer applications, till recently not closely associated with these enzymes (such as the management of neuropathic pain, cerebral ischemia and inflammation, among others) will be dealt with (Supuran, 2021b). Furthermore, the presence of CAs in many pathogenic agents (bacteria, fungi, protozoans, nematodes, etc.) opened new possibilities for the management of drug resistant such infections and the design of anti-infectives with a new mechanism of action (Capasso and Supuran, 2015; Supuran and Capasso, 2021; Flaherty et al., 2021; Supuran, 2023e). On the other hand, several clinically used agents, discovered for other pathologies/drug targets than CAs, were shown to possess relevant CA inhibitory effects and a rather interesting polypharmacology due to their interaction with various CAs, leading in several cases to drug repurposing (Supuran, 2024a). These agents (topiramate, zonisamide, sulpiride, veralipride, celecoxib, polmacoxib, pazopanib and famotidine) will be also discussed in the review article.

2. CAs as drug targets: overview of mammalian, eukaryotic and prokaryotic enzymes

α-CAs are present in many prokaryotic and eukaryotic organisms, among which vertebrates, protozoans, some fungi, algae, cytoplasm of green plants and in many bacteria (Aspatwar et al., 2022b; Capasso and Supuran, 2015; Supuran, 2016a), whereas the β-CAs are found in algae,

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chloroplasts of mono- and dicotyledon plants, in many fungi, *Archaea* and most Gram negative and positive bacteria (Capasso and Supuran, 2015; DiMario et al., 2018; Schlicker et al., 2009; Supuran, 2016a). The γ-CAs are present in cyanobacteria, bacteria and *Archaea* (Alber and Ferry, 1994; Capasso and Supuran, 2015; De Luca et al., 2016), the δ-, ζ-, and θ-CAs were described at the moment only in marine diatoms (Cox et al., 2000; Kikutani et al., 2016; Xu et al., 2008), whereas the η-CAs were found to date only in some protozoan species (Del Prete et al., 2014). The ι-CAs are present in bacteria (Nocentini et al., 2021b), diatoms (Jensen et al., 2018), cyanobacteria and algae (Hirakawa et al. 2021).

Fig. 3 here

Fig. 3 shows an example of a phylogenetic tree for a restricted number of CAs belonging to the mainly investigated genetic families, the α-, β- and γ-CAs from several bacteria, algae, plants, fungi and humans. As mentioned earlier, the different CA classes evolved separately through convergent evolution and are not much related with each other. On the other hand, in each specific genetic family, the enzymes are rather conserved and even between highly distant oragnisms, such as *Homo sapiens* and bacteria encoding α-CAs, there is a high level of conserved amino acid residues, and tight structural similarities (Aspatwar et al., 2022b). The same may be said for the β-CAs from various organisms, although the plant, bacterial and fungal enzymes cluster on different branches of the tree (Fig. 3). The α- and γ-CAs seem to have a more recent common ancestor compared to the β-CAs (Capasso and Supuran, 2015), as obvious from data of Fig. 3 (Supuran, 2023a).

Vertebrates encode only for α-CAs (Aspatwar et al., 2022b), and for a long period only such enzymes were considered as drug targets (Supuran, 2008). This is of course still a reality, but over the last two decades the CAs present in other organisms, among which pathogenic bacteria, fungi and protozoans, many of which belonging to the β-, γ- and η-classes, started to be considerd and in some cases validated as drug targets (Capasso and Supuran, 2015; Flaherty et al., 2021; Supuran,

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2023e; Supuran and Capasso, 2021). Thus, the family of possible CA drug targets is greatly enlarged compared to the first years of drug design research for obtaining CAIs (Maren, 1967). This offered interesting opportunities, but also many challenges, since as mentioned above, bacterial, fungal or protozoan α-CAs show a great homology and similar active site architecture to human enzymes (Supuran, 2016a), of which, as already mentioned, 15 diferent isoforms are known, 12 possessing catalytic activity for CO_2 hydration (Aspatwar et al., 2022b; Supuran, 2008) - Table 1.

Table 1 here

Table 1 shows the 12 catalytically active hCA isoforms, hCA I – XIV. It should be mentioned that isoforms VIII, X and XI (also called CA-related proteins, or CARPs) are devoid of catalytic activity since one (or two) of their zinc-binding His ligands are absent and as thus, they do not bind Zn(II) and are catalytically inactive for the CO_2 hydration reaction (Aspatwar et al., 2022b). Although they seem to be involved in some pathologies (Aspatwar et al., 2013; Karjalainen et al., 2018) they will be not discussed here.

As seen from Table 1, some isoforms are widespread in many tissues, such as for example hCA I and II, whereas others have a more restricted distribution in few organs and tissues (e.g., hCA III in skeletal muscle and adipocytes; hCA VII in the CNS, hCA VA in the liver and brain). Furthermore, their subcellular localization is also variable, with cytosolic, mitochondrial, membrane-bound, transmembrane and secreted isoforms (Table 1) (Nocentini et al., 2021a; Supuran, 2021b). Their catalytic activity (for the CO_2 hydration or bicarbonate dehydration) is also variable, with hCA III being around 1% as active as a catalyst for CO_2 hydration compared to hCA II and IX, which are the catalytically most effective hCAs, with the other isoforms having an activity between these two extremes (Nocentini et al., 2021a; Supuran, 2008; 2016a,b). The role of these isoforms in the cells, tissues and organs where they are present is multiple, and range from pH regulation, electrolyte secretion, sensing and transport of CO_2/bicarbonate, to biosynthetic reactions (lipogenesis, gluconeogenesis, urea synthesis) and internal/external pH homeostatic control (Theparambil et al.,

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2024). CAs are thus involved in fundamental physiologic processes, among which respiration, excretion of cations and anions, pH homeostasis, electrolytes secretion, bone resorption, calcification, etc. (Aspatwar et al., 2022b; Maren, 1967; Supuran, 2021b;2022; 2023,a,b). Dysregulation of such physiological activities due to a too high (or too low) activity of these enzymes may lead to a range of disorders, which are shown in Table 1. They include edema, glaucoma, epilepsy, altitude sickness, retinis pigmentosa, obesity, cerebrovascular disease, oxidative stress, cariogenesis, sterility, but also inflammation, retinopathies, Alzheimer's disease and tumors (Aspatwar et al., 2022b; Maren, 1967; Provensi et al., 2019; Supuran, 2021b,2022, 2023,a,b). In most cases the precise isoform(s) involved in each disorder is clear and the targeting well understood. For example, the renal isoforms hCA I, II and IV are involved in the diuretic effects of the sulfonamide CAIs (Maren, 1967; Supuran 2016c; 2021), whereas targeting the overexpressed hCA IX/XII in hypoxic tumors (McDonald et al., 2022) leads to anticancer effects of these pharmacological agents. However, in other cases the involvement of more than one isoform seems to be associated with a disease, e.g., hCA I, II, IV and XIV in some retinopathies; hCA II and VII in neuropathic pain; hCA VII and XIV in epilepsy; hCA IV, IX and XII in some inflammatory pathologies (Bua et al., 2020; Margheri et al., 2016; Mishra et al., 2021; Supuran, 2016c). In few cases, such as idiopathic intracranial hypertension (IIH) (Supuran, 2015) or Alzheimer's disease (Provensi et al., 2019) for which increasing evidences demonstrate the efficacy of CAIs in their management, the involved isoform(s) and the precise mechanism of action of their inhibition are still largely unknown and/or debated (Supuran, 2021b).

A crucial question thus raises: why are there 12 catalytically active CAs, with a rather superimposable distribution (in many tissues/organs) and even similar catalytic activity, when most other pharmacologically relevant enzymes have usually only 2 or 3 different isoforms? The monoamine oxidase, MAO, has just two human isoforms MAO-A and MAO-B which metabolize the three major monoamine neurotransmitters (Giovannuzi et al., 2024); the cyclooxygenase (COX)

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three isoforms (Romanelli, 2024), but only COX-1 and COX-2 are involved in pharmacological effects of the NSAIDs; the angiotensin-converting enzyme ACE has again only two isoforms, ACE-1 and ACE-2 (Arrighi et al., 2024) and the list may continue with many other such examples. These enzymes (MAO, COX or ACE) play highly relevant physiological functions in many cells, tissues and organs, which are carried out by a limited number of 2-3 isoforms. Why is the situation for the CAs so different? For the moment no clear-cut reply to this question emerged, and this may represent a serious complication from the pharmacological viewpoint, due to the fact that isoform-selective inhibitors for 12 similar enzymes are needed (Alterio et al., 2012) in order to have useful pharmacological agents, which is not a facile task for medicinal chemists and pharmacologists. On the other hand, the large number of isoforms and their slightly different properties, offer the opportunity for many different pharmacological and clinical applications for inhibitors of these enzymes, in diseases not at all related to each other, as shown in this discussion and illustrated in Table 1. This is probably a unique case in which the same drug target, an enzyme possessing 12 slightly different isoforms, leads to so many pharmacological applications in very diverse fields, ranging from acid base balance and metabolism, to epilepsy, obesity, cancer, inflammatory conditions, neurodegeneration and all the other pathologies mentioned above.

When considering CAs from pathogenic organisms, the targeting is apparently easier, but also in these cases many complications may emerge, which are outlined below: (i) pathogenic organisms may encode for CAs belonging to more than one class and frequently possess different isoforms (but usually not as many as the vertebrates ones mentioned above for hCAs) with different inhibition mechanisms or sensitivity to inhibitors (Capasso and Supuran CT, 2015); (ii) in case of organisms possessing α-CAs, the homology with some of the human enzymes is quite high, which may lead to inhibitors targeting both the host and the parasite enzyme, and as thus, to side effects of the potential drugs; (iii) most CAIs are highly polar molecules which do not penetrate membranes easily, and thus do not arrive in high enough concentrations at the parasite enzyme, for exerting

their antiinfective action; (iv) many fungal or protozoan parasites have different developmental stages which are not particularly easy to target by using CAIs since the expression of these enzymes in all such stages is not known or highly variable (Capasso and Supuran, 2023; Fisher et al., 2017; Vermelho et al., 2020); (v) few animal models for studying antiinfective CAIs are available to date, although relevant progress has been registered ultimately for some bacterial infections (Abutaleb et al., 2022a,b).

Thus, apart the 12 hCAs, considered as the classical drug targets among these enzymes (and which continue to be highly investigated to date), the last decade saw the emergence of many CAs from other organisms, belonging to the α-, β-, γ- and η-families as potential antiinfective targets. Many of these drug targets and the pharmacological agents modulating them will be discussed in this review.

3. CA inhibitor (CAI) classes

CAs are inhibited by a large number of different chemotypes, shown in Table 2 (Angeli et al., 2022a; Schulze Wischeler et al., 2010; Supuran, 2016a,b; Mueller et al., 2021; Chrysanthopoulos et al., 2017; Pustenko et al., 2020;2023; Grandane et al., 2020; Winum and Supuran, 2015; Winum et al., 2009).

Table 2 here

There are in fact four CA inhibition mechanisms (for the metallo-CAs, i.e., α-θ-class enzymes), which have been rationalized by considering the interaction (or the lack of interaction) between the inhibitor and the metal ion from the active site of the enzyme (Supuran, 2016b). They are:

(i) binding to the active site metal ion, these inhibitors being termed metal ion binders, as they incorporate a metal-binding group (usually a zinc-binding group, ZBG, as most CAs investigated in deatil for pharmacological applications contain this cation). The coordination is usually achieved as

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anions (for example the primary sulfonamides bind as sulfonamidates, RSO_2NH^-), monodentately (the metal ion being in tetrahedral geometry) or more rarely, bidentately (the active site cation is in trigonal bipyramidal geometry) (Alterio et al., 2012). At least 25 different chemotypes bind CAs in this way (Table 2) and for most of them there are X-ray crystal structures of complexes of the inhibitors with many human or non-human enzymes belonging to the α-, β-, γ- and ζ-CA classes (around 1000 different crystallographic structures were deposited in the Protein Data Base) (Alterio et al., 2012; Angeli et al., 2019; Costa et al., 2016; De Simone and Supuran, 2012; De Simone et al., 2013; Tanini et al., 2020). The primary sulfonamides and their isosteres (sulfamates and sulfamides) are by far the most investigated such CAIs (Supuran, 2021b), also due to their pharmacological activity and clinical use for decades, see later in the text;

(ii) inhibitors that anchor to the metal ion coordinated water molecule/hydroxide ion. The phenol was the first inhibitor for which this mechanism has been reported for hCA II (Simonsson et al., 1982; Nair et al., 1994), followed thereafter by many other chemotypes, such as polyamines (Carta et al., 2010), sulfocoumarins (Tars et al., 2013) and all other derivatives mentioned in Table 2, second column, for which X-ray crystal structures are available mainly in adduct with hCAs (Nair et al., 1994; Carta et al., 2013; Supuran, 2016b). These 7 chemotypes possess an anchoring moiety (e.g., phenol/alcohol OH, primary amine, sulfonate, etc.) which hydrogen bonds to the solvent molecule coordinated to the metal cation and participates in other favorable interactions with residues from the enzyme active site (Supuran, 2016b; D'Ambrosio et al., 2020);

(iii) the third CA inhibition mechanism, the occlusion of the active site entrance, has been reported in 2009 (Maresca et al., 2009) for coumarins, which have been shown to act as "prodrug inhibitors" of hCAs, undergoing an active site mediated hydrolysis of the lactone ring, followed by the binding of the obtained 2-hydroxy-cinnamic acids at the entrance of the active site (crystal structures of two different coumarins bound to hCA II were reported so far) (Maresca et al., 2009; 2010). Other CAIs of this type were thereafter reported (see the third column of Table 2), most of which are coumarin

derivatives, but also monocyclic (thio)lactones (Carta et al., 2012); isocoumarins (Onyılmaz et al., 2022; 2024); homocoumarins (Grandane et al., 2020), phosphocoumarins (Pustenko et al., 2023) and also the antiepileptic drug lacosamide (Temperini et al., 2010), which although not structurally related to the coumarins, binds in the same active site region as the hydrolyzed coumarins, as demonstrated by kinetic and X-ray crystallographic studies (Temperini et al, 2010);

(iv) the fourth inhibition mechanism was observed only for one compound, and it was named "out of the active site binding" (D'Ambrosio et al., 2015). Indeed, 2-benzylsulfinylbenzoic acid was observed bound out of the active site of hCA II, in an adjacent hydrophobic pocket near the entrance to the active site, position in which allows the inhibitor to participate to hydrogen bonds, through a bridging water molecule, with His64, the proton shuttling residue of this enzyme, thus leading to the collapse of the catalytic cycle, as demonstrated again by kinetic and crystallographic studies (D'Ambrosio et al., 2015).

Thus, more than 40 different chemotypes (Table 2) show significant CA inhibitory properties against enzymes belonging to all 8 genetic families, since a large number of representative enzymes belonging to various classes (including the ι-CAs) were investigated in detail for their inhibition with different such classes of inhibitors (Supuran 2008; 2016a,b; 2023a). It is interesting to note that for the ι-CAs, Hirakawa et al (2021) observed by X-ray crystallography bicarbonate and iodide (anion inhibitors of metallo-CAs) bound in the enzyme active site, although no metal ion is present in this class of enzymes, as mentioned above. Furthermore, other studies demonstrated that both inorganic/organic anions and sulfonamides, do inhibit bacterial ι-CAs, although the inhibition mechanism is not well understood at the moment (De Luca et al., 2021; Petreni et al., 2021).

It should be also mentioned that there are inhibitors for which the CA inhibition mechanism is unknown (Supuran, 2016b). They include some tertiary sulfonamides (Le Darz et al., 2015), probenecid amide derivatives (Carradori et al., 2015) and kinase inhibitors such as imatinib or nilotinib (Parkkila et al., 2009), for which solution (kinetic) studies revealed relevant inhibitory

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activity (against hCAs of physiological relevance) but no X-ray crystal structures of such inhibitors bound to any CAs are available to date.

Not all CA inhibitory chemotypes of Table 2 were investigated for their detailed pharmacological activity in cell or animal models so far, but some of them, among which the primary sulfonamides, sulfamates and sulfamides, several dithiocarbamates and benzoxaboroles, phenols/catechols and coumarins are present in clinically used drugs interacting with these enzymes and they will be discussed in detail in the next sections.

4. The tail approach for obtaining isoforms-selective CAIs

Sulfanilamide (4-amino-benzenesulfonamide) was the first organic CAI ever discovered (Mann and Keilin, 1940). Other aromatic and heterocyclic (primary) sulfonamides were soon thereafter investigated, being noted that heterocyclic derivatives were more effective CAIs compared to aromatic compounds (Krebs, 1948). The first drug design studies in the field were shortly thereafter reported (Miller et al.,1950) which led to the discovery and launch of the first clinically used CAI diuretic, acetazolamide in 1954, and in the next years of the other first generation sulfonamides (Maren, 1967) that will be discussed in the Diuretics/Antiglaucoma sections shortly. However, all drugs of the first (and actually also second) generation of CAIs were aromatic/heterocylic sulfonamides incorporating simple and compact scaffolds that assured a potent binding to all known CA isoforms, but as thus, they were pan-inhibitors, presenting a large number of side effects when used systemically (Maren, 1967; Supuran, 2008). This considerably limited the use of the entire class of such pharmacological agents which were considered problematic drugs, with a too wide range of undesired side effects, due to the inhibition of CAs in many organs, other than the target one (Maren, 1967; Wistrand, 1984). Thus, the need to design isoform-selective CAIs was stringent in the late '90s, when an increasing number of human isoforms was constantly being discovered and

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the available CAIs at that time were inhibiting undiscriminately most of them (Lindskog, 1997; Supuran and Scozzafava, 2000).

Fig. 4. here

The tail approach thus emerged as a solution for resolving the problem of obtaining isoform-selective CAIs for all human isoforms (Scozzafava et al., 1999a,b). The original idea was to obtain elongated molecules (actually primary sulfonamides) which could interact not only with the bottom and middle parts of the CA active site (similar to the clinically used agents, e.g., acetazolamide, methazolamide), but also with the entrance in the cleft (Fig. 4A). The attachment of moieties named "tails" on the scaffold of simple aromatic/heterocyclic sulfonamides (easily derivatizable at their amino or hydroxyl moieties) may thus induce not only the desired physico-chemical properties (e.g., enhanced hydro- or liposolubility to the CAI) but also the possibility that the tail interacts with the middle and outer rim (the entrance) of the active site cavity (Scozzafava et al., 1999b). It has been known for a long time that the bottom and partially also the middle parts of the hCA active sites are rather conserved among the isoforms, with many identical amino acid residues being involved in the catalytic and inhibition mechanisms (Lindskog, 1997). However, the rim part of the active site is more variable among the different hCAs, differentiating them in their interaction with modulators of activity, as shown by a large number of solution and crystallographic studies (Scozzafava et al, 1999a,b; Menchise et al., 2005; Algterio et al., 2006; Di Fiore et al., 2007; Winum et al., 2006). Indeed, the rather high variability of many amino acid residues in those parts of the active site make possible favorable or clash interactions between the inhibitor and the enzyme, potentially leading to isoform-selective inhibitors, as again demonstrated in a multitude of kinetic and crystallographic studies on various human isoforms (Alterio et al., 2012; Pacchiano et al., 2010; 2011; Winum et al., 2006). Detailed kinetic (and in some cases X-ray crystallographic) studies were thereafter performed on over 10,000 different sulfonamide derivatives obtained according to the tail approach in our and many other laboratories worldwide, which demonstrated

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not only that the initial hypothesis was correct but also that it is a facile and versatile modality to generate a large number of CAIs by tailoring the dimensions (length, bulkiness, etc.) as well as the chemical nature of the various tails, leading to compounds with a good selectivity for all human isoforms of interest (reviewed in Supuran, 2023a and Kumar et al., 2022). Furthermore, the tail approach was shown to be extendable to many other chemotypes apart the sulfonamides acting as CAIs (Table 2), both belonging to the metal ion binders or to the inhibitors which anchor to the metal ion coordinated water molecule (Supuran. 2023a). As the active sites of many CA isoforms are rather spacious, the idea to introduce motre than one tail, of different natures (e.g., one hydrophobic and one hydrophilic; two hydrophobic, two hydrophilic, more than two, etc.) appeared of interest, and resulted in highly effective and isoform-selective CAIs, by the so-called "two tails" (Tanpure et al., 20155; Fares et al., 2020) – see Fig. 4B and "three tails approaches (Bonardi et al., 2020;2022) – Fig. 4C. After its discovery in 1999 (Scozzafava et al., 1999b), the tail approach and its spinoffs (two- and three tails) was virtually the only drug design strategy used to obtain CAIs, being adopted by research groups all over the world (Supuran, 2023a; Kumar et al., 2022). For space reasons the many hundreds of such drug design strategies and the huge number of effective CAIs thus obtained, cannot be mentioned here.

5. Classical pharmacological applications of the CAIs used clinically

In this section I will review the classical pharmacological applications and the available drugs with CA inhibitory action, acting on different isoforms and possessing thus various actions, as diuretics, antiglaucoma/ocular drugs, antiepileptics, antiobesity agents, drugs for the management of acute mountain sickness, or idiopathic intracranial hypertension agents. I will therefater deal in the next section with the latest developments in the field, i.e., the developments of CAIs acting as antitumor agents targeting hypoxic tumors as well as "less classical" applications of these pharmacological agent, in the management of neuropathic pain, cerebral ischemia and some forms of inflammation.

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5.1. Diuretics

Acetazolamide (compound **1**, Fig. 5) was the first modern diuretic ever, being launched for clinical use in 1954, replacing the rather toxic mercurials employed till then (Maren, 1967). It is still a clinically used compound, as it will be mentioned in the review, albeit more rarely as a diuretic, nowadays. However, it was the lead molecule which led to the discovery of many widely used diuretics, such as the thiazides **2**, the thiazide-like drugs **3-6** as well as the high-ceiling diuretics furosemide **7**, azosemide **8** and bumethanide **9** (Fig. 5), all of which incorporate the primary sulfonamide moiety, responsible for CA inhibition (Supuran, 2021b; Ferraroni et al., 2022b; Temperini et al, 2008;2009; Carta and Supuran, 2013).

The diuretic effects of acetazolamide are due to the inhibition of renal CA isoforms, since both cytosolic (CA I, II) as well as membrane-bound (CA IV and XII) such enzymes are abundant in all segments of the nephron, being involved in urine acidification (Maren, 1967; Wistrand, 1980). When the renal enzyme is inhibited, an increased amount of bicarbonate is excreted, which leads to urine alkalinization, together with an increased loss of Na^+, K^+ and osmotically obligated water (Garvey and Maude, 1981) with the concomitant instauration of a mild metabolic acidosis. This phenomenon is due to the fact that bicarbonate eliminated in the urine, is formed from CO_2 in equivalent amounts with H^+ ions in the CA catalyzed process, and the acid is taken by the blood, leading thus to acidosis (Maren, 1967; Tsikas, 2024). This is also the reason why the diuretic effect of acetazolamide alone is low and tolerance develops in few weeks, although the precise mechanisms by which this occurs is still debated (Maren, 1967; Tsikas, 2024). However, very recently, this diuretic has been "resurrected", since it has been observed that the combination of acetazolamide and high ceiling diuretics of types **7-9** was synergistic and efficient in the management of patients with acute decompensated heart failure (Mullens et al., 2022). This randomized, multicenter, double-blind, placebo-controlled trial, demonstrated that in patients

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suffering of acute decompensated heart failure, a condition with few therapeutic options, the i.v. administration of acetazolamide plus the loop diuretics mentioned above, was more efficient in decongestion of the patients, compared to each drug alone, leading also to a higher cumulative natriuresis and urine output (Mullens et al., 2022). These findings were confirmed in other studies (Lim, 2022; Cuthbert and Cleland, 2023; Meekers et al., 2023) demonstrating thus the clinical utility and "renaissance" of this old diuretic. This discovery is not only clinically relevant, but also counter-intuitive, since it has been considered for a long period that the combination of acetazolamide with other sulfonamide diuretics (thiazides, loop diuretics, etc.) may lead to serious drug-drug interaction manifested by electrolyte abnormalities and enhanced metabolic acidosis (Supuran, 2020a; 2024).

Fig. 5 here

It is well-known that the sulfonamide drugs **2-9** (Fig. 5) exert their diuretic action by targeting other proteins than the CAs (e.g., Na^+/Cl^- transporters, $Na^+/K^+/Cl^-$ cotransporters, such as NKCC1, etc.) (Supuran, 2018b,c), but they also possess significant inhibitory action against many CA isoforms (Temperini et al., 2008,2009), in some cases in the low nanomolar range (see Supuran, 2008, for the precise K_I values of all drugs discussed here against all human isoforms) which might be useful for their repurposing for other therapeutic applications, such as antitumor agents, as already proposed for some of them, e.g., benzthiazide (Lee et al., 2012).

It has recently been demonstrated that acute ingestion of acetazolamide **1** (250 mg) temporarily increased also the excretion of two amino acids (Leu and Ile), not only of bicarbonate and cations, as well as that of nitrite and nitrate, with a concomitant decreased excretion of other amino acids (Tsikas, 2024). In the same study, acetazolamide was shown to decrease the urinary excretion of malondialdehyde, a biomarker of oxidative stress (Tsikas, 2024). The urine alkalinization induced by acetazolamide is on the other hand useful also for the management of kidney injury in patients treated with methotrexate (Reed et al., 2019; Truong et al., 2024), for the reduction of lithium-

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induced nephrogenic diabetes insipidus (de Groot et al., 2016), for the management of cystinuria (Tiselius, 2010), or in patients with uric acid/cystine kidney stones (Sterrett et al., 2008).

5.2. Antiglaucoma agents and other applications of CAIs in ocular diseases

Glaucoma, an eye condition characterized by elevated intraocular pressure (IOP) is a neuropathy leading to blindness if untreated (Mincione et al., 2021). CAIs constitute a cornerstone in its ttreatment, starting with the '50s, when it has been discovered that aqueous humor in the anterior chamber of the eye is rich in bicarbonate, which in turn is generated through the activity of ocular CAs (isoforms I, II, IV, XII), highly abundant in the ciliary processes (Wistrand, 1951; Maren, 1967).

Fig. 6 here

Inhibition of CA isoforms within the ciliary processes of the eye (with acetazolamide **1**) has been shown to decreased bicarbonate formation in the anterior chamber (Becker, 1955) which was responsible of the pharmacologic effect of decreased IOP, with **1** and several other CAIs approved for clinical use in the first decades of CA research, e.g., methazolamide **10**, ethoxzolamide **11** and dichlorophenamide **12** – Fig. 6 (Kinsey and Reddy, 1955; Maren, 1967; Carta et al., 2012; Masini et al., 2013; Bua and Supuran, 2019). The lowered IOP is beneficial for the patients, as the damage of the optic nerve is directly proportional to the high pressure in the eye (Mincione et al., 2021). Drugs **1** and **10-12** are effective only by systemic administration as antiglaucoma agents, due to their improper physico-chemical properties, among which a low water solubility, too low lipophilicity, and the impossibility to be administered as eye drops (Maren 1967, 1997; Supuran, 2008). Methazolamide **10**, ethoxzolamide **11** and dichlorophenamide **12** are highly effective *in vitro* CAIs (similar to acetazolamide **1**) but are more lipophilic compared to acetazolamide and also show more CNS side effects compared to acetazolamide, due to their easier penetration through membranes

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(Maren, 1967). However, like **1**, they are also administered only systemically in order to exert their ocular pharmacological activity. This administration modality led to serious side effects due to the inhibition of CAs in other organs than the eye, such as the gastro-intestinal tract, kidneys, lungs, blood, etc. (Maren 1967, 1997; Supuran, 2008) limiting thus their usefulness. In the '90s, the second generation antiglaucoma agents dorzolamide **13** and brinzolamide **14** (Fig. 6) have been developed, which are water-soluble, can be administered as eye drops, and are efficient IOP lowering agents (Ponticello et al., 1998; Silver, 1998). Both drugs are used alone or more frequently in combination with other antiglaucoma agents, such as β-blockers, α-adrenergic agonists, prostaglandin receptor antagonists, rho-kinase inhibitors or nitric oxide donors (Angeli and Supuran, 2019; Berrino and Supuran, 2019; Nocentini and Supuran, 2019; Mincione et al., 2021; Supuran et al., 2019). Thus, acetazolamide **1** and the remaining first generation CAIs **10-12** are no longer used as systemic drugs for the treatment of glaucoma, being replaced by the topically-acting agents dorzolamide **13** and brinzolamide **14**, but there are many recent reports highlighting their usefulness (alone, or more frequently in combination with other drugs) for the management of other ocular diseases, among which X-linked retinoschisis (Wey et al., 2023); macular edema secondary to retinal vein occlusion, in combination with the VEGF drug bevacizumab (Karimi et al., 2023); macular edema, in combination with topical NSAIDs (Aljundi et al., 2022) as well as age-related macular degeneration (Supuran, 2019), cases in which antagonistic interactions between these CAIs and NSAIDs did not manifest (Supuran, 2020a;2024).

5.3. Antiepileptics

Acetazolamide **1**, sulthiame **15**, topiramate **16** and zonisamide **17** (Fig. 7) are clinically used agents for the management of several types of epilepsy (Supuran, 2021b).

Fig. 7 here

Acetazolamide **1** has a long story in the treatment of epilepsy, dating back to the '50s (Merlis, 1954) although its mechanism of action and its efficacy in various epilepsy forms were and are rather controversial (Ansel and Clarke, 1956; Oles et al., 1989; Reiss and Oles, 1996). pH regulation in the brain is potently correlated with the neuronal function, and since 11 CA isoforms are present in the CNS (Table 1), their inhibition/activation as well as the activity of other voltage- and ligand-gated ion channels/gap junctions, may synergistically lead to pH changes which affect neuron excitability (Ruusuvuori and Kaila K, 2014). Indeed, alkaline shifts were shown to induce an increase in excitability which may trigger epileptiform activity, whereas acidosis had the opposite effect (Ruusuvuori and Kaila K, 2014). The highly CNS-abundant cytosolic isoforms hCA II and VII, as well as the transmembrane hCA XIV (Table 1) are considered the most probable targets on which CAIs act exerting an antiepileptic activity (Thiry et al., 2008; Aggarwal et al., 2013; De Simone et al., 2009). Indeed, it is ascertained that acetazolamide is effective in patients with refractory epilepsy to other medical treatments (Oles et al., 1989; Reiss and Oles, 1996; Aribi and Stringer, 2002). Sulthiame **15** is a highly potent CAI for all brain CA isoforms (Leniger et al., 2002; Temperini et al., 2007) and was shown to interfere with the pH regulation in CNS inducing a modest but pharmacologically significant intracellular acidosis of central neurons (Leniger et al., 2002) together with CO_2 retention, which probably also interferes with pH regulation in this organ, and explains its antiepileptic effect (Shi et al., 2017). Topiramate **16**, a sulfamate and zonisamide **17**, an aliphatic sulfonamide (Fig. 7), are two newer, widely used antiepileptics which show significant CA inhibitory effects (Leniger et al., 2004; Leppik, 2004; Thiry et al., 2008; Aggarwal et al., 2013; De Simone et al., 2009). However, both of them have a rather complex pharmacology (similar to many other antiepileptics), since their anti-seizure effects are due to interaction with other targets apart the brain CAs (Leniger et al., 2004; Leppik, 2004; Thiry et al., 2008), among which voltage-gated sodium channels, high-voltage-activated calcium channels, $GABA_A$ receptors, AMPA/kainate receptors, for topiramate (Leniger et al., 2004) whereas for zonisamide sodium and T-type calcium channels (Leppik, 2004). The diuretic bumethanide **9** (Fig. 5) was also shown

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recently to possess antiepileptic action in neonatal seizure (Kaila and Löscher, 2022) although, as for the previously discussed agents, it targets not only brain CAs but also the Na-K-2Cl cotransporter, NKCC1. These drugs and other antiseizure CAIs, primarily sulfonamides and carbamates acting by inhibiting the CNS isoforms CA II, VII and XIV (Bibi et al., 2019; Odi et al., 2021; Mishra et al., 2021) were investigated experimentally in various seizure models, leading to effective anticonvusant effects. However, similar to many other antiepileptics, CAIs **1, 9, 15-17** may show a considerable number of drug-drug interactions with other antiepileptics and different other pharmacological agents (Arman and Haghshenas, 2022; Ji et al., 2022; Pan et al., 2021; Sarayani et al., 2023; Willems et al., 2023).

5.4. Antiobesity agents

Obesity is considered nowadays as a chronic, degenerative disease, characterized by excessive fat accumulation, and constitutes a challenging medical problem worldwide, with a large number of affected people and few available efficient drugs, which in addition possess a large number of side effects (Müller et al., 2022; Supuran, 2022). CAs were demonstrated to possess a relevant role in several metabolic processes in various cells, including fatty acid biosynthesis and *de novo* lipogenesis (DNL) (Supuran, 2022). Both fatty acid biosynthesis and DNL involve mitochondrial and cytosolic steps, in which participate several enzymes implicated in the Krebs cycle as well as DNL, such as pyruvate carboxylase (PC) and acetyl-coenzyme A carboxylase (ACC), which both employ bicarbonate and not CO_2 as substrate (De Simone et al., 2008; Supuran, et al., 2008; Supuran, 2012; Scozzafava et al., 2013). In order to achieve the rapid interconversion between these two species (CO_2 and bicarbonate), highly catalytically active CA isoforms (among which hCA II in the cytosol and hCA VA/VB in the mitochondria) are needed (Alldred and Reilly, 1997; Atwood, 1995). It has been demonstrated already in the 90s that these CAs participate in biosynthetic pathways, also being observed that inhibition of mitochondrial/cytosolic CAs interferes with both

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fatty acid biosynthesis and DNL in various cells, tissues and animal models (Lynch et al., 1995; Chegwidden and Spencer, 1996; Hazen et al., 1996). The metabolism of Krebs cycle intermediates, such as pyruvate, acetate, and succinate were examined thereafter in the presence of specific sulfonamide hCA VA/B – selective inhibitors using electrode wired mitochondria, and measuring metabolic energy conversion (Arechederra et al., 2013). Several potent, nanomolar hCA VA/B inhibitors showed a broad spectrum inhibition of metabolism, whereas others only had significant effects on pyruvate and fatty acid metabolism, but such data conclusively demonstrated the crucial role of mitochondrial CAs in the biosynthesis of fatty acid, and as thus in lipogenesis. Evenso, the idea to use CAIs for obtaining antiobesity drugs started to be considered only in 2000s (Antel and Hebebrand, 2012) by examining weight loss side effects of some antiepileptics, such as topiramate and zonisamide (compounds **16** and **17**, Fig. 7). The use of **16** and **17** (and to a lower extent also of acetazolamide **1**) as anti-obesity drugs may be thus considered as one of the first example of drug repurposing among clinically used CAIs (Supuran, 2022). The use of such CAIs alone or in combination with other agents (phentermine + topiramate, bupropion + zonisamide, metformin in combination with both CAIs, etc.) was demonstrated to induce weight loss in many patients, also improving their blood glucose levels (Aronne et al., 2013; Gadde et al., 2003; 2011; Garvey et al., 2012; Lévy et al., 2007; Mahmood et al., 2013; Schneiderhan and Marvin, 2007; Muñoz et al., 2018; Sari et al., 2021).

How do these agents exert their antiobesity effects? Although the pharmacology of **16** and **17** is rather complex (see discussion in the previous section), both of them and also acetazolamide **1**, are effective CAIs against hCA isoforms involved in fatty acid biosynthesis/DNL, exerting probabaly in this way their antiobesity action (Supuran, 2022). The increased use of topiramate (and zonisamide) as antiobesity agents in the last 5 years also led to the observation of side effects typical of systemic CAIs, such as abnormalities in the serum bicarbonate concentration (Naps et al., 2023); dysgeusia with carbonated drinks, presumably due to salivary CA VI inhibition

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(Charbonneau et al., 2020), as well as type II renal tubular acidosis (Chahin et al., 2020). However, several different studies demonstrated that the elevated glucose-induced mitochondrial respiration followed by formation of reactive oxygen species (ROS), typical of type II diabetes, may be reversed by pharmacological inhibition of mitochondrial CAs with topiramate **16**, in a mouse cerebral pericytes model of the disease, allowing thus for potential therapeutic applications of the CAIs in other metabolic disorders, e.g., diabetes (Gamal et al., 2024; Shah et al., 2013; Salameh et al., 2019).

5.5. Acute mountain sickness drugs

Acute mountain sickness commonly affects high altitudes travelers, with debilitating symptoms such as dizziness, headache, insomnia, nausea, anorexia, dyspnea, vomiting, peripheral edema, and retinal hemorrhage (Bradwell et al., 1992; Porcelli and Gugelchuk, 1995). Pulmonary and/or cerebral edema may also be rather common in severe cases. The reduced oxygen supply at high altitudes seems to be the triggering event of this condition, which induces hypoxemia and hypoxia in many tissues and organs. CA inhibition has been reported several decades ago to represent one of the few useful therapeutic approaches for the management of acute mountain sickness (Bradwell et al., 1992). The usefulness of CA inhibition in the treatment of this condition is not fully understood, but the diuresis and bicarbonate excretion induced by CAIs, such as acetazolamide **1** or methazolamide **10**, leading also to a mild metabolic acidosis, might explain the phenomenon (West, 2004). In addition, CA inhibition in peripheral chemoreceptors mediates ventilatory optimization by receding the hypoxic and hypercapnic sensitivity (Ainslie et al., 2013). This also leads to an alteration in cerebral blood flow, that in turn is responsible for the control of cerebral oxygenation, leading to the relieve of many symptoms associated with this condition, which may become life-threatening if not treated (Teppema et al., 2007). It has been also demonstrated that cerebral intraventricular administration of acetazolamide **1** induced an enhancement of cerebrospinal fluid

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(CSF) bicarbonate concentrations, with consequences in the modulating of CSF pH values during respiratory acidosis in experimental animals (Kazemi and Choma, 1977). Acetazolamide **1** was among the most popular and well-studied drug for the management of this condition (Carlsten et al., 2004), but methazolamide **10** seems to be even more effective due to its increased lipophilicity (Doherty et al., 2023). At 250 mg/day acetazolamide **1** significantly reduced symptoms of high altitude sickness (Carlsten et al., 2004; Burtscher et al., 2016) whereas methazolamide may be used at a lower dosage of 100 mg/day (Doherty et al., 2023; Swenson, 2022).

5.6. Idiopathic intracranial hypertension agents

Idiopathic intracranial hypertension (IIH) is characterized by an increased intracranial pressure in the absence of brain tumors, condition that occurs through a poorly understood mechanism, but presumably involves cerebrospinal fluid (CSF) secretion, process in which several brain CA isoforms are involved (Maren, 1967; Supuran, 2015; Uldall et al., 2017). Acetazolamide **1** is known for decades to constitute one of the few effective therapies for its treatment (Maren, 1967). Acetazolamide, a low nanomolar inhibitor of all CA isoforms involved in CSF formation, e.g., hCA II, IV, VA and XII (Supuran, 2008) leads to a decreased CSF secretion and an efficient control of the intracranial pressure in many of the affected patients after systemic administration (Maren, 1967; Supuran, 2015; Uldall et al., 2017). Apart **1**, currently used for the treatment of IIH (although it has many side effects, as mentioned above), the more lipophilic CAIs methazolamide **10** (Nia et al., 2022) and topiramate **16** (Goyal and Zarroli, 2023) were recently shown to lead to an even more effective control of the increased intracranial pressure compared to **1**, presumably due to their enhanced lipophilicity compared to acetazolamide, and easier access to the brain enzymes. The combination of acetazolamide **1** with glucagon-like peptide-1 (GLP-1) receptor agonists, such as semaglutide, tirzepatide, and other similar drugs, was on the other hand very recently shown to be synergistic [118] and to be more effective for the management of IIH than **1** alone, also leading to a

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significant weight loss, which is a beneficial "side effect" for patients suffering of this disease, who frequently are obese (Krajnc et al., 2023). Thus, there seem to be highly synergistic interactions between CAIs and GLP-1 mimic drugs, which need to be studied in more detail, considering that these are relatively new drugs, highly effective for the manegement of diabetes and obesity, but their safety profile and especially drug-drug interactions are poorly investigated at the moment (Supuran, 2024a).

6. Pharmacological applications of CAIs in clinical trials/preclinical development

6.1. Antitumor agents targeting hypoxic tumors

Connection between CA inhibition and cancer started to emerge in the '90s, when Teicher et al (1993) showed that acetazolamide **1** may sensitize cancer cells to other chemotherapeutic agents, whereas other researchers observed that this sulfonamide inhibits the growth of gastric cancers in patients treated with high doses of the drug for the management of ulcers (Puscas et al., 1994). Such reports were considered anecdotal until 1994 when a cancer-associated CA isoforms, CA IX has been discovered (Pastorek et al., 1994), followed by the report of a second such enzyme, CA XII, in 1998 (Tureci et al., 1998). Both of them are multi-domain transmembrane isoforms with the CA domain situated outside the cell, are present in many tumors and possess a rather limited distribution in normal tissues (Kivelä et al., 2000; Leppilampi et al., 2003; McDonald et al., 2020, 2022; Angeli et al., 2020; Supuran, 2020b). Both proteins are under the control of a transcription factor, hypoxia inducible factor (isoforms HIF-1 or HIF-2), which, by acting as O_2 sensors, induce CA IX/XII overexpression in hypoxic tumors as a consequence of an activation cascade which upregulates several proteins involved in pH regulation, metabolism, angiogenesis, ferroptosis and other physiologic/pathologic processes (Semenza, 2019; Neri and Supuran, 2011; Pugh and Ratcliffe, 2017; Pettersen et al., 2015; Chafe et al., 2021). As shown schematically in Fig. 8, CA IX (and also CA XII, which is however less widespread in tumors compared to isoform IX) is involved

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not only in intra-/extracellular pH regulation and metabolism, but also in survival, proliferation, and migration of the cancer cells, as well as in angiogenesis and ferroptosis, phenomena which cancer cells exploit in order to overcome the Darwinian competition with their normal counterparts and thus, thrive (Semenza, 2019; Neri and Supuran, 2011; Pugh and Ratcliffe, 2017; Pettersen et al., 2015; Chafe et al., 2021).

Work from several laboratories demonstrated that inhibition of CA IX, CA XII or both enzymes, with compounds acting as potent (usually low nanomolar inhibitors of both enzymes), such as derivatives **18-26** shown in Fig. 9, leads to a potent inhibition of growth of primary tumors and metastases, in several cell cultures/animal models of cancer (Svastová et al., 2004; Ahlskog et al., 2009; Pacchiano et al., 2011; Lou et al., 2011; Lounnas et al., 2013; Gieling et al., 2012). As seen from Fig. 9, all types of CAIs (sulfonamides, such as **18, 19, 25** and **26**; sulfamates, such as **22-24**, and coumarins, such as **20** and **21**) possess significant antitumor activity (reviewed in Supuran, 2020b, 2021, 2023d).

Figs. 8 and 9 here

CA IX/XII inhibition with such compounds (and also with monoclonal antibodies, mAbs, which will be not discussed here, as they were reviewed recently, see Supuran, 2020b) leads to interference with pH regulation (Neri and Supuran, 2011; Svastova et al., 2004), affects the metabolism of the cancer cells, as less bicarbonate is available for biosynthetic reactions (Santi et al., 2013), enhances ferroptosis (Chafe et al., 2021) and also diminishes the number of cancer stem cells (Lock et al., 2013), all antitumor/antimetastatic mechanisms which explain the significant biological effects observed with them. One of the main hurdles in validating CAIs as antitumor/antimetastatic agents was the lack of CA IX/XII-selective inhibitors till recent dates (Kumar et al., 2022; Supuran, 2023a; Angeli and Supuran, 2023; Bendi et al., 2024). However, by using the tail approach (discussed above) and the many new chemotypes with CA inhibitory action discovered (e.g., coumarins, benzoxaboroles, etc.), many highly isoform-selective such compounds

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were obtained (Supuran, et al., 2018), among which the sulfonamide **19** (SLC-0111), which is one such interesting example, acting as a low nanomolar hCA IX/XII inhibitor, without potently inhibiting the remaining hCAs (Pacchiano et al., 2010,2011). SLC-0111 is the first new generation CAI to progress to clinical trials for the management of hypoxic metastatic tumors (Table 3) (McDonald et al., 2020).

Table 3 here

In fact apart SLC-0111, the pan-inhibitors acetazolamide **1**, and benzolamide (in combination with various other anticancer drugs, see Table 3) are nowadays investigated in several clinical trials, but as they are strong inhibitors of all human CAs, most of the preclinical and clinical work has been performed with compound **19** (Andreucci et al., 2023; Boyd et al., 2017; Eloranta et al., 2023; Grajek and Poleszczuk, 2023; McDonald et al., 2019; 2020; 2022; McDonald and Dedhar, 2023; Mussi et al., 2022; Peppicelli et al., 2020; Sarnella et al., 2022). SLC-0111 is nowadays in Phase Ib/II clinical trials for pancreatic cancer in combination with gemcitabine (NCT03450018) but several other Phase II clinical trials are scheduled for the near future. The potential of CAIs for the imaging of hypoxic tumors is also relevant, and the field has been reviewed recently and will be not discussed in detail here (see Burianova et al., 2021; Nerella et al., 2023).

It should be also mentioned that a large number of CA IX/XII inhibitors were reported by several groups worldwide in the last decade, which used SLC-0111 as a lead molecule, but they are not discussed here as they were reviewed recently (Kumar et al., 2022; Bendi et al., 2024; Elsawi et al., 2023). Nowadays there is a large number of CAIs (mainly sulfonamides, coumarins and sulfocoumarins) for which significant antitumor effects were proved in many cell/animal models, and presumably some of them will enter in clinical trials soon.

It should be mentioned that several groups reported aplastic anemia-like syndromes in patients with various malignancies (Lakota et al., 2012; Jankovicova, 2013; Menteşe et al., 2015). In all cases it has been discovered that an immune response against red blood cell isoforms hCA I

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and II developed, leading to auto-antibodies against these isoforms which interfere with their physiolopgical function, whereas isoforms CA IX and CA XII seem to be not implicated in these forms of anemia (Lakota et al., 2012; Jankovicova, 2013; Menteşe et al., 2015).

6.2. New pharmacological applications of CAIs

In the last decade, several proof-of-concept studies have emerged on the applications of CAIs in conditions usually not associated with CA modulation, such as neuropathic pain (Supuran, 2016c), cerebral ischemia (Bulli et al., 2021), rheumatoid arthritis (Bua et al., 2017), neurodegenerative conditions, such as Alzheimer's (Provensi et al., 2019) and Parkinson's diseases (Murata et al., 2020) and more generally, oxidative stress (Del Giudice, 2013).

6.2.1. Neuropathic pain

Advances in understanding pathological mechanisms related to neuropathic pain, for which few therapies are available (Supuran, 2016c) and the biochemical/pharmacological characterization of novel drug targets, provided evidence that CA inhibition might be a novel approach for obtaining agents useful for the management of this condition (Assiedu et al., 2010; Carta et al. 2015). The rationale for using CAIs for the management of neuropathic pain is based on the fact that peripheral nerve injury negatively influences spinal GABA-ergic networks via a reduction in the neuron-specific potassium-chloride (K^+-Cl^-) cotransporter (KCC2) which induces neuropathic allodynia (Assiedu et al., 2010). CAIs inhibit enzymes present in these tissues and reduce the bicarbonate-dependent depolarization of GABA$_A$ receptors by diminishing the production of bicarbonate in SNC and peripheral nervous system, thus leading to an analgesic effect (Assiedu et al., 2010). hCA II and VII are most probably the enzymes involved in these phenomena, both at central and peripheral levels (Supuran, 2016c; Carta et al., 2015). Sulfonamide hCA II/VII inhibitors such as

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derivative **27** (Fig. 10) showed efficacy in animal models of neuropathic pain (Carta et al., 2015), better than acetazolamide **1** (Assiedu et al., 2010), putting the basis for the development of a specific therapy based on selective hCA II/VII inhibition. Many other CAIs apart **27** were investigated to date for their efficacy in the management of neuropathic pain, with promising and long lasting effects being noticed (Angeli et al., 2023), but no derivative reached clinical trials at the moment.

6.2.2. Cerebral ischemia

The current pharmacological options for cerebral ischemia are few and have a limited impact on ischemic damage. Hypoxia, which is a consequence of the ischemic event, triggers overexpression of isoforms hCA IX/XII (as discussed above), and their inhibition was recently demonstrated to represent a new possibility for the management of this condition (Di Cesare Manelli et al., 2016; Bulli et al., 2021). The pharmacological evaluation of several sulfonamide and coumarin CAIs (such as compounds **28** and **29,** Fig. 10) in an animal model of cerebral ischemia, i.e., rats with a permanent middle cerebral artery occlusion (pMCAO), afforded the proof-of-concept study that CA IX/XII inhibition is useful to treat ischemia, since the neurological score of pMCAO rats was dramatically reduced 24 h after occlusion, and repeated subcutaneous injections of CAIs **28** or **29** (at 1 mg/kg) increased the neurological score by 40% (Di Cesare Manelli et al., 2016). Coumarin **28,** acting as a low nanomolar hCA IX/XII-selective inhibitor also reduced the volume of hemisphere infarction, whereas acetazolamide **1** used as standard drug was completely ineffective. The ability of novel CAIs to improve neurological functionalities after cerebral ischemic events may thus open the path to a novel pharmacologic treatment of the condition (Dettori et al., 2021).

Fig. 10 here

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6.2.3. Rheumatoid arthritis

Margheri et al (2016) reported that isoforms hCA IX and XII are also overexpressed in some forms of arthritis. Thus, the rationale was that inhibition of these enzymes with CAIX/XII-selective inhibitors might provide the the proof-of-concept that these isoforms may be new drug targets for the management of arthritis (Margheri et al., 2016). The drug design involved the synthesis of hybrid compounds incorporating non-steroidal anti-inflammatory drugs (NSAIDs) belonging to the carboxylic acid derivatives (Fig. 10) and sulfonamide or coumarins CAIs (Akgul et al., 2018; Bua et al., 2017) leading to compounds of types **30** and **31**. These hybrids showed effective inhibitory profiles against the target isoforms hCA IX and XII, and had a potent and long-lasting antihyperalgesic effects in a rat model of arthritis and may thus have a future for the management of this disease (Akgul et al., 2018; Bua et al., 2017).

6.2.4. Neurodegenerative diseases and oxidative stress

Fossati's group demonstrated that classical CAIs such as acetazolamide **1** and methazolamide **10** show effectiveness in the prevention of mitochondrial dysfunction, caspase activation and cell death associated with amyloid β (Aβ) formation in animal models of Alzheimer's disease (AD) (Fossati et al., 2016; Solesio et al., 2018). Acetazolamide was 10 times more effective than methazolamide in inhibiting such toxic mitochondrial mechanisms induced by Aβ in neuronal and vascular cells in an *in vitro* model of the disease showing also neuroprotective activity. The two compounds reduced memory impairment and Aβ pathology in a transgenic mouse model of amyloidosis, also ameliorating cerebrovascular health and glial fitness (Canepa et al., 2023; Fossati et al., 2016; Solesio et al., 2018), which led to the proposal of clinical trials to be scheduled for their evaluation in patients with AD (Canepa et al., 2023). Another approach recently proposed consisted in the conjugation of reversible MAO-B inhibitors of the coumarin/chromone types with sulfonamide CAIs, leading to hybrids which showed efficacy in preventing Aβ-related neurotoxicity, reverting

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ROS formation, and restoring mitochondrial functionality in an SH-SY5Y cell model of AD (Giovannuzzi et al., 2024).

Recently, Murata et al. (2020) reported that zonisamide **17** is useful in the management of Parkinson's disease (PD), in combination with levodopa or other PD drugs (Murata et al., 2020; Tsuboi et al., 2021). Zonisamide significantly improved wearing off without exacerbating dyskinesia in patients with PD, presumably due to its inhibitory efects on MAO-B and brain CA isoforms, and several clinical trials nowadays investigate in detail these drug regimens for a neurodegeneration for which few pharmacological options are available in late stages of the disease (Tohge et al., 2021; Odawara et al., 2022).

More generally speaking, some CA isoforms among which hCA III and VII, were shown to be involved in oxidative stress, being associated with inflammatory diseases such as systemic lupus erythematosus, diabetes, hypertensive renal disease, myasthenia gravis, rheumatoid arthritis, all conditions in which the redox state of the cell is perturbed (Del Giudice, 2013). hCA III, although exhibiting a very low CO_2 hydratase activity, possesses a relevant phosphatase activity, is abundant in skeletal muscles, where free radical production is increased during physical exercise, and probably plays a role in shielding cells against oxidative damage and free radicals formation through reaction of an exposed cysteine residues (Cys186) with glutathione (Cabiscol and Levine, 1996). A similar role has been demonstrated for hCA VII, which undergoes S-glutathionylation at two residues, Cys183 and Cys217, without any loss of its high catalytic activity for CO_2 hydration to bicarbonate, being also proposed that the enzyme may function as an oxygen radical scavenger for protecting cells from oxidative stress (Monti et al., 2017; Truppo et al., 2012).

7. Antiinfective CAIs

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As discussed in the Introduction (see Fig. 3 and Table 4), CAs belonging to diverse genetic families are widespread in many organisms, including pathogenic bacteria, fungi, protozoans and invertebrates (nemtaodes, for example) (Capasso and Supuran, 2015; 2023; Supuran, 2021b). In the last decade, several proof-of-concept studies demonstrated the possibility to consider CAs present in such organisms as antiinfective drug targets, with relevant results being obtained mainly for antibacterials and antifungals, with some interesting data being also obtained on antiprotozoan effects of some CAIs.

7.1. Antibacterials

Bacteria encode for four CA classes, the α-, β-, γ- and ι-CAs (Capasso and Supuran, 2015; Nocentini et al., 2021b), but presently only enzymes belonging to the first three classes have been investigated in detail and validated as antiinfective drug targets (Capasso and Supuran, 2024; Supuran, 2021b; 2024b). Table 4 shows some bacterial pathogenic species which produce a range of diseases in humans, some of which mild or realtively easy to be treated, whereas others (among which gonorrhoea, tuberculosis, but also the infections due to *Helicobacter pylori, Enterococcus* spp., *Streptococcus pneumoniae, Pseudomonas aeruginosa*) characterized in the last decades by the emergence of drug resistant strains to most classes of available antibiotics. This leads to significant difficulties in treating the infected patients and a worldwide medical emergency with unpredictable consequences, considering that the phenomenon is in expansion (Salam et al., 2023). In species shown in Table 4 (as well as in many others which will be not discussed here) the various CAs have been biochemically characterized in detail in the last decade, and for some of them effective and selective inhibitors were detected. In few cases, the *in vitro* and *in vivo* validation that bacterial CAs may be alternative antiinfective targets to classical antibiotics, have also been reported (Abutaleb et al., 2022b; Flaherty et al., 2021).

Table 4 here

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N. gonorrhoeae encodes for two CAs, an α- and a β-CA, of which the first one (NgCAα) was the most investigated enzyme. Acetazolamide (**1**), the clinically used sulfonamide CAI discussed extensively in the article was used as starting point in the design of compounds (sulfonamides **32**) targeting this bacterial enzyme– Table 5 (Hewitt et al., 2021; Marapaka et al., 2022).

Table 5 here

Some of the sulfonamides **32** showed low nanomolar activity against NgCAα, and MIC values for the inhibition of the bacterial growth of 0.25 – 4 µg/mL (for the CDC 181 strain of *N. gonorrhoeae*, see Table 5, and for some drug resistant strains, data not shown but available in Hewitt et al., 2021). Furthermore, FDA-approved sulfonamides, such as **1** but also ethoxzolamide **11** (Fig. 6) showed effective *in vitro* inhibition of NgCAα and MIC values in the range of 0.015 – 4 µg/mL against a range of various drug-resistant strains of *N. gonorrhoeae* (Abutaleb et al., 2022a) leading to the proposal of the repurposing of these sulfonamides as antibacterials for the management of this infection. Seleem's group thereafter also demonstrated the *in vivo* efficacy of **1**, using a mouse model of *N. gonorrhoeae* infection (Abutaleb et al., 2022b). Administration of **1** for three days reduced by 90% the gonococcal burden in vagina of infected mice when 50 mg/kg drug was used, and as **1** is a safe, inexpensive and well-known sulfonamide CAI drug, these data represent a strong basis for using such agents in the management of drug-resistant gonorrhea (Abutaleb et al., 2022b). It should be mentioned that the β-CA from this pathogen was less investigated for the moment (as a drug target), but it seems to be involved in the drug resistance of the pathogen to many classes of antibiotics (Rubin et al., 2023) and presumably detailed investigation will be performed soon on its inhibition.

Helicobacter pylori is a Gram-negative neutralophilic bacterium, considered to be the principal cause of gastric ulcer, gastritis and gastric cancer (Buzas and Birinyi, 2023). It is adapted to live in the highly acidic environment of the stomach, and it was observed already more than two decades ago to encode for three CAs belonging to the α-, β- and γ-classes, but so far, only HpCAα

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and HpCAβ were cloned and investigated in detail (Campestre et al., 2021; Nishimori et al., 2006, 2007). Acetazolamide **1** has been used for at least two decades as an antiulcer drug by Puscas and his group starting with the '70s, with the rationale that inhibition of gastric mucosa CAs induces a reduction of gastric acid secretion (Puscas, 1984; Buzas and Supuran, 2016). However, decades later, it has been demonstrated that the antiulcer effects are due to the inhibition of the bacterial enzymes HpCAα/β (not of the human ones present in the gastric mucosa), which impaires the ability of the bacterium to survive in the acidic environment within the stomach (Marcus et al., 2005; Buzas and Supuran, 2016). HpCAs are involved together with the urease in the acclimation of *H. pylori* in the acidic environment in which it lives, whereas the inhibition of these enzymes disturbs the process, leading to the death and decolonization of the stomach from the pathogen (Marcus et al., 2005; Buzas and Supuran, 2016). Roujeinikova's group evaluated ethoxzolamide **11** on various *H. pylori* strains *ex vivo*, in cell cultures, in order to understand whether drug resistance to this sulfonamide may emerge after repeated passages (Modak et al., 2019; Rahman et al., 2020). For strains SS1 and 26695, it has been observed that resistance did not develop easily, and a quite low rate of spontaneous resistance acquisition of $< 10^{-8}$ has been reported (Modak et al., 2019). Acquisition of resistance was associated with mutations in three genes in strain SS1, and six different genes in strain 26695, but the bacterial CA genes were not among them. In fact, the resistant isolates had mutations in genes involved in bacterial cell wall synthesis, gene expression control but remained susceptible to inhibition by ethoxzolamide (Modak et al., 2019; Rahman et al., 2020).

Acetazolamide **1** was again used as lead molecule for drug design studies of HpCAα/β inhibitors, also considering the fact that the first enzyme has been crystallized, alone or in complex with several inhibitors, including acetazolamide (Modak et al., 2015,2016) – Table 6. Compound **35** (and acetazolamide **1**) were the most effective inhibitors of both bacterial enzymes, (K_Is < 50 nM) but it has been observed that only **35** was a more effective HpCAα than hCA II inhibitor (Modak et al.,

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2016). The compounds were medium-high nanomolar bacterial CA inhibitors, apart deacetylated acetazolamide (compound **33**) which was the most ineffective inhibitor (micromolar inhibitor for the bacterial α- and β-CAs). These differences in inhibition between these sulfonamides were rationalized by the report of their X-ray crystal structures in adduct with the bacterial (and human) enzymes (Modak et al., 2015,2016; Supuran, 2024b). The importance of these studies was that for the first time it has been demonstrated that it is possible to design bacterial CA-selective inhibitors (over the human CAs) and that some of these compounds do have anti-*H. pylori* effects, without leading easily to the development of drug resistance in the bacterial CA genes (Supuran, 2024b).

Table 6 here

Different species belonging to the genus *Enterococcus*, among which *E. faecium* and *E. faecalis*, gram-positive bacteria commonly found in the gastrointestinal tract (GIT) microbioma, cause disease in hospitalized/immunosupressed patients, ranging from mild to serious, with few therapeutic options for their treatment, as the pathogens became highly multi-drug resistant to most antibiotics used clinically, including vancomycin (Geraldes et al., 2022; O'Toole et al., 2023). These bacteria are commonly reffered to as vancomycin-resistant enterococci (VRE) and only two therapies for their treatment, i.e., linezolid, and a combination of quinupristin and dalfopristin (which leads to serious toxicity problems) are available to date (Geraldes et al., 2022; O'Toole et al., 2023).

CAs present in these bacteria (α- and a γ-class enzymes) were recently proposed as alternative antimicrobial drug targets, with some remarkable results being reported by inhibiting them with sulfonamide CAIs (Kaur et al., 2020; An et al., 2022). *E. faecium* CAs (EfCAα and EfCAγ) have been used as model enzymes, being cloned, purified and characterized biochemically and for their inhibition profiles with sulfonamides and other CAIs (An et al., 2022). Acetazolamide **1** has been used again as investigational inhibitor, being observed that it possesses a rather effective MIC against *E. faecium* strain HM-965 (of 2 μg/mL) as well as against several multidrug-resistant strains

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of the bacterium (Kaur et al., 2020; An et al., 2022) Analogs of **1** in which the acetamide moiety of the lead molecule has been modified to a variety of other amides (compounds **36a-h**), led to CAIs which showed both effective MIC values for the inhibition of *E. faecium* HM-965 strain, and also inhibited *in vitro* the two CAs from the pathogen (Table 7).

Table 7 here

The lowest MIC values against *E. faecium* strain HM-965 were observed for the *tert*-butylacetamido (**36g**), cyclohexylcarboxamido (**36a**), cyclohexylmethylcarboxamido (**36c**), cyclohexylethylcarboxamido (**36e**), and phenethylcarboxamido (**36d**) derivatives, for which MIC-s in the range of 0.007 – 0.25 μg/mL were determined. Acetazolamide **1**, the benzoyl derivative **36b** or the morpholinyl-methyl- derivative **36h** were also active, with MIC-s in the range of 1-2 μg/mL (Kaur et al., 2020; An et al., 2022). The possibility of using such bacterial CAIs for the gut decolonization of VRE in an animal model of the disease has recently been reported (Abutaleb et al., 2023). Acetazolamide **1** and three of its derivatives (**36e, 36g** and **36h**) were used in such experiments, all of which had MIC-s in the range of 0.007 -0.060 μg/mL and were also effective inhibitors of the two CAs present in *E. faecium*. Mice infected with *E. faecium* strain HM-952 have been used in the experiments and their GIT bioburden was quantified in the fecal pellets after treatment for 8 days with 20 mg/kg CAIs. The treated animals showed significantly reduced *E. faecium* fecal bioburden compared to those treated with the vehicle or linezolid as control drug. After three days of treatment with CAIs, the *E. faecium* fecal bioburden was already reduced by 90 %, whereas the highest effect was observed after 5 days of treatment, with the bioburden being reduced by 92.6−99.3%, with **1** which was the most effective inhibitor among the four investigated compounds (Abutaleb et al., 2023). Of note, the effect of linezolid was of only 86.5 % reduction of the fecal bioburden (after 8 days of treatment), demonstrating thus that sulfonamide CAIs were by far more effective as decolonization agents against VRE as compared to the standard, clinically used drug (Abutaleb et al., 20239.

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Vibrio cholerae is a Gram-negative bacillus possessing several serogroups, of which two, the O1 and O139, cause cholera. The disease is spread in poor countries, being characterized by a massive loss of water and electrolytes, which leads to death, if untreated. *V. cholerae* encodes for three CAs, belonging to the α-, β- and γ-classes, denominated VchCAα, VchCAβ and VchCAγ, which were cloned and characterized biochemically over the last decade, with many effective *in vitro* inhibitors being discovered (Del Prete et al., 2012; 2016a,b; Fantacuzzi et al., 2023). Sulfonamide CAIs structurally related to SLC-0111 (compound **19**, Fig. 9), which efficiently inhibited the three *V. cholerae* CAs *in vitro*, were recently investigated *in vivo* for their antibacterial effects on different clinical isolates of the bacterium, i.e., SI-Vc22, SI-Vc71, and SI-Vc912, but their MICs were > 64 µg/mL (although inhibition of growth of the bacterium has been observed, without the possibility to determine the precise MIC values) (Fantacuzzi et al., 2023). Thus, more detailed studies of the three CAs present in this pathogen and better inhibitors are needed in order to validate these enzymes as antibacterial drug targets.

M. tuberculosis, the pathogen provoking tuberculosis, encodes for three β-CAs which have been well characterized [99-103], and also a γ-class enzyme, which has not yet been cloned and characterized (Minakuchi et al., 2009; Nishimori et al., 2009; Carta et al., 2009). The three β-CAs, sometimes called with names derived from the genes encoding them, as Rv1284, Rv3588c and Rv3273, but also as mtCA1, mtCA2 and mtCA3, have been investigated in detail for their catalytic activity and inhibition with sulfonamides and other classes of compounds, such as phenols, carboxylic acids, natural products, dithiocarbamates, with many effective, low nanomolar *in vitro* inhibitors detected (Aspagtwar et al., 2019). However, many such compounds were ineffective i*n vivo/ex vivo*, especially the sulfonamides, presumably due to their low penetrability through the complex membranes of the bacterium, whereas the dithiocarbamates, such as derivatives **37** and **38** (Fig. 11) showed significant anti-mycobacterial activity *in vivo*, in zebra fish larvae infected with *M. marinum* (Aspatwar et al., 2017, 2018).

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Fig. 11 here

Apart the enzymes discussed above, many other CAs have been described in other pathogenic bacterial species, such as *Brucella suis*, *Salmonella enterica* serovar *Typhimurium*, *Legionella pneumophila*, *Porphyromonas gingivalis*, *Clostridium perfringens*, *Streptococcus mutans*, *Burkholderia pseudomallei*, *Francisella tularensis*, *Escherichia coli*, *Mammaliicoccus (Staphylococcus) sciuri*, *Acinetobacter baumanii*, etc. (Capasso and Supuran, 2015, 2024; De Luca et al., 2024b). For many of them, detailed catalytic activity and inhibition data with various classes of compounds are available, but *in vivo/ex vivo* validation studies are missing. Future studies and the growing problem of resistance to clinically used drugs might contribute to considering some of them as potential antiinfective drug targets.

7.2. Antifungals

A number of pathogenic fungi/yeasts, such as *Cryptococcus neoformans*, *Candida albicans*, *C. glabrata*, *Malassezia globosa*, *M. restricta*, *M. pachydermatis, M. furfur, Saccharomyces cereviasiae*, etc., encode for β-CAs (one of the most relevant such gene being denominated Nce103, Aguilera et al., 2005) which are involved in CO_2-sensing mechanisms together with an adenylate cyclase enzyme (Klengel et al., 2005; Martin et al., 2017; Mogensen et al., 2006; Supuran and Capasso, 2021). This bi-component enzyme system is crucial for fungal growth, differentiation, but also determines the expression of phenotypic features essential for virulence of these pathogens (Klengel et al., 2005; Innocenti et al., 2009; Isik et al., 2010; Martin et al., 2017; Supuran and Capasso, 2021; Schlicker et al., 2009). Some non-pathogenic fungi, such as *Sordaria macrospora* also encode for β-CAs which have also been investigated (Lehneck et al., 2014; Vullo et al., 2020). In fact, the genomes of basidiomycetous and hemiascomycetous yeasts encode only for one *β*-CA, as mentioned above, whereas filamentous ascomycetes contain multiple *β*-CA genes (*S.*

macrospora has three such enzymes, Vullo et al., 2020; Lehneck et al., 2014). Furthermore, in some ascomycetes, genes encoding for *α*-CAs were also reported and such enzymes characterized (Cuesta-Seijo et al., 2011). However, at the moment only fungal β-CAs have been investigated in detail as potential antifungal drug targets (Supuran and Capasso, 2021; Angeli et al., 2022c). These enzymes have been investigated for their inhibition with many classes of CAIs, such as sulfonamides, dithiocarbamates, carboxylates, boronic acids, etc., and many low nanomolar *in vitro* inhibitors were thus detected for many enzymes form various pathogens (*C. albicans, C. glabrata, Malassezia* spp., etc.) (Supuran and Capasso, 2021; Angeli et al., 2022c). The most investigated fungal CAs were however those from *Malassezia* spp., as potential anti-dandruff agents (Hewitson et al., 2012; Supuran and Capasso, 2021; Angeli et al., 2022b). Simple aromatic sulfonamides such as **39** and **40** (Fig. 12) acted as low nanomolar *in vitro* inhibitor for the CA from *M. globosa* (MgCA) and also showed MIC values in the medium potency range, of 10 – 120 μg/mL for inhibiting the growth of various *Malassezia* spp. (Hewitson et al., 2012). In a mouse model of *M. pachydermatis* skin infection, compound **40** showed an efficacy similar to that of the azole antifungal drug ketoconazole, leading to hypha fragmentation, indicative of an effect impairing the fungal growth of the CAI (Hewitson et al., 2012).

Fig. 12 here

More recently, a large series of selenoureas of which **41a,b** (Fig. 12) are two representatives, showed much better *in vitro* fungal CA inhibition and MIC values (in the range of 0.25 – 32 μg/mL) against various strains of M. *pachydermatis, C. albicans* and *C. glabrata* (Angeli et al., 2022c). These seleno-containing sulfonamides showed appreciable antifungal activity, comparable to the standard-of-care antifungal drugs for which drug resistance emerged, which was suppressed when the selenium was replaced with its cognate isosteric elements sulfur or oxygen. Furthermore, some of these compounds showed excellent selectivity against *M. pachydermatis* over its related genera

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M. furfur and *M. globosa* (Angeli et al., 2022c), making them of interest for the development of novel antifungals.

7.3. Antiprotozoal agents

Protozoans encode for CAs belonging to several genetic families, with α-, β-, γ- and η- classes enzymes described so far in several pathogenic species (Supuran 2023e; Capasso and Supuran, 2023; Caroli et al., 2023; Mansoldo et al., 2020; Vermelho et al., 2020; D'Ambrosio et al., 2018; De Simone et al., 2015; Vermelho et al., 2017). CAs were characterized in the following protozoans, producing diseases with various degrees of danger, from mild to very serious: *Trichomonas vaginalis* (Urbański et al., 2020,2021,2022); *Plasmodium falciparum*, the most difficult to treat malaria-provoking protozoan (Krungkrai et al., 2005; De Simone et al., 2015; Fisher et al., 2017; D'Agostino et al., 2023); *Leishmania donovanii* the parasite provoking visceral leishmaniasis (Syrjänen et al., 2013; da Silva Cardoso et al., 2017), *Trypanosoma cruzi,* the etiological agents of Chagas disease, for which few therapeutic options are available (Pan et al., 2013; Vermelho et al., 2018; 2020; Mansoldo et al., 2020) and *Entamoeba histolytica* (Bua et al., 2018; Haapanen et al., 2018). As for bacterial and fungal CAs, also in thre case of the protozoan enzymes mentioned above, a large number of effective *in vitro* inhibitors were detected, but it has been more challenging to evidence antiprotozoal action *in vivo* (Supuran, 2023e). The reasons are multiple, and are mainly due to the fact that most protozoans have complicated life cycles, with many different stages and also more than one host, with the human one being generally just one of the links in their cycle. For example *Plasmodia* have at least 6 different stages/phases during their life cycle, with the various forms of the pathogen present in different organs and tissues, but also with many different genes which are expressed/silenced in the different phases, and thus, with a substantial capability to evade the host immune defences (Supuran, 2023e). However, some successful studies have recently emerged for protozoan CAs as potential antiinfective drug targets. Sulfonamide CAI

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nanoformulations for the management of *Leishmania* and *T. cruzi* infections have been obtained in clove (*Eugenia caryophyllus*) oil, which showed low micromolar efficacy in inhibiting the growth of *Leishmania infantum* MHOM/BR/1974/PP75 and *Leishmania amazonensis* IFLA/BR/1967/PH8 strains (da Silva Cardoso et al., 2017) and *T. cruzi* strains Y and DM28c (Vermelho et al., 2018). Both benzenesulfonamide and 1,3,4-thiadiazole-2-sulfonamides obtained by the tail approach showed such promising activity (da Silva Cardoso et al., 2017; Vermelho et al., 2018), although they were ineffective in normal formulation not envisaging the use of nanoemulsions (Vermelho et al., 2018). Many other pathogenic protozoans, among which *Cryptosporidium* spp., *Giardia lamblia*, *Entamoeba* spp. or *Acanthamoeba castellanii* encode for CAs (Supuran, 2023e) and they started to be investigated very recently, with some promising results being reported for example for the ocular infections caused by the last pathogen, *A. castellanii* (Haapanen et al., 2024). Although this amoeba encodes for at least eight CAs belonging to the α-, β- and γ-classes, all attempts to clone them were not successful for the moment (Haapanen et al., 2024). However, a drug screening assay recently developed, evidenced that clinically used CAIs such as acetazolamide **1**, ethoxzolamide **11**, and dorzolamide **13** showed promising antiamoebic effects *in vivo* (Haapanen et al., 2024). *Toxoplasma gondii* also encodes for an α-CA which was recently cloned and characterized, being observed that the enzyme was effectively inhibited by acetazolamide, sulfamide and sulfamic acid (De Luca et al., 2024a). Thus, the more detailed investigation of protozoan CAs may lead to innovative approaches for the management of these difficult to treat diseases.

7.4. Antinematode agents

CAs are probably present in all nematodes, but they have been investigated in some detail only in few species, among which the model organism *Caenorhabditis elegans,* which encodes both for α- (Hall et al., 2008) and β-CAs (Fasseas et al., 2010) and in several parasitic organisms, among which

Ascaris lumbricoides (Zolfaghari Emameh et al., 2015,2016), *Schistosoma mansoni* (Da'dara et al., 2019; Haapanen et al., 2023) and *Gyrodactylus salaris* (Aspatwar et al., 2022a, 2023). The role of these enzymes in worms are poorly understood, but in *C. elegans* it has been demonstrated that they are involved in pH regulation and adaptation to the environment/niches in which these organisms live. Indeed, unlike many other organisms, this nematode is able to maintain > 90% survival in pH conditions ranging from 3 to 10 (Hall et al., 2008). A transcriptional analysis identified genes differentially regulated by pH, and among them a gene encoding for two (splicing variants) α-CAs (*cah-4*), was observed to be five-fold upregulated in alkaline environment. CAH-4b was also shown to be a highly active enzyme for the CO_2 hydration reaction, being effectively inhibited by suflonamide CAIs (Hall et al., 2008; Crocetti et al., 2009; Güzel et al., 2009). Such data constituted the proof-of-concept that nematode CAs might be considered as drug targets. Indeed, in the intravascular parasitic worm *Schistosoma mansoni* which causes schistosomiasis, a disease of great global public health significance, two CAs were identified, belonging to the α- and β-classes (Da'dara et al., 2019; Haapanen et al., 2023). The first enzyme has been investigated in detail, with several effective *in vitro* inhibitors discovered (sulfonamides, boronic and arsonic acids) which showed a modest but significant inhibition of growth of the pathogen (Angeli et al., 2021,2022b; Ferraroni et al., 2022a). The salmon plathelminth parasite *Gyrodactylus salaris* encodes for a β-CA which has been cloned and characterized in detail recently (Aspatwar et al., 2022a, 2023). Several effective *in vitro* inhibitors of this enzyme were also detected, belonging to the sulfonamide class, but no *in vivo* studies were performed to date for validating this protein as a potential antiinfective drug target (Aspatwar et al., 2022a, 2023). The challenges to obtain a potent antiinfective effect with such CAIs is mainly due to the highly polar nature of these molecules and their difficulty to cross the rather thick tegument (and the membranes it contains) of nematodes.

8. Polypharmacology of CAIs

Some drugs which have been discovered for treating conditions normally not associated with CA inhibition, were subsequently shown to possess potent inhibitory action against some isoforms of this enzyme, and are thus considered to possess a polypharmacology in which CA may play a relevant role which explains their activity and in some cases, also their side effects. The examples which will be discussed here are the antiepileptics topiramate **16** and zonisamide **17** (Fig. 7), the antipsychotic acting as dopamine D_2 receptors antagonists sulpiride **42** and veralipride **43**, the NSAIDs acting as COX-2 selective inhibitors celecoxib **44** and polmacoxib **45**, the pan-kinase inhibitor pazopanib **46** and the histamine H_2 receptor antagonist famotidine **47** (Fig. 13). As it will be discussed shortly, all these clinically used drugs were shown to possess relevant CA inhibitory activity against most human CA isoforms, and in all cases except one (pazopanib), X-ray crystal structures of their adducts with some hCAs were also reported (see discussion in the next paragraphs).

Fig. 13 here

8.1. Topiramate and zonisamide

Topiramate **16**, an antiepileptic drug belongimg to the sugar sulfamates class was discovered by Maryanoff's group in the 80s by using the maximal electroshock seizure (MES) test, an empirical approach widely employed for testing anticonvulsants (Maryanoff et al., 1987), Topiramate is an orally active agent, useful for the management of various forms of epilepsy, refractory to other medication, has a high bioavailability, rapid absorption and long duration of action (Rosenfeld, 1997). The drug is not structurally related to any other antiepileptic used clinically, such as the phenytoins, carbamazepines, barbiturates, benzodiazepines or GABA-scaffold derived antiepileptics. It has some similarity to zonisamide **17** (Fig. 7), as both drugs contain the sulfamate/sulfonamide ZBG known to coordinate to the Zn(II) ion of CAs (Alterio et al., 2012). The mechanism of action of this drug is not fully understood yet (Supuran, 2008; De

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Simone et al., 2009) and seems to be rather complex, including apart CA inhibition, enhancement of GABA-ergic transmission (Rosenfeld, 1997; Herrero et al., 2002), antagonism of kainate/AMPA receptors (Gibbs et al., 2000; Zullino et al., 2003), inhibition of action potentials creation in neurons via antagonizing the activation of Na^+ channels (Perucca, 1997; Taverna et al., 1999). Initially topiramate was reported to be a very weak (millimolar) CAI (Maryanoff et al., 1987) but later it has been ascertained by several techniques that topiramate is a potent CAI against most CA isoforms present in the SNC (Casini et al., 2003; Supuran, 2008), and its crystal structure in complex with several isoforms has also been reported (Casini et al., 2003; Alterio et al., 2010). These findings match with many clinically observed side effects of topiramate, which are in agreement with the typical pharmacological profiles of strong sulfonamide CAIs used as systemic antiglaucoma agents/diuretics and include paresthesias, nephrolithiasis, metabolic acidosis, weight loss, carbonation dysgeusia (bitter taste perception of carbonated drinks, due to salivary CA VI inhibition), etc. (Charbonneau et al., 2020; Chahin et al., 2020; Naps et al., 2023). Topiramate inhibits eight CA isoforms with inhibition constants < 65 nM, it inhibits hCA I with a K_I of 250 nM whereas only three isozymes are less inhibited (hCA III, IV and XIV, with K_Is in the range of 1460 nM – 0.78 mM) (Supuran, 2008) - see Table 8. Many CA isozymes involved in crucial physiologic functions, such as hCA II, hCA VA, hCA VB, hCA VI, hCA VII, hCA IX, hCA XII and hCA XIII are highly inhibited by this drug, sometimes with K_Is in the subnanomolar range (e.g., hCA VII, K_I of 0.9 nM, Supuran, 2008). Apart its use as an antiepileptic, topiramate has been repurposed as an antoiobesity agent (Supuran, 2022) as discussed earlier in this review, but also as an anti-migraine drug (Pearl et al., 2023).

Zonisamide **17** (Fig. 7), is another widely used antiepileptic drug (Leppik, 2004). Zonisamide, as discussed above, is an efficient sulfonamide CAI, but also blocks repetitive firing of voltage-sensitive sodium channels and reduces voltage-sensitive T-type calcium currents without affecting L-type calcium currents (Perucca, 1997; Leppik, 2004). This rather complex mechanism of action probably explains its efficacy in patients resistant to other antiepileptic drugs, and its

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pharmacokinetic profile is also favorable, since the drug is rapidly and completely absorbed, has a long half-life (63-69h), which allows twice- or once-daily dosing (Perucca, 1997; Leppik, 2004). Zonisamide has been investigated for the inhibition of CA already by its discoverers (Masuda and Karasawa, 1993), being concluded that although it binds significantly to erythrocytes (where two CA isozymes, hCA I and II are highly abundant – Maren, 1967) its CA inhibitory properties are rather weak, and thus, this phenomenon does not play any role in the anticonvulsant activity of the drug (Masuda et al., 1994). However subsequent studies showed zonisamide to be a potent CAI for many CNS-present CA isoforms such as hCA I, II, VA, VI, VII and IX (which are inhibited with $K_{IS} < 120$ nM) (De Simone et al., 2005; Supuran, 2008). The X-ray crystal structure of zonisamide bound to hCA II has also been reported, which explained its potent inhibition of this and other CA isoforms (De Simone et al., 2005). Apart its use as an antiepileptic, as for topiramate, it has been proposed a repurposing of zonisamide as an antiobesity agent (Gadde et al., 2003; Supuran, 2022) and for the management of Parkinson's disease (Murata et al., 2020), a conditions in which CAs seem definitely to be involved (Supuran, 2008; 2023a).

8.2. Sulpiride and veralipride

Sulpiride **42** and veralipride **43** (Fig. 13) are atypical antipsychotics acting as dopamine D_2 receptors antagonist and belong to the benzamide class of such drugs (Furtado and Srihari, 2008; Masdrakis and Baldwin, 2021). They are widey eployed for the management of schizophrenia, depression but also as anticonvulsants (Furtado and Srihari, 2008; Masdrakis and Baldwin, 2021). Both compounds possess a primary sulfonamide moiety, which is known to be involved in CA inhibition as one of the most effective ZBGs described to date (Alterio et al., 2012; Supuran, 2008). Thus, both drugs have been tested as potential inhibitors of all hCAs and their X-ray crystal structures in adducts with the major isofororms, hCA I and II, were also reported (Abbate et al., 2004; Angeli et al., 2024). Sulpiride **42** is a highly effective inhibitor of hCA II, VB, VI, IX, XII

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and XIV (K_Is of 0.8 – 110 nM), being less potent against hCA I, III, IV and VII, with K_Is in the micromolar range (Abbate et al., 2004; Supuran, 2008). Veralipride **43** also showed effective inhibitory activity against hCA VB, VI, IX, XII and XIII (K_Is ranging between 24 – 92 nM) being less effective against the remaining isoforms (K_Is of 0.15 – 2.7 μM against hCA I, II, IV, VA, VII and XIV, and > 10 μM against hCA III, Angeli et al., 2024). It is not known at the moment whether the CA inhibitory effects of these drugs may have a relevance for their clinical efficacy in schizophrenia. In fact, older studies showed acetazolamide to possess some effectiveness for the management of this diseases (Inoue et al., 1984) whereas a more recent study proposed the repurposing of CAIs, including **1** (as well as topiramate), for the management of schizophrenia based on interactome analysis (Karunakaran et al., 2019). Thus, we hypothesize that CAIs may have a future for the manegement of this condition too.

8.3. Celecoxib and polmacoxib

COXs catalyze a commited step in the conversion of arachidonic acid to prostaglandins (PGs) and thromboxane, with at least three distinct isozymes (COX-1 - COX-3) known to date (Romanelli et al., 2024). Isoforms COX-1 and COX-2 have been considered to be responsible for different effects, benefficial or not, of PGs in various tissues (Fitzgerald, 2003; Romanelli et al., 2024). The development of COX-2 specific inhibitors, collectively called coxibs in the late '90s, was initially considered as a breakthrough in anti-inflammatory therapy due to the fact that this inducible isoform was considered to play a major role in inflammation (Fitzgerald, 2003), but the withdrawal of some of these drugs in 2004/2005 due to severe adverse side effects completey redimensioned the field (Dognè et al., 2005). In fact, the development of the coxibs, was based on the erroneous hypothesis that only COX-2 mediates inflammation in several organs via the biosynthesis of prostaglandins E_2 and I_2 and that COX-1 was the source of prostaglandins in the gastric epithelium, acting as cytoprotective mediators (Dognè et al., 2005). The severe cardiac side

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effects of some of the coxibs (such as rofecoxib) demonstrated that this was not the real situation (Epstein, 2001), as both COX-1 and COX-2 participates to the inflammatory PG biosynthesis, and thus, the only two drugs of the class still in clinical use are those which were less COX-2 selective, i.e., celecoxib **44** and polmacoxib **45** (Dogné et al., 2008; Kim et al., 2016; Supuran, 2020a). However, celecoxib **44** and polmacoxib **45** contain a primary sulfamoyl moiety which is known to interact with the zinc ion from CAs (Alterio et al., 2012). Celecoxib has been investigated as CAI against all human enzymes in 2004, and its X-ray crystal structure in adduct with hCA II was also reported (Weber et al., 2004). Isoforms hCA II, VB, IX and XII were inhibited with $K_{I}S < 100$ nM by celecoxib, with CA IX and XII showing the most efective inhibition, in the low nanomolar range ($K_{I}S < 40$ nM) (Weber et al, 2004). Based on the potent inhibitory activity of celecoxib **44** on the tumor-associated isoforms hCA IX/XII, there are various studies suggesting its repurposing as an antitumor agent (Supuran et al., 2004; Alian et al., 2024; Berckmans et al., 2023; Xu et al., 2023). Polmacoxib (also known as GCG100649) was reported to show high affinity for hCA I and II (Hirankarn et al., 2013). The drug, considered a dual inhibitor of COX-2 and hCA I/II, was approved in 2022 for the treatment of osteoarthritis in South Korea (Cho et al., 2022).

8.4. Pazopanib

Pazopanib **46** (Fig. 13) is an antitumor drug belonging to the class of tyrosine kinase inhibitors, approved for clinical use for the treatment of solid tumors in 2010 (Schutz et al., 2011; Pick and Nystrom, 2012). It is a pan-kinase inhibitor, binding to several different proteins involved in angiogenesis, tumor growth and cell survival, such as the vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), c-Kit, and it is mainly used for the management of renal cell cancer, breast cancer, soft tissue sarcoma, thyroid cancer, hepatocellular cancer and cervical cancer (Jones et al., 2022; Schutz et al., 2011; Peerzada et al., 2021; Pick and Nystrom, 2012, Wang et al., 2024). As many drugs discussed in this section, also

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pazopanib incorporates the primary sulfonamide moiety associated with effective CA inhibitory action. Thus, the drug was investigated for its interaction with all catalytically active hCAs (Winum et al., 2012). Pazopanib was observed to be a highly effective CAI of all hCAs except for hCA III, which is anyhow inhibited in the low micromolar range (K_I of 4.5 μM). The remaining isoforms were inhibited with K_Is ranging between 0.88 nM to 78 nM, and the most pazopanib-avid isoforms were hCA VA, VB, IX and XII (K_Is of 0.88-9.1 nM) (Winum et al., 2012). Thus, it has been hypothesized that the effective antitumor effects of the compounds are due to both its kinase and CAs inhibitory activities (Winum et al., 2012; Supuran, 2021a)

8.5. Famotidine

Famotidine **47** (Fig. 13) is a H_2 receptor antagonists of third generation, used for the treatment of gastric and duodenal ulcers, gastroesophageal reflux, pathological acid hypersecretory conditions (e.g., Zollinger-Ellison syndrome) and NSAIDs-induced gastric injury (Campoli-Richards et al., 1986; Sakurai et al., 2012; Zhang et al., 2021). Like other drugs discussed in this section, famotidine incorporates a putative ZBG present in CAIs, not of the sulfonamide but of the sulfamide type. Thus, the drug has been assayed for the inhibition of human and bacterial CAs, including those found in the gastric pathogen *H. pylori* (Angeli et al., 2018). Famotidine was observed to be an effective inhibitor of hCA II, VI, VII, IX, XII and XIII, with K_Is in the range of 3.0 – 171 nM, whereas it was a submicromolar or micromolar inhibitor of hCA I, IV, VA, VB and XIV (K_Is of 0.67 – 5.3 μM), with only hCA III being poorly inhibited (K_I > 10 μM) (Angeli et al., 2018). The drug was highly effective as an inhibitor of the two CAs from *H. pylori* with K_Is of 21-50 nM (Angeli et al., 2018). The X-ray crystal structures of famotidine bound to hCA I and II were also reported, explaining at molecular level the binding efficacy of the drug to these targets (Angeli et al., 2018). As discussed in this article, bacterial CA inhibition may constitute a novel antibacterial mechanism, and considering that other CAIs were demonstrated to inhibit the growth of *H. pylori*

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(Modak et al., 2019) it has been proposed a polpypharmacological effect also for explaining the action of this drug, which probably exerts its antiulcer action both by inhibiting hydrochloric acid secretion through the H_2 receptors blockade, as well as the growth of *H. pylori* by interfering with mechanisms of acid acclimation of the bacterium within the stomach (Angeli et al., 2018).

9. Conclusions and future prospects

CAIs are pharmacological agents with a multitude of clinical applications, some of them known for decades (as diuretics, antiglaucoma, anti-acute mountain sickness or antiepleptic drugs – Maren, 1967), others, more recent, such as for the manegement of obesity or hypoxic tumors (Supuran, 2021b;2022). Even newer pharmacological applications have recently become feasible, such as for example in the management of neuropathic pain, cerebral ischemia, inflammation, and neurodegenerative conditions, e.g., Alzheimer's and Parkinson's disease (Supuran, 2021b). The presence and detailed biochemical/pharmacological investigations of CAs in pathogens (bacteria, fungi, protozoans, nematodes) opened the possibility to design antiinfectives with a new mechanism of action, considering the crucial roles these enzyme play in such organisms (Supuran, 2023a,b,c). Relevant progress has been reached indeed in the last few years in the validation of some of these enzymes as antibacterials, mainly for drug resistant pathogens such as *Neisseria gonorrhoea* or vancomycin resistant enterococci (Flaherty et al., 2021; Abutaleb et al., 2022a,b). However, only α- and few β-/γ- bacterial CAs have been investigated in detail for obtaining antibacterials, whereas the number of pathogenic bacteria encoding such enzymes is high, and presumably the future will see the emergence of effective inhibitors for some of them. The ι-CAs are also present in many pathogenic bacteria and were scarcely investigated for the moment (Nocentini et al., 2021b). Furthermore, the bacterial CAs present in microbiota organisms, as well as the differences between healthy/diseased microbiotas (and their enzymes), started to be investigated only recently (Amedei et al., 2021), but the field may lead to relevant discoveries for the pharmacology of inhibitors of

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these enzymes and more generally for the management of antibiotics, which may damage the "good" bacteria from the microbiota, with deleterious effects for the patient (Amedei et al., 2021).

Although viruses do not encode for CAs, there may be interesting connections between some of them, such as for example SARS CoV-2, the causative agent of COVID-19, and CAs. Indeed, it has been reported already in 2021 that blood CA activity was 2-3 times higher than normal in patients with acute COVID-19 as well as in the first weeks after infection, leading to the proposal that CAIs such as acetazolamide **1** may be useful in the management of these patients (Deniz et al., 2021). These results were validated in another study, which reported CA I as a candidate biomarker in platelets of COVID-19 patients, with a significant increase of the level of this isoform in such patients (Wolny et al., 2023) or in those who were dead of COVID-19 infection (Eltobgy et al., 2024), although the mechanisms responsible for this increased CA I expression are not known at the moment. Kugler et al (2024) reported on the other hand that CA XIV levels were decreased in patients with worsening SARS CoV-2 infection compared to those whose infection improved or compared to non-infected subjects. Muñoz-Prieto et al. (2022) observed downregulation of CA VI in saliva of patients with COVID-19, which may explain the abnormalities in taste perception observed for some infected subjects with SARS CoV-2.

Many advances in the CAI field were possible because highly isoform-selective inhibitors started to be available in the last two decades, most of which emerged after the tail-approach has been discovered (Scozzafava et al., 1999b) for generating large libraries of CAIs but also through the discovery of various new chemotypes possessing efficient inhibitory action against these enzymes (Supuran, 2016b). In fact, apart sulfonamides and their isosteres, known since the '40s and dominating the field till the 2000s, which are still pharmacologically relevant and clinically used CAIs, nowadays more than 40 different chemotypes with effective CA inhibitory patterns were disclosed, many of which amenable to drug development programs.

Table 8 here

Table 8 shows the CA inhibition data of 9 clinically used drugs and one compound in clinical trials against the twelve catalytically active human isoforms, hCA I – XIV (Table 8) (Supuran, 2008). It may be observed that the first and second generation drugs (from acetazolamide **1** to zonisamide **17**) potently inhibit almost all isoforms, except hCA III, which is less prone to inhibition by sulfonamides/sulfamates. For example acetazolamide **1** has K_Is ranging from 2.5 to 74 nM for 10 of the 12 considered isoforms, which explain the many side effects of the drug, that have been discussed here. The other drugs presented in the table show more or less the same range of promiscuous inhibition as acetazolamide (Table 8). On the other hand, the third generation compound **19** (SLC-0111) which has been designed as a selective agent for targeting CA IX and XII by the tail approach, is a potent inhibitor of these two isoforms (K_Is of 4.5 – 45 nM) whereas it acts as a poor inhibitor of the remaining 10 CAs. Most probably, the future drugs belonging to this class of pharmacological agents will be designed by using this type of strategy, for potently inhibiting only the isoforms of interest and not the offtarget ones, e.g., CA I and II, which are abundant in many organs and their inhibition is responsible for many of the side effects of the first and second generation such drugs (Supuran, 2021a,b).

Another much investigated drug design strategy in the last decade was that of obtaining hybrid drugs incorporating CAI and other pharmacological agents, by the so-called multitargeting approach (Supuran, 2021c). Indeed, sulfonamides, coumarins or sulfocoumarins were hybridized with NO donors, CO donors, prostaglandin analogs, β-adrenergic blockers, non-steroidal anti-inflammatory drugs, and a variety of anticancer agents (cytotoxic drugs, kinase/telomerase inhibitors, P-gp and thioredoxin inhibitors, etc.) leading to compounds with an interesting pharmacology and biological activity, most of the times better than that of the parent drugs or their combinations. These derivatives were not dealt with in this review, as the field is very ample and was reviewed recently (Supuran, 2021c), but this strategy may lead to relevant progress, mainly for

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developing more effective anticancer drugs and antiinfectives (De Simone and Supuran, 2024; Capasso and Supuran, 2024).

Considering the many topics treated here, a hyperkink to CAs in the the International Union of Basic and Clinical Pharmacology (IUPHAR) /British Pharmacological Society (BPS) database is provided here https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=842, which may be helpful for those interested to read more on this argument.

CAs have as substrate CO_2, a hot house gas which by accumulating in the atmosphere may induce irreversible climate change in a planet of > 8 billion inhabitants, and there are recent attempts for using these enzymes for carbon capture (Migliardini et al., 2014). Plant/algal CAs are involved in photosynthesis and there are attempts of crop engineering consisting in inserting CA genes in cultivars such as rice, corn and other cereals, for boosting agricultural yields (Findinier and Grossman, 2023; Supuran, 2023b). Although these aspects are not directly connected with the CA pharmacological applications discussed in this review, the detailed knowledge acquired by studying these enzymes as drug targets and the design of their inhibitors with clinical applications led to relevant discoveries that may relieve (or resolve, if one is optimistic) some of the huge problems which humankind faces in the 21 century: climate change, insufficient resources, pandemics. Whether this will be feasible depends not only of our scientific achievements for understanding in more details these enzymes and their inhibitors, but also of the wisdom of our globalized society, which hopefully will be good enough for the purpose.

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Tables

Table 1. Human CA (hCA) isoforms, their distribution, localization/activity level, and involvement in various diseases.

Isoform	Organ/tissue distribution	Subcellular localization/activity level	Diseases in which is involved
hCA I	Erythrocytes, gastrointestinal tract, eyes	Cytosolic/low	Retinal/cerebral edema
hCA II	Erythrocytes, eyes, gastrointestinal tract, bone osteoclasts, kidneys, lungs, testis, brain	Cytosolic/high	Glaucoma, edema, epilepsy, altitude sickness, gastric cancer
hCA III	Skeletal muscle, adipocytes	Cytosolic/very low	Oxidative stress, obesity
hCA IV	Kidneys, lungs, pancreas, brain capillaries, colon, heart muscle, eyes	Membrane-bound/high	Glaucoma, retinis pigmentosa
hCA VA	Liver, brain	Mitochondrial/moderate	Obesity, diabetic cerebrovascular disease; Alzheimer's disease ? Parkinson's diseases ?
hCA VB	Heart and skeletal muscle, pancreas, kidneys, spinal cord, gastrointestinal tract	Mitochondrial/high	Obesity Alzheimer's disease ?
hCA VI	Salivary and mammary glands	Secreted/moderate	Cariogenesis
hCA VII	Brain	Cytosolic/high	Epilepsy, neuropathic pain
hCA IX	Tumors, gastrointestinal mucosa	Transmembrane/high	Hypoxic tumors, inflammation
hCA XII	Kidneys, intestine, reproductive epithelia, eyes, tumors	Transmembrane/high	Cancer, glaucoma, inflammation
hCA XIII	Kidneys, brain, lung, gut, reproductive tract	Cytosolic/low	Sterility ?
hCA XIV	Kidneys, brain, liver, skeletal muscle	Transmembrane/medium	Epilepsy, rethinopathies

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Table 2. CAI chemical classes belonging to three inhibition mechanisms: (i) metal binders, (ii) inhibitors which anchor to the metal coordinated water, and (iii) occluders of the active site entrance.

Metal ion binders	Anchoring to the metal coordinated water	Occlusion of the active site
Sulfonamides (primary) Sulfamates Sulfamides Thiols Boronic acids Dihiocarbamates Monothiocarbamates Xanthates Trithiocarbonates Carboxylates (Thio)hydroxamates Tropolones Selenols Ninhydrins Carbamates Secondary sulfonamides Phosphonamidates Benzoxaboroles Carbamimidothioates Hydantoins 2,4-Oxazolidinediones Thiazolidinediones Selenocarbamates Inorganic anions	Phenols Catechols Polyamines Sulfocoumarins 2-Thioxo-coumarins Alcohols Carboxylates	Coumarins Thiocoumarins Selenocoumarins Benzoxepinones Monocyclic lactones Monocyclic thiolactones Isocoumarins Phosphocoumarins 3 H-1,2-benzoxaphosphepine 2-oxides 1,2,3-benzoxathiazine 2,2-dioxides Lacosamide

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Table 3. Clinical trials of anticancer/antimetastatic CAIs, alone or in combination with other agents.

CAI	Combination drug	Phase	ClinicalTrials.gov Identifier
Acetazolamide	Temozolamide	I	NCT03011671 (malignant glioma)
Acetazolamide	Etoposide + Platinum derivatives	I	NCT03467360 (small cells lung cancer)
SLC-0111	Safety trial	I (completed)	NCT02215850 (advanced solid tumors)
SLC-0111	Gemcitabine	Ib/II	NCT03450018 (pancreas, metastatic)
Benzolamide	Temozolamide	II	NCT04209790 (glioblastoma)

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Table 4. CAs present in several pathogenic bacterial species which have been investigated as antiinfective targets and the diseases/infections they produce (Capasso and Supuran, 2015; Supuran, 2023c).

Bacterial pathogen	CA family	Disease	Infection(s)
Neisseria gonorrhoeae	*α*, β	gonorrhoea	sexually transmitted disease
Helicobacter pylori	*α*, *β* and *γ*	gastritis and gastric ulcers	inflammation of the stomach lining, may lead to gastric cancer
Enterococcus spp.	α and *γ*	no specific name	vancomycin resistant enterococci (VRE) involved in nosocomial infections
Vibrio cholerae	*α*, *β* and *γ*	cholera	severe diarrhea.
Mycobacterium tuberculosis	*β* and *γ*	tuberculosis	infection attacking lungs or other organs.
Francisella tularensis	*β* and *γ*	tularemia	debilitating febrile illness
Clostridium perfringens	*β* and *γ*	food poisoning	death as a result of food poisoning.
Streptococcus pneumoniae	*β* and *γ*	pneumonia	inflammatory condition of the lung
Streptococcus mutans	*β* and *γ*	dental caries	infectious of the dental hard tissues
Salmonella enterica	*β*	salmonellosis	diarrhea, fever, vomiting, and abdominal cramps
Haemophilus influenzae	*β*	influenza	nausea,vomiting, gastroenteritis
Porphyromonas gingivalis	*β* and *γ*	periodontitis, rheumatoid arthritis	inflammatory diseases affecting the tissues around teeth; rheumatoid arthritis
Legionella pneumophila	*β*	legionellosis	pneumonia
Pseudomonas aeruginosa	*β* and γ	no specific name	infections in cystic fibrosis patients
Escherichia coli	*β* and *γ*	no specific name	diarrhea (for some strains)
Brucella suis	*β* and *γ*	brucellosis	contagious zoonosis (ingestion of unpasteurized milk or undercooked meat)

Table 5. Acetazolamide **1** and sulfonamides **32a-32i** effectively inhibit the bacterial enzyme NgCAα but also the human isoform hCA II. The minimum inhibitory concentration (MIC) against strain CDC 181 of *N. gonorrhoeae* are also shown (Abbutaleb et al., 2022a).
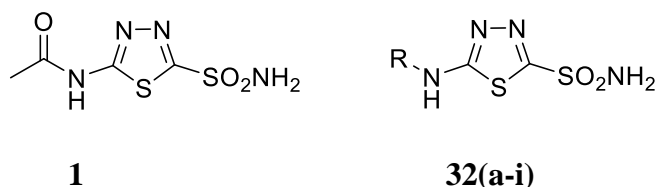
Compound	R	K_I (nM)		MIC
		hCA II	NgCAα	(µg/mL)
1	MeCO	12.5	74	4
32a	H	60	73	4
32b	Et	47	74	>64
32c	Cyclohexyl-CO	20	9.8	2
32d	Cyclohexyl-CH$_2$	78	42	>64
32e	PhCO	29	79	4
32f	Cyclohexyl-CH$_2$CO	24.5	37	0.5
32g	Cyclohexyl-CH$_2$CH$_2$	38	66	>64
32h	PhCH$_2$CH$_2$CO	8.1	8.3	0.25
32i	Cyclohexyl-CH$_2$CH$_2$CO	0.32	0.7	1

Table 6. HpCAα, HpCAβ, hCA I and hCA II inhibition data for acetazolamide **1**, methazolamide

10, ethoxzolamide **11** and thiadiazole-sulfonamides **33-35** (Modak et al., 2016).
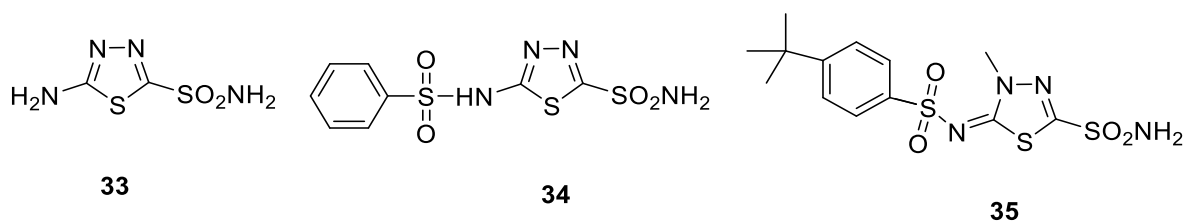
Compound	K_I (nM)			
	HpCAα	HpCAβ	hCA I	hCA II
1	21	40	250	12
10	225	176	50	14
11	193	33	25	8
33	323	2590	8600	60
34	315	54	15	9
35	8	40	338	24

Table 7. Inhibitory effects of hCA II, EfCAα and EfCAγ with sulfonamides **1, 36a-h** incorporating the 1,3,4-thiadiazole-2-sulfonamide scaffold and their MIC values against *E. faecium* strain HM-965 is also shown (An et al., 2022).
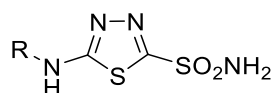
1, 36a-h

Compound	R	K_I (nM)			MIC
		hCA II	EfCAα	EfCAγ	(µg/mL)
1 (AAZ)	MeCO	12.5	56.7	323	2
36a	Cyclohexyl-CO	20.2	49.3	131	0.25
36b	PhCO	29.0	11.7	310	2
36c	Cyclohexyl-CH$_2$CO	24.5	14.5	305	0.06
36d	PhCH$_2$CH$_2$CO	8.1	6.4	148	0.06
36e	Cyclohexyl-CH$_2$CH$_2$CO	0.32	66.9	346	0.007
36f	EtCO	37.2	37.5	250	1
36g	*t*-Bu-CH$_2$CO	7.3	69.6	390	0.015
36h	O[CH$_2$CH$_2$]$_2$NCH$_2$CO	0.9	20.1	56.4	1

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Table 8: Inhibition data with some of ten sulfonamides/sulfamates **1-19** in clinical use/clinical trials against hCA isozymes I – XIV (Supuran, 2008).

Isozyme	K_I (nM)									
	1	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**19**
	(acetazolamide)	(metazolamide)	(ethoxzolamide)	(dichlorophenamide)	(dorzolamide)	(brinzolamide)	(sulthiame)	(topiramate)	(zonisamide)	(SLC-0111)
hCA I	250	50	25	1200	50000	45000	374	250	56	5080
hCA II	12	14	8	9	9	3	10	38	35	960
hCA III	2.10^5	7.10^5	$1\ 10^6$	$6.8.\ 10^5$	$7.7.10^5$	$1.1.10^5$	$6.3.10^5$	$7.8.\ 10^5$	$2.2.\ 10^6$	7920
hCA IV	74	6200	93	15000	8500	3950	95	4900	8590	286
hCA VA	63	65	25	630	42	50	81	63	20	2545
hCA VB	54	62	19	21	33	30	91	30	6033	910
hCA VI	11	10	43	79	10	0.9	134	45	89	6490
hCA VII	2.5	2.1	0.8	26	3.5	2.8	6	0.9	117	8550
hCA IX	25	27	34	50	52	37	43	58	5.1	45
hCA XII	5.7	3.4	22	50	3.5	3.0	56	3.8	11000	4.5
hCA XIII	17	19	50	23	18	10	1450	47	430	8755
hCA XIV	41	43	25	345	27	24	1540	1460	5250	257

Figure legends

Fig. 1. A. Physiologic reaction catalyzed by the CAs (equation *1*) and the catalyutic mechanism of metallo-CAs, equations *2* and *3*. B. Catalytic mechanism of ι-CAs: activation of the water molecule for the nucleophilic attack upon CO_2 is achieved by hydrogen-bonds formation with a triad of hydrophilic amino acid residues, Thr106, Ser199 and Tyr124, whereas the proton transfer is achieved presumably by His197.

Fig. 2. A. Human (h) CA isoform II (hCA II) active site: the zinc ion (gold), its three His ligands (sky blue) and a coordinated water molecule (red) are shown, together with the proton shuttle residue His64 and the His cluster (in green) positioned at the exit of the active site cavity. The hydrophobic residues Phe131 and Gln92 involved in the binding of many inhibitor classea are also highlighrted in green. B. hCA II with superimposed CO_2 (in green) and bicarbonate (pink) bound within the active site, as determined by X-ray crystallography (pdb files 2VVA and 2VVB, respectively). The metal ion, which is zinc in α-CAs is the gray sphere, and its three coordinated amino acid ligands (green), are His94, His96 and His119. Residues involved in the binding of the substrates/inhibitors (Glu106, Thr199, Leu198, Val121, Val143, Thr200 and Trp209) are also shown (hCA II numbering system).

Fig. 3. Phylogenetic tree of α-, β- and γ-CAs from bacteria, algae, plants, fungi and vertebrates (left panel). The program PhyML 3.0 was used to build the tree and branch support values are reported at branch point as red figures. The enzyme family, organisms in which they are present and accession numbers of the genetic sequences are shown in the right panel.

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Fig. 4. The tail approach (A), the two- (B) and three-tails (C) approaches exemplified for inhibitors binding to the zinc ion in the active site of a hCA isoform (hCA II numbering of amino acid residues); (ZBG = zinc-binding group).

Fig. 5. Chemical structuress of sulfonamide CAIs used as diuretics: acetazolamide **1**, thiazides (**2a-e**), thiazide-like drugs (quinethazone **3**, metholazone **4**, chlorthalidone **5**, indapamide **6**) and the high-ceiling diuretics furosemide **7,** azosemide **8** and bumetanide **9.**

Fig. 6. Antiglaucoma CAIs: the first generation drugs include (apart acetazolamide **1**) methazolamide **10**, ethoxzolamide **11** and dichlorophenamide **12** whereas the second generation ones are dorzolamide **13** and brinzolamide **14**.

Fig. 7. Antiepileptics with CA inhibitory activity: sulthiame **15**, topiramate **16** and zonisamide **17.**

Fig. 8: Role of hypoxia-induced CA IX in pH regulation, ferroptosis, redox homeostasy, angiogenesis, survival, proliferation and migration of cancer cells.

Fig. 9. Antitumor/antimetastatic CAIs **18-26**.

Fig. 10. CAIs used for the proof-of-concept studies of the involvement of these enzymes in neuropathic pain (**27**), cerebral ischemia (**28** and **29**) and rheumatoid arthritis (**30** and **31**).

Fig. 11. Dithiocarbamates **37** and **38** with antimycobacterial activity *in vivo.*

Fig. 12. Sulfonamides investigated as antifungals *in vitro/in vivo* (in **41a**, F in *para*; **41b**, F in *ortho*).

Fig. 13. Clinically used drugs **42-47** possessing polypharmacology connected to CA inhibition.

A

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \qquad (1)$$

$$EM^{2+} - OH^- + CO_2 \rightleftharpoons EM^{2+} - HCO_3^- \overset{H_2O}{\rightleftharpoons} EM^{2+} - OH_2 + HCO_3^- \qquad (2)$$

$$EM^{2+} - OH_2 \rightleftharpoons EM^{2+} - OH^- + H^+ \qquad (3)$$

M may be Zn, Fe, Co, Cd

B

Fig. 1

A   B
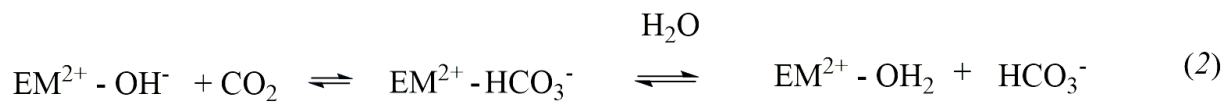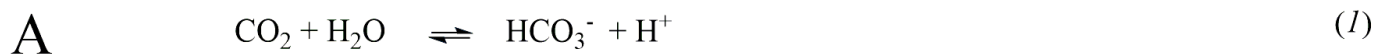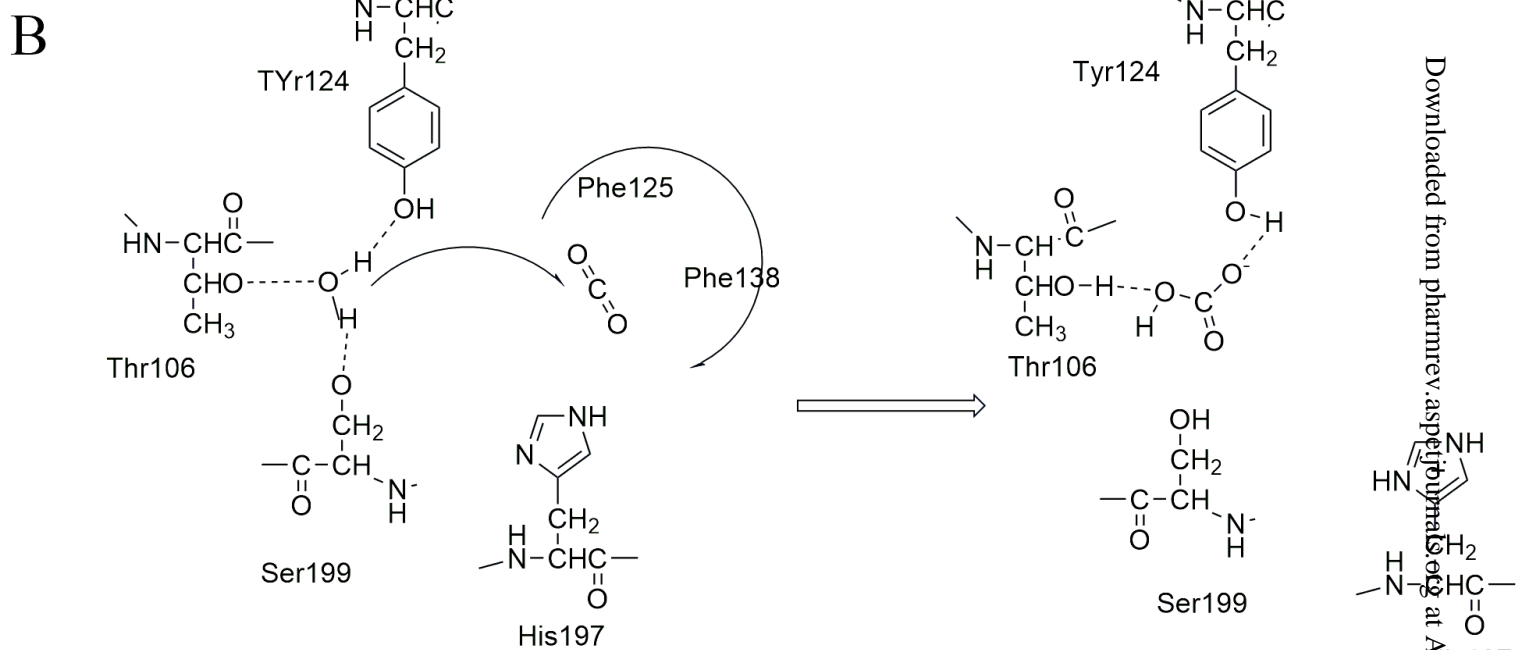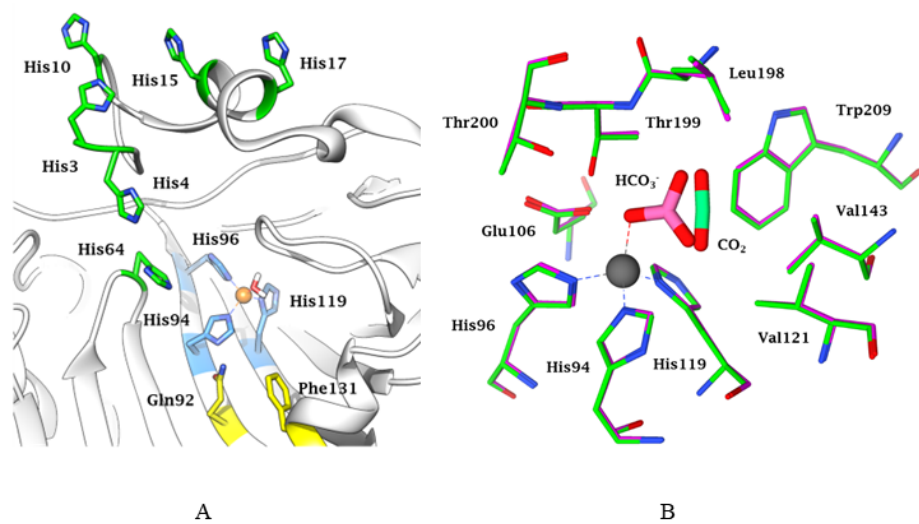
Fig. 2
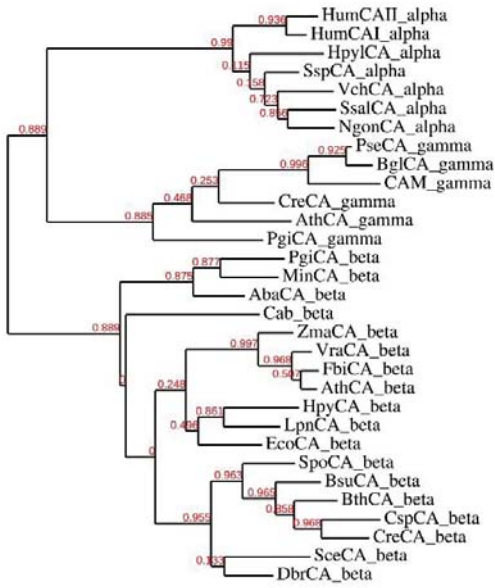
CA class	Organism	Accession number	Cryptonym
alpha	*Helicobacter pylori J99*	NP_223829.1	HpylCA_alpha
	Homo sapiens, isoform II	AAI11949.1	HumCAII_alpha
	Homo sapiens, isoform I	NP_001158302.1	humCAI_alpha
	Sulfurihydrogenibium yellowstonense YO3AOP1	ACD66216.1	SspCA_alpha
	Streptococcus salivarius PS4	EIC81445.1	SsalCA_alpha
	Vibrio Cholerae	AFC59768.1	VchCA_alpha
	Neisseria gonorrhoeae	CAA72038.1	NgonCA_alpha
beta	*Schizosaccharomyces pombe*	CAA21790	SpoCA_beta
	Brucella suis 1330	NP_699962.1	BsuCA_beta
	Burkholderia thailandensis Bt4	ZP_02386321	BthCA_beta
	Coccomyxa sp.	AAC33484.1	CspCA_beta
	Chlamydomonas reinhardtii	XP_001699151.1	CreCA_beta
	Acinetobacter baumannii	YP_002326524	AbaCA_beta
	Porphyromonas gingivalis	YP_001929649.1	PgiCA_beta
	Myroides injenensis	ZP_10784819	MinCA_beta
	Zea mays	NP_001147846.1	ZmaCA_beta
	Vigna radiata	AAD27876	VraCA_beta
	Flaveria bidentis, isoform I	AAA86939.2	FbiCA_beta
	Arabidopsis thaliana	AAA50156	AthCA_beta
	Helicobacter pylori	BAF34127.1	HpyCA_beta
	Legionella pneumophila 2300/99	YP_003619232	LpnCA_beta
	Escherichia coli	ACT70660	EcoCa_beta
	Methanobacterium thermoautotrophicum	GI:13786688	Cab_beta
	Saccharomyces cerevisiae	GAA26059	SceCA_beta
	Dekkera bruxellensis AWRI1499	EIF49256	DbrCA_beta
gamma	*Pseudomonas sp. PAMC 25886*	ZP_10427314.1	PseCA_gamma
	Burkholderia gladioli BSR3	YP_004359911.1	BglCA_gamma
	Methanosarcina thermophila	ACQ57353.1	CAM_gamma
	Chlamydomonas reinhardtii	XP_001703237.1	CrcCA_gamma
	Arabidopsis thaliana	NP_564091.1	AthCA_gamma
	Porphyromonas gingivalis	YP_001929649.1	PgiCA_gamma

Fig. 3

A

Hydrophilic half
of active site

middle rim

TAIL

outer rim

Glu106

O

O⁻

H

O

H

N H

ZBG

SCAFFOLD

Hydrophobic half
of active site

inner rim

Thr199

Zn²⁺ — His119

His94   His96

B

Hydrophilic half
of active site

middle rim

TAIL

TAIL

outer rim

Glu106

O

O⁻

H

O

H

N H

ZBG

SCAFFOLD

Hydrophobic half
of active site

inner rim

Thr199

Zn²⁺ — His119

His94   His96

C

Hydrophilic half
of active site

middle rim

TAIL

TAIL

outer rim

TAIL

Glu106

O

O⁻

H

O

H

N H

ZBG

SCAFFOLD

Hydrophobic half
of active site

inner rim

Thr199

Zn²⁺ — His119

His94   His96

Fig. 4

1

2

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: $R_2 = Me$, $R_3 = CH_2SCH_2CF_3$, $R_6 = Cl$, Polythiazide

3

4

5

6

7

8

9

Fig. 5

10

11

12

13

14

Fig. 6

15

16

17

Fig. 7

PROLIFERATION

ANGIOGENESIS

SURVIVAL

MIGRATION

FERROPTOSIS
REDOX HOMEOSTASY

$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$

H^+

H^+

CA IX

HCO_3^-

Extracellular pH

$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$

CA II

HCO_3^- HCO_3^- HCO_3^- HCO_3^-

Intracellular pH

Hypoxia-induced
pathway

↑ CA IX

HIF1β

HIF1α

Oncogenic
pathways
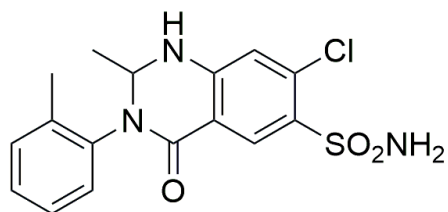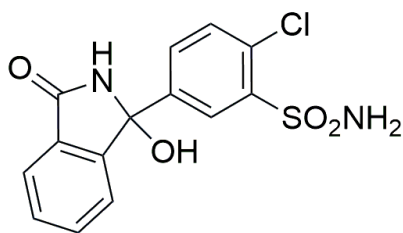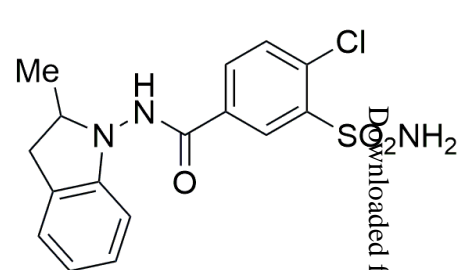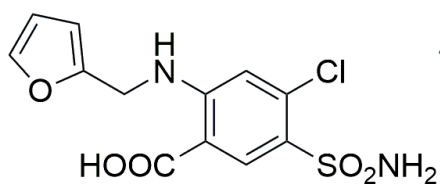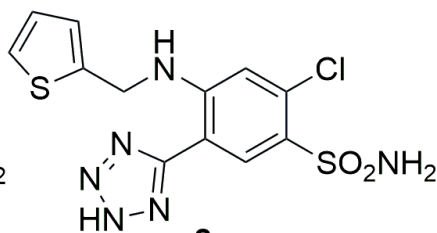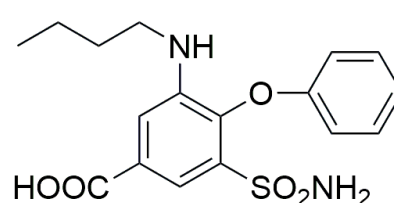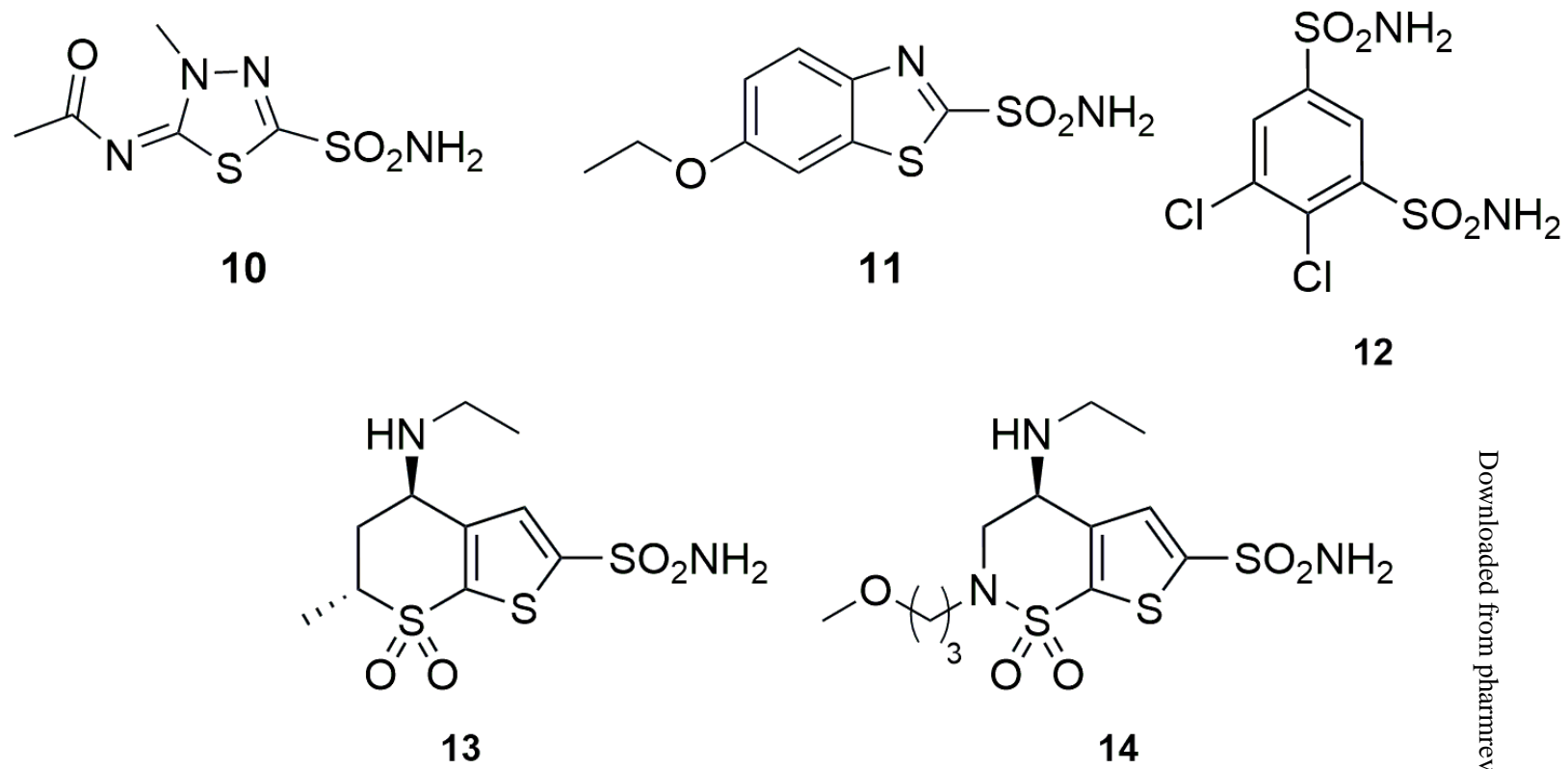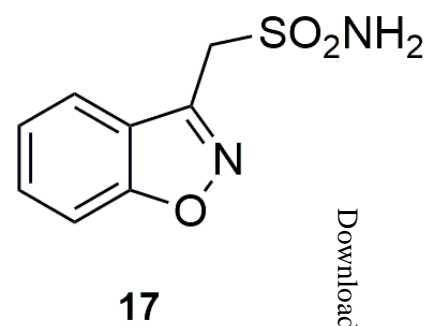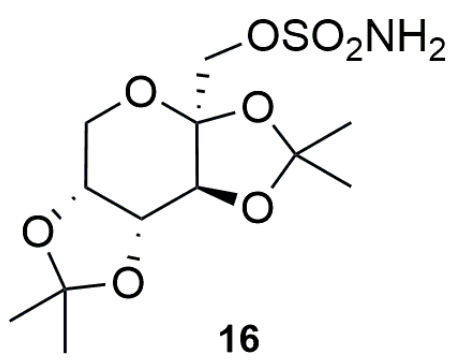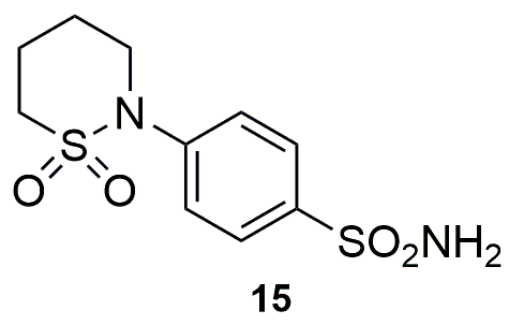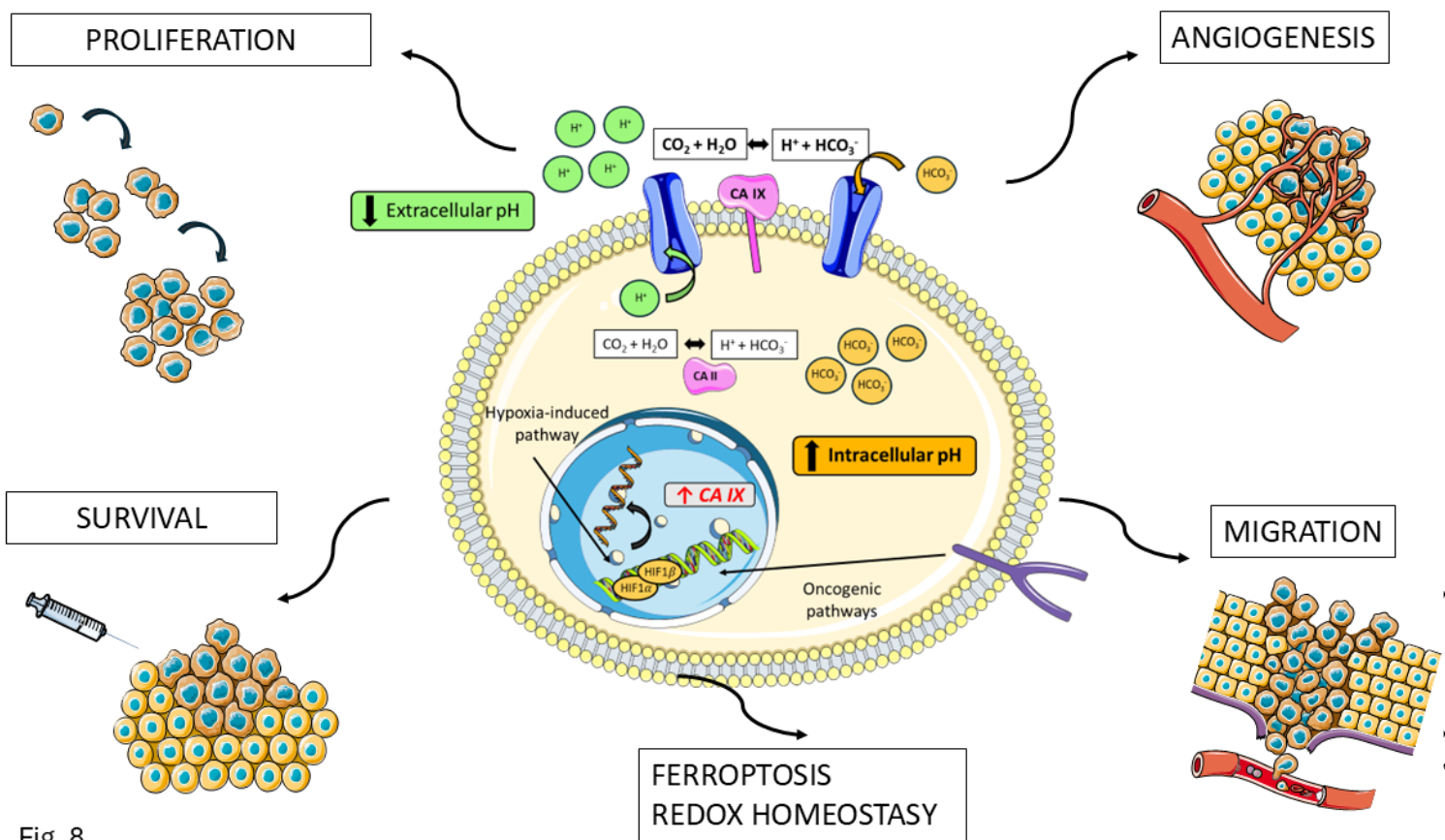
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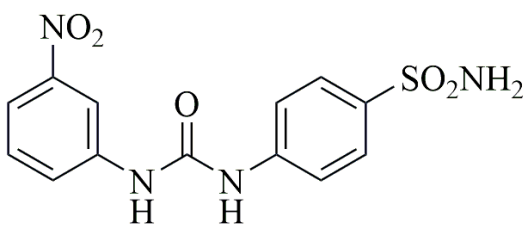
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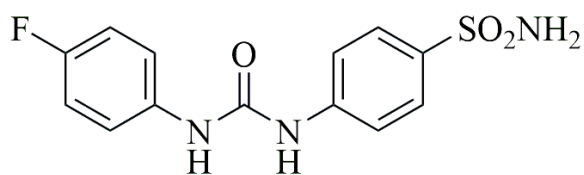
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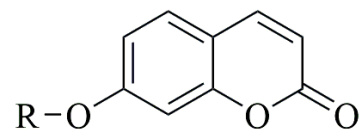
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21: R = galactosyl
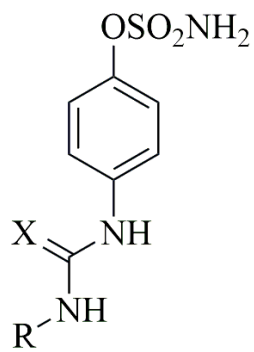
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23: X = S
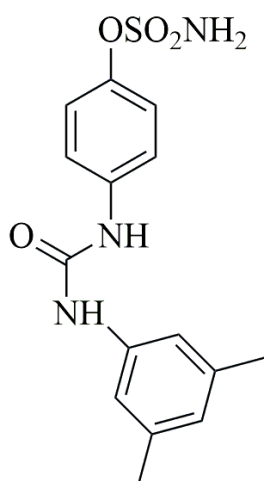
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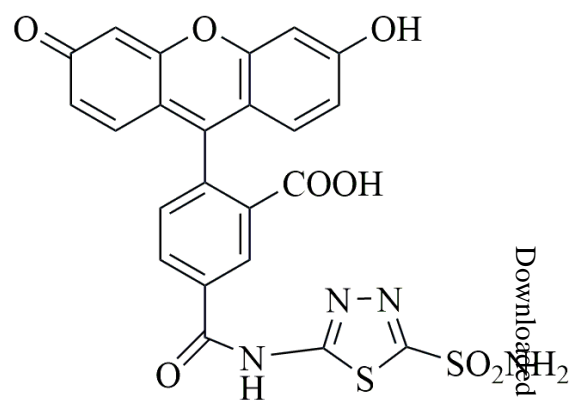
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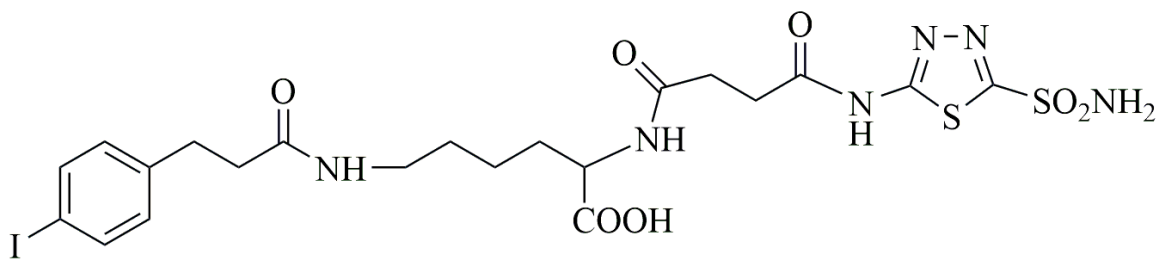
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Fig. 9
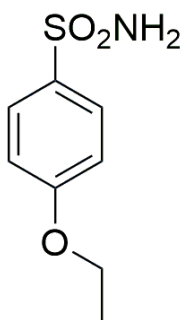
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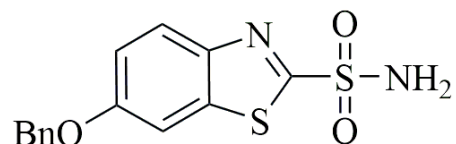
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31
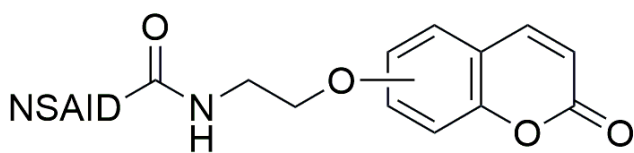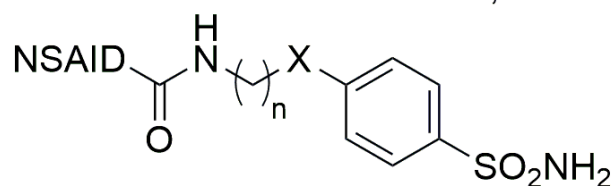
n = 0-4; X = none or O

NSAID: Indomethacin, Sulindac, Ketoprofen, Ibuprofen, Diclofenac, Flurbiprofen,Ketorolac, Naproxen

Fig. 10
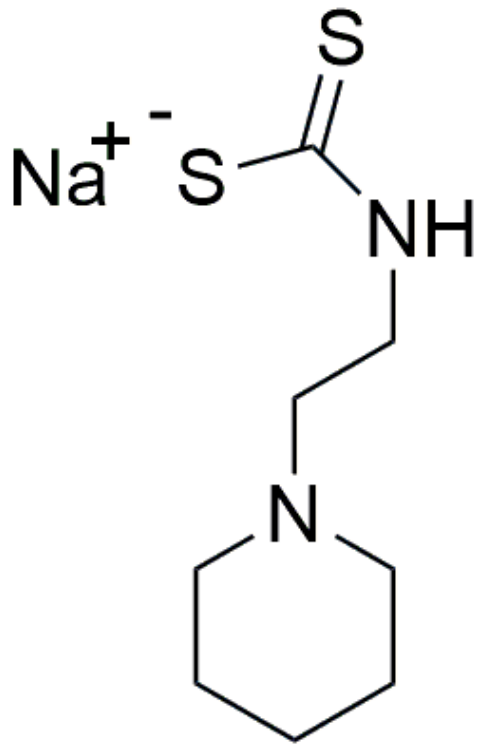
37
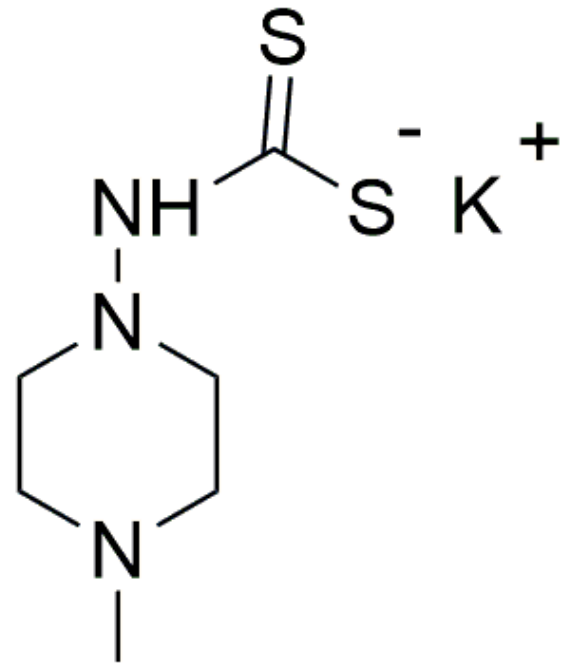
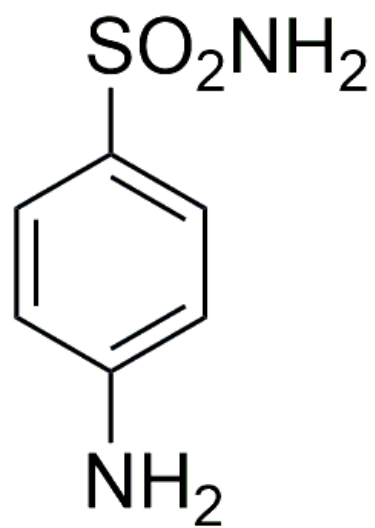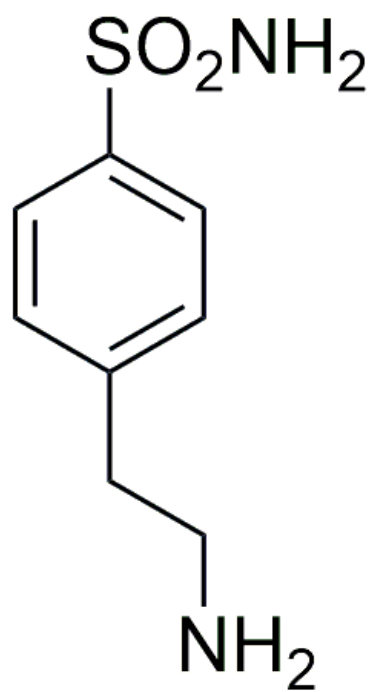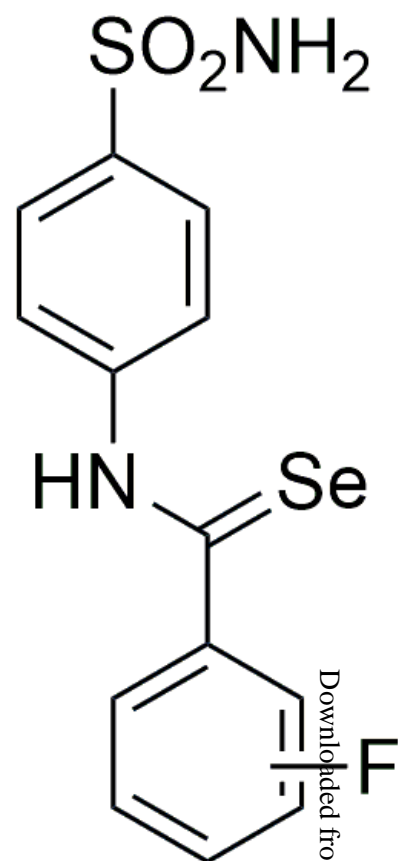**38**

Fig. 11

39

40

41a,b

Fig. 12

Fig. 13