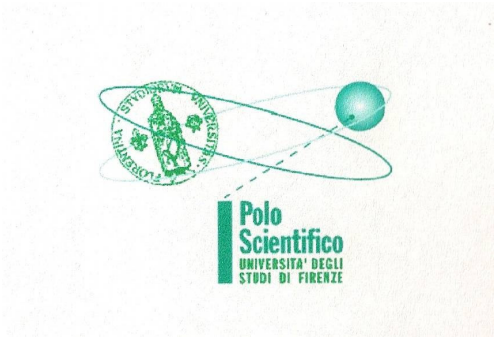

Università degli Studi di Firenze

Facoltà di S.F.M.N. – Dipartimento di Chimica

Dottorato di Ricerca in Scienze Chimiche XXIII ciclo

Dr. Fabio Pacchiano

Design, synthesis and biological evaluation of novel carbonic anhydrase inhibitors against mammalian, bacterial and fungal isoforms.

CHIM 03

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INDEX

1.0 INTRODUCTION

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) catalyzes a very simple but essential physiological reaction, carbon dioxide hydration to bicarbonate and protons [1–3]. This reaction also occurs without a catalyst but it is too slow. As CO_2, bicarbonate, and protons are essential molecules/ions in many important physiologic processes in all life kingdoms (Bacteria, Archaea, and Eukarya), throughout the phylogenetic tree, and relatively high amounts of them are present in different tissues/cell compartments of all such organisms; it is no wonder that CAs evolved independently at least five times, with five genetically distinct enzyme families known to date: the α-, β-, γ-, δ-, and ζ-CAs [1–6]. All of them are metalloenzymes, but whereas α-, β-, and δ-CAs use Zn(II) ions at the active site [1, 4], the γ-CAs are probably Fe(II) enzymes (but they are active also with bound Zn(II) or Co(II) ions) [5], whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis [6]. The 3D-fold of the five enzyme classes is very different from each other, as it is their oligomerization state: α-CAs are normally monomers and rarely dimers; β-CAs are dimers, tetramers, or octamers; γ-CAs are trimers, whereas the δ- and ζ-CAs are probably monomers but in the case of the last family, three slightly different active sites are present on the same protein backbone which is in fact a pseudotrimer [1–6]. Many representatives of all these enzyme classes have been crystallized and characterized in detail, except the δ-CAs [1–6].

The α-CAs are present in vertebrates, protozoa, algae, and cytoplasm of green plants, and in some bacteria [1–3], the β-CAs are predominantly found in bacteria, algae, and chloroplasts of both mono- and dicotyledons, but also in many fungi and some Archaea [4, 5]. The γ-CAs were found in Archaea and some Bacteria [1, 4, 5], whereas the δ- and ζ-CAs seem to be present only in marine diatoms [6].

In many organisms these enzymes are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate, pH, and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes (thoroughly studied in vertebrates) [1–3, 7–10], whereas in algae, plants, and some bacteria they play an important role in photosynthesis and other biosynthetic reactions [1, 4, 5, 11]. In diatoms δ- and ζ-CAs play a crucial role in carbon dioxide fixation [6].

1.1.0 *CATALYTIC MECHANISM OF CAs*

The catalytic mechanism of CAs is understood in detail (see after **Fig. 1**) [1, 2, 12]. In all enzyme classes, a metal hydroxide species (L_3–M^{2+}–OH) of the enzyme is the catalytically active species, acting as a strong nucleophile (at neutral pH) on the CO_2 molecule bound in a hydrophobic pocket nearby [2d]. This metal hydroxide species is generated from water coordinated to the metal ion, which is found at the bottom of the active site cavity. The active center normally comprises M(II) ions in tetrahedral geometry, with three protein ligands (L) in addition to the water molecule/hydroxide ion, but Zn(II) and Co(II) were also observed in trigonal bipyramidal or octahedral coordination geometries, at least in γ-CAs [5]. In many enzymes, generation of the metal hydroxide species from the metal-coordinated water is the rate determining step of the catalytic turnover, which for some α- and ζ-CAs achieve kcat/KM values $>10^8$ M^{-1} s^{-1}, making CAs among the most effective catalysts known in nature [1, 2]. The metal ion ligands are three His residues in α-, γ-, and δ-CAs or one His and two Cys residues in β- and ζ-CAs [1–6]. Some β-class enzymes have four protein zinc ligands, that is, one His, two Cys, and one Asp coordinated to Zn(II) [13].

For these enzymes no water coordinated to the metal ion is present at pH values <8, as shown in an excellent crystallographic work from Jones' group on the mycobacterial enzymes mtCA 2, encoded by Rv3558c gene and mtCA 1 encoded by Rv1284 (**Fig. 2**) [13].

However, at pH values >8, a conserved Arg residue in all β-CAs investigated so far (belonging to a so- called catalytic dyad) [13] makes a salt bridge with the Asp coordinated to Zn(II), liberating the fourth Zn(II) coordination position, which is then occupied by an incoming water molecule/hydroxide ion [13].

The inhibition and activation of CAs are also well understood processes, with most classes of inhibitors binding to the metal center [1–4, 12], and activators binding at the entrance of the active site cavity and participating in the proton shuttling between the metal ion—bound water molecule and the environment [14]. This leads to the enhanced formation of the metal hydroxide, catalytically active species of the enzyme [14, 15].

1.1.1 The α-CAs

Sequencing of eukaryotic/prokaryotic genomes, in particular of pathogens causing widespread diseases (for example, malaria, tuberculosis, as well as other bacterial and fungal infections), has revealed that CAs are also present in these organisms [1, 3e, 4a, 5]. However, although vertebrates possess only CAs belonging to the α-class, such non-vertebrate organisms have enzymes belonging to the same α-CA family as: a CAs from protozoan *P. falciparum*, named (pfCA); a CAs from *H. pylori,* named (hpCA).

Moreover, 16 different α-CA isoforms were isolated and characterized in mammals, where they play important physiological roles, as briefly outlined above.

Some of them are cytosolic (CA I, CA II, CA III, CA VII, CA XIII), others are membrane-bound (CA IV, CA IX, CA XII, CA XIV and CA XV), CA VA and CA VB are mitochondrial, and CA VI is secreted in saliva and milk. Three acatalytic forms are also known, the CA-related proteins (CARP), CARP VIII, CARP X, and CARP XI which seem to be cytosolic proteins too [1–3]. The mammalian CAs were the first such enzymes isolated and studied in detail [1, 12], and many of them are established therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders [1–3, 7–10, 12–15].

The metal ion (which is a Zn^{2+} ion in all α-CAs investigated up to now) is essential for catalysis. X-ray crystallographic data show that the ion is situated at the bottom of a 15 Å deep active-site cleft (shown as a pink sphere in the figure **1**; left side), and is coordinated by three histidine residues (His94, His96 and His119 shown in green in the figure) and a water molecule/hydroxide ion. The histidine cluster involved in the proton shuttling processes between the active site and the environment, comprising residues His64, His4, His3, His17, His15 and His10, is also evidenced. Amino-acid residues 92 and 131 involved in the binding of many sulphonamide/ sulphamate inhibitors are shown in yellow. The zinc-bound water is also engaged in hydrogen-bond interactions with the hydroxyl moiety of Thr199, which in turn is bridged to the carboxylate moiety of Glu106; these interactions enhance the nucleophilicity of the zinc-bound water molecule, and orient the substrate (CO_2) in a favourable location for nucleophilic attack (figure **1**; right side). The active form of the enzyme is the basic one, with hydroxide bound to Zn^{2+} (**A**). This strong nucleophile attacks the CO_2 molecule that is bound in a hydrophobic pocket in its neighbourhood (the elusive substrate-binding site comprises residues Val121, Val143 and Leu198 in the case of the human isozyme CA II) (**B**), leading to the formation of bicarbonate coordinated to Zn^{2+} (**C**). The bicarbonate ion is then displaced by a water molecule and liberated into

solution, leading to the acid form of the enzyme, with water coordinated to Zn^{2+} (**D**), which is catalytically inactive.

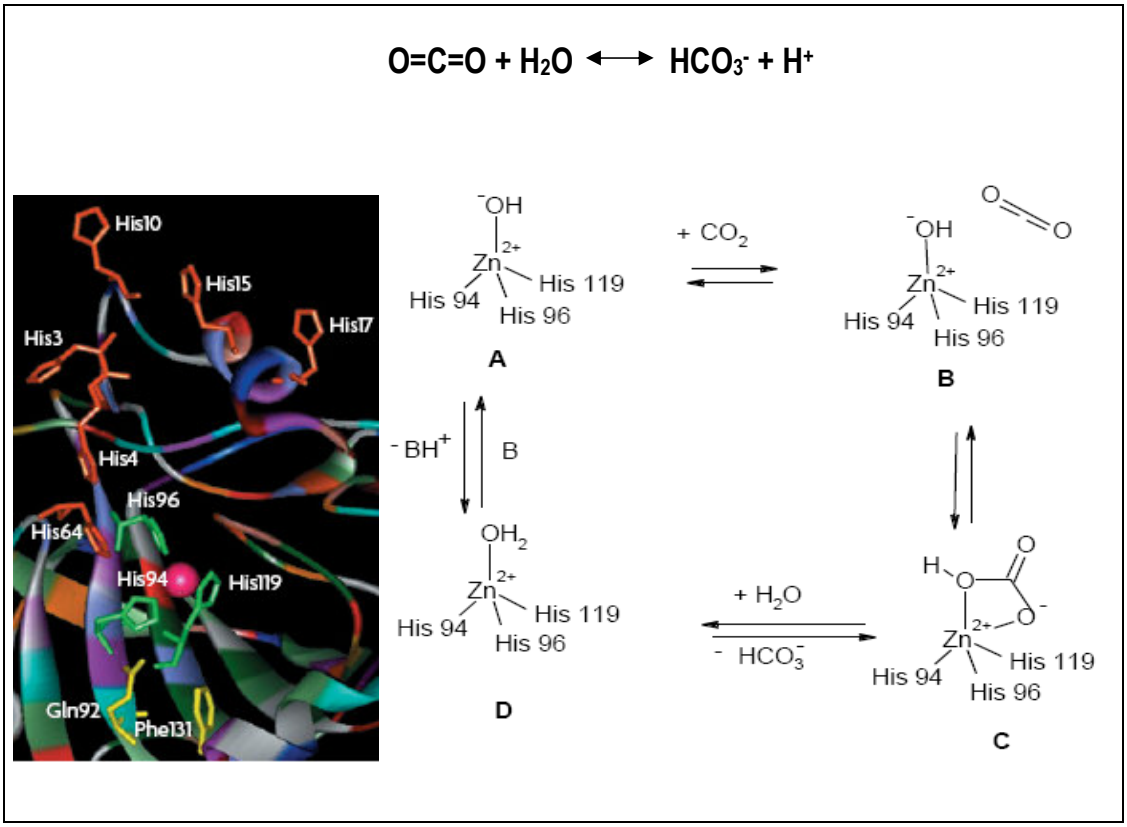


Fig. 1 hCA II active site and Mechanism of action of carbonic anhydrases

To regenerate the basic form (**A**), a proton transfer reaction from the active site to the environment takes place, which may be assisted either by active-site residues (such as His64, the proton shuttle in isozymes I, II, IV, VI, VII, IX and XII–XIV among others) or by buffers present in the medium. The process may be schematically represented by reactions (2) and (3):

$$E–Zn^{2+}–OH^- \xleftrightarrow{H_2O} E–Zn^{2+}HCO_3^- \longleftrightarrow E–Zn^{2+}–OH_2 + HCO_3^- \quad (2)$$

$$E–Zn^{2+}–OH_2 \longleftrightarrow E–Zn^{2+}–OH^- + H^+ \quad (3)$$

The rate-limiting step in catalysis is reaction 3, that is the proton transfer that regenerates the zinc-hydroxide species of the enzyme. In the catalytically very active isozymes, such as CA II, IV, VI, VII, IX, XII, XIII and XIV, the process is assisted by a histidine residue placed at the entrance of the active site (His64), or by a cluster of histidines (figure **1**, left side), which protrudes from the rim of the active site to the surface of the enzyme, thus assuring efficient proton-transfer pathways. This may explain why CA II is one of the most active enzymes known (with a kcat/Km of 1.5 x 10^8 M^{-1} s^{-1}), approaching the limit of diffusion-controlled processes.

1.1.2 The β-CAs

In the last years, some scientists have demonstrated that enzymes present in other organisms, such as the bacterial ones from *Helicobacter pylori* [4a], *Mycobacterium tuberculosis* [4d, 13], *Brucella suis* [16], and many other pathogenic bacteria, as well as pathogenic fungi such as *Cryptococcus neoformans* [17], *Candida albicans* [17], *Candida glabrata* [18], and *Saccharomyces cerevisiae* [4c], constitute new drug targets with the potential to design anti-infectives (antibacterial, and antifungal agents) possessing a new mechanism of action [1]. These enzymes belong to the β-CA family, which is not present in humans [1, 4,16–18].

The new found β-CAs have proved to be crucial for the virulence, growth of these pathogens [19–20]. The β-CAs posses high CO_2 hydration activity comparable to the most active α-CA isoforms, but are less strongly inhibited by sulphonamides. The principale difference between these enzymes and α-CAs is that β-CAs are usually oligomers, generally formed of two or six monomers of molecular weigh 25 to 30 kDa each.The Zn (II) ion is essential for the catalysis in both families of enzymes, but its coordination is different and rather variable for β-CAs (**Fig. 2**, **3**).

Infact, from *Mycobacterium tuberculosis* we can compare the active sites of two β-CAs; in the (named mtCA 1) enzyme, encoded by the gene Rv 1284, there are two cysteinate residues (Cys35, Cys91), an imidazole from a His88 residue and a water molecule which coordinate the metal ion, whereas the neighboring Asp37 and Arg39 residues form a well-defined salt bridge; in the (named mtCA 3) enzyme, encoded by gene Rv3588c, the active site is characterized by a residue Asp53, which displaces the water molecule and coordinates directly to the zinc ion, thus breaking a potential salt link to Arg55 (**Fig. 2**).

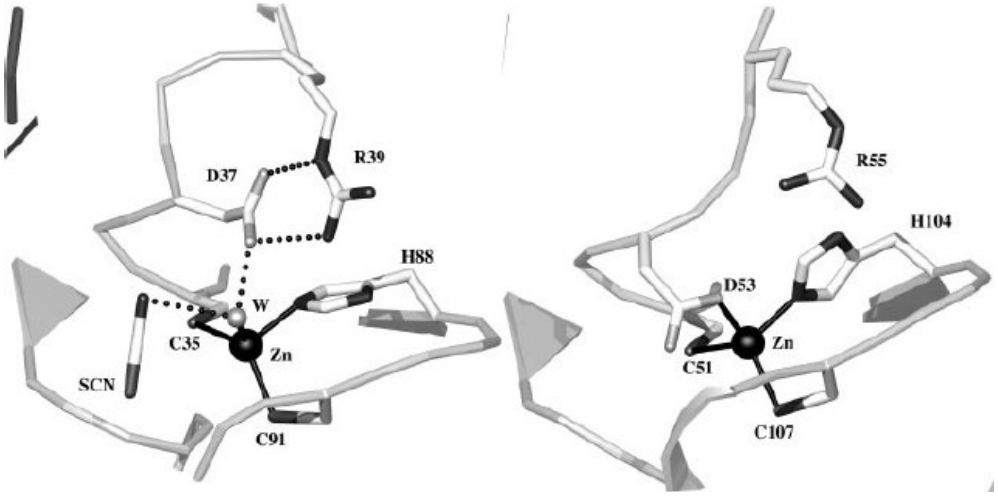


Fig. 2 Active sites of two of *β*-class carbonic anhydrases isoforms, isolated from *Mycobacterium tuberculosis* [13]. Residues involved in metal chelation in mtCA 1 (**A**) and mtCA 2 (**B**) are shown.

In summary, differences in coordination of the active site metal are observed; in mtCA 2 (**B**), an aspartic acid side chain displaces a water molecule and coordinates directly to the zinc ion, thereby closing the zinc coordination sphere and breaking the salt link to a nearby arginine that is a feature of mtCA 1 (**A**). The two β- carbonic anhydrases, thus exhibit both of the metal coordination geometries that have previously been observed for structures in this family [13].

The active site of fungal β-CAs (named Can2) from *Cryptococcus neoformans*, is characterized by a similar metal coordination geometry, in which the catalytic zinc ion is coordinated by the side chains of Cys68, His124, and Cys127 (**Fig. 3**). The fourth coordination site is occupied by a water molecule with an oxygen–metal distance of 2.07 Å. The position of the water molecule is stabilized through a hydrogen bond to the conserved residue Asp70, with a short distance of 2.62 Å [4b], indicating a strong interaction. Asp70 is part of an Asp/Arg pair (Asp70/Arg72 in Can2) conserved in all β-CAs sequenced so far.

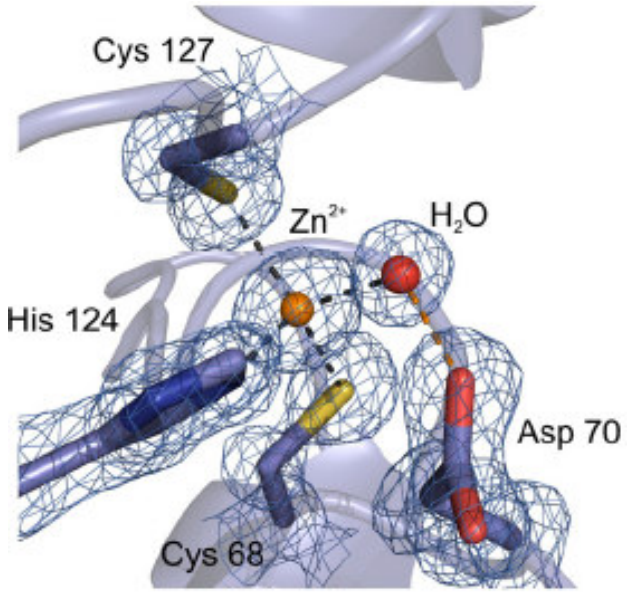


Fig. 3 Active site of the Carbonic Anhydrase (Can2) isolated from the pathogenic *Cryptococcus neoformans* [4b].

1.2.0 *PHYSIOLOGICAL FUNCTION OF CAs*

In prokaryotes, CAs possess two general functions: (1) transport of CO_2/bicarbonate between different tissues of organism; (2) provide carbon dioxide/HCO_3^- for enzymatic reactions [21]. Recently, it has been proved that non-vertebrate CAs, representative of the α- and β- families in several pathogens, are crucial for the growth or virulence [19, 20]. In the aquatic photosynthetic organism, an additional role is that of a CO_2-concentrating mechanism, which helps overcome carbon dioxide limitation in the environment.

In higher organism, including vertebrates, the physiological function of CAs have been widely investigated over the last 70 years [2c, 22, 23].Thus, isozyme I, II, and IV are involved in respiration and regulation of the acid/base homeostasis. These complex processes involve both the transport of CO_2/bicarbonate between metabolizing tissues and excretion sites (lungs, kidneys), facilitated CO_2 elimination in capillaries and pulmonary microvasculature, elimination of H^+ ions in the renal tubules and collecting ducts, as well as reabsorption of bicarbonate in the brush border and thick ascending Henle loop in kidneys [2c, 23].

By producing the bicarbonate-rich aqueous humor secretion (mediated by ciliary processes isozymes CA II and CA IV) within the eye, CAs are involved in vision, and their misfunctioning leads to high intraocular pressure and glaucoma.

The highly active CA II is abundant in the bone, and is present only in osteoclasts at concentrations of the same order of magnitude as those present in the kidneys [24]. Its role there is to provide hydrogen ions, formed from the hydration of CO_2, to an ATP-dependent proton pump, which uses them in the mobilization of calcium from the bone. Thus, CA II is involved in bone development and function, such as differentiation of osteoclasts.

CAs are involved in the secretion of electrolytes in many other tissues and organs, such as: CSF formation, by providing bicarbonate and regulating the pH in the choroids plexus; saliva production in acinar and ductal cells [25]; gastric acid production in the stomach parietal cells; bile production; pancreatic juice production; intestinal ion transport.

CAs play an important physiological role in: 1) protection of the gastrointestinal tract from extreme pH conditions; 2) regulation of pH and bicarbonate concentration in the seminal fluid; 3) muscle functions; 4) adaptation to cellular stress [2c, 23, 25].

Some CA isozymes are involved in the carboxylation of pyruvate to oxaloacetate (mitochondrial isoforms CA VA and VB) and of acetylcoenzyme A to malonylcoenzyme A (cytosolic isoform CA II), as shown schematically (**Fig 4**) [1]; thus in fatty-acid biosynthesis and lipogenesis.

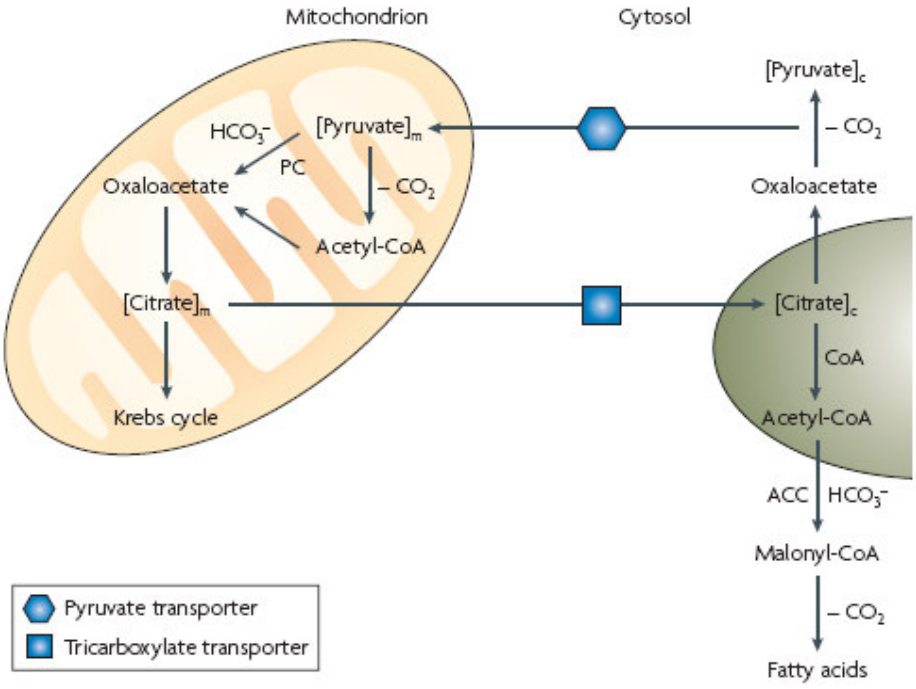


Fig. 4 The transfer of acetyl groups from the mitochondrion to the cytosol (as citrate) for the provision of substrate for de novo lipogenesis [1]. All steps involving bicarbonate also need the presence of CA isozymes: CA VA and CA VB in the mitochondrion and CA II in the cytosol.

Some isozymes (CA IX, CA XII) are predominantly in tumor cells and show a restricted expression in normal tissues [1, 2, 7, 10]. It has been recently proved that by efficiently hydrating carbon dioxide to protons and bicarbonate, these CAs contribute significantly to the extracellular acidification of solid tumors, whereas their inhibition reverts this phenomenon to a certain extent [7b]. CA IX and XII are overexpressed in many such tumors in response to the hypoxia inducible factor (HIF) pathway, and research on the involvement of these isozymes in cancer has progressed significantly in recent years.

At present we know that CA IX, is a transmembrane protein involved in solid tumor acidification through the HIF-1α activation cascade. CA IX has a very high catalytic activity for the hydration of carbon dioxide to bicarbonate and protons, even at acidic pH values (of around 6.5), typical of solid, hypoxic tumours, which are largely unresponsive to classical chemo- and radiotherapy. Furthermore, in addition to the central CA domain, CA IX contain a transmembrane anchor followed by short C-terminal cytoplasmic tail (**Fig. 5**). The N-terminal side of CA IX molecule is extended with a so-called proteoglycan-like (PG) region that is homologous to keratin sulphate attachment domain of a large proteoglycan aggrecan [9g].

Thus, CA IX is used as a marker of tumor hypoxia and as a prognostic factor for many human cancers. CA IX is involved in tumorigenesis through many pathways, such as pH regulation and cell adhesion control.

The report of the X-ray crystal structure of CA IX [9c], which is a dimeric protein with a quaternary structure not evidenced earlier for this family of enzymes (**Fig. 6**), allows for structure-based drug design campaigns of inhibitors against this novel antitumor target.

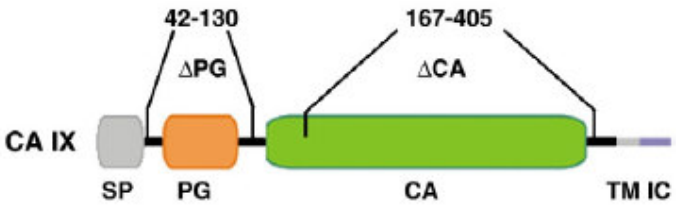


Fig. 5 Domain organization of the CA IX protein: SP=signal peptide; PG=proteoglycan-like domain; CA=catalytic domain; TM=transmembrane segment; IC=intracellular, cytosolic tail.

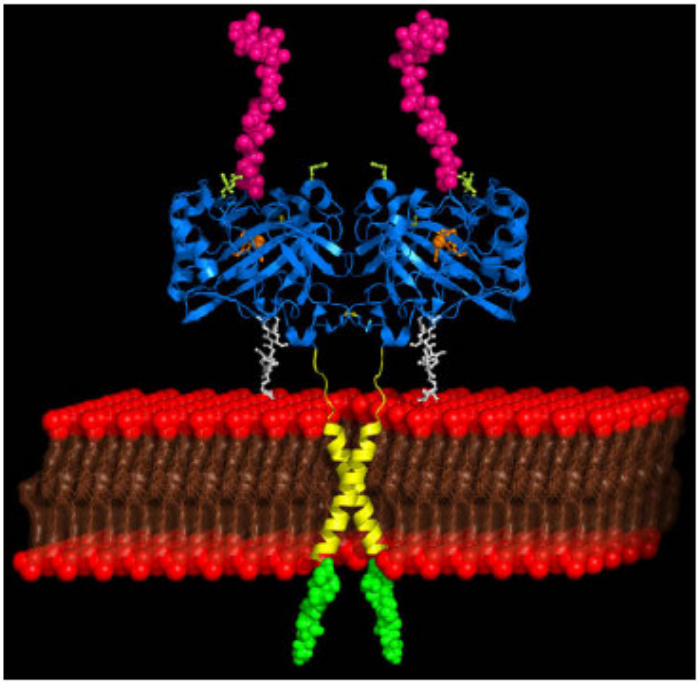


Fig. 6 Cartoon representation of the CA IX dimeric protein: in green the short intracytosolic tail, in yellow the transmembrane region, in blue the CA domain as obtained by X-ray crystallography [**9c**] and in magenta the proteoglycan (PG) domain.

2.0 CARBONIC ANHYDRASE INHIBITORS

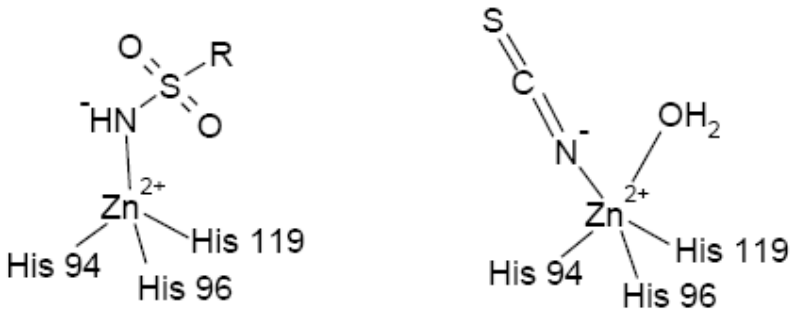
2.1.0 *INHIBITION BY SULFONAMIDES*

The recent years saw the discovery of many new chemotypes (such as coumarins [27] phenols, fullerenes) showing significant CA inhibitory activity and a novel mechanism of action, in addition to the classical sulfonamides and bioisosteres CAIs (sulfamates, sulfamides).

That, prove how dynamic this research field is. The two main classes of CA inhibitors (CAIs) are: the metal complexing anions and the unsubstituted sulfonamide and their bioisosteres. These inhibitors bind to the Zn^{2+} ion of the enzyme either by substituting the non-protein zinc ligand to generate a tetrahedral adduct (Equation 1) or by addition to the metal coordination sphere to generate trigonal-bypiramidal species (Equation 2) [2c, 7a–9a, 21, 22, 26]. The unsubstituted sulfonamides and their bioisosteres, which are the most important CAIs, bind in the tetrahedral geometry of the Zn^{2+} ion of the enzyme (**Fig. 7A**), in deprotonated state, with the nitrogen atom of the sulfonamide moiety coordinated to Zn^{2+}, while the anions add to the metal coordination sphere, generating trigonal-bypiramidal specie , such as the thiocyanate adduct shown in figure (**Fig. 7B**).

$$E–Zn^{2+}–OH_2 + I \longleftrightarrow E–Zn^{2+}–I + H_2O \qquad \text{(Substitution reaction)} \qquad (1)$$

$$E–Zn^{2+}–OH_2 + I \longleftrightarrow E–Zn^{2+}–OH_2\,(I) \qquad \text{(Addition reaction)} \qquad (2)$$



Tetrahedral adduct (sulfonamides) **A**

Trigonal-bypiramidal adduct (thiocyanate) B

Fig. 7 Mechanism of inhibition of carbonic anhydrase: **A**) Unsubstituted sulfonamides bind to the the Zn^{2+} ion of the enzyme by substituting the non-protein zing ligand to generate a tetrahedral adduct. **B**) Anions inhibitors add to the metal coordination sphere, generating trigonal bypiramidal adducts.

At present, several X-ray crystallographic structures are available for many adducts of the main CAIs classes known, above all those with sulfonamide inhibitors and isozymes CA I, II, IV, IX [9c, 22, 28, 29]. Here, it is reported X-ray structure of the catalytic domain of CA IX in complex with a classical, clinically used sulfonamide inhibitor, acetazolamide (**Fig. 8**). Generally, in these adducts, the inhibitors bind within the enzyme active site, coordinating with the deprotonated sulfonamide moiety to the catalytically Zn^{2+} ion and generally its NH moiety participates in an hydrogen bond with the Oγ of Thr199. Furthermore, one of the oxygen atoms of the SO_2NH_2 moiety also participate in an hydrogen bond with the backbone NH moiety of Thr199.

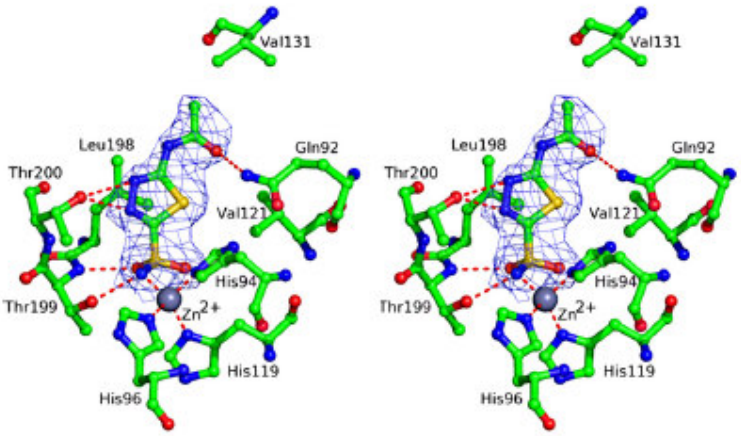


Fig. 8 Active site region of the hCA IX/AAZ complex, showing residues participating in the AAZ recognition

A general pharmacophore (**Fig. 9**) for the compounds acting as carbonic anhydrase inhibitors has been reported by Thiry et al. [30] from the analysis of the CA active site and from the structure of the inhibitors described in the literature [2c]. This pharmacophore includes the structural elements that are required to be present in the compounds in order to act as CA inhibitors. This includes the presence of a sulfonamide moiety which coordinates with the zinc ion of the active site of the CA and the sulfonamide is attached to a scaffold which is usually a benzene ring. The side chain might posses a hydrophilic link able to interact with the hydrophilic part of the active site and a hydrophobic moiety which can interact with the hydrophobic part of the CA active site.

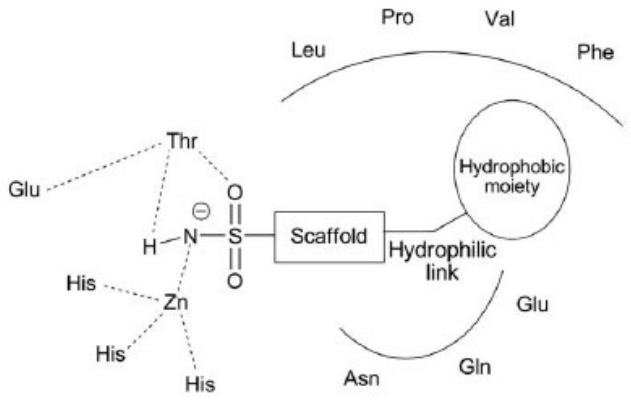
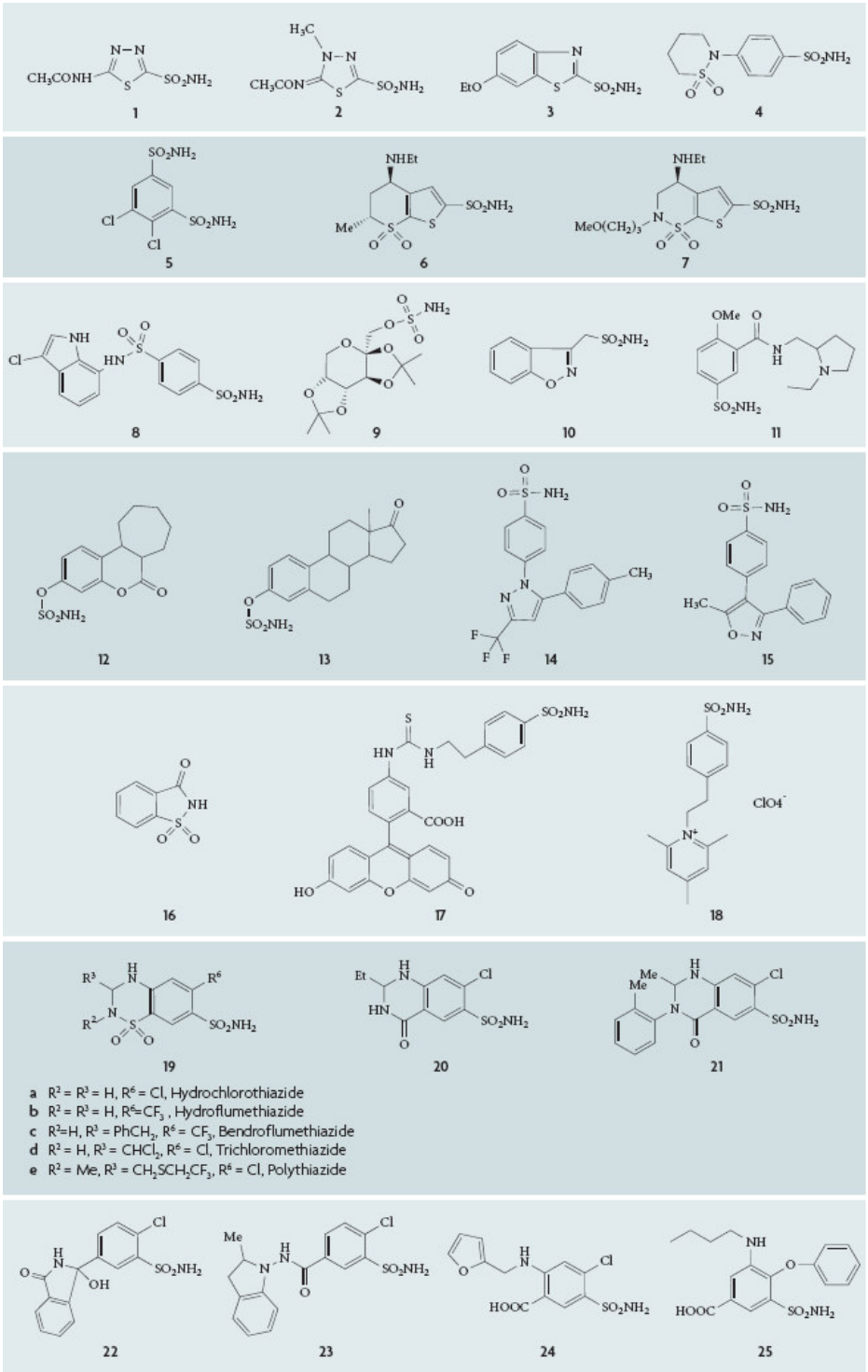


Fig. 9 Structural elements of CA inhibitors in the CA enzymatic active site.

The classical CA inhibitors (CAIs) are the primary sulfonamides, RSO_2NH_2, which are in clinical use for more than 50 years as diuretics and systemically acting antiglaucoma drugs [1–3, 12]. In fact there are around 30 clinically used drugs (or agents in clinical development) belonging to the sulfonamide or sulfamate class, of types **1–25**, which show significant CA inhibitory activity (**Tab. 1**) [1].



Tab.1 Structures of carbonic anhydrase inhibitors **1-25**

In addition to the established role of these CAIs as diuretics and antiglaucoma agents, it has recently emerged that they have potential as anticonvulsant, antiobesity, anticancer, antipain, and antiinfective drugs [1–3, 12]. However, critical barriers to the design of CAIs as therapeutic agents are related to the high number of isoforms in humans (i.e., 16 CAs, of which 13 have catalytic activity), their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors of the sulfonamide/ sulfamate type [1–3]. Infact, among derivatives **1–25** mentioned before, there are no compounds which selectively inhibit some CA isoforms with therapeutic value, although their inhibition profiles against the 13 mammalian isozymes are highly variable and can be used for the structure-based drug design of novel generation, isoform-selective inhibitors (inhibition data of **1–25** against all human (h) CA isoforms are provided in reference) [1].

Acetazolamide (1) was the first non mercurial diuretic to be used clinically in 1956. The best-studied drug is acetazolamide, which is frequently administered long term owing to its efficient reduction of IOP, minimal toxicity and ideal pharmacokinetic properties. However, as CAs are ubiquitously expressed in vertebrates, the systemic administration of sulphonamides leads to nonspecific CA inhibition and is associated with undesired side effects. The development of water-soluble sulphonamide CAIs to be used as eye drops began in the 1990s, and by 1995 the first such pharmacological agent, dorzolamide (compound 6), was launched [31] by Merck under the trade name Trusopt. A second structurally similar compound, brinzolamide (compound 7), has also been approved (by Alcon under the tradename Azopt) for the topical treatment of glaucoma [31]. Dorzolamide and brinzolamide are potent water-soluble CAIs that are sufficiently liposoluble to penetrate the cornea, and may be administered topically as the hydrochloride salt (at a pH of 5.5) or as the free base, respectively [31].

The two drugs are effective in reducing IOP and show fewer side effects as compared with systemically used drugs. Acetazolamide, dorzolamide or brinzolamide

probably act as local vasodilators, improving blood flow in this organ and consequently clearing metabolic waste products and drusen. Vision following such treatment (in early phases of the disease) is markedly improved [31, 32].

Thus, acetazolamide represents the prototype of a class of pharmacological agents with relatively limited therapeutic use, but which played a major role in the development of fundamental renal physiology and pharmacology, and in the design of many of the current widely used diuretic agents, such as the thiazide and the high-ceiling (loop) diuretics. Acetazolamide (compound 1), methazolamide (compound 2), ethoxzolamide (compound 3) and dichlorophenamide (compound 5) are used to treat oedema due to congestive heart failure and for drug-induced oedema [33-34]. Thiazide and high-ceiling diuretics, such as trichloromethiazide 19d, chlorthalidone 22, indapamide 23, and furosemide 24, have been discovered in the 60s–70s, when little was known about the various CA isozymes, and these drugs were considered not to interact substantially with the mammalian CAs [29]

Indisulam (compound 8), a sulfonamide derivative (originally called E7070) with powerful anticancer activity, is in phase II clinical trials as anticancer sulfonamide with a complex mechanism of action, which involve the inhibition of several enzymes including the carbonic anhydrases (CA IX, CA XII), which participate in tumour genesis; recently it was also shown to act as a nanomolar inhibitor of CA IX [35].

Recently, our group reinvestigated their interactions with the 13 catalytically active mammalian CAs and reported the X-ray crystal structures of their adducts with hCA II [29]. The newly evidenced binding modes of these diuretics may be thus exploited for designing better CA II inhibitors as well as compounds with selectivity/affinity for various isoforms with medicinal chemistry applications.

CA II is the physiologically dominant isoform, which is catalytically highly efficient, widespread in many cell types and thus involved in many physiological processes, and also easily crystallisable [1, 2d, 36]. Thus, most of knowledge in the design of CAIs with pharmacological applications are based on detailed CA II crystallographic studies. CA II has been crystallized in adducts with the main classes of inhibitors, sulfonamides and their bioisosters [10a, 36-38], anions [39], phenols [40], coumarins [27a], and polyamines [41]. Among all these classes of CAIs, the sulfonamides continue to be of great interest, considering the many drugs currently used clinically as antibacterial, hypoglycaemic, anti-hypertensive and antiviral and recently as anticancer agent [1, 2c, 42].

In summary, all the best derivatives, used clinically incorporate in their molecules a common chemical motif of aromatic/heteroaromatic sulfonamide and have unsubstituted primary sulfonamide moieties acting as effective zinc-binding moieties; X-ray crystallography of CA inhibitors in a complex with enzymes, is an important tool in drug design of zinc-enzyme inhibitors, using such structures for the rational modelling, drug design of more selective and potent CAIs for several disease applications [10a].

3.0 AIM OF THE WORK

The overall aim of the present thesis is to study the interaction between several isoforms of carbonic anhydrase from human, bacteria, fungi and new obtained sulfonamides derivatives and their bioisosteres.

The development of more specific agents is required because of the high number of isozymes present in the human body as well as the isolation of many new representatives of CAs from all Kingdoms. This is possible only understanding in detail the catalytic and inhibition mechanism of these enzymes.

3.1.0 *HUMAN α-CA INHIBITION*

The h-CAs control a remarkable range of fundamental physiological processes in the organism and various tissue types [2c]. These include acid-base balance, bone resorption, calcification, several biosynthetic pathways and a variety of processes involving ion, gas and fluid transfer. Recent evidence demonstrates involvement of CAs in cell growth and signalling of hypoxia with implication for cancer development and progression [8a]. They differ by tissue distribution, localization, kinetic properties, sensitivity to inhibitors and presence of CA unrelated domains. Now it is emerging that each CA isoform is associated with specific biochemical pathways. According to the newest research data, some CAs interact with other proteins to form large complexes called metabolons in which they improve the biosynthetic reactions or ion transport efficiency. Clearly, carbonic anhydrases are the major players in mammalian physiology, but still in many cases the series of biochemical events are far from being

fully elucidated as well as are still unknown the partners of the different isoforms associate to many physiological/pathological processes.

Basic research is still necessary to clarify fundamental physiological processes, whose understanding is a prerequisite for approaching diseases.

A completely new and very promising aspect of CAs physiology has been recently found and refers to over expression of some CA isoforms in cells as a consequence of reduced oxygen afflux (hypoxia). This situation is frequently met in tumour tissues were the fast growing rate favours anoxic conditions. The isoforms over expressed (CA IX, CA XII) are membrane located with the active site exposed toward the exterior part thus representing a possible target for new therapies and or diagnostic tools.

It is well known that aberrant expression of CAs and excess of their enzyme activity are common in various pathological situations. For example, increased CA II activity contributes to glaucoma and osteoporosis, CA II, VII and XIV are suspected to participate in neurological disorders including brain oedema and epilepsy, CAII/CA IV play a role in kidney dysfunction, CA V appears involved in obesity and CA IX/CA XII have been implicated in cancer progression.

Therefore, the control of the activity of isoforms involved in a particular disease represents a promising therapeutic option.

Inhibitors of carbonic anhydrase (CAI), mainly of sulphonamide type, are widely used as therapeutic agents in the management or prevention of several diseases, but they were developed years ago when there was no clear idea of the different and specific role played by each isozyme [2c].

As a consequence, the available drugs possess many undesired side effects due to their lack of selectivity, i.e. indiscriminate inhibition of all CA isozymes. For instance, side effects of a recently used antiglaucoma drug, dorzolamide, include contact allergy, nephrolitiasis, anorexia, depression and dementia.

The advancements in the comprehension of the role of the different CAs isoforms and of their spatial localization on one side as well as the progress in the X ray crystallographic techniques at high and ultrahigh resolution, and in virtual screening methods make more realistic the perspective to design new CA inhibitors with differential selectivity toward the different isoforms. Indeed some important achievements have been already obtained by some of the groups involved in this research field [9c-f]. The main goal is on one hand to improve the knowledge of the role of each CAs isoform and, on one hand, to develop inhibitors with higher specific isoform selectivity, in order to obtain drugs with reduced side effects and/or new diagnostic tools.

3.1.1 *hCA I and hCA II inhibition*

These two isoforms are expressed in several tissues (erythrocytes, eye, GI tract, bone osteclasts, kidney, lung, testis, brain) and are included in the group of intracellular CAs (cytosolic isoforms). Carbonic anhydrase II is one of the fastest enzymes known; it performs major role in respiration and acid-base balance. In erythrocytes, it catalyzes the hydration CO_2 to HCO_3^- ions, while in renal tubule it is important for the acidification of urine.

Although CA I appears to contribute about 50% of CO_2 hydration activity of most mammalian erythrocytes, the available evidence suggest that this isozyme is abundant provided; presumably it is present more than CA II; however, the catalytic activity of CA I is lower than CA II (about 10% of the catalytic activity).

The physiological function of the major red cell isozyme, CA I (present in concentration of up to 150 μM in the blood) is still unknown.

Red blood cell (RBC) CA contributes to CO_2 excretion in vertebrates by accelerating the rate of conversion between molecular CO_2, (the form in which CO_2 can readily diffuse across membranes) and HCO_3^- (the form in with CO_2 is transported in the circulation).

Inhibition of CA has been investigated in the treatment of disease as glaucoma. The treatment of this disease with inhibitors of the CAs [2c] is very effective in reducing elevated intraocular pressure (IOP) characteristic of this pathology. At present are available for clinic use two topical acting inhibitors (dorzolamide DZA and Brinzolamide, BRZ) [31], in addition to the classical inhibitors used via systemic route of administration (acetazolamide AAZ, methazolamide MZA, ethoxzolamide EZA, and dichlorophenamide DCP) [43].

However, all these compounds clinically used have a lot of side effects and lack of selectivity.

3.1.2 *hCA VA and hCA VB inhibition*

Between the intracellular hCAs are included also two mitochondrial (VA and VB) isoforms; the two homologues of CA V possess a leader sequence that target them to the mitochondria [44]. These isozymes were shown to be involved in several biosynthetic processes, such as ureagenesis [45], gluconeogenesis [46], and lipogenesis, both in vertebrates(rodents) as well as invertebrates (locust) [47-49]. Indeed, in several important biosynthetic processes involving pyruvate carboxylase, acetylCoA carboxylase, and carbamoyl phosphate synthetases I and II, bicarbonate, not carbon dioxide is the real substrate of these carboxylating enzymes, and the provision of enough bicarbonate is assured mainly by the catalysis involving the mitochondrial

isozyme hCA V (probably assisted by the high activity cytosolic isozyme hCA II) [50-52].

Recently, some of the side-effects observed in obese epileptic patients treated with topiramate 9 or zonisamide 10 consisted of a significant weight loss [1, 53-55]. This has been rationalized [1] as being due to the lipogenesis inhibition mediated by these two agents, which in turn is mediated by inhibition of some CA isozymes involved in the carboxylation of pyruvate to oxaloacetate (mitochondrial isoforms CA VA and CA VB) and of acetylcoenzyme A to malonylcoenzyme A (cytosolic isoform CA II), as already shown, schematically in Figure **4**.

A possible new approach for the treatment and prophylaxis of obesity is based on the inhibition of carbonic anhydrases enzymes involved in several steps of de novo lipogenesis, both in the mitochondria and the cytosol of cells. Topiramate and zonisamide are two antiepileptic drugs that were shown to induce persistent weight loss in obese patients, but their mechanism of action is largely unknown. We demonstrated strong CA inhibitory properties for these two drugs, by means of kinetic studies in solution and X-ray crystallography, against several physiologically relevant isoforms, such as CA II, VA and VB. It has been proved that topiramate also inhibits lipogenesis in adipocytes, similarly to other sulfonamide CA inhibitors investigated earlier. A large number of new sulfonamides have been synthesized and assayed as possible inhibitors of CA isoforms involved in lipogenesis. This is the beginning of a very new and promising approach for the treatment of obesity [57].

These compounds have good affinity, but as well as all clinically used drugs (Tab.1) [1], are without selectivity. Thus, the objective is to discovery of compounds with high affinity and accepted selectivity to understand the role of CA V and the real possibility to use CAIs as potential drugs in therapy. The overall effect is a potent inhibition of lipogenesis. Presently, a combination of topiramate 9 (sustained release

form) with phentermine, Qnexa, is in Phase III clinical trials for the treatment of obesity [56] making it the first in a class drug, with a novel mechanism of action [1].

In previous contributions from our laboratories [54] we have shown that both CA VA and CA VB are druggable targets. Rather large libraries of various sulfonamides and sulfamates have been assayed as inhibitors of these mitochondrial enzymes, with several low nanomolar inhibitors being detected [54]. However, an important drawback of most of these compounds is represented by the rather low selectivity for inhibiting the mitochondrial CAs over the cytosolic/membrane-bound ubiquitous isoforms such as CA I, II (cytosolic) and CA IV (extracellular, membrane-associated isozyme) [1,54].

3.1.3 *hCA IX and hCA XII inhibition*

CA IX, originally named MN protein, has been the first detected with a monoclonal antibody M75 in a human carcinoma cell line HeLa as a cell density-regulated plasma membrane antigen [50b]. At that time, this was the ninth mammalian CA identified and thus the MN protein was renamed CA IX [50c].

Sequence of the cDNA encoding transmembrane isozyme CA XII has been published in 1998 by two independent groups [50d-e]. After the identification of these two transmembrane isoforms, it has been shown that CA IX is almost exclusively associated with tumours, while CA XII is over expressed in some tumour types.

Carbonic anhydrase (CA, EC 4.2.1.1) IX (CA IX) has recently been shown to be a druggble target for imaging and treatment of hypoxic tumours over expressing this protein [1, 7b, 9c, 9e-f, 58-62]. Indeed, CA IX is the most strongly over expressed gene product, in response to hypoxia in human cancer cells [7b, 9c, 9e-f, 60].This enzyme is a multidomain protein [9c] with the CA subdomain situated outside the cell

and possessing a very high CO_2 hydrase catalytic activity [9d], making it a key player in the regulation of tumour pH [1, 7b, 9c, 9e-f, 58-62].

CA IX expression is strongly increased in many types of solid tumours, such as gliomas/ependymomas, mesotheliomas, papillary/follicular carcinomas, as well as carcinomas of the bladder, uterine cervix, kidneys, esophagus, lungs, head and neck, breast, brain, vulva, and squamous/basal cell carcinomas, among others [8b, 50b, 63-64]. Furthermore, such hypoxic tumours do not generally respond to the classic chemo- and radiotherapy [8b, 50b, 63-64].

Pouyssegur's group showed recently [61] that in hypoxic LS174Tr tumour cells expressing either CA IX or both CA IX and XII isoforms, in response to a CO_2 load, both enzymes contribute to extracellular acidification and to maintaining a more alkaline resting intracellular pH (pHi), an action that preserves ATP levels and cell survival in a range of acidic outside pH (6.0-6.8) and low bicarbonate medium. In vivo experiments showed that silencing of CA IX alone leads to a 40% reduction in xenograft tumour volume, with up-regulation of the second gene, that encoding for CA XII. Silencing of both CA IX and CA XII gave an impressive 85% reduction of tumor growth [61]. Thus, hypoxia-induced CA IX and CA XII are major tumor prosurvival pH-regulating enzymes, and their combined targeting (i.e., inhibition) held potential for the design of anticancer drugs with a novel mechanism of action. The in vivo proof of concept that sulfonamide CA IX inhibitors may indeed show antitumor effects, has been only very recently published by Neri's group [62a]. By using membrane-impermeant derivatives based on the acetazolamide **AAZ** scaffold to which either fluorescein-carboxylic acid or albumin-binding moieties were attached, this group demonstrated the strong tumour growth retardation in animals treated for one month with these CA inhibitors (CAIs) in mice with xenografts of a renal clear cell carcinoma line, SK-RC-52 [62a].

Such data show indeed the great promise of tumour growth inhibition with sulfonamides acting as CA IX/XII inhibitors, making thus probable the development of alternative anticancer drugs based on this approach [1]. The same group also reported the proof-of-concept study showing that human monoclonal antibodies targeting CA IX can also be used for imaging of hypoxic tumours [62b]. The generation of high-affinity human monoclonal antibodies (A3 and CC7) specific to hCA IX, using phage technology has been recently reported.

These antibodies were able to stain CA IX ex vivo and to target the cognate antigen in vivo. In one animal model of colorectal cancer studied (LS174T), CA IX imaging closely matched pimonidazole staining, with a preferential staining of tumour areas characterized by little vascularity and low perfusion [62b].

The same conclusion has been reached by our group, by using small molecule CA IX-selective inhibitors of the type **1** [7b, 9e-f] Fluorescent sulfonamides **1** with a high affinity for CA IX have been developed and shown to bind to cells only when CA IX protein was expressed and while cells were hypoxic. NMRI-nu mice subcutaneously transplanted with HT-29 colorectal tumours were treated with 7% oxygen or with nicotinamide and carbogen and were compared with control animals. Accumulation of CAI compound **1** was monitored by non-invasive fluorescent imaging. Specific accumulation of **1** could be observed in delineated tumour areas as compared with a structurally similar non-sulfonamide analogue incorporating the same scaffold **2**. Administration of nicotinamide and carbogen, decreasing acute and chronic hypoxia, respectively, prevented accumulation of **1** in the tumour. When treated with 7% oxygen breathing, a 3-fold higher accumulation of **1** was observed. Furthermore, the bound inhibitor fraction was rapidly reduced upon tumour re oxygenation [7b, 9e-f]. Such *in vivo* imaging results confirm previous *in vitro* data demonstrating that CAI binding and retention require exposure to hypoxia. Labelled sulfonamide CAIs may thus provide a powerful tool to visualize hypoxia response in solid tumours.

An important step was thus made towards clinical applicability, indicating the potential of patient selection for CA IX-directed therapies [7b, 9e-f].

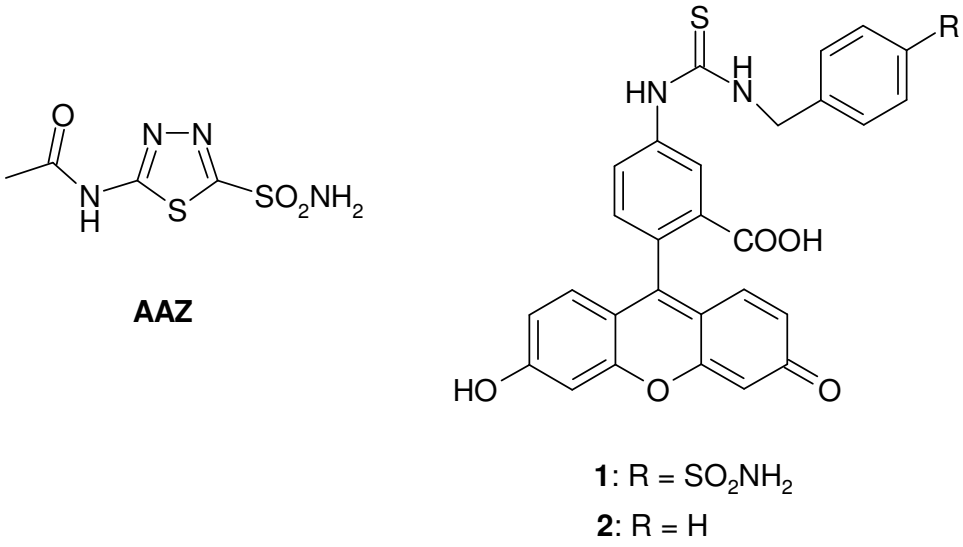


Fig. 10 AAZ and Fluorescent sulfonamides **1**

One main draw-back of classical sulfonamide CAIs (such as **AAZ**) is the lack of selectivity for inhibiting CA IX over the other CA isoforms present in humans (fifteen isoforms, CA I – XIV, are present in primates and 16 isoforms (i.e., CA I – CA XV in other vertebrates) [1]. Thus, it is crucial to explores chemotypes possessing a more selective inhibition profile against the target isoforms, e.g., with selectivity for the inhibition of the tumor-associted over other CA isoforms.

Indeed, it has been known that many classes of aromatic/heterocyclic sulfonamides and sulfamates have good affinity for this isoform, [1-3, 7–8, 9a-c, 10] but generally they do not show specificity for the inhibition of the tumour-associated isoform versus the remaining CA isozymes (CA I–VII and XII–XV) found in mammals.

Several approaches were reported in the last years for obtaining compounds that specifically target the tumour-associated isoforms: (i) the fluorescent sulfonamides (such as compound 17 (Tab.1) or see above (type **1)**, used for imaging purposes and for determining the role of CA IX in tumour acidification [1,7, 9e-f]; (ii) the positively such as 18 (Tab.1) [65] or negatively charged compounds, which cannot cross plasma membranes due to their charged character and thus inhibit selectively only extracellular CAs, among which CA IX and XII [1]; (iii) the hypoxia-activatable compounds, which exploit the reducing conditions of hypoxic tumours to convert an inactive prodrug into an active CAI [66]; (iv) the sugar-containing sulfonamides/sulfamates/sulfamides, which due to their highly hydrophilic character do not easily cross membranes and thus possess an enhanced affinity for extracellular CAs such as CA IX and XII [67]; (v) the nanoparticles coated with CAIs [68] and (vi) diverse chemotypes than the sulfonamides and their bioisosteres, such as the phenols, protein tyrosine kinase inhibitors, coumarins, fullerenes, and other compounds recently investigated as alternative CAIs to the classical types of inhibitors [27, 69-71].

At present, one of the most interesting compound is named Indisulam. It is a sulfonamide derivative with powerful anticancer activity and recently shown to act as nanomolar inhibitor of CA IX (in vitro), but without selectivity for some isoforms [35].

Indisulam showed *in vivo* efficacy against human tumour xenograft in nude mice, exhibiting a significant antitumour affect and progressing to Phase I and II clinical trials for the treatment of solid tumours. Its detailed mechanism of action is not clear, involving the inhibition of several enzymes including the carbonic anhydrase (CA IX, CA XII).

Thus, it seem to be necessary to obtain again a better affinity-selectivity profile with new compounds to understand the role of CA IX in tumour and the possible applications of CAIs as anticancer agents in therapy.

3.2.0 BACTERIAL, FUNGAL *β-CA INHIBITION*

The β-CAs from various fungal [4b-d,17,18] (*C. neoformans* [4c] , *C. albicans* [17], *C. glabrata* [18] or *S. cerevisiae* [4c]) and bacterial [13,16], (*M. tuberculosis* [13], and *B. suis* [16]) pathogens started to be investigated more recently. Potent inhibitors targeting these enzymes were evidenced among sulfonamides, sulfamates, boronic acids, and carboxylates [4,13–18].

However, *in vivo* the inhibition of the pathogen growth has been observed only for some fungi [4b] and bacteria as H. pylori [4a]. In the case of *M. tuberculosis* no *in vivo* inhibition of growth was evidenced so far, probably due to the difficulty of the inhibitor to cross the mycolic acid membrane typical of this bacterium [13] Thus, future work should address the permeability of CAIs which in many cases are difficult to be delivered at the sites where the enzymes are present in these prokaryotes. On the other hand, more bacterial/fungal genomes are being constantly sequenced and the finding of novel targets belonging to the various CA families is constant, leading to a certain degree of optimism that anti-infectives based on CAIs can be developed.

The emergence of resistance to the currently used drugs in several human pathologies such as Tuberculosis or Candidosis, force to develop of new antiifective agents with a novel mechanism of action as isoform-selective CA inhibitors that target a particular CA isoform to maximize therapeutic efficacy and to minimize the side effects.

3.2.1 *β-CA from Mycobacterium tuberculosis inhibition*

Three β-carbonic anhydrases (CAs, EC 4.2.1.1), (mtCA 1, mtCA 2, mtCA 3) respectively encoded by the gene Rv1284, Rv3588c and Rv3273, are present in the human pathogen *Mycobacterium tuberculosis*. These enzymes were cloned and they showed appreciable catalytic activity for CO_2 hydration. The Rv3273 gene product (mtCA 3) is predicted to be a 764 amino acid residues polypeptide, consisting of a sulfate transporter domain (amino acids 121-414) in addition to the β-CA mentioned above (which is encoded by residues 571-741). All these enzymes were inhibited appreciably by many sulfonamides and sulfamates, in the nanomolar-micromolar range, whereas some subnanomolar inhibitors were also reported for two of them (mtCA 1 and mtCA 3) [13c]. Screening analysis for genes specifically required for the mycobacterial growth showed that Rv3588c is required but Rv3273 is not essential for the bacterial growth in vivo [20a-b]. However, such findings can not conclusively exclude the biological significance of Rv3273 in the survival and/or pathogenicity of *M. tuberculosis*.

An elegant study by Miltner et al. [20c], identified six invasion-related genes of these bacteria. Constitutive expression of the proteins encoded by these genes showed significantly increased ability of the bacterial invasion into HEp-2 and HT-29 intestinal epithelial cells. One of these genes is homologous to *M. tuberculosis* Rv3273, which showed 1.4-1.6 times increase of infected cell numbers by *M. avium* over expressing its gene product. These findings indicate that Rv3273 gene plays a role in the bacterial infection process, which is however poorly understood at the present time. Thus, inhibition of the three CAs present in this pathogen (mtCA 1, mtCA 2, mtCA 3) may have relevance for the design of compounds with anti-TB activity possessing a novel mechanism of action, devoid of resistance problems encountered with classical antibiotics. Rv3273 is predicted to code a bifunctional transmembrane

protein that consists of an N-terminal sulfate transporter domain and a C-terminal CA domain, and we herein demonstrated the significant catalytic activity of this CA domain.

Although it is uncertain how the CA activity of the Rv3273 gene product (mtCA 3) works in the bacterial infection process, it is not improbable that effective CAIs may prevent bacterial infection [72]. In addition to confirm the sulfate transporter function of the amino-terminal end of mtCA 3, further studies are warranted for understanding the biological role of this bifunctional transmembrane protein.

As sulfonamides also efficiently inhibit dehydropteroate synthetase (DHPS), the contribution of mtCAs and DHPS inhibition to a possible antimycobacterial action of these drugs must be better understood. It has been however proved that mtCAs are druggable targets, with a real potential for developing antimycobacterial agents with a diverse mechanism of action compared to the clinically used drugs for which many strains exhibit multi-drug resistance and extensive multi-drug resistance, although for the moment no in vivo inhibition of the bacteria could be evidenced with the presently available drugs due to lack of penetrability through the mycolic acid cell wall of *M. tuberculosis*.

3.2.2 *β-CA from Candida albicans and Candida glabrata inhibition*

C.albicans and *C. glabrata* are important fungal pathogens of humans. In healthy individuals *C. albicans* can be frequently found as a member of the gastrointestinal flora and rarely causes infections. However, when individuals become immuno-suppressed secondary to chemotherapy, AIDS, or even as a consequence of age, *C. albicans* is able to disseminate and cause chronic and potentially life threatening systemic disease [73a-b].

One important factor that contributes to *C. albicans* pathogenicity is its polymorphic nature. Indeed, this ascomycete may grow as yeast, pseudohyphal, true hyphal and chlamydospore forms. The ability to switch between the yeast and hyphal forms is regulated by a wide range of host environmental factors including serum, pH, temperature, CO_2, and available carbon sources [19a,73]. *C. albicans* is the predominant cause of both superficial and invasive forms of candidosis.

Candida glabrata is a pathogenic fungi which, similar to *C. albicans*, possesses a recently characterized β-CA isoform (named CgCA) encoded by the nce103 gene [74-75]. *C. glabrata* is a common cause of mucosal and invasive, systemic infections, accounting for 15% of Candida infections in the general patient population [76-77] Furthermore, this pathogen is innately resistant to azole antifungal agents and is less susceptible to commonly used in clinical practice antifungals and resistance to these agents in *C. glabrata* has been frequently reported as well [78].

The most interesting findings in this field regard the signalling role of CAs that is important for the virulence of fungal pathogens such as *C. albicans* and *C. neoformans*, as identified by Muhlschlegel's and Heitman's groups [19]. It has been demonstrated that physiological concentrations of CO_2/HCO_3^- induce filamentation in *C. albicans* by a direct stimulation of the adenylyl cyclase activity AC (Cdc35), producing a second messenger cAMP. The second protein involved in this phenomenon dubbed "CO_2 sensing" in pathogenic fungi is a carbonic anhydrase, belonging to the β-family encoded by the gene nce103, named here (Nce103p) [1,79].

Recently our group has shown that *C. glabrata* has evolved additional signaling mechanisms to permit it to respond to elevated CO_2 concentrations, compared to *C. albicans* and *C. neoformans* [75]. In the same study it has been shown that CA expression in *C. glabrata* is tightly controlled in accordance with the availability of bicarbonate, which is essential as a carbon source for the intermediate metabolism [75].

4.0 CARBONIC ANHYDRASE ENZYMATIC ACTIVITY: SPECTROPHOTOMETRY AND STOPPED FLOW ASSAY

The stopped flow technique is a useful method for following the kinetics of reactions in solution (usually in the millisecond time range) in which two reactant solutions are rapidly mixed by being forced through a mixing chamber. The flow of the mixed solution along a uniform tube is then suddenly arrested. At a fixed position along the tube the solution is monitored (as a function of the time following the stopped of the flow) by absorption method with a rapid response (spectrophotometry); there is a variable slope (decrease) of the absorption curves of CA II alone and with increasing amount of the inhibitor.

The SX.18MV-R Applied Photophysics stopped-flow instrument directly gives the rate value for the reaction (CO_2 hydration activity) catalyzed by CAs [80].

The equation used by the instrument for the data analysis is the following:

$$Y = A_0\, e^{-Kt} + mX + c \quad \Longrightarrow \quad P(1)\, e^{-P(2)\, t} + P(3)\, X + P(4)$$

P(1) is A_0 (Amplitude)
P(2) is K (Rate)
P(3) is m (Slope)
P(4) is c (Intercept)

We obtain the Amplitude, Rate, Slope and intercept value directly from the instrument once chosen the steady state with single exponential function for the analysis.

The main advantages of this method are:

I) use of CA physiological substrate (saturated CO_2 solution)

II) pH an temperature under physiological conditions

III) reaction times and reagent amounts reduction

IV) data reliability

4.1.0 *REAGENTS*

The standard protocol used to carry out all the experiment is reported.

1. *Buffers*: 4-(hydroxyethyl)-1-Piperazine-ethanesulphonic acid (HEPES) 10-20 mM as buffer, and Na_2SO_4 anhydrous (to maintain the ionic strength) 10-20 mM at (pH 7.5 for α-CAs) or TRIS 10-20 mM as buffer and 10-20 mM NaCl (for maintaining constant the ionic strength) at (pH 8.3 for β-CAs).

2. *Phenol red* (at the concentration of 0.2 mM) used as indicator (pH 6.8-8.4), working at the maximum absorbance of 557 nm.

3. *CO_2 solution*: obtained by bubbling pure CO_2 in bidistilled water solution at 20° C until the saturation (usually more than 35 minutes). After that, the solution is kept in a tightly closed vessel. If the measurement takes more than 2 hours, another 15 min bubbling is performed, in order to maintain the CO_2 concentration constant. Depending on the temperature the solubility of CO_2 in water is known and we can calculate the molarity of the substrate. At 20° C this is 17 mM. When working at variable substrate concentration is required, this saturated solution is diluted with bidistilled water till the desired molarity (CO_2 concentration ranged from 1.7-17 mM).

4. *CA stock solution*: enzyme concentrations were prepared in a range between 0.1 μM (CA I, CA II) to 1 μM (CA V, CA IX, CAXII) depending on the specific activity of the isoform (i.e less active they are, more concentrated are the used solutions).

5. *Inhibitor stock solutions*: the stock solution (10 mM) were prepared in distilled-deionized water with 10-20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter. Four different concentrations of inhibitors for each enzyme were used. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex.

4.2.0 *DATA ANALYSIS*

The collected data from the stopped-flow Assay are plotted, as inhibition percentage of the enzyme, versus the inhibitors concentrations using a sigmoidal dose-response curve (**Fig. 11**)

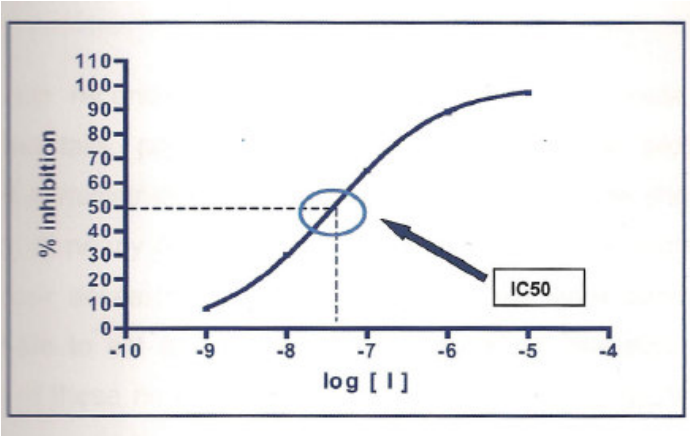


Fig. 11 Sigmoidal dose-response curve of CA II against acetazolamide

From the data analysis it is easy measured the IC50 value using a Prism 3.0 Software.

95% Confidence Intervals	
LOGEC50	-7.449 to -7.385
HILLSLOPE	0.6174 to 0.6729
EC50	3.554e-008 to 4.121e-008
Goodness of Fit	
Degrees of Freedom	3
R^2	0,9997
Absolute Sum of Squares	0,7546
Sy.x	0,5015
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	5
Number of Y replicates	1
Total number of values	5
Number of missing values	0

The simplified Michaelis-Menten equation for steady state is used to calculate the specific used inhibitor Ki.

$$K_i = \frac{IC_{50}}{1 + \frac{[Substrate]}{K_M}}$$

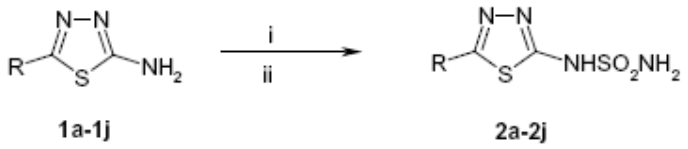
5.0 α-CA INHIBITION: CAIs FOR DISEASE APPLICATIONS

The effort is being directed toward the development of isoform-selective CA inhibitors that target a particular CA isoform to maximize therapeutic efficacy while minimizing unwanted side effects.

5.1.0 NEW *SULFAMIDES AS POTENTIAL ANTIOBESITY DRUGS*

Considering the interest in CA VA/VB—selective inhibitors which might be developed as antiobesity agents, we explore here a less investigated class of CA inhibitors (CAIs) for obtaining compounds targeting the mitochondrial CAs, that is, the sulfamides [67a, 81-82]. Furthermore, it has been proved that the sulfamide moety is an effective zinc binding group for obtaining CAIs, similarly to the bioisosteric sulfonamide and sulfamate ones [81].

Here we report the synthesis of 1,3,4-thidiazole sulfamides possessing various 2-substituents (Scheme 1) [83a-b]. This scaffold has been chosen as it is present in one of the most investigated and powerful CAI, acetazolamide AAZ, used clinically since 1956 [1], and also because its binding to the enzyme is effective, as shown for many AAZ derivatives for which the X-ray crystal structure has been resolved in adduct with different CA isoforms [38, 84]. Furthermore, all the compounds have been physico-chemically characterized by ^{1}H, ^{13}C and ^{19}F NMR and Melting points (m.p.) analysis.



1a-1j **2a-2j**

Scheme 1. Synthesis of 2-substituted-1,3,4-thiadiazole-5-sulfamide 2a — 2j; reagents and conditions:(I) *t*-BuOH, $ClSO_2NCO$, NEt_3, CH_2Cl_2; (II) TFA 95% in CH_2Cl_2

The general procedure for the preparation of thiadiazole-sulfamides 2 was the following: to a solution of 2-substituted-5-amino-1,3,4-thiadiazole 1 in methylene chloride and 1.1 equiv of triethylamine was added dropwise a solution of tert-butoxycarbonylamino sulfonyl chloride (prepared ab initio by reacting 1 equiv of tert-butanol and 1 equiv of chlorosulfonyl isocyanate in methylene chloride at 0°C) [83a]. The mixture was stirred 1 h at room temperature, and then concentrated under vacuum. The residue is purified on silica gel column chromatography using ethyl acetate-petroleum ether 7–3 as eluent to give the Boc-protected sulfamide in good yield (75–80%). This compound was then deprotected using a solution of trifluoroacetic acid in methylene choride (1:1, v–v).

Starting with the commercially available 2-substituted-5-amino-1,3,4-thiadiazoles 1, which have been sulfamoylated with in situ generated sulfamoyl chloride, by a method reported earlier by one of this groups [83a], the sulfamides 2a–j have been prepared with excellent yields [83b]. The various substituents in position 2 of the heterocyclic ring of the new compounds 2a–j were chosen in such a way as to have a rather comprehensive SAR insight for this class of CAIs. Thus, starting with the unsubstituted parent compound (R = H, 2a), both aliphatic (Et, t-Bu, etc), aromatic (Ph, substituted phenyl, etc) as well as sulfide- and sulfone incorporating such moieties have been introduced in this position. X-ray crystallographic data on many CA—sulfonamide/sulfamate/sulfamide adducts revealed that in addition to the zinc

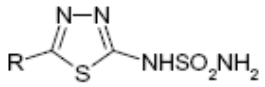
binding group (a sulfamide one for derivatives 2) and organic scaffold (1,3,4-thiadiazole for 2), the tails present in CAIs are critical both for the isozyme inhibition profile as well as for modulating the physico-chemical properties of such inhibitors, which may thus lead to various pharmacological applications [1, 10b, 38, 84–87]. This explains the choice of the tail groups present in derivatives 2a–j reported here. Inhibition data against five physiologically relevant CA isozymes, that is the cytosolic hCA (human CA) I and II, the membrane-associated hCA IV as well as the two mitochondrial isoforms hCA VA and VB, are presented in **Tab. 2**. Three standard CAIs, that is, AAZ, ZNS and TPM, have been assayed in the same conditions in order to allow a better understanding of the inhibition profile of the new compounds 2a–2j investigated here. Data of **Tab. 2** allow the following SAR to be evidenced for the new sulfamides 2 reported here:

(i) Against hCA I, the sulfamides 2 showed a moderate-weak inhibitory activity, with inhibition constants in the range of 102 nM–7.42 μM, bing thus, with two exceptions (compounds 2h and 2j) much weaker inhibitors as compared to the clinically used drugs AAZ-TPM (K_Is in the range of 56–250 nM). The least active hCA I inhibitor was the parent, unsubstituted compound 2a, whereas introduction of various substituents in position 2 of the thiadiazole ring enhances activity. The groups leading to best activity were 4-methoxyphenyl and methylsulfonyl (2h and 2j).

(ii) Isozyme hCA II was also weakly inhibited by the new sulfamides 2, with K_Is in the range of 0.54–1.13 μM, whereas the clinically used drugs were much stronger inhibitors of this ubiquitous isoform (K_Is in the range of 10–35 nM). It may be observed a very flat SAR for sulfamides 2 in inhibiting hCA II, with the nature of groups substituting in 2 the thiadiazole ring, having a small influence on the inhibitory power (**Tab. 2**).

(iii) Sulfamides 2 act as quite weak inhibitors of the membrane-associated isoforms hCA IV, with K_Is in the range of 4.32– 10.05 μM, unlike AAZ which is a strong inhibitor (K_I of 74 nM) but similarly to ZNS and TPM (K_Is of 4.90–8.59 μM).

(iv) Excellent inhibitory properties were evidenced for derivatives 2 against the two mitochondrial CA isozymes, hCA VA and hCA VB. Indeed, these compounds showed K_Is in the range of 4.2–28.3 nM against hCA VA, and of 1.3–74 nM against hCA VB, respectively. It may be observed that compounds 2 are much better inhibitors of the mitochondrial CAs as compared to the three clinically used drugs (K_Is in the range of 20–63 nM against hCA VA, and of 30–6033 nM against hCA VB, respectively (**Tab. 2**). For hCA VA, the substitution patterns of the heterocyclic ring leading to the best inhibitors included the trifluoromethyl, thioethyl-, aryl and methylsulfonyl moieties (K_Is < 10 nM) whereas the remaining ones generated slightly less effective inhibitors. For hCA VB, only three compounds (2a, 2b and 2f) showed K_Is > 10 nM, all the other substitution patterns leading to derivatives with excellent activity (K_Is < 7.5 nM).



2a-2j

N_0	R	K_I (nM)				
		hCA I	hCA II	hCA IV	hCA VA	hCA VB
2a	H	7420	970	8910	28.3	74
2b	Et	7010	950	8604	18.7	63
2c	*t*-Bu	5540	900	8400	10.4	2.8
2d	CF_3	6860	1080	4320	7.3	3.9
2e	MeS	2400	902	8300	32	2.9
2f	EtS	1890	1130	7580	9.3	23.1
2g	Ph	1660	870	5540	9.2	7.5
2h	4-$MeOC_6H_4$	102	540	8760	8.0	1.3
2i	4-Br-C_6H_4	5850	820	10050	4.2	4.5
2j	$MeSO_2$	103	940	6100	8.7	2.7
AAZ	–	250	12	74	63	54
ZNS	–	56	35	8590	20	6033
TPM	–	259	10	4900	63	30

Tab. 2 Inhibition data (K_I) of human CA isozymes I, II (cytosolic), IV (membrane-associated), VA and VB (mitochondrial), with compounds 2a-j and standard inhibitors (acetazolamide AAZ, zonisamide ZNS and topiramate TPM) by a stopped flow, CO_2 hydration assay [80].

These are infact the compounds with the best inhibitory activity ever reported against the mitochondrial enzymes CA VA and CAVB [83b].

(v) Another very interesting property of the newly described sulfamides 2 is related to their selective inhibition of the mitochondrial isozymes (CA VA and VB) over the cytosolic and membrane-associated isoforms (CA I, II and IV). Especially CA II constitutes a problem when designing various CAIs targeting other isozymes, because CA II has generally a very high affinity for sulfonamides, sulfamates and sulfamides, and it is also a ubiquitous enzyme in vertebrates, including humans [1] As seen from data of **Tab. 2**, the three clinically used compounds mentioned here (but also the other CAIs in clinical use) [1] are generally much better CA II than CA VA/VB inhibitors (except for zonisamide against CA VA). For example the selectivity ratio of AAZ for inhibiting CA VA over CA II is of 0.19, and for inhibiting CA VB over CA II is of 0.22. Basically AAZ has a much higher affinity for the cytosolic isoform CA II than for the mitochondrial ones. However, all compounds 2 reported here showed a much better inhibitory activity against the mitochondrial isozymes than against the cytosolic (or membrane-associated) ones. Thus, for example, 2h has a selectivity ratio for inhibiting CA VA over CA II of 67.5, and for inhibiting CA VB over CA II of 415. For 2i, these ratios are of 195 and of 182, respectively. Thus, these compounds are 67.5–415-times better inhibitors of the mitochondrial over the cytosolic isozymes, which is a very interesting result, never evidenced earlier for any other class of CAIs [83b].

In conclusion, we prepared a small series of 2-substituted-1,3,4-thiadiazole-5-sulfamides and assayed for the inhibition of five physiologically relevant isozymes, the cytosolic CA I and II, the membrane-associated CA IV and the mitochondrial CA VA and VB. The new compounds showed weak inhibitory activity against human isoforms: a) hCA I with inhibition constants in the range (K_Is of 102 nM–7.42 μM); b) hCA II (K_Is of 0.54–1.13 μM); c) hCA IV (K_Is of 4.32–10.05 μM); but were low nanomolar inhibitors

of hCA VA and hCA VB, with inhibition constants in the range of 4.2–32 nM and 1.3–74 nM, respectively.

Furthermore, the selectivity ratios for inhibiting the mitochondrial enzymes over CA II were in the range of 67.5–415, making these sulfamides the first selective CA VA/VB inhibitors.

5.2.0 NEW *SULFONAMIDES AS POTENTIAL ANTICANCER DRUGS*

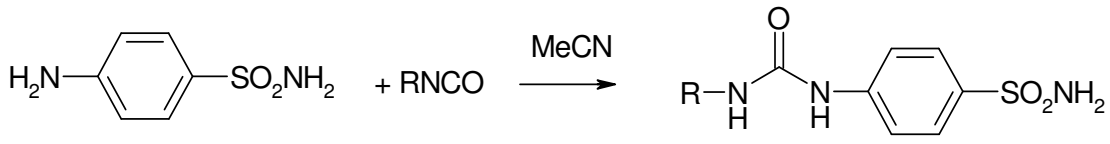
To the beginning of my thesis work, we decided to prepare new substituted-pyridinium derivatives of aromatic sulfonamides for two reasons: 1) this type of inhibitors were the first non-polymeric membrane-impermeant at low molecular weight sulfonamide possessing in vitro and *in vivo* selectivity for the membrane-bound isozyme (CA IV) versus cytosolic isozyme (CA I, II). Due to the salt like character, are unable to penetrate through biological membranes as shown by *ex vivo* (human red blood cells as model system) and *in vivo* studies (perfusion experiments in rats) discriminating and selectively inhibiting only CA IV [88]. Recently other representatives of these inhibitor's type have been shown to act selectively versus transmembrane isozyme CA IX in vitro [89]; 2) because there is a common project with Bayer Sharing Pharma about the possible use as intermediate compounds, leading to the PET agents for possible applications *in vivo*.

However, considering that there are patenting reasons, thus obviously the secret and that at present, none of these compounds obtained with this therapeutic approach has been tested *in vivo* so far, we decided to prepare a new series of ureido-substituted benzene sulfonamides that have a better bioavailability in comparison with

them, and are as diverse ureido-substituted benzene sulfonamides, which have been shown earlier to be isoform-selective for some isozyme as CA I [90-91].

We observed that they may lead to isoform-selective compounds [91]; thus, we decided to investigate in detail such derivatives. We report here the synthesis of a large series of 4-substituted-ureido-benzenesulfonamides **4-30** prepared by reaction of sulfanilamide **3** with aryl/alkyl-isocyanates **A1-A27** (**Scheme 2**). The chemical diversity was generated by varying the nature of the starting isocyanate **A1-A27**. Generally Sulfanilamide **3** (2.90 mmols) was dissolved in acetonitrile (20-30 mL) and then treated with a stoichiometric amount of isocyanates **A1-A27**. The mixture was stirred at r.t. or heated at 50 °C for 2 h, until completion (TLC monitoring). The heavy precipitate formed was filtered-off, washed with diethyl ether (100 ml) and dried *under vacuo.*

In order to characterize the compounds, ^{1}H, ^{13}C and ^{19}F spectra were recorded using a Bruker Advance III 400 MHz spectrometer. The chemical shifts are reported in parts per million (ppm) and the coupling constants (*J*) are expressed in Hertz (Hz). Infrared spectra were recorded on a Perkin Elmer Spectrum R XI spectrometer as solids on KBr plates. Melting points (m.p.) were measured in open capillary tubes, unless otherwise stated, using a Büchi Melting Point B-540 melting point apparatus and are uncorrected.

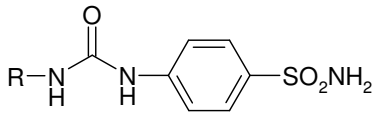


3 **A1-A27** **4-30**

Scheme 2: Preparation of the 4-ureidosubstituted-benzenesulfonamides **4-30** from sulfanilamide **3** and isocyanates **A1-A27**.

In a recent work [92], we showed that benzenesulfonamides incorporating 4-substituted ureido moieties acts as CA II inhibitors with potencies which correlate well with the orientation of the R moiety present in the ureido tail of the compounds. Indeed, in a congeneric series of five such derivatives investigated as inhibitors of the cytosolic isoform hCA II (h =human), it has been observed that the potency varied between 3.3 and 226 nM, and by means of X-ray crystallography it has been evidenced a very variable orientation of the R-ureido moiety, when the inhibitor was bound within the enzyme active site [92].

A strong correlation has been observed between the binding pattern and R group orientation for this small library of compounds [92]. Since, as shown by the X-ray crystallographic work [92], it is the nature of the tail R greatly influencing the binding to the enzyme (**Tab. 3**). As the R moiety binds towards the more external part of the enzyme active site, where there are major differences in the amino acids constituting the active site between the various isoforms [10a], this may also lead to the generation of compounds with a better selectivity profile for inhibiting various isoforms. This has been infact reported earlier by our group, [91], with the observation that some ureido-substituted sulfonamides show selective inhibition of isoform hCA I over the dominant one hCA II.



4-30

No	R	K_I (nM)			
		hCA I	hCA II	hCA IX	hCA XII
4	Ph	760	3730	575	67.3
5	$PhCH_2$	92	2200	41.4	49.5
6	Ph_2CH	83	3725	58.8	64.5
7	4-FC_6H_4	5080	96	45.1	4.5
8	4-Cl-C_6H_4	2150	781	58.0	5.3
9	4-BrC_6H_4	1465	1290	69.3	7.9
10	4-IC_6H_4	5500	2634	24.5	4.3
11	4-$CF_3C_6H_4$	9.7	1150	6.2	2.3
12	3,5-$(CF_3)_2C_6H_3$	3690	75	53	39
13	C_6F_5	2395	50	5.4	5.1
14	2-$MeOC_6H_4$	92	4070	465	61.2
15	4-AcC_6H_4	388	1060	5.4	4.6
16	2-i-PrC_6H_4	9.0	3.3	0.5	4.2
17	4-i-PrC_6H_4	4330	5005	541	49.7
18	4-n-BuC_6H_4	5530	2485	376	28.5
19	4-n-$BuOC_6H_4$	11.3	2.1	0.8	2.5
20	4-n-octyl-C_6H_4	536	9600	47.1	52.8
21	4-NCC_6H_4	57.0	64.7	6.0	6.5
22	2-NCC_6H_4	10.9	2.4	0.3	4.6
23	4-$PhOC_6H_4$	604	85	69.1	7.1
24	2-PhC_6H_4	1170	9.7	65.7	65.1
25	3-$O_2NC_6H_4$	23.4	15	0.9	5.7
26	4-MeO-2-MeC_6H_3	89.2	3310	73.3	6.0
27	9H-fluoren-2-yl	1700	908	102	55.4
28	cyclopentyl	470	226	7.3	7.0
29	3,5-$Me_2C_6H_3$	6530	1765	6.9	6.2
30	indan-5-yl	9.8	8.9	7.0	2.5
-	AAZ	250	12	25	5.7

Tab.3 Inhibition of hCA I, II (cytosolic isoforms) and hCA IX and XII (transmembrane, tumor-associated enzymes) with ureido sulfonamides **4-30** and acetazolamide **AAZ** as standard [80]

As shown in **Scheme 2** and **Tab 3**, a large variety of aromatic and aliphatic R moieties have been incorporated in the new CAIs **4-30** reported here, in order to have an extensive structure-activity relationship (SAR) insight regarding their interactions with various CA isoforms with medicinal chemistry relevance.

The inhibition data of four physiologically relevant α -CA isoforms with compounds **4-30** and acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide, **AAZ**, a clinically used drug) are presented before in (**Tab. 3**); hCA I and II (cytosolic, widespread enzymes) as well as hCA IX and XII (transmembrane, tumor-associated CAs) have been included in this study due to their relevance as targets/offtargets when developing CAIs. Indeed, CA II for example is the drug target for developing antiglaucoma CAIs [1, 58, 59], but it is an offtarget when considering CA IX/XII inhibition [1, 7b, 9c,e,f, 58, 59]. In this latter case, only the transmembrane, tumor-associated isozymes (IX and XII) should be inhibited, as CA II may have the function of housekeeping enzyme, and its inhibition may lead to side effects [1, 7b, 9c,e,f, 58, 59].

The following SAR can be observed from data of **Tab 3**, for the series of ureido-sulfonamides **4-30** investigated here:

a) the slow cytosolic isoform hCA I was inhibited by compounds 4-30 with a very variable potency, with inhibition constants in the range of 9.0 – 5530 nM. Potent hCA I inhibition has been observed with the following compounds: **11, 16, 19, 22,** and **30**. These compounds incorporate 2- or 4-substuted phenyl moieties (such as 4-trifluoromethylphenyl, 2-isopropylphenyl, 4-n-butoxyphenyl, and 2-cyanophenyl), except **30** which possesses an indane moiety. It is thus clear, as in the crystallographic work reported earlier [92] for 5 of the 27 derivatives reported here, that the nature of the (R) moiety substituting the second ureido nitrogen is the determining factor in controlling the inhibitory power, probably due to the flexibility of

the ureido linker and the possibility of this moiety to orientate in different subpockets of the active site cavities of these enzymes investigated CA isoforms.

Indeed, a number of these derivatives, including **5, 6, 14, 4, 25** and **26** behaved as medium potency hCA I inhibitors with K_Is in the range of 23.4 – 92 nM. Again the nature of the R moieties present in these compounds was rather variable (benzyl, diphenylmethyl and range of 388 – 5530 nM. **AAZ** is similar to these compounds, with an inhibition constant of 250 nM against hCA I (**Tab. 3**).

(**b**) the second offtarget isoform, the cytosolic hCA II has also been inhibited with potencies ranging from the low nanomolar to the micromolar by ureidosulfonamides **4-30** (**Tab. 3**). Thus, compounds **16, 19, 22, 24** and **30** were very potent hCA II inhibitors, with K_Is in the range of 2.1 – 9.7 nM. These derivatives incorporate 2- or 4-substituted phenyl moieties or the indane ring. It is interesting to note that **16** and **19** contain in their molecule the rather bulky, lipophilic i-Pr, n-BuO or biphenyl moieties, which one could considered to be too bulky to fit well within the hCA II active site. However as shown here and in the crystallographic, preliminary communication (see forward the **Fig. 12D**) [92], the i-Pr moiety of **16** makes hydrophobic contacts in a patch within the enzyme active site never seen earlier to accommodate inhibitors, also making a stacking with Phe131. This particular binding mode explains thus the excellent hCA II inhibitory of **16**. However, four other derivatives of the series investigated here, i.e., **7, 13, 25** and **28**, exploited different binding pockets within the enzyme active site (see forward the **Fig 13**) [92], and also possessed diverse inhibitory power. Another series of compounds showed medium potency hCA II inhibition, with K_Is in the range of 15 – 96 nM. These compounds (**7, 12, 13, 21, 23** and **25**) also possess aromatic groups at the second nitrogen ureido group, such as 4-fluorophenyl, 3,5-di(trifluoromethyl)phenyl, pentafluorophenyl, 4-cyanophenyl, 4-phenoxyphenyl or 3-nitrophenyl.

The remaining derivatives showed lower hCA II inhibitory properties, with K_Is in the range of 226 – 9600 nM. The least effective hCA II inhibitor was the derivative incorporating the long 4-n-octylphenyl moiety (**20**).

(**c**) the tumor-associated hCA IX was generally better inhibited by these compounds compared to hCA I and II discussed before, with K_Is in the range of 0.3 – 575 nM. Only few weak hCA IX inhibitor were detected (e.g., 4, 14, 18 and 27), with K_Is in the range of 102 – 575 nM, whereas all other derivatives presented inhibition constants < 100 nM. Compounds such as **11, 13, 15, 16, 19, 21, 22, 25, 28** and **29** had excellent hCA IX inhibiting properties, with K_Is in the range of 0.3 – 7.3 nM.

Thus, substitution patterns favourable to potent hCA IX inhibition include the presence of 4-trifluoromethylphenyl, pentafluorophenyl, 4-acetylphenyl, 2-i-propylphenyl, 4-n-butoxyphenyl, 2- and 4-cyanophenyl, 3-nitrophenyl, cyclopentyl and 3,5-dimethylphenyl moieties. It should be however noted that most of the other substitution patterns present in the remaining compounds lead to quite effective hCA IX inhibitors, with K_Is in the range of 24.5 – 73.3 nM. **AAZ** is an effective hCA IX inhibitor too, with a Ki of 25 nM (**Tab. 3**).

(**d**) hCA XII was also effectively inhibited by sulfonamides **4-30**, with K_Is in the range of 2.3 – 67.3 nM. Thus, this is the isoform with the highest affinity for these compounds, as all of them are excellent inhibitors. Inhibition constants < 10 nM have been observed with a rather large number of compounds, among which **7-11, 13, 15, 16, 19, 21, 25, 26, 28-30** and **AAZ**. Thus many substitution patterns lead to very effective hCA XII inhibitors in this congeneric series investigated here.

The excellent inhibition of all CA isoforms investigated here but also the variability in inhibitory power for the various members of this congeneric series, are phenomena never observed earlier for any class of CAIs investigated in detail, and is probably due to the presence of the ureido linker in these molecules, which leads to the binding of the compound in quite multiple ways to the enzymes active sites.

(**e**) the selectivity ratio for inhibiting the target over the offtarget isoforms for this congeneric series of sulfonamides is rather complex, just because of the factors mentioned above.

However, some interesting findings emerged. For example, compound **15** has a selectivity ratio of 71.8 for inhibiting CA IX over CA I and of 196.3 for inhibiting CA IX over CA II (the corresponding ratios for inhibiting CA XII over CA I and II are even better), being thus a selective inhibitor of the tumour-associated isoforms over the cytosolic ones. Compound **25**, is a more stronger inhibitor of the cytosolic isoforms compared to **15**, but has anyhow rather good selectivity ratio for inhibiting the tumor-associated over the cytosolic isoforms, i.e., of 26 for the inhibition of CA IX over CA I, of 16.7 for the inhibition of CA IX over CA II.

It should be noted that most of the CAIs of sulfonamide type investigated earlier contained CONH or SO_2NH linkers between the sulfonamide head moiety and the tail, instead of the ureido one present in **4-30**. These different linkers allow however less flexibility for the inhibitor scaffold, and probably this is the reason why most such sulfonamides bind in the cannonical hydrophobic pocket of hCA II and also generally do not show isoform selectivity [10a, 92,93]. In contrast, for derivatives incorporating the ureido linker (of the type 4-30), it has been shown [92] that the substituted ureido tails are found in various hydrophobic pockets/regions of the CA II active site in the enzyme-inhibitor adduct. As seen from the crystallographic data [92], the torsion angles between these two fragments of the scaffold (indicated by orange and blue arrows, respectively) of this type of sulfonamides CAI were quite different for the five compounds investigated by X-ray crystallography (i.e., compounds 7, 13, 16, 25, 28) (**Fig.12**) [92]. This probably allows the flexibility of the inhibitors to select the hydrophobic pocket in such a way as to avoid steric clashes [10a,92,93]. And to make as many as possible favorible interactions with amino acid residues within the enzyme

cavity. In particular, only the compound **25** (see after **Fig 12E**) differs from other compounds **7**, **13**, **16**, and **28** in that a direct hydrogen bond is observed between the inhibitor and the active site, in addition to those linking the sulfonamide to the surrounding amino acids.

This interaction which does not involve a solvent bridge , is between Gln92 NE2 and O8 of the inhibitor at the distance of 2.8 Å (**Fig. 12E**). Again, compound **25** exhibits nonplanarity with the C5–C4–N7–C8 and C8–N9–C10–C15, torsion angles being –33.91° and –19.11°, respectively. This allows the terminal nitro atoms N16, O16 and O17 to make hydrophobic contacts with side chain carbon atoms of Ile91, Glu69, and Gln92 (see **Fig. 12E**).

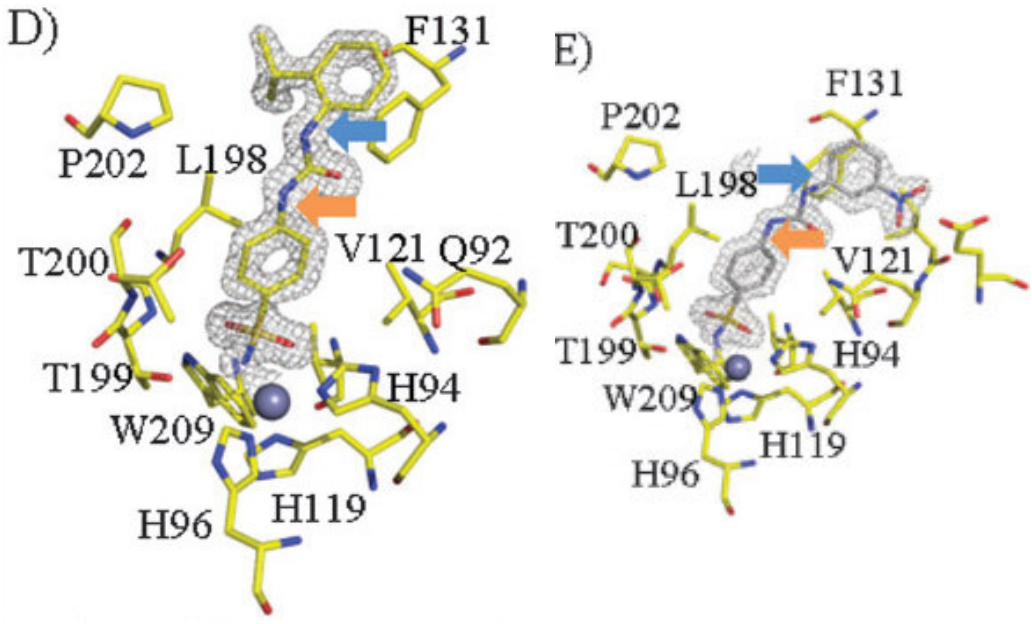


Fig. 12 Compound 16 (**D**) and 25 (**E**) within the enzyme active site of CA II.

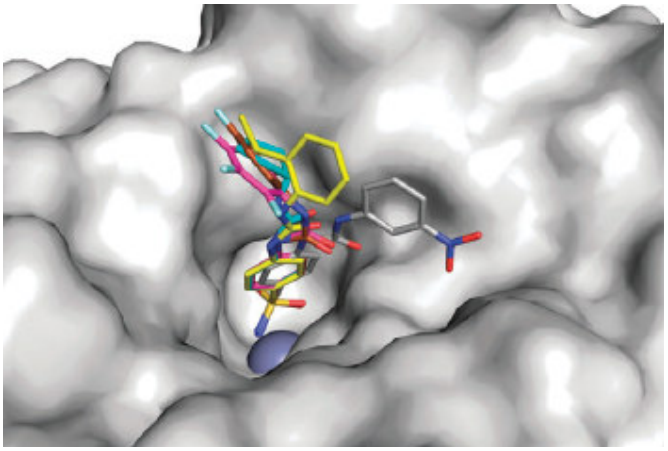


Fig. 13 View of compound 7 (orange), compound 13 (pink), 16 (yellow), 25 (grey), 28 (cyan) superimposed in the active site of CA II.CA II is depicted as a grey surface representation. The active site is depicted as a black sphere. The positions of oxygen and nitrogen atoms of the compounds are colourated red and blue, respectively.

We report a new series of ureido substituted sulfonamides CAIs which possess strong affinity for CA IX, an acceptable selectivity profile for inhibiting in vitro the tumour associated-isoforms CA IX, CA X II over the cytosolic one CA I, CA II.

The previous inhibition data were obtained for the purified recombinant catalytic domains of CA IX, CA XII and therefore it is difficult to directly anticipate their biological effects either in context of living cells in culture or in tumour tissue.

As recently demonstrated by Svastova *et al*. and Cecchi *et al*. some sulfonamide derivatives represented by fluorescent-conjugated thioureido-homosulfanilamide, bind only to hypoxic MDCK cells that ectopically express CA IX, but neither to CA IX-positive normoxic cells nor the CA IX-negative MDCK controls. One of the most promising compound developed so far is derivative fluorescent sulfonamide **1** [(4-sulfamoylphenylethylthioureido) fluorescein] (**Fig. 10**)

It was shown to bind only to hypoxic tumour overexpressing the cancer-associated isozyme, which makes it an important candidate for imaging purposes of this type of cancer [7b, 9e, 94]. This compounds presents a K_I against the CA IX f 24 nM and shows a membrane-impermeant property assessed through an ex vivo model of red blood cells membranes [94]. This compound was able to reduce the extracellular acidification of Madin–Dardy canine Kidney CA IX (MDCK-CA) cells in hypoxia, and their effect on the normoxic extracellular pH was negligible. Furthermore, recently it has shown *in vivo* imaging studies (cells in xenograft tumour model) to be an useful diagnostic tool for imaging hypoxic tumour [9e], visualizing hypoxia response.

Thus, considering all these important findings, in the effort directed toward the development of isoform-selective CA inhibitors suitable for the management of cancer, we decided to investigate some compounds (compouds 7, 10, 13, 15, 16, 25) as valuable candidates within this large series, for *in vivo* studies. Infact, there are still many questions that remain to be answered, such as th sensitivity of tumour-associated carbonic anhydrases to different sulfonamide inhibitors in various cell culture setting under different physiological conditions and to *in vivo* efficacy as antitumour drugs.

A major challenge of modern chemotherapy is the development of drugs that selectively target cancer cells, overcome chemoresistant tumor cells, and have limited toxic effects.

Thus, these compouds were evaluated for their possible antitumour/antimetastasis activity on human breast cancer cell line (MDA-MB-231), on (4T1) mouse mammary tumour cell line, which produces spontaneously metastatis in mice and on (67NR) mouse breast tumour cell line, which forms primary tumours in mice without metastasis; the six compouds have been selected due to their selective inhibitory activity for CA IX, to investigate them against a peculiar tumour cell lines resistant to chemotherapeutics and hypoxia *in vivo* Assays.

These are *in vivo* methods of screening investigative anticancer drugs, biologic response modifiers or radiotherapies. Human tumour tissue or cells are transplanted into mice or rats followed by tumor treatment regimens. A variety of outcomes are monitored to assess antitumor effectiveness.

CA IX is highly expressed in breast malignancies [95] and studies have demonstrated that CA IX and CA XII are variably expressed in breast cancer cell lines [8b, 50a, 63a-b]. Furthermore, CA IX is a poor prognostic marker for patients with breast cancer [96] and it is significantly associated with distant metastasis [99]. Therefore, we have chosen breast cancer as a model malignancy in which to test, in vivo, the anti-metastatic activity of some of the CA IX inhibitors described here. Specifically, we have used the 4T1 syngeneic mouse mammary tumor model [97]. The 4T1 cells are highly tumorigenic and metastasize spontaneously to multiple sites in a manner similar to that for human breast cancer following orthotopic implantation into an immunocompetent host [97,98]. We have shown recently that 4T1 tumors overexpress CA IX [99], and that these cells induce CA IX in hypoxia in vitro. These attributes make the 4T1 mouse mammary tumor cells a good model in which to examine the effects of inhibiting CAIX activity on breast cancer metastasis. Although the 4T1 tumor model can be used to evaluate spontaneous metastatic events subsequent to tumor implantation and growth, such studies are lengthy and require surgical removal of the primary tumor. Because we were interested in evaluating the effect of the compounds on metastasis specifically, we elected to test the inhibitors using an experimental metastasis approach. We injected 4T1 cells expressing luciferase intravenously and assessed the ability of the cells to form lung metastases after treating with compounds **15** (MST-109, **Figure 14A**) and **25** (MST-119, **Figure 15A**) using bioluminescent imaging techniques. Mice were treated with the inhibitors or equal amounts of vehicle beginning 24 hours post-injection of cells and metastases were imaged the day following the final dose of inhibitor.

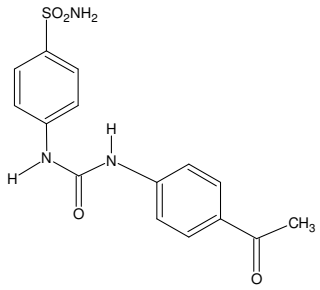
We found that whereas treatment with MST-109 was not able to decrease the metastatic burden relative to the vehicle control group (**Figure 14B**), treatment with MST-119 was effective in limiting colonization of the cells in the lungs in a dose-dependent fashion (**Figure 15B**). Quantification of the bioluminescent signal revealed a statistically significant decrease in the formation of metastases in the mice administered MST-119 (**Figure 15C**), while no significant differences were observed among the vehicle and MST-109-treated groups (**Figure 14C**) [100a].

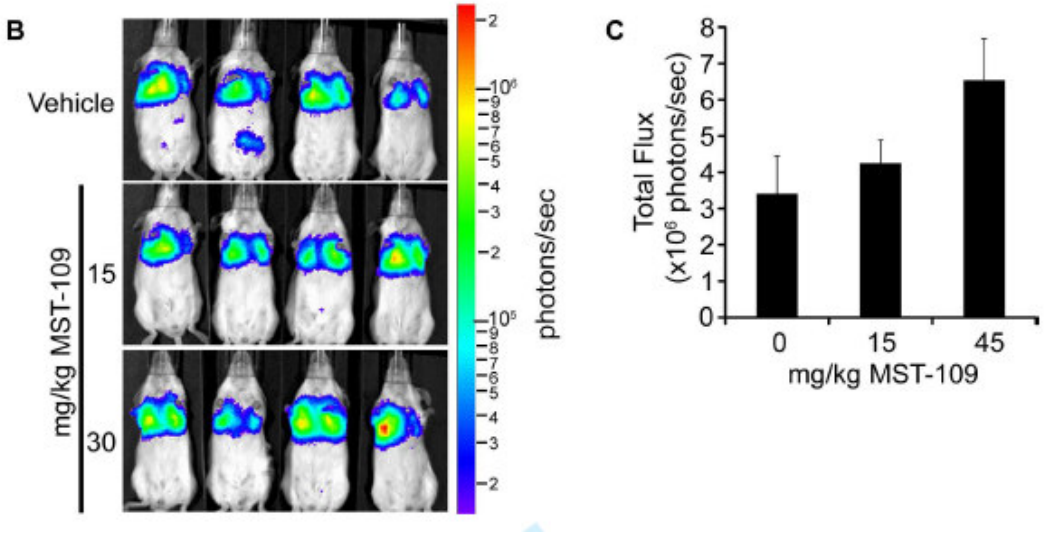
All animal studies and procedures were carried out in accordance with protocols approved by the Institution Animal Care Committee at the BC Cancer research Centre and the University of British Columbia (Vancouver, BC, Canada). 4T1 cells ($5x10^5$/mouse) were injected directly into the tail vein of 7-9 week-old female BALB/c mice. Metastatic burden was monitored and quantified using bioluminescent imaging as previously described [99, 101]. Briefly, Mice were imaged 24 hours following the final dose of a given compound. Images showing tumor burden are displayed using a log-scale color range and data were quantified using images acquired for 1 minute at a binning level of 8.

Data were analyzed using the contour ROI feature in the Living ImageR software (Xenogen). Results were subjected to statistical analysis using the Data Analysis ToolPack in Excel software.Two-tailed p values were calculated using Student's t-test. Data were considered significant for $p<0$.

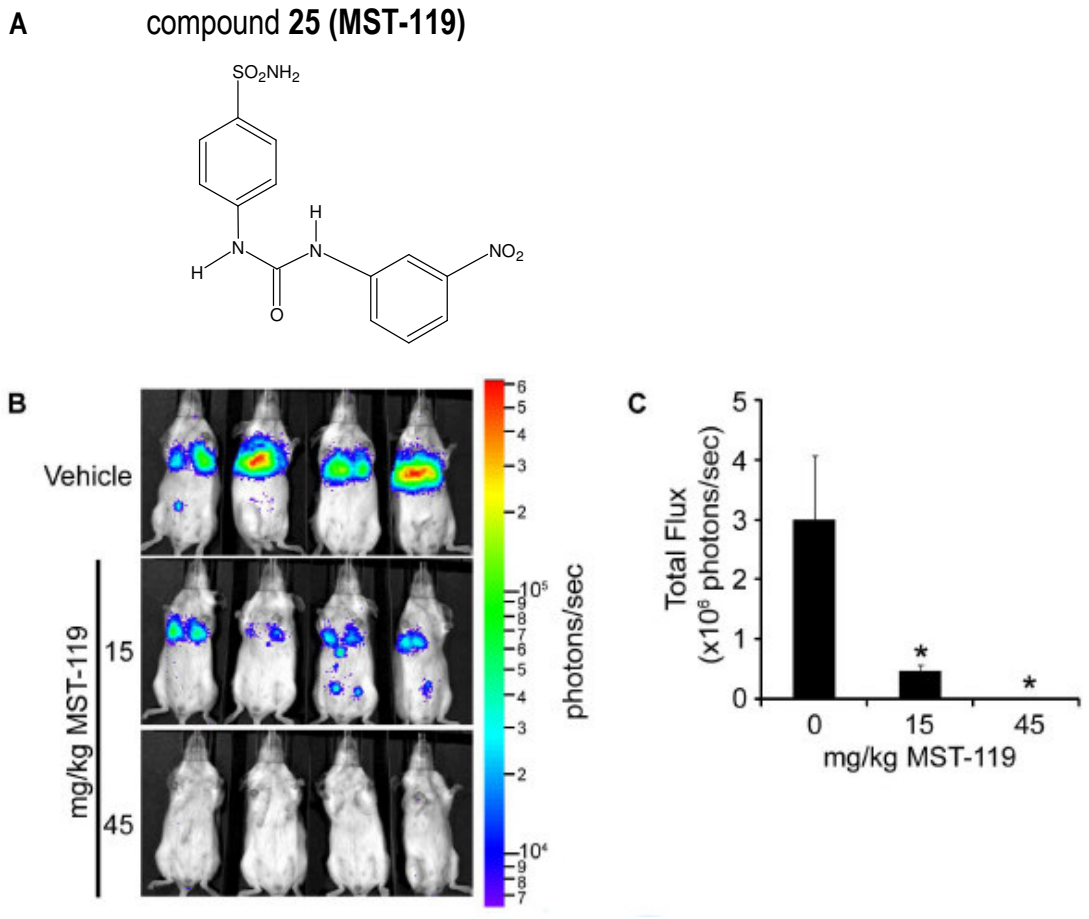
For in vivo studies, compound 25 (MST-119) was solubilized in 37.5% PEG400/12.5% ethanol/50% saline prior to injection, while compound 15 (MST-109) was solubilized in 37.5% PEG400/12.5% ethanol/10% Cremophor/40% saline. Drug aliquots were made fresh daily from powder. Drugs were administered by i.p. injection using a volume of200 µl for a 20 g mouse. Specific dosing schedules are described in the appropriate figures.

A compound **15 (MST-109)**





14. Ureido-sulfonamide MST-109 did not reduce the formation of metastases by 4T1 mammary tumor cells. (A) Chemical structure of MST-109. (B) 4T1 cells were injected directly into the tail vein of BALB/c mice. Mice then received vehicle or MST-109 starting 24 hours after injection of cells. Animals received therapy every day and 5 doses were provided. Bioluminescent images were acquired 24 hours after the final dose of inhibitor. (C) Quantification of bioluminescence. n = 4 per group. No significant differences were observed among the vehicle inhibitor-treated groups.



15. Ureido-sulfonamide MST-119 inhibits the formation of metastases by 4T1 mammary tumor cells. (A) Chemical structure of MST-119. (B) 4T1 cells were injected directly into the tail vein of BALB/c mice. Mice then received vehicle or MST-119 starting 24 hours after injection of cells. Animals received therapy every other day and 3 doses were provided. Bioluminescent images were acquired 24 hours after the final dose of inhibitor. (C) Quantification of bioluminescence. n = 4 per group. *P<0.05.

Furthermore, recently in these pharmacolgical studies, it has been tested the effect of an other selective ureido-sulfonamide inhibitor for CA IX (compound 7), on primary breast tumor growth (using a highly metastatic variant of the MDA-MB-231 cell line, which induce robustly CA IX in hypoxia) [100b]. Therefore, tumor volume measurement showed significant inhibition of primary tumor growth in the mice treated with the compound MTS-104 (compound 7) compared to vehicle controls (**Fig. 16**) [100b]. Having demonstrated that selective sulfonamide-based compounds inhibit the growth of primary breast tumors, we next tested MST-104 for its ability to inhibit metastasis formation in the 4T1 experimental metastasis model. Intravenous injection of 4T1 cells into mice and subsequent daily treatment of these animals beginning 24 hours post-injection with MST-104 resulted in inhibition of metastases formation (**Figure 17A**). Quantification of the bioluminescent signal revealed a statistically significant decrease in the formation of metastases in the treated mice (**Figure 17B**).

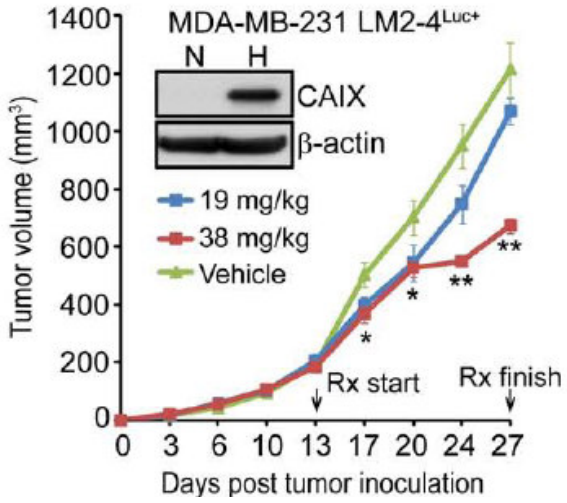
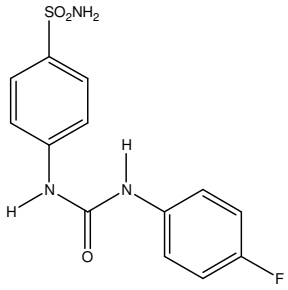
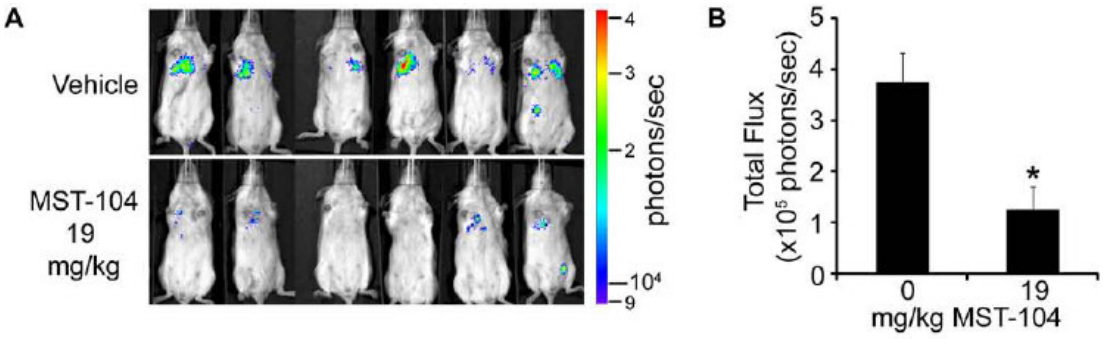


Fig.16 MDA-MB-231 LM2-4Luc+ cells were implanted orthotopically into NOD/SCID mice. When tumors reached an average of 200 mm^3, animals received the indicated doses of MST-104 (compound 7) daily and tumor growth was monitored. n = 8/group. *P<0.03, **P<0.001. Inset, hypoxia-induced CAIX expression by LM2-4Luc+ cells.

Compound **7 (MST-104)**





17. Ureido-sulfonamide MST-104 inhibits the formation of metastases by 4T1 mammary tumor cells. (A) 4T1 cells were injected directly into the tail vein of BALB/c mice. Mice then received vehicle or MST-104 starting 24 hours after injection of cells. Animals received therapy every day and 5 doses were provided. Bioluminescent images were acquired 24 hours after the final dose of inhibitor (B) Quantification of bioluminescence. n = 6 per group. *P<0.01

These results suggest that CAIX-specific inhibition may be useful in treating metastatic disease in breast cancer. These data show that some of the ureido-sulfonamide inhibitors of CA IX reported here are effective in attenuating formation of metastases in a model of aggressive breast cancer.

In conclusion, we report here a series of sulfonamides that was prepared by reaction of sulfanilamide with aryl/alkyl isocyanates. These ureido-substituted benzenesulfonamides showed a very interesting profile for the inhibition of several human carbonic anhydrases (hCAs, E.C. 4.2.1.1), such as hCA I, II (cytosolic isoforms) and hCA IX and XII (transmembrane, tumor-associated enzymes). Excellent inhibition of all these isoforms has been observed with various members of the series, depending on the substitution pattern of the urea moiety. Several low nanomolar CA IX/XII inhibitors also showing good selectivity for the transmembrane over the cytsosolic isoforms have also been discovered.

One of them, 4-{[(3'-nitrophenyl)carbamoyl]amino}benzenesulfonamide, completely inhibited the formation of metastases by the highly aggressive 4T1 mammary tumor cells at pharmacologic concentrations of 45 mg/kg, constituting thus an interesting candidate for the development of conceptually novel antimetastatic drugs.

Therefore, we showed that inhibiting CA IX with selective inhibitors suitable is possible to manage the breast cancer *in vivo* experiments.

6.0 β-CA INHIBITION: CAIs AS ANTIINFECTIVE DRUGS

The infection with *Mycobacterium tuberculosis* and related *Mycobacteria* affects much of the word population, with an estimated 9.2 million new cases each year, of which many lead to death. Furthermore, multidrug resistant and extensively multidrug resistant tuberculosis (MDR-TB) is present in 50 countries posing serious concern to the global healthcare system. Indeed, the combination therapy used to treat TB is based on agents developed in the 1960-1980s, with no new drugs launched for the past 30 years. At present, there is a huge need for anti-TB drugs with novel mechanism of action, and several agents belonging to the fluoroquinolone, oxazolidinone, diarylquinoline, and nitroimidazole-oxazole/-oxazine classes are in *M. tuberculosis* genome in 1998 greatly facilitated the identification of possible new drug targets which may lead to the development of compounds possessing a new mechanism of action and thus resolve the drug resistance problem mentionated above. Among the new proteins identified after the *M. tuberculosis* genome was published, there are three carbonic anhydrases (CAs, EC 4.2.1.1) belonging to the β-class.

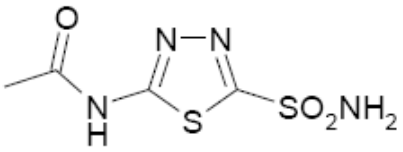
The emergence of resistance to the currently used drugs (e.g. azoles, polyenes, caspofungin and amphotericin B), as well as changes in the spectrum of *Candida* infections have led to an increased interest in susceptibility testing of new antifungal, possibly possessing a different mechanism of action.

Thus, the goal is the structure-base design of possible inhibitory molecules (isoform-selective CA inhibitors) against new proteins targets, which are in *C. albicans* and *Mycobacterium tuberculosis*, beeing today a global drug-resistant emergence around these eziologic agents of human Candidosis and Tuberculosis. The catalytic activity and inhibition with sulfonamides/sulfamates of all these β-class enzymes from

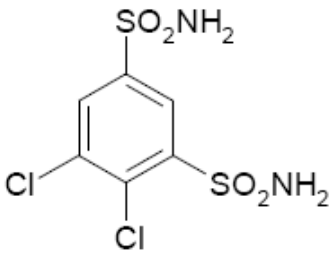
bacterial and fungal parasites have been reported, but very few low nanomolar inhibitors were detected so far for most of them [13c,72,102d-e].

Sulfonamide/sulfamate CAIs targeting various mammalian α-CAs, such as acetazolamide **1**, dichlorophenamide **2** or sulfanilamide **3**, have been in clinical use for more than 50 years [1], but such compounds generally show much less effective inhibition of the β-class enzymes [102a-c]. Furthermore, they generally do not possess the appropriate pharmacological properties (e.g., good penetration through bacterial walls/membranes) to effectively impair the growth of many such pathogens (or they show weak such properties, as shown by work on *H. pylori* and *B. suis* enzymes) [4a, 16a,b].

Thus, in order to understand whether β-CAs may constitute drug targets for developing antiinfective agents, it is essential to design compounds with different affinities and pharmacological properties compared to the classical CAIs of types **1-3**.



1



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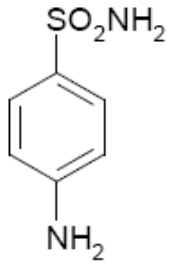


3

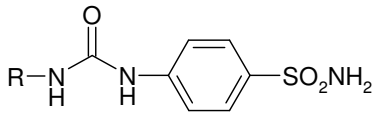
6.1.0 *SULFONAMIDES AGAINST CANDIDOSIS AND TUBERCULOSIS*

The fungal β-CAs from *Candida albicans* [19a] and *Cryptococcus neoformans* [4b, 17a-c] (as well as the related enzyme present in *Saccharomyces cerevisiae*) [4c, 102a-c] were also characterized, being shown to be susceptible to inhibition with the main classes of CA inhibitors (CAIs), i.e., the inorganic anions and the sulfonamides and their bioisosteres [4b,c, 16a,b, 17a-c, 19a, 102a-c].

Two other β-CAs from *Mycobacterium tuberculosis* were successfully cloned and crystallized by Suarez Covarrubias *et al*. [12,13] being denominated mtCA1 and mtCA 2. Our group identified, cloned and characterized the third such enzyme (mtCA 3), encoded by the gene Rv3273 [14].

Here we report the synthesis and inhibition studies of several β-CAs from fungal or bacterial pathogens with a large series of ureido-substituted sulfonamides obtained from sulfanilamide **3** as lead molecule. A large series of 4-ureidosubstituted-benzenesulfonamides **4-30** was prepared by reaction of sulfanilamide **3** with aryl/alkyl-isocyanates **A1-A27** (Scheme 2, pag.48) The chemical diversity of these derivatives was generated by varying the nature of the starting isocyanate **A1-A27**, since, as shown by a recent X-ray crystallographic work on five of these derivatives for which the structure in complex with the human isoforms hCA II has been reported [92], was determined that it is the nature of the R group that greatly influences the binding to the enzyme. For compounds **7, 13, 16, 25** and **28** [92] in adduct with hCA II, it has been observed that the benzenesulfonamide fragment binds in the usual manner, coordinating to the zinc ion as SO_2NH- moiety. The phenylsulfamoyl part of the inhibitor was superimposable between all these adducts and bound in the usual way [10a], filling the middle of the active site cavity [10a, 92].

However the different R moieties present in these compounds were observed to bind towards the external part of the enzyme active site, occupying various sub-pockets, and none of them was superimposable with each other. This phenomenon is probably due to the rather flexible nature of the ureido linker connecting the benzenesulfonamide with the the R group in this series of compounds, and may also explain a fact reported earlier by our group [91] that such ureido-substituted sulfonamides (different of the compounds investigated here) may show selective inhibition of isoform hCA I over the dominant one hCA II. Normally, it is hCA II having higher affinity for sulfonamides compared to hCA I, at least for most of the clinically used derivatives [1, 2c, 3a,g, 5b,e, 103]. A large variety of aromatic and aliphatic R moieties have been incorporated in the new CAIs **4-30** reported here, in order to expand the structure-activity relationship (SAR) insight regarding their interactions with various CA isoforms belonging to the β-class. The synthesis of such compounds is a one-step process, generally occurring in high yield, being possible to apply it for the preparation of a large number of derivatives. It should be noted that most of the CAIs of sulfonamide type investigated earlier [1, 2c, 3a,g, 5b,e, 103] contained CONH or SO_2NH linkers between the sulfonamide head moiety and the R group, instead of the ureido one present in **4-30**. These different linkers allow less flexibility for the inhibitor scaffold, and this is probably the reason why most such sulfonamides bind in the cannonical hydrophobic pocket of hCA II and also generally do not show isoform selectivity (**Tab. 4**) [10a, 91, 93].



4-30

No	R	K_I (nM)			
		hCA II	Nce103p	mtCA 1	mtCA 3
3	**- (sulfanilamide)**	240	1086	9230	6240
4	Ph	3730	395	356	758
5	$PhCH_2$	2200	547	440	717
6	Ph_2CH	3725	3970	4330	6380
7	4-FC_6H_4	96	42.1	6.4	53.0
8	4-ClC_6H_4	781	62.0	4.8	63.1
9	4-BrC_6H_4	1290	45.0	7.5	87.1
10	4-IC_6H_4	2634	42.5	6.8	56.4
11	4-$CF_3C_6H_4$	1150	61.0	69.3	533
12	3,5-$(CF_3)_2C_6H_3$	75	58.5	65.7	481
13	C_6F_5	50	38.0	5.0	6.4
14	2-$MeOC_6H_4$	4070	702	473	603
15	4-AcC_6H_4	1060	29.1	470	74.6
16	2-i-PrC_6H_4	3.3	3450	5610	6850
17	4-i-PrC_6H_4	5005	355	5690	57.5
18	4-n-BuC_6H_4	2485	49.6	4760	63.7
19	4-n-$BuOC_6H_4$	2.1	50.6	35.2	63.0
20	4-n-octyl-C_6H_4	9600	45.9	6500	81.2
21	4-NCC_6H_4	64.7	53.5	50.2	64.4
22	2-NCC_6H_4	2.4	326	534	463
23	4-$PhOC_6H_4$	85	3.4	560	818
24	2-PhC_6H_4	9.7	297	5590	748
25	3-$O_2NC_6H_4$	15	40.1	67.1	6.5
26	4-MeO-2-MeC_6H_3	3310	53.2	49.1	70.3
27	9H-fluoren-2-yl	908	573	728	370
28	cyclopentyl	226	565	509	733
29	3,5-$Me_2C_6H_3$	1765	65.9	315	768
30	indan-5-yl	8.9	71.8	486	632
-	**AAZ**	12	132	481	104

Tab.4 Inhibition of the β-CAs enzyme (Nce103p) from *C. albicans*, the mycobacterial enzymes from *Mycobacterium tuberculosis* (mtCA 1 and mtCA 3) and human hCA II with ureido sulfonamides **4-30**, sulfanilamide **3** and acetazolamide **AAZ**, by a stopped-flow CO_2 hydrase assay [80]

We have investigated the inhibition of the dominant, human isoform hCA II (offtarget) as well as three β-CAs from pathogenic organisms (fungi and bacteria) with derivatives **4-30** reported here (**Tab. 4**) [4b,19a, 80] The following SAR could be observed from data of **Tab.4**:

(i) the offtarget isoform, the cytosolic hCA II, was inhibited with potencies ranging from the low nanomolar to the micromolar by ureidosulfonamides **4-30**. Compounds **16, 19, 22, 24** and **30** were very potent hCA II inhibitors, with K_Is in the range of 2.1 – 9.7 nM. These derivatives incorporate 2- or 4-substituted phenyl moieties or the indane ring. It is interesting to note that **16** and **19** contain in their molecule the rather bulky, lipophilic i-Pr, n-BuO or biphenyl moieties, which might be considered too bulky to fit well within the hCA II active site. However as shown here and in the crystallographic, preliminary communication [92], the i-Pr moiety of **16** makes favorable hydrophobic contacts in a patch within the enzyme active site never seen earlier to accommodate inhibitors, also participating to a π stacking with Phe131. This particular binding mode explains the excellent hCA II inhibitory properties of **16** (K_I of 3.3 nM). However, four other derivatives of the series investigated here, i.e., **7, 13, 25** and **28**, exploited different binding pockets within the hCA II active site [92], and also possessed diverse inhibitory power compared to **16**, with inhibition constants in the range of 15-226 nM (**Tab. 1**). Another series of investigated sulfonamides showed medium potency hCA II inhibition, with K_Is in the range of 15 – 96 nM. These compounds (**7, 12, 13, 21, 23** and **25**) also incorporate aromatic R groups, such as 4-fluorophenyl, 3,5-di(trifluoromethyl)phenyl, pentafluorophenyl, 4-cyanophenyl, 4-phenoxyphenyl or 3-nitrophenyl. The remaining derivatives in this series showed lower hCA II inhibitory properties, with K_Is in the range of 226 – 9600 nM. The least effective hCA II inhibitor was the derivative incorporating the long 4-n-octylphenyl moiety (**20**). It should be also noted that sulfanilamide **3** was a medium potency hCA II inhibitor (K_I of 240 nM) whereas acetazolamide **1** a potent one (K_I of 12 nM).

(ii) the β-CA from *C. albicans* was inhibited by the ureido-sulfonamides **4-30** with a variable potency, with inhibition constants in the range of 3.4 – 3970 nM, compared to sulfanilamide **3** which was a weak inhibitor (K_I of 1086 nM) and acetazoalmide **1** which was a medium potency inhibitor (K_I of 132 nM). Thus, strong inhibition has been observed for derivatives **7-13, 15, 18-21, 23, 25, 26, 29** and **30**, which possessed inhibition constants in the range of 3.4 – 71.8 nM. These compounds incorporate 3- or 4-substituted phenyl moieties as R groups, such as 4-halogenophenyl, 4-trifluoromethylphenyl, 4-acetylphenyl-, 4-n-butyl/butoxyphenyl, 4-cyanophenyl-, 4-phenoxyphenyl-, 3-nitrophenyl-, as well as 3,5- or 2,4-disubstituted-phenyl moieties (except **30** which possesses an indane moiety and **13** which possesses the pentafluorophenyl moiety). It is obvious that a rather large number of substitution patterns lead to effective *C. albicans* β-CA inhibitors. The best such inhibitor was **23**, incoporating the elongated 4-phenoxyphenylureido moiety which, with an inhibition constant of 3.4 nM, is the best inhibitor of this enzyme ever reported in the literature [4b,17c]. Other substitution patterns present in these compounds led to less effective inhibitors. For example, derivatives **4-6, 14, 16, 17, 27** and **28**, having aryl, aralkyl or cycloalkyl R moieties, are weak *C. albicans* CAIs, with inhibition constants in the range of 297 – 3970 nM (**Tab. 4**). As for hCA II, small variations in the nature of the R moiety leads to very different inhibitory properties for this series of compounds, with highly effective, medium potency and ineffective CAIs detected in this congeneric series of sulfonamides.

(iii) the mycobacterial enzyme mtCA 1 was very weakly inhibited by sulfanilamide **3** and weakly by acetazolamide **1**, with inhibition constants in the range of 481-9230 nM (**Tab. 4**). However the ureido-benzenesulfonamides **4-30** reported here generally showed a better inhibitory action against this enzyme, with KIs in the range of 4.8 – 6500 nM. Thus, a number of derivatives, such as **7-10**, and **13**, were the best mtCA 1 inhibitors in this series, with K_Is in the range of 4.8 – 7.5 nM.

All of them incorporate 4-halogenosubstituted phenyl or pentafluorophenyl R moieties. Another subseries of these sulfonamides, such as **11, 12, 19, 21, 25** and **26**, showed slightly less effective inhibitory activity, with K_Is in the range of 35.2 – 69.3 nM. They incorporate mono- or disubstituted phenyl R moieties containing trifluoromethyl, nitro, cyano, and alkoxy groups. Medium potency inhibition has been observed with compounds such as **4, 5, 14, 15, 22, 23, 27-30**, which incorporate aryl, aralkyl or cycloalkyl R moieties. The least effective inhibitors were **6, 16-18, 20** and **23**, most of them incorporating rather bulky R groups (diphenylmethyl, 2-i-Pr-phenyl, 4-n-Bu-phenyl, 4-noctyl- phenyl, etc), which showed micromolar affinity for this enzyme (K_Is in the range of 4.33 – 6.50 μM).

(iv) the mycobacterial enzyme mtCA 3 was inhibited by the new compounds **4-30** reported here with K_Is in the range of 6.4 – 6850 nM, whereas the lead **3** and **AAZ** were ineffective and medium potency inhibitors, respectively (K_I of 6.24 μM for **3** and of 104 nM for **1**). The most effective CAIs against this enzyme were **13** and **25**, with K_Is in the range of 6.4-6.5 nM. The incorporate the pentafluorophenyl and 3-nitrophenyl R moieties, respectively. A rather large groups of compounds showed medium potency inhibitory activity against mtCA 3 , with K_Is in the range of 53.0 – 87.1 nM, and they include the halogenophenyl-substituted derivatives **7-10**, the 4-acetylphenyl group of **15**, and the alkyl-/alkoxyphenyl or cyanophenyl moities present in **17-21**. The disubtituted compound **26** also belong to this category. Again a rather large number of R moieties lead to highly enhanced β-CA inhibitory properties compared to the lead **3**, which was a very ineffective inhibitor of this enzyme (**Tab. 4**). Several of the new derivatives were medium potency (**4, 5, 11, 12, 14, 22- 24** and **27-30**) or ineffective (**6** and **16**) inhibitors of the mtCA 3 enzyme. As for the other α- or β-CAs investigated here, it may be observed that all types of activities were detected against mtCA 3 for the sulfonamides synthesized in this work.

(v) the selectivity ratios of the new sulfonamides investigated here, for the inhibition of the target versus offtarget CAs is a rather complex issue, due to the particular SAR discussed above for each particular enzyme. However, there are interesting aspects that may be evidenced. For example, several compounds such as **16, 22** and **24** showed low nanomolar hCA II inhibitory activity but were much less effective as β-CA inhibitors and can be considered as selective for the inhibition of the α- versus β-CAs. Sulfonamide **23** on the other hand was a *C. albicans* CA selective inhibitor, with selectivity ratios for inhibiting the fungal enzyme over the mammalian one hCA II of 25, and for inhibiting the fungal over mycobacterial enzymes of 164.7 and of 240.5, respectively.

An inhibitor which was selective for the mtca 1 enzyme has also been discovered, **8**, which had selectivity ratios for inhibiting mtCA 1 over hCA II of 162.7, for inhibiting mtca 1 over the fungal enzyme of 12.9 and for inhibiting mtCA 1 over mtCA 3 of 13.1. For mtCA 3 the most selective inhibitor was **25**, with selectivity ratios of 2.3 over hCA II, of 6.1 over the *C. albicans* enzyme, and of 10.3 over mtCA 1. These selectivity ratios are less high than for the other two investigate β-CAs, but these are the first ever reported CAIs showing this interesting profile and may be useful for understanding in greater detail the physiological role of some of these enzymes.

In conclusion, we report here a series of sulfonamides prepared by reaction of sulfanilamide with aryl/alkyl isocyanates. The ureido-substituted benzenesulfonamides showed a very interesting profile for the inhibition of hCAII and three β-CAs from pathogenic fungal or bacterial species. The *C. albicans* enzyme was inhibited with potencies in the range of 3.4 – 3970 nM, whereas the mycobacterial enzymes mtCA 1 and mtCA 3 with K_Is in the range of 4.8 – 6500 nM and of 6.4 – 6850 nM, respectively. The structure-activity relationship for this class of inhibitors is rather complex but the main features associated with effective inhibition of both α- and each β-CAs investigated here have been delineated.

The nature of the R moiety substituting the second ureido nitrogen is the determining factor in controlling the inhibitory power, probably due to the flexibility of the ureido linker and the possibility of the R group to orientate in different subpockets of the active site cavity of these enzymes.

7.0 CONCLUSIONS

The overall aim of the present thesis was to study the interaction between several isoforms of carbonic anhydrase from human, bacteria, fungi with new series of sulfonamides and their bioisosteres.

The development of more specific agents is required because of the high number of isozymes present in the human body as well as the isolation of many new representatives of CAs from all Kingdoms. This is possible only understanding in detail the catalytic and inhibition mechanism of these enzyme. The carbonic anhydrases are the major players in mammalian physiology; thus the control of the activity in a particular disease represents a promising therapeutic option.

We obtained the discovery of some different and more specific agents (CAIs). Thus, they may be used, on one hand, to improve the knowledge of the role of each CAs isoform and, on one hand, to develop carbonic anhydrase inhibitors with higher specific isoform selectivity for (CA VA, CA VB, CA IX), in order to have potentially drugs with reduced side effects and/or new diagnostic tools for medicinal chemistry applications.

7.1.0 α-CAs INHIBITION

We began our work synthesizing before bioisosteres (sulfamides) and then sulfonamides, analysing the their inhibitory activity versus human carbonic anhydrases, which were described with very different subcellular localization and tissue distribution.

7.1.1 hCA VA and hCA VB inhibition

A possible new approach for the treatment and prophylaxis of obesity is based on the inhibition of carbonic anhydrases enzymes involved in several steps of de novo lipogenesis, both in the mitochondria and the cytosol of cells. Topiramate and zonisamide are two antiepileptic drugs that were shown to induce persistent weight loss in obese patients, but their mechanism of action is largely unknown.

This has been rationalized [1] as being due to the lipogenesis inhibition mediated by these two agents, which in turn is mediated by inhibition of some CA isozymes involved in the carboxylation of pyruvate to oxaloacetate (mitochondrial isoforms CA VA and CA VB) and of acetylcoenzyme A to malonylcoenzyme A (cytosolic isoform CA II).

These compounds (topiramate and zonisamide) have good affinity, but as well as all clinically used drugs (Tab.1) [1], are without selectivity.

Considering the interest in CA VA/VB—selective inhibitors which might be developed as antiobesity agents, we explored here a less investigated class of CA inhibitors (CAIs) for obtaining compounds targeting the mitochondrial CAs, that is, the sulfamides [67a, 81-82].

Furthermore, it has been proved that the sulfamide moety is an effective zinc binding group for obtaining CAIs, similarly to the bioisosteric sulfonamide and sulfamate ones [81].

Thus, we prepared a small series of 2-substituted-1,3,4-thiadiazole-5-sulfamides, which was prepared and assayed as inhibitors of several carbonic anhydrase (CA, EC 4.2.1.1) isoforms: the cytosolic CA I and II, the membrane-associated CA IV and the mitochondrial CA VA and VB. The new compounds showed weak inhibitory activity against hCA I (K_Is of 102 nM–7.42 μM), hCA II (K_Is of 0.54–1.13 μM) and hCA IV (K_Is of 4.32–10.05 μM), but were low nanomolar inhibitors of hCA VA and hCA VB, with

inhibition constants in the range of 4.2–32 nM and 1.3–74 nM, respectively.

Furthermore, the selectivity ratios for inhibiting the mitochondrial enzymes over CA II were in the range of 67.5–415, making these sulfamides the first selective CA VA/VB inhibitors.

Concluding, we discovered compouds with the best inhibitory activity ever reported against the mitochondrial CA VA and CA VB. Furthermore they showed an interesting selective inhibition profile for the mitochondrial isoforms versus cytosolic and membrane-bound isoforms; thus, these results indicate the new synthesized sulfamides as useful tools to understand the exact role of the CA VA and CA VB in lipogenesis as well as the potentiality of CAIs as antiobesity drugs.

7.1.2 hCA I, hCA II, hCA IX, CA XII inhibition

A completely new and very promising aspect of CAs physiology has been recently found and refers to over expression of some CA isoforms in cells as a consequence of reduced oxygen afflux (hypoxia). This situation is frequently met in tumour tissues were the fast growing rate favours anoxic conditions. The isoforms over expressed (CA IX, CA XII) are membrane located with the active site exposed toward the exterior part thus representing a possible target for new therapies and or diagnostic tools.

CA IX and XII are overexpressed in many such tumors in response to the hypoxia inducible factor (HIF) pathway, and research on the involvement of these isozymes in cancer has progressed significantly in recent years.

Carbonic anhydrase (CA, EC 4.2.1.1) IX (CA IX) has recently been shown to be a druggble target for imaging and treatment of hypoxic tumours over expressing this protein.

At present we know that CA IX, is a transmembrane protein involved in solid tumor acidification through the HIF-1α activation cascade. CA IX has a very high catalytic activity for the hydration of carbon dioxide to bicarbonate and protons, even at acidic pH values (of around 6.5), typical of solid, hypoxic tumours, which are largely unresponsive to classical chemo- and radiotherapy. CA IX is involved in tumorigenesis through many pathways, such as pH regulation and cell adhesion control.

The advancements in the comprehension of the role of the different CAs isoforms and of their spatial localization on one side as well as the progress in the X ray crystallographic techniques make more realistic the perspective to design new CA inhibitors with differential selectivity toward the different isoforms.

X-ray crystallography of CA inhibitors in a complex with enzymes, is an important tool in drug design of zinc-enzyme inhibitors, using such structures for the rational modelling, drug design of more selective and potent CAIs for several disease applications [10a].

Thus, we started our work analysing the α-CA inhbition with a new series of ureido substituted benzene sulfonamide derivatives, prepared by reaction of sulfanilamide with aryl/alkyl isocyanate. The ureido-substituted benzenesulfonamides showed a very interesting profile for the inhibition of several carbonic anhydrases (CAs, E.C. 4.2.1.1) such as the human hCA II.

Recently, our group investigated their interactions with the 4 catalytically active mammalian CAs and reported the X-ray crystal structures of five selected compounds of this series (4-substituted-ureido-benzenesulfonamides), in complex with hCA II [92]; it was determined that it is the nature of the R group (e.g the moiety substituting the second ureido nitrogen) that greatly influences the binding to the enzyme. Thus, the nature of the R moiety is the determining factor in controlling the inhibitory power, probably due to flexibility of the ureido linker and the possibility of the R group to orientate in different subpockets of the active site cavity of these enzymes.

All the X-ray and in vitro inhibitory activity data collected, can be used for the structure-based drug design of other new similar inhibitors as their isomers (3-substituted-ureido-benzenesulfonamides) related to CAIs class; 6 isomers synthesized and tested have already shown to be isoform-selective inhibitors of CA IX (data not reported); that to individuate other possible compouds with a different and better affinity-selectivity profile for CA IX, CA XII over CA II.

Due to the structural similarity but different localization and tissue distribution of α-CA isoforms, these findings can be thus extended also to other classes of CAIs, and probably to other CA isoforms less well investigated than CA II, with the possibility of designing inhibitors (CAI) with a better selectivity/affinity for various isoforms with medicinal chemistry applications.

At present, CAI mainly of sulfonamides used as therapeutic agents possess many undesired side effects due to their lack of selectivity, i.e. indiscriminate inhibition of all CA isozymes. One of the most interesting compound is named Indisulam. It is a sulfonamide derivative with powerful anticancer activity and recently was shown to act as nanomolar inhibitor of CA IX (in vitro), but without selectivity for some isoforms [35].

Indisulam showed *in vivo* efficacy against human tumour xenograft in nude mice, exhibiting a significant antitumour affect and progressing to Phase I and II clinical trials for the treatment of solid tumours. Its detailed mechanism of action is not clear, involving the inhibition of several enzymes including the carbonic anhydrase (CA IX, CA XII).

Thus, in the effort directed toward the development of isoform-selective CA inhibitors suitable for the management of cancer we decided to investigate some compounds (7, 10, 13, 15, 16, 25), selected considering several parameters as aqueous solubility, affinity for CA IX and selectivity ratios for inhibiting CA IX over CA II, to evaluate the possible antitumor/antimetastasis activity *in vivo* breast tumour model.

In these experiments, the compounds (25) inside the series of ureido-substituted sulfonamide CAIs which possess good selectivity for CA IX and CA XII enzyme over the cytosolic enzymes, showed excellent *in vivo* antimetastatic effects in breast cancer model. In particular, treating of mice harbouring CAIX-positive 4T1 mammary tumours with novel CAIX-specific small molecule inhibitors that mimicked the effects of CAIX depletion in vitro resulted in attenuation of primary tumor growth and metastasis formation in both spontaneous and experimental models of metastasis, inhibitory effects on CAIX-negative tumors. Similar inhibitory effects on primary tumor growth were observed in mice harbouring orthotopic tumors comprised of lung metastatic MDA-MB-231 LM2-4Luc+ cells. These findings demonstrate that CAIX is vital for growth and metastasis of hypoxic breast tumors and is a specific , biomarker for breast cancer metastasis

In conclusion, we report the synthesis of a series of ureido-substituted sulfonamide CAIs which possess strong affinity for CA IX, an acceptable selectivity profile for inhibiting the tumor-associated isoforms CA IX and XII over the cytosolic enzymes CA I and II, as well as excellent *in vivo* antimetastatic effects in a mice breast cancer model. We have indentified a series *of* ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antitumour/antimetastatic activity in a breast cancer models.

These results show how the carbonic anhydrase sulfonamides may be really used alone and/or in association with other antitumour agents, in surrounding of multidrug resistance to the usually therapies, because they have a different action, which answer to the hypoxia, considered the main environmental factor of resistance.

7.2.0 β-CA INHIBITION

The emergence of resistance to the currently used drugs in several human pathologies such as Tuberculosis or Candidosis, force to develop of new antiifective agents with a novel mechanism of action.

At present, more bacterial/fungal genomes are being constantly sequenced and the finding of novel targets belonging to the various CA families is constant, leading to a certain degree of optimism that anti-infectives based on CAIs can be developed.

The development of isoform-selective CA inhibitors that target a particular CA isoform could maximize therapeutic efficacy and to minimize the side effects.

7.2.1 *β-CA from Mycobacterium tuberculosis and C. albicans inhibition*

In order to understand whether β-CAs may constitute drug targets for developing antiinfective agents, it is essential to design compounds with different affinities and pharmacological properties compared to the classical CAIs (SA, AAZ).

We report a series of sulfonamides prepared by reaction of sulfanilamide with aryl/alkyl isocyanates. The ureido-substituted benzenesulfonamides showed a very interesting profile for the inhibition of hCAII and three β-CAs from pathogenic fungal or bacterial species.

The *C. albicans* enzyme was inhibited with potencies in the range of 3.4 – 3970 nM, whereas the mycobacterial enzymes mtCA 1 and mtCA 3 with K_Is in the range of 4.8 – 6500 nM and of 6.4 – 6850 nM, respectively. flexibility of the ureido linker and the possibility of the R group to orientate in different subpockets of the active site cavity of these enzymes.

We discovered three sulfonamides isoform-selective inhibitors for β-CA (fungal, bacterial enzymes) versus α-CA (human enzymes):

- Sulfonamide **23** was a *C. albicans* CA selective inhibitor, with selectivity ratios for inhibiting the fungal enzyme over the mammalian one hCA II of 25, and for inhibiting the fungal over mycobacterial enzymes of 164.7 and of 240.5, respectively.
- Sulfonamide **8** which was an selective inhibitor for the mtCA 1 enzyme from Mycobacterium tuberculosis; it showed a selectivity ratios for inhibiting mtCA 1 over hCA II of 162.7, for inhibiting mtCA 1 over the fungal enzyme of 12.9 and for inhibiting ntCA 3 over mtCA 3 of 13.1.

- Sulfonamide **25** was the most selective inhibitor for mtCA 3, with selectivity ratios of 2.3 over hCA II, of 6.1 over the *C. albicans* enzyme, and of 10.3 over mtCA 1; These selectivity ratios are less high than for the other two investigate β-CAs, but these are the first ever reported CAIs showing this interesting profile and may be useful for understanding in greater detail the physiological role of some of these enzymes.

The structure-activity relationship for this class of inhibitors is rather complex and variable. We have delineated the main features associated with effective inhibition of both α- and each β-CAs investigated, and understood the motif of this phenomenon. Infact, as shown by a recent X-ray crystallographic work on five of these derivatives for which the structure in complex with the human isoforms hCA II has been reported [92], it was determined that is the nature of the R group (e.g the moiety substituting the second ureido nitrogen) that greatly influences the binding to the enzyme.

Thus, the nature of the R moiety is the determining factor in controlling the inhibitory power, probably due to flexibility of the ureido linker and the possibility of the R group to orientate in different subpockets of the active site cavity of these enzymes.

In conclusion we synthesized, designed new sulfonamide compounds with different affinities, selectivities and pharmacological properties to understand whether β-CAs may constitute drug targets to develop antiinfective agents with a new mechanism of action.

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9.0 ARTICLES LIST

1) F.-Z. Smaine, **Fabio Pacchiano**, M. Rami, V. Barragan-Montero, D. Vullo, A. Scozzafava, J.-Y Winum, C. T. Supuran, *Carbonic anhydrase inhibitors: 2-Substituted-1,3,4-thiadiazole-5-sulfamides act as powerful and selective inhibitors of the mitochondrial isozymes VA and VB over the cytosolic and membrane-associated carbonic anhydrases I, II and IV*, Bioorg. Med. Chem. Lett., 2008, 18, 6332-6335.

2) **Fabio Pacchiano**, M. Aggarwal, B. S. Avvaru, A. H. Robbins, A. Scozzafava, R. McKenna, C. T. Supuran, *Selective hydrophobic pocket binding observed within the carbonic anhydrase II active site accomodate different 4-substituted-ureido-benzenesulfonamides and correlate to inhibitor potency*, Chem. Commun. (Camb.), 2010, 46, 8371-8373.

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Fabio Pacchiano *"Design, synthesis and biological evaluation of novel carbonic anhydrase inhibitors against mammalian, bacterial and fungal isoforms"*

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Carbonic anhydrase inhibitors: 2-Substituted-1,3,4-thiadiazole-5-sulfamides act as powerful and selective inhibitors of the mitochondrial isozymes VA and VB over the cytosolic and membrane-associated carbonic anhydrases I, II and IV

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ABSTRACT

A series of 2-substituted-1,3,4-thiadiazole-5-sulfamides was prepared and assayed as inhibitors of several carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the cytosolic CA I and II, the membrane-associated CA IV and the mitochondrial CA VA and VB. The new compounds showed weak inhibitory activity against hCA I (K_Is of 102 nM–7.42 μM), hCA II (K_Is of 0.54–7.42 μM) and hCA IV (K_Is of 4.32–10.05 μM) but were low nanomolar inhibitors of hCA VA and hCA VB, with inhibition constants in the range of 4.2–32 nM and 1.3–74 nM, respectively. Furthermore, the selectivity ratios for inhibiting the mitochondrial enzymes over CA II were in the range of 67.5–415, making these sulfamides the first selective CA VA/VB inhibitors.

Among the sixteen α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms found in animals, two CA isozymes, VA and VB, are present in mitochondria.[1–3] These isozymes are involved in several biosynthetic processes, such as ureagenesis, gluconeogenesis, and lipogenesis, both in vertebrates as well as in invertebrates.[1,4,5] The provision of enough of the substrate, bicarbonate, in several biosynthetic processes involving pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC), and carbamoyl phosphate synthetases I and II, is assured mainly by the catalysis involving the mitochondrial isozymes CA VA and CA VB, probably assisted by the high activity cytosolic isozyme CA II, as shown schematically in Figure 1.[1,2] CAs thus play a key role in fatty acid biosynthesis. Mitochondrial pyruvate carboxylase (PC) is needed for efflux of acetyl groups from the mitochondria to the cytosol where fatty acid biosynthesis takes place.[1] Pyruvate is carboxylated to oxaloacetate in the presence of bicarbonate and under the catalytic influence of the mitochondrial isozymes CA VA and/or CA VB. The mitochondrial membrane is impermeable to acetyl-CoA which reacts with oxaloacetate, leading to citrate, which is thereafter translocated to the cytoplasm by means of the tricarboxylic acid transporter. As oxaloacetate is unable to cross the mitochondrial membrane, its decarboxylation regenerates pyruvate which can be then transported into the mitochondria by means of the pyruvate transporter (Fig. 1). The acetyl-CoA thus generated in the cytosol is in fact used for the de novo lipogenesis, by carboxylation in the presence of ACC and bicarbonate, with formation of malonyl-CoA, the conversion between CO_2 and bicarbonate being assisted by CA II. Subsequent steps involving the sequential transfer of acetyl groups lead to longer chain fatty acids.[1–5] Therefore, several CA isozymes are critical to the entire process of fatty acid biosynthesis: VA and/or VB within the mitochondria (to provide enough substrate to PC), and CA II within the cytosol (for providing sufficient substrate to ACC). Inhibition of CAs by clinically used sulfonamides such as acetazolamide **AZA**, zonisamide **ZNS** or the sulfamate topiramate, **TPM**, can decrease lipogenesis in adipocytes in cell culture.[1–3] Furthermore, among some of the side effects of these drugs is also the weight loss,[6] which may in fact lead to novel anti-obesity therapies.[7]

In previous contributions from our laboratories[8–10] we have shown that both CA VA and CA VB are druggable targets. Rather large libraries of various sulfonamides and sulfamates have been assayed as inhibitors of these mitochondrial enzymes, with several low nanomolar inhibitors being detected.[8–10] However, an important drawback of most of these compounds is represented by the rather low selectivity for inhibiting the mitochondrial CAs over

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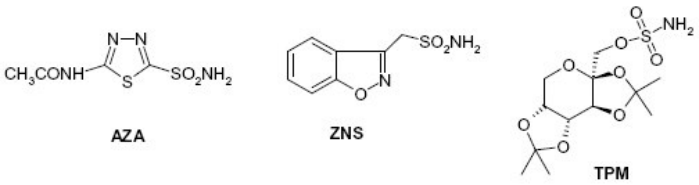
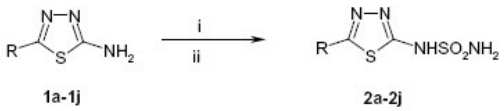


the cytosolic/membrane-bound ubiquitous isoforms such as CA I, II (cytosolic) and CA IV (extracellular, membrane-associated isozyme).[1,8–10] Considering the interest in CA VA/VB–selective inhibitors which might be developed as antiobesity agents, we explore here a less investigated class of CA inhibitors (CAIs) for obtaining compounds targeting the mitochondrial CAs, that is, the sulfamides.[11,12] Indeed, aromatic, heterocyclic and sugar sulfamides have been reported earlier to generate effective inhibitors of several CA isozymes (mainly CA I, II and IX),[11,12] and the X-ray crystal structures for two such compounds complexed within the CA II active site are also available,[12c,12d] proving the sulfamide moiety to be an effective zinc binding group for obtaining CAIs, similarly to the bioisosteric sulfonamide and sulfamate ones.[11]

Here we report the synthesis of 1,3,4-thidiazole sulfamides possessing various 2-substitutents.[13,14] This scaffold has been chosen as it is present in one of the most investigated and powerful CAI, acetazolamide **AZA**, used clinically since 1956,[1] and also because its binding to the enzyme is effective, as shown for many **AZA** derivatives for which the X-ray crystal structure has been resolved in adduct with different CA isoforms[15] (Scheme 1).

Starting with the commercially available 2-substituted-5-amino-1,3,4-thiadiazoles **1**, which have been sulfamoylated with in situ generated sulfamoyl chloride, by a method reported earlier by one of this groups,[14] the sulfamides **2a–j** have been prepared with excellent yields.[13] The various substituents in position 2 of the heterocyclic ring of the new compounds **2a–j** were chosen in such a way as to have a rather comprehensive SAR insight for this



Scheme 1. Synthesis of 2-substituted-1,3,4-thiadiazole-5-sulfamide **2a–2j**: Reagents and conditions: (i) *t*-BuOH, $ClSO_2NCO$, NEt_3, CH_2Cl_2. (ii) TFA 95% in CH_2Cl_2.

class of CAIs. Thus, starting with the unsubstituted parent compound ($R = H$, **2a**), both aliphatic (Et, *t*-Bu, etc), aromatic (Ph, substituted phenyl, etc) as well as sulfide- and sulfone incorporating such moieties have been introduced in this position. X-ray crystallographic data on many CA–sulfonamide/sulfamate/sulfamide adducts revealed that in addition to the zinc binding group (a sulfamide one for derivatives **2**) and organic scaffold (1,3,4-thiadiazole for **2**), the tails present in CAIs are critical both for the isozyme inhibition profile as well as for modulationg the physico-chemical properties of such inhibitors, which may thus lead to various pharmacological applications.[1,15–18] This explains the choice of the tail groups present in derivatives **2a–j** reported here.

Inhibition data against five physiologically relevant CA isozymes, that is, the cytosolic hCA (human CA) I and II, the membrane-associated hCA IV as well as the two mitochondrial isoforms hCA VA and VB, are presented in Table 1.[19] Three standard CAIs, that is, **AZA**, **ZNS** and **TPM**, have been assayed in the same conditions in order to allow a better understanding of the inhibi-

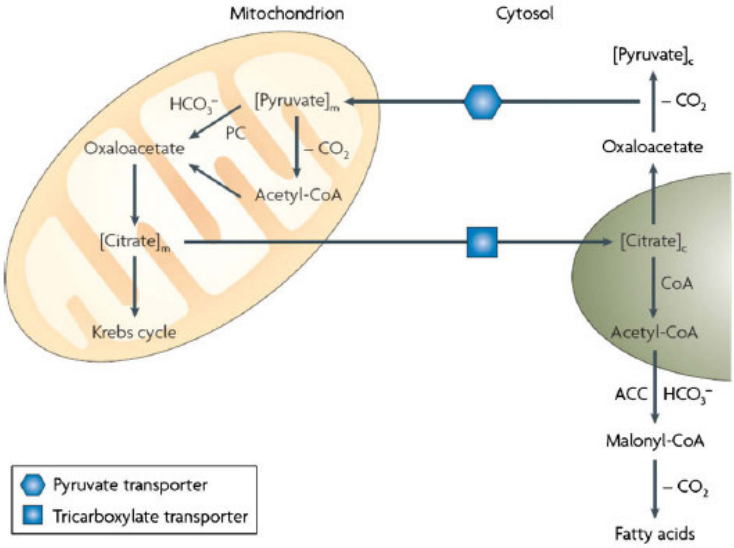
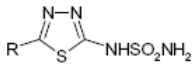


Figure 1. The transfer of acetyl groups from the mitochondrion to the cytosol (as citrate) for the provision of substrate for *de novo* lipogenesis.[1] All steps involving bicarbonate need the presence of at least two CA isozymes: CA VA/VB in the mitochondrion and CA II in the cytosol (see discussion in the text).

Table 1
Inhibition data of human CA isozyms I, II (cytosolic), IV (membrane-associated) and VA, and VB (mitochondrial) with compounds **2a–2j** and standard inhibitors (acetazolamide **AZA**, zonisamide **ZNS** and topiramate **TPM**), by a stopped-flow, CO_2 hydration assay.[19]



2a-2j

No	R	K_I*				
		hCA I[a] (μM)	hCA II[a] (μM)	hCA IV[b] (μM)	hCA VA[c] (μM)	hCA VB[c] (μM)
2a	H	7.42	0.97	8.91	28.3	74
2b	Et	7.01	0.95	8.64	18.7	63
2c	*t*-Bu	5.54	0.90	8.40	10.4	2.8
2d	CF_3	6.86	1.08	4.32	7.3	3.9
2e	MeS	2.40	0.92	8.30	32	2.9
2f	EtS	1.89	1.13	7.58	9.3	23.1
2g	Ph	1.66	0.87	5.54	9.2	7.5
2h	4-$MeOC_6H_4$	0.102	0.54	8.76	8.0	1.3
2i	4-Br-C_6H_4	5.85	0.82	10.05	4.2	4.5
2j	$MeSO_2$	0.103	0.94	6.10	8.7	2.7
AZA	–	0.250	0.012	0.074	63	54
ZNS	–	0.056	0.035	8.59	20	6033
TPM	–	0.250	0.010	4.90	63	30

* Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[19]
[a] Human, recombinant isozymes.
[b] Truncated cloned human isoform lacking the first 20 aminoterminal amino acids.
[c] Full length, recombinant human isoforms.[8–10]

tion profile of the new compounds **2a–2j** investigated here. Data of Table 1 allow the following SAR to be evidenced for the new sulfamides **2** reported here:

(i) Against hCA I, the sulfamides **2** showed a moderate-weak inhibitory activity, with inhibition constants in the range of 102 nM–7.42 μM, bing thus, with two exceptions (compounds **2h** and **2j**) much weaker inhibitors as compared to the clinically used drugs **AZA-TPM** (K_Is in the range of 56–250 nM). The least active hCA I inhibitor was the parent, unsubstituted compound **2a**, whereas introduction of various substituents in position 2 of the thiadiazole ring enhances activity. The groups leading to best activity were 4-methoxyphenyl and methylsulfonyl (**2h** and **2j**).

(ii) Isozyme hCA II was also weakly inhibited by the new sulfamides **2**, with K_Is in the range of 0.54–1.13 μM, whereas the clinically used drugs were much stronger inhibitors of this ubiquitous isoform (K_Is in the range of 10–35 nM). It may be observed a very flat SAR for sulfamides **2** in inhibiting hCA II, with the nature of groups substituting in 2 the thiadiazole ring, having a small influence on the inhibitory power (Table 1).

(iii) Sulfamides **2** act as quite weak inhibitors of the membrane-associated isoforms hCA IV, with K_Is in the range of 4.32–10.05 μM, unlike **AZA** which is a strong inhibitor (K_I of 74 nM) but similarly to **ZNS** and **TPM** (K_Is of 4.90–8.59 μM).

(iv) Excellent inhibitory properties were evidenced for derivatives **2** against the two mitochondrial CA iozymes, hCA VA and hCA VB. Indeed, these compounds showed K_Is in the range of 4.2–28.3 nM against hCA VA, and of 1.3–74 nM against hCA VB, respectively. It may be observed that compounds **2** are much better inhibitors of the mitochondrial CAs as compared to the three clinically used drugs (K_Is in the range of 20–63 nM against hCA VA, and of 30–6033 nM against hCA VB, respectively, Table 1). For hCA VA, the substitution patterns of the heterocyclic ring leading to the best inhibitors included the trifluoromethyl, thioethyl-, aryl and methylsulfonyl moieties (K_Is < 10 nM) whereas the remaining ones generated slightly less effective inhibitors. For hCA VB, only three compounds (**2a**, **2b** and **2f**) showed K_Is > 10 nM, all the other substitution patterns leading to derivatives with excellent activity (K_Is < 7.5 nM). These are in fact the compounds with the best inhibitory activity ever reported against the mitochondrial enzymes CA VA and CA VB.

(v) Another very interesting property of the newly described sulfamides **2** is related to their selective inhibition of the mitochondrial isozymes (CA VA and VB) over the cytosolic and membrane asociated isoforms (CA I, II and IV). Especially CA II constitutes a problem when designing various CAIs targeting other izoymes, because CA II has generally a very high affinity for sulfonamides, sulfamates and sulfamides, and it is also a ubiquitoous enzyme in vertebrates, including humans.[1–3] As seen from data of Table 1, the three clinically used compounds mentioned here (but also the other CAIs in clinical use)[1] are generally much better CA II than CA VA/VB inhibitors (except for zonisamide against CA VA). For example the selectivity ratio of **AZA** for inhibiting CA VA over CA II is of 0.19, and for inhibiting CA VB over CA II is of 0.22. Basically **AZA** has a much higher affinity for the cytosolic isoform CA II than for the mitochondrial ones. However, all compounds **2** reported here showed a much better inhibitory activity against the mitochondrial isozymes than against the cytosolic (or membrane-associated) ones. Thus, for example, **2h** has a selectivity ratio for inhibiting CA VA over CA II of 67.5, and for inhibiting CA VB over CA II of 415. For **2i**, these ratios are of 195 and of 182, respectively. Thus, these compounds are 67.5–415-times better inhibitors of the mitochondrial over the cytosolic isozymes, which is a very interesting result, never evidenced earlier for any other class of CAIs.

In conclusion, we prepared a small series of 2-substituted-1,3,4-thiadiazole-5-sulfamides and assayed them for the inhibition of five physiologically relevant isozymes, the cytosolic CA I and II, the membrane-associated CA IV and the mitochondrial CA VA and VB. The new compounds showed weak inhibitory activity against hCA I (K_Is of 102 nM–7.42 μM), hCA II (K_Is of 0.54–7.42 μM) and hCA IV (K_Is of 4.32–10.05 μM) but were low nanomolar inhibitors of hCA VA and hCA VB, with inhibition constants in the range of 4.2–32 nM and 1.3–74 nM, respectively. Furthermore, the selectivity ratios for inhibiting the mitochondrial enzymes over CA II were in the range of 67.5–415, making these sulfamides the first selective CA VA/VB inhibitors.

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13. General procedure for the preparation of thiadiazole-sulfamides **2**: To a solution of 2-substituted-5-amino-1,3,4-thiadiazole **1** in methylene chloride and 1.1 equiv of triethylamine was added dropwise a solution of *tert*-butoxycarbonylamino sulfonyl chloride (prepared ab initio by reacting 1 equiv of *tert*-butanol and 1 equiv of chlorosulfonyl isocyanate in methylene chloride at 0 °C).[14] The mixture was stirred 1 h at room temperature, and then concentrated under vacuum. The residue is purified on silica gel column chromatography using ethyl acetate-petroleum ether 7–3 as eluent to give the Boc-protected sulfamide in good yield (75–80%). This compound was then deprotected using a solution of trifluoroacetic acid in methylene choride 50–50 v–v. **2a**: mp 151–154 °C; MS (ESI⁻, 20eV): *m/z* 203.17 [M+Na]⁻, 383.10 [2M+Na]⁻ ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.63 (s, 1H), 6.77 (s, 2H), 3.16 (s, 1H). **2b**: mp 100–110 °C; MS (ESI⁻, 20eV): *m/z* 231.1 [M+Na]⁻; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.71 (s, 2H), 3.16 (s, 1H), 2.78 (q, 2H), 1.2 (t, 3H).**2c**: mp 150–153 °C; MS (ESI⁻, 20eV): *m/z* 259.23 [M+Na]⁻; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.73 (s, 2H), 3.16 (s, 1H), 1.30 (s, 9H). **2d**: mp 179-180°C; MS (ESI⁻, 20eV): *m/z* 247.06 [M−H]⁻; ^{1}H NMR (400MHz, DMSO-d_6) δ 7.23 (s, 2H), 3.16 (s, 1H). **2e**: mp 150–153 °C; MS (ESI⁻, 20 eV): *m/z* 249.09 [M+Na]⁻; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.85 (s, 2H), 3.16 (s, 1H), 2.61 (s, 3H). **2f**: mp 145–148 °C; MS (ESI⁻, 20 eV): *m/z* 263.20 [M+Na]⁻; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.86 (s, 2H), 3.16 (s, 1H), 3.12 (q, 2H), 1.31 (t, 3H). 2 g: mp 170–172 °C; MS (ESI⁻, 20 eV): *m/z* 279.15; [M+Na]⁻; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.78 (s, 2H), 7.53 (s, 3H), 6.88 (s, 2H), 3.16 (s, 1H). **2h**: 171–174 °C; MS (ESI⁻, 20 eV): *m/z* 309.17 [M+Na]⁻; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.72 (d, 2H), 7.06 (d, 2H), 6.82 (s, 2H), 3.81 (s, 3H), 3.16 (s, 1H). **2i**: mp 173–176 °C; MS (ESI⁻, 20 eV): *m/z* 359.06 [M+Na]⁻; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.74 (m, 4H), 6.89 (s, 2H), 3.16 (s, 1H). **2j**: 154–156 °C; MS (ESI⁻, 20 eV): *m/z* 281.15 [M+Na]⁻; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.14 (s, 2H), 3.5 (s, 3H), 3.16 (s, 1H).
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19. Khalifah, R.G. *J. Biol. Chem.*, **1971**, *246*, 2561. An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction.[18] The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, and represent the mean from at least three different determinations.

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Selective hydrophobic pocket binding observed within the carbonic anhydrase II active site accommodate different 4-substituted-ureido-benzenesulfonamides and correlate to inhibitor potency†

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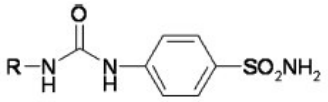
4-Substituted-ureido benzenesulfonamides showing inhibitory activity against carbonic anhydrase (CA, EC 4.2.1.1) II between 3.3–226 nM were crystallized in complex with the enzyme. Hydrophobic interactions between the scaffold of the inhibitors in different hydrophobic pockets of the enzyme were observed, explaining the diverse inhibitory range of these derivatives.

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread enzymes in all organisms, catalyzing CO_2 hydration to bicarbonate and a proton.[1,2] Their inhibition has been exploited clinically for decades for various classes of diuretics and systemically acting antiglaucoma agents. In the last few years novel applications of CA inhibitors (CAIs) have emerged, such as topically acting antiglaucoma, anticonvulsants, antiobesity, antipain and antitumor agents/diagnostic tools. Such CAIs target diverse isozymes of the 13 catalytically active α-CA isoforms present in mammals.[1,2] However, the physiologically dominant isoform is CA II, which is catalytically highly efficient, widespread in many cell types and thus involved in many physiological processes, and also easily crystallizable.[1–3] Thus, most of our knowledge in the design of CAIs with pharmacological applications are based on detailed CA II crystallographic studies. CA II has been crystallized in adducts with the main classes of inhibitors, sulfonamides and their bioisosters,[3–6] anions,[7] phenols,[8] coumarins,[9] and polyamines.[10] Among all these classes of CAIs, the sulfonamides continue to be of great interest, with more than 30 such drugs being currently used clinically.[1]

Continuing our interest in the structure-based drug design of sulfonamide CAIs we report here the X-ray crystal structure of five substituted-ureido benzenesulfonamides, compounds **1–5**, which show varying CA II inhibitory activity (Fig. 1).[11] These compounds possess various substituents at the ureido moiety and the common benzenesulfonamide head. Their inhibition varies from 3.3 nM for the isopropyl-substituted compound **3** to 226 nM for the cyclopentyl substituted compound **5**. The scope of this work was to understand how



	K_I (nm)
1: R = 4-F-C_6H_4	96 ± 8
2: R = C_6F_5	50 ± 4
3: R = 2-iso-Pr-C_6H_4	3.3 ± 0.07
4: R = 3-O_2N-C_6H_4	15 ± 0.8
5: R = cyclopentyl	226 ± 14

Fig. 1 Structure of sulfonamides **1–5** and their inhibition against CA II (K_Is, in nM, mean ± standard error, from 3 different assays).

the substitution pattern at the ureido moiety is able to induce such a large variation in inhibition, a factor of 68, for such a congeneric series of sulfonamides.

The crystal structures of CA II complexed with compounds **1–5** have been determined using methods as previously described by us[12,13] between 1.65 and 1.5 Å resolution (Supplementary text and Table S1, ESI†). All five compounds were well ordered and refined with full occupancy, with B factors that were comparable to the solvent within the active site (Fig. 2 and Table S1, ESI†). Although not shown, as in other CA II complexes, for all five compounds, additional electron density consistent with a bound glycerol molecule, from the cryoprotectant solution used during data collection, was observed adjacent to the aromatic ring of the benzene sulfonamide.

Similar to other sulfonamide inhibitors of CA II,[4–7,12,13] the ureido-substituted sulfonamides are buried deep in the active site, displacing solvent molecules (Fig. 2A), with the sulfonamide amine nitrogen binding directly to the active site zinc atom (distance of ~1.9–2.0 Å) and accepting a hydrogen bond from OG1 of Thr199 and with the O2 of the sulfonamide accepting a hydrogen bond from the main-chain nitrogen atom of Thr199 at a distance of ~2.9–3.0 Å. The overall $Zn(N)_4$ coordination can be described as a distorted tetrahedron. The compound moieties protrude out of the active site and are stabilized by both hydrophilic and hydrophobic residues (Fig. 2 and 3).

For all five compounds the benzene core is at van der Waals distance from the side chain of Leu198, and is sandwiched on the other side by the glycerol molecule, and atoms from Val121 and Gln92. Hydrogen bonds from the O1 and O2 hydroxyl groups of the glycerol anchor it to the side chain atoms of Asn67 and Asn62. Other than compound **4** there are

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† Electronic supplementary information (ESI) available: full characterization of compounds, assay method and details of crystallographic experiments are available. See DOI: 10.1039/c0cc02707c

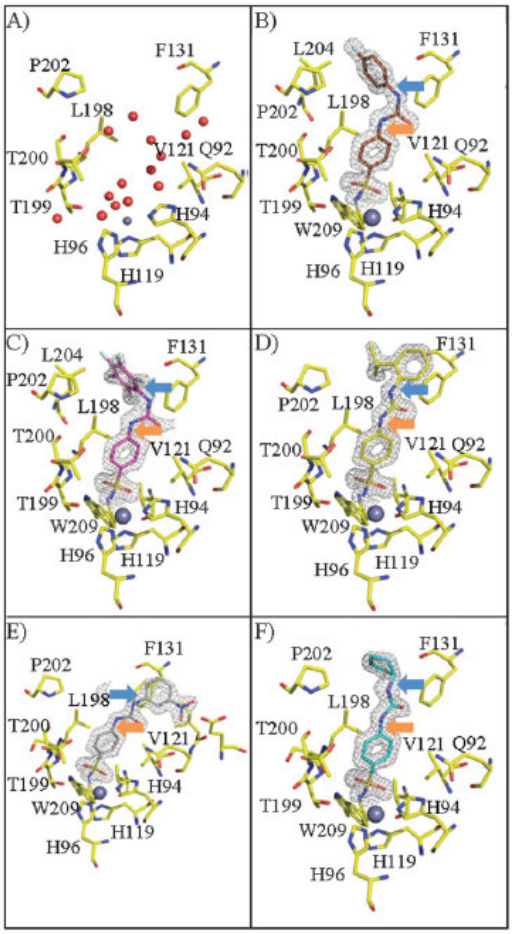

Fig. 2 CAII active site (A) unbound (PDB:2ILI) (*5*), (B) with compound **1** (orange, PDB:3N4D), (C) **2** (pink, PDB:3N0N), (D) **3** (yellow, PDB:3N3J), (E) **4** (grey, PDB:3N2P), and (F) **5** (cyan, PDB:3MZC). The zinc is depicted as a black and solvent as red spheres. The electron density is represented by a σ-weighted $2F_o - F_c$ Fourier map (grey mesh). Amino acids are as labelled. The torsion angles C5–C4–N7–C8 and C8–N9–C10–C15(C11) are indicated by orange and blue arrows, respectively.

no direct hydrogen bonds between the compounds and the active site other than those linking the sulfonamide to the surrounding amino acids. Two bridging solvent molecules link O8 to NE2 of Gln92 and N7 to the main chain carbonyl oxygen of Pro201, respectively. All the compounds are non-planar and exhibit a twisted shape that can be ascribed to two torsion angles, C5–C4–N7–C8 (orange arrow) and C8–N9–C10–C15(C11) (blue arrow) (Fig. 2).

For compound **1**, the C5–C4–N7–C8 and C8–N9–C10–C15 torsion angles are −29.6° and −38.2°, respectively. This allows the terminal fluorine atom F13 to make hydrophobic contacts with side chain carbon atoms of Pro202 and Leu204. Other hydrophobic interactions are between N9, C10 and C15 with the CE2 carbon atom of Phe131 in a face to edge interaction,

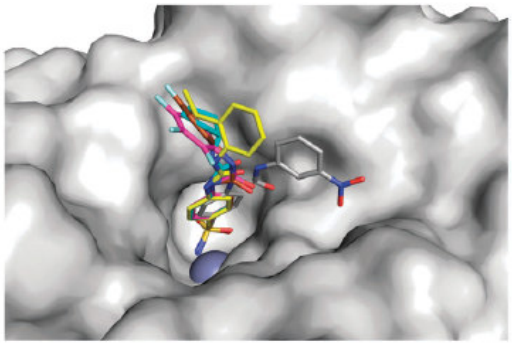

Fig. 3 View of compounds **1** (orange), **2** (pink), **3** (yellow), **4** (grey), and **5** (cyan) superposed in the active site of CA II. CA II is depicted as a grey surface representation. The active-site zinc is depicted as a black sphere. The positions of oxygen and nitrogen atoms of the compounds are coloured red and blue, respectively. Figure made using PyMOL (DeLano Scientific).

C14 with CB and CG of Pro202 and CD1 of Leu198, and C13 with CB and CG of Pro202 (Fig. 2B). For compound **2**, the C5–C4–N7–C8 and C8–N9–C10–C11, torsion angles are −18.0° and −45.7°, respectively. This allows the pentafluorophenyl atoms C12, C13, F12, and F13 to make hydrophobic contacts with the CB and CG atoms of Pro202 (Fig. 2C). With compound **3**, the C5–C4–N7–C8 and C8–N9–C10–C11, torsion angles are 5.5° and −66.1°, respectively. The overall shape of the inhibitor brings the isopropyl methyl carbon atom C18 into hydrophobic contacts with methyl carbon atoms of Val135 and the CD1 atom of Leu198, respectively. Other hydrophobic interactions are between the ring atoms of the isopropylphenyl group with the CE2 carbon atom of Phe131 in a face-to-edge interaction (Fig. 2D). Compound **4** differs from compounds **1**, **2**, **3** and **5** in that a direct hydrogen bond is observed between the inhibitor and the active site, in addition to those linking the sulfonamide to the surrounding amino acids. This interaction, which does not involve a solvent bridge, is between Gln92 NE2 and O8 of the inhibitor at a distance of 2.8 Å (Fig. 2E). Again, compound **4** exhibits non-planarity with the C5–C4–N7–C8 and C8–N9–C10–C15, torsion angles being −33.9° and −19.1°, respectively. This allows the terminal nitro atoms N16, O16 and O17 to make hydrophobic contacts with side chain carbon atoms of Ile91, Glu69, and Gln92 (Fig. 1E). Other hydrophobic interactions for compound **4** are observed between the aromatic ring of the nitrophenyl group and a dimethyl sulfoxide (DMSO) C1 carbon atom (used to dissolve the compounds). The ring is also in a partial stacking interaction with Phe131, which includes N9, C8 and O8 of the urea linker (see Fig. S1, ESI†). These interactions make the orientation of compound **4** uniquely different from the other compounds (Fig. 2E and 3). With compound **5**, the C5–C4–N7–C8 and C8–N9–C10–C11, torsion angles are −163.9° and −175.7°, respectively. The torsion angles allow the cyclopentyl group of compound **5** to make hydrophobic contacts with side chain carbon atoms of Pro202 and Phe131. Both N9 and N7 are within hydrogen bond distance of a solvent molecule (water 30), which also is a bridge solvent to the main chain Pro201 O atom (Fig. 2F).

The X-ray data for the five CA II adducts of sulfonamides **1–5** presented above show that the benzenesulfonamide part of the molecules is very much superposable between the five adducts, whereas the ureido and aryl/cycloalkyl moieties are much less so. Data of Fig. 3 clearly show that 3 of the 5 compounds (**1**, **2** and **5**) bind in a rather similar manner, with the 4-fluorophenyl, pentafluorophenyl and cyclopentyl moieties observed in the same active site region. This is a rather well defined hydrophobic pocket observed in other CA II–sulfonamide adducts,[12,13] which we have defined in a recent paper hydrophobic pocket 2.[12] In contrast to these three inhibitors, the 3-nitrophenyl substituted compound **4** binds in a less utilized hydrophobic pocket, which we have defined pocket 1,[12] these two pockets being separated by Phe131, an amino acid residue critical for orientating inhibitors in most CA isoforms.[14] Few inhibitors observed to date to bind in the hydrophobic pocket 1, compound **4** being one of the 6 cases detected so far.[12] What is even more interesting, the 2-iso-propylphenyl derivative, **3**, exploits yet another hydrophobic patch, which is an intermediate location between pockets 1 and 2 mentioned above, which we tentatively assign the name hydrophobic region 3. At this point, by comparing the inhibition constants of the five inhibitors (Fig. 1) with their superposition when bound to the enzyme (Fig. 3), a very interesting fact emerges: the three inhibitors binding in the hydrophobic pocket 2 are the weaker ones (K_I*s* in the range of 50–226 nM) in the small series of derivatives investigated here. The compound located in the hydrophobic pocket 1, compound **4**, is a much more effective CA II inhibitor (K_I of 15 nM) whereas the one binding in the intermediate hydrophobic patch, between the two pockets, observed for the first time here, is an excellent CAI, with a K_I of 3.3 nM against CA II. As above, the differences of activity between the most active (derivative **3**) and the least active (compound **5**) sulfonamides investigated here are significant, a factor of 68, and this difference can be directly attributed to the rather diverse binding of the substituted ureido tails of these compounds in the various hydrophobic pockets/regions of the enzyme. Another aspect that should be stressed here, which probably explains the rather variable binding patterns of these structurally similar compounds to CA II, is related to the presence of the ureido fragment (NHCONH) which connects the benzenesulfonamide part of the molecule to the aryl/cycloalkyl tails. As seen from the crystallographic data, the torsion angles between these two fragments of the scaffold are different in the five compounds investigated here. This probably allows the flexibility of the inhibitor to select the most energetically favourable hydrophobic pocket to bind into and avoid steric clashes[15] and/or to make as many as possible favorable interactions with amino acid residues within the enzyme cavity. Most of the CAIs of sulfonamide type investigated earlier contained CONH or SO_2NH linkers instead of the ureido one present in **1–5**. These two different linkers allow less flexibility for the inhibitor scaffold, and probably this is the reason why most of those compounds bind in the cannonical hydrophobic pocket 2. It is thus rather obvious that even a very minor moiety (in this case the linker) from the scaffold of a CAI may contribute significantly to the overall potency of the compound.[16-22]

In conclusion, this study demonstrates the importance of the hydrophobic pockets/regions within the CA II active site for the binding of a series of structurally related sulfonamides possessing various ureido substitutions. These findings can be extended to other classes of CAIs, and probably also to other CA isoforms, less well investigated than CA II, with the possibility of designing inhibitors with a better selectivity for the various isoforms with medicinal chemistry applications.

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Inhibition of β-carbonic anhydrases with ureido-substituted benzenesulfonamides

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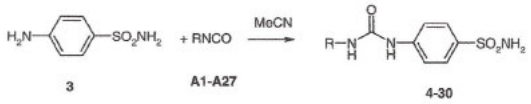

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K_I (hCA II) = 2.1–9600 nM; K_I (*C. albicans*) = 3.4–3970 nM; K_I (Rv1284) = 4.8–6500 nM; K_I (Rv3273) = 6.4–6850 nM.

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Inhibition of β-carbonic anhydrases with ureido-substituted benzenesulfonamides

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ABSTRACT

A series of sulfonamides was prepared by reaction of sulfanilamide with aryl/alkyl isocyanates. The ureido-substituted benzenesulfonamides showed a very interesting profile for the inhibition of several carbonic anhydrases (CAs, EC 4.2.1.1) such as the human hCA II and three β-CAs from pathogenic fungal or bacterial species. The *Candida albicans* enzyme was inhibited with potencies in the range of 3.4–3970 nM, whereas the *Mycobacterium tuberculosis* enzymes Rv1284 and Rv3273 were inhibited with K_Is in the range of 4.8–6500 nM and of 6.4–6850 nM, respectively. The structure–activity relationship for this class of inhibitors is rather complex, but the main features associated with effective inhibition of both α- and β-CAs investigated here have been delineated. The nature of the moiety substituting the second ureido nitrogen is the determining factor in controlling the inhibitory power, probably due to the flexibility of the ureido linker and the possibility of this moiety to orientate in different subpockets of the active site cavities of these enzymes.

The carbonic anhydrases (CAs, EC 4.2.1.1) enzymes are widely distributed throughout the phylogenetic tree, with five genetically unrelated classes (α-, β-, γ-, δ- and ζ-) known to date.[1] These proteins which catalyze the interconversion between carbon dioxide and bicarbonate, with release of a proton, are not only involved in pH homeostasis and regulation, but also in biosynthetic reactions, such as gluconeogenesis and ureagenesis among others (in animals), CO_2 fixation (in plants and algae), electrolyte secretion in a variety of tissues/organs, with many of the 16 mammalian CA isozymes being established drug targets for the design of diuretics, antiglaucoma, antiepileptic, antiobesity and/or anticancer agents.[1–3] While the α-CA family is mainly present (but not exclusive to) in mammals and it has been thoroughly investigated from the drug design viewpoint,[1–3] only recently CAs belonging to the β- and γ-CA families, which are widespread in bacteria and fungi (the β-CAs) and *Archaea* (the β- and γ-CAs), respectively, started to be considered for such a purpose. Thus, a β-CA present in the gastric pathogen *Helicobacter pylori* was recently shown to be a possible target for gastric drugs,[4,5] with several low nanomolar inhibitors detected, which effectively inhibited the in vitro and in vivo growth of the pathogen,[4,5] whereas another enzyme from *Brucella suis* was also shown to be inhibited strongly by some sulfonamide derivatives, leading to impairment of bacterial growth.[6] The fungal β-CAs from *Candida albicans*[7] and *Cryptococcus neoformans*[8,9] (as well as the related enzyme present in *Saccharomyces cerevisiae*)[10,11] were also characterized, being shown to be susceptible to inhibition with the main classes of CA inhibitors (CAIs), that is, the inorganic anions and the sulfonamides and their bioisosteres.[6–11] Two other β-CAs from *Mycobacterium tuberculosis* were successfully cloned and crystallized by Suarez Covarrubias et al.[12,13] being denominated Rv1284 and Rv3588c. Our group identified, cloned and characterized the third such enzyme, encoded by the gene Rv3273.[14] The catalytic activity and inhibition with sulfonamides/sulfamates of all these β-class enzymes from bacterial and fungal parasites have been reported, but very few low nanomolar inhibitors were detected so far for most of them.[11,14,15] Sulfonamide/sulfamate CAIs targeting various mammalian α-CAs, such as acetazolamide **1**, dichlorophenamide **2** or sulfanilamide **3**, have been in clinical use for more than 50 years,[1] but such compounds generally show much less effective inhibition of the β-class enzymes.[11] Furthermore, they generally do not possess the appropriate pharmacological properties (e.g., good penetration through bacterial walls/membranes) to effectively impair the growth of many such pathogens (or they show weak such properties, as shown by work on *H. pylori* and *B. suis* enzymes mentioned above).[5,6] Thus, in order to understand whether β-CAs may constitute drug targets for developing antiinfective agents, it is essential to design compounds with different affinities and pharmacological properties compared to the classical CAIs of types **1**–**3**.

Here we report the synthesis and inhibition studies of several β-CAs from fungal or bacterial pathogens with a large series of ureido-substituted sulfonamides obtained from sulfanilamide **3** as lead molecule. A large series of 4-ureido-substituted benzenesulfonamides **4**–**30** was prepared by reaction of sulfanilamide **3**

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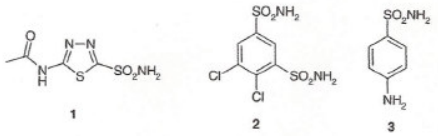

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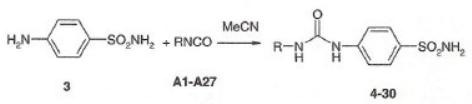
with aryl/alkyl-isocyanates **A1-A27** (Scheme 1).[16] The chemical diversity of these derivatives was generated by varying the nature of the starting isocyanate **A1-A27**, since, as shown by a recent X-ray crystallographic work on five of these derivatives for which the structure in complex with the human isoforms hCA II has been reported,[17] was determined that it is the nature of the R group that greatly influences the binding to the enzyme. For compounds 7, **13, 16, 25** and **28**[17] in adduct with hCA II, it has been observed that the benzenesulfonamide fragment binds in the usual manner, coordinating to the zinc ion as SO_2NH^- moiety. The phenylsulfamoyl part of the inhibitor was superimposable between all these adducts and bound in the usual way,[18] filling the middle of the active site cavity.[17,18]

However the different R moieties present in these compounds were observed to bind towards the external part of the enzyme active site, occupying various subpockets, and none of them was superimposable with each other. This phenomenon is probably due to the rather flexible nature of the ureido linker connecting the benzenesulfonamide with the R group in this series of compounds, and may also explain a fact reported earlier by our group,[19] that such ureido-substituted sulfonamides (different of the compounds investigated here) may show selective inhibition of isoform hCA I over the dominant one hCA II. Normally, it is hCA II having higher affinity for sulfonamides compared to hCA I, at least for most of the clinically used derivatives.[1–3]

A large variety of aromatic and aliphatic R moieties have been incorporated in the new CAIs **4–30** reported here, in order to expand the structure–activity relationship (SAR) insight regarding their interactions with various CA isoforms belonging to the β-class. The synthesis of such compounds is a one-step process, generally occurring in high yield, being possible to apply it for the preparation of a large number of derivatives.[16] It should be noted that most of the CAIs of sulfonamide type investigated earlier[1–3] contained CONH or SO_2NH linkers between the sulfonamide head moiety and the R group, instead of the ureido one present in **4–30**. These different linkers allow less flexibility for the inhibitor scaffold, and this is probably the reason why most such sulfonamides bind in the canonical hydrophobic pocket of hCA II and also generally do not show isoform selectivity.[18,20] We have investigated the inhibition of the dominant, human isoform hCA II (offtarget) as well as three β-CAs from pathogenic organisms (fungi and bacteria) with derivatives **4–30** reported here (Table 1).[21,22] The following SAR could be observed from data of Table 1:

(i) the offtarget isoform, the cytosolic hCA II, was inhibited with potencies ranging from the low nanomolar to the micromolar by ureidosulfonamides **4–30**. Compounds 16, **19, 22, 24** and **30** were very potent hCA II inhibitors, with K_is in the range of 2.1–9.7 nM. These derivatives incorporate 2- or 4-substituted phenyl moieties or the indane ring. It is interesting to note that **16** and **19** contain in their molecule the rather bulky, lipophilic *i*-Pr, *n*-BuO or biphenyl moieties, which might be considered too bulky to fit well within the hCA II active site. However as shown here and in the crystallographic, preliminary communication,[17] the *i*-Pr moiety of **16** makes favorable hydrophobic contacts in a patch within the enzyme active site never seen earlier to accommodate inhibitors, also participating to a Π stacking with Phe131. This particular binding mode explains the excellent hCA II inhibitory properties of **16** (K_i of 3.3 nM). However, four other derivatives of the series investigated here, that is, 7, 13, **25** and **28**, exploited different binding pockets within the hCA II active site,[17] and also possessed diverse inhibitory power compared to **16**, with inhibition constants in the range of 15–226 nM (Table 1). Another series of investigated sulfonamides showed medium potency hCA II inhibition, with K_is in the range of 15–96 nM. These compounds (7, **12, 13, 21, 23** and **25**) also incorporate aromatic R groups, such as 4-fluorophenyl, 3,5-di(trifluoromethyl)phenyl, pentafluorophenyl, 4-cyanophenyl, 4-phenoxyphenyl or 3-nitrophenyl. The remaining derivatives in this series showed lower hCA II inhibitory properties, with K_is in the range of 226–9600 nM. The least effective hCA II inhibitor was the derivative incorporating the long 4-*n*-octylphenyl moiety (**20**). It should be also noted that sulfanilamide **3** was a medium potency hCA II inhibitor (K_i of 240 nM) whereas acetazolamide **1** a potent one (K_i of 12 nM).



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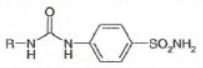
Scheme 1. Preparation of ureidosulfonamides **4–30** from sulfanilamide **3**.

Table 1
Inhibition of the β-CAs from *C. albicans*, the mycobacterial enzymes Rv1284 and Rv3273, and human hCA II with ureido-sulfonamides **4–30**, sulfanilamide **3** and acetazolamide **AAZ**, by a stopped-flow CO_2 hydrase assay[21]



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No.	R	K_i[a] (nM)			
		hCA II	*C. albicans*	Rv1284	Rv3273
3	–(Sulfanilamide)	240	1086	9230	6240
4	Ph	3730	395	356	758
5	$PhCH_2$	2200	547	440	717
6	Ph_2CH	3725	3970	4330	6380
7	4-FC_6H_4	96	42.1	6.4	53.0
8	Cl-FC_6H_4	781	62.0	4.8	63.1
9	4-BrC_6H_4	1290	45.0	7.5	87.1
10	4-IC_6H_4	2634	42.5	6.8	56.4
11	4-$CF_3C_6H_4$	1150	61.0	69.3	533
12	3,5-$(CF_3)_2C_6H_3$	75	58.5	65.7	481
13	C_6F_5	50	38.0	5.0	6.4
14	2-$MeOC_6H_4$	4070	702	473	603
15	4-AcC_6H_4	1060	29.1	470	74.6
16	2-*i*-PrC_6H_4	3.3	3450	5610	6850
17	4-*i*-PrC_6H_4	5005	355	5690	57.5
18	4-*n*-BuC_6H_4	2485	49.9	4760	63.7
19	4-*n*-$BuOC_6H_4$	2.1	50.6	35.2	63.0
20	4-*n*-octyl-C_6H_4	9600	45.9	6500	81.2
21	4-NCC_6H_4	64.7	53.5	50.2	64.4
22	2-NCC_6H_4	2.4	326	534	463
23	4-$PhOC_6H_4$	85	3.4	560	818
24	2-PhC_6H_4	9.7	297	5590	748
25	3-$O_2NC_6H_4$	15	40.1	67.1	6.5
26	4-MeO-2-MeC_6H_3	3310	53.2	49.1	70.3
27	9H-Fluoren-2-yl	908	573	728	370
28	Cyclopentyl	226	565	509	733
29	3,5-$Me_2C_6H_3$	1765	65.9	315	768
30	Indan-5-yl	8.9	71.8	486	632
–	AAZ	12	132	481	104

[a] Errors were in the range of ±10% of the reported data, from three different assays.

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(ii) the β-CA from *C. albicans* was inhibited by the ureido-sulfonamides 4–**30** with a variable potency, with inhibition constants in the range of 3.4–3970 nM, compared to sulfanilamide **3** which was a weak inhibitor (K_I of 1086 nM) and acetazoalmide **1** which was a medium potency inhibitor (K_I of 132 nM). Thus, strong inhibition has been observed for derivatives 7–**13, 15, 18–21, 23, 25, 26, 29** and **30**, which possessed inhibition constants in the range of 3.4–71.8 nM. These compounds incorporate 3- or 4-substituted phenyl moieties as R groups, such as 4-halogenophenyl, 4-trifluoromethylphenyl, 4-acetylphenyl-; 4-*n*-butyl/butoxyphenyl, 4-cyanophenyl-; 4-phenoxyphenyl-, 3-nitrophenyl-, as well as 3,5- or 2,4-disubstituted-phenyl moieties (except **30** which possesses an indane moiety and **13** which possesses the pentafluorophenyl moiety). It is obvious that a rather large number of substitution patterns lead to effective *C. albicans* β-CA inhibitors. The best such inhibitor was **23**, incorporating the elongated 4-phenoxyphenylureido moiety which, with an inhibition constant of 3.4 nM, is the best inhibitor of this enzyme ever reported in the literature.[8] Other substitution patterns present in these compounds led to less effective inhibitors. For example, derivatives 4–**6, 14, 16, 17, 27** and **28**, having aryl, aralkyl or cycloalkyl R moieties, are weak *C. albicans* CAIs, with inhibition constants in the range of 297–3970 nM (Table 1). As for hCA II; small variations in the nature of the R moicty leads to very different inhibitory properties for this series of compounds, with highly effective, medium potency and ineffective CAIs detected in this congeneric series of sulfonamides.

(iii) the mycobacterial enzyme Rv1284 was very weakly inhibited by sulfanilamide **3** and weakly by acetazolamide **1**, with inhibition constants in the range of 481–9230 nM (Table 1). However the ureido-benzenesulfonamides 4–**30** reported here generally showed a better inhibitory action against this enzyme, with K_Is in the range of 4.8–6500 nM. Thus, a number of derivatives, such as 7–**10**, and **13**, were the best Rv1284 inhibitors in this series, with K_Is in the range of 4.8–7.5 nM. All of them incorporate 4-halogenosubstituted phenyl or pentafluorophenyl R moieties. Another subseries of these sulfonamides, such as 11, **12, 19, 21, 25** and **26**, showed slightly less effective inhibitory activity, with K_Is in the range of 35.2–69.3 nM. They incorporate mono- or disubstituted phenyl R moieties containing trifluoromethyl, nitro, cyano, and alkoxy groups. Medium potency inhibition has been observed with compounds such as 4, **5, 14, 15, 22, 23, 27–30**, which incorporate aryl, aralkyl or cycloalkyl R moieties. The least effective inhibitors were 6, **16–18, 20** and **23**, most of them incorporating rather bulky R groups (diphenylmethyl, 2-*i*-Pr-phenyl, 4-*n*-Bu-phenyl, 4-*n*-octyl-phenyl, etc.), which showed micromolar affinity for this enzyme (K_Is in the range of 4.33–6.50 μM).

(iv) the mycobacterial enzyme Rv3273 was inhibited by the new compounds 4–**30** reported here with K_Is in the range of 6.4–6850 nM, whereas the lead **3** and **AAZ** were ineffective and medium potency inhibitors, respectively (K_I of 6.24 μM for **3** and of 104 nM for **1**). The most effective CAIs against this enzyme were **13** and **25**, with K_Is in the range of 6.4–6.5 nM. The incorporate the pentafluorophenyl and 3-nitrophenyl R moieties, respectively. A rather large groups of compounds showed medium potency inhibitory activity against Rv3273, with K_Is in the range of 53.0–87.1 nM, and they include the halogenophenyl-substituted derivatives 7–**10**, the 4-acetylphenyl group of **15**, and the alkyl-/alkoxy-phenyl or cyanophenyl moieties present in 17–**21**. The disubstituted compound **26** also belong to this category. Again a rather large number of R moieties lead to highly enhanced β-CA inhibitory properties compared to the lead **3**, which was a very ineffective inhibitor of this enzyme (Table 1). Several of the new derivatives were medium potency (4, **5, 11, 12, 14, 22–24** and 27–**30**) or ineffective (**6** and **16**) inhibitors of the Rv3273 enzyme. As for the other α- or β-CAs investigated here, it may be observed that all types of activities were detected against Rv3273 for the sulfonamides synthesized in this work.

(v) the selectivity ratios of the new sulfonamides investigated here, for the inhibition of the target versus offtarget CAs is a rather complex issue, due to the particular SAR discussed above for each particular enzyme. However, there are interesting aspects that may be evidenced. For example, several compounds such as 16, **22** and **24** showed low nanomolar hCA II inhibitory activity but were much less effective as β-CA inhibitors and can be considered as selective for the inhibition of the α- versus β-CAs. Sulfonamide **23** on the other hand was a *C. albicans* CA selective inhibitor, with selectivity ratios for inhibiting the fungal enzyme over the mammalian one hCA II of 25, and for inhibiting the fungal over mycobacterial enzymes of 164.7 and of 240.5, respectively. An inhibitor which was selective for the Rv1284 enzyme has also been discovered, **8**, which had selectivity ratios for inhibiting Rv1284 over hCA II of 162.7, for inhibiting Rv1284 over the fungal enzyme of 12.9 and for inhibiting Rv1284 over Rv3273 of 13.1. For Rv3273 the most selective inhibitor was **25**, with selectivity ratios of 2.3 over hCA II, of 6.1 over the *C. albicans* enzyme, and of 10.3 over Rv1284. These selectivity ratios are less high than for the other two investigate β-CAs, but these are the first ever reported CAIs showing this interesting profile and may be useful for understanding in greater detail the physiological role of some of these enzymes.

In conclusion, we report here a series of sulfonamides prepared by reaction of sulfanilamide with aryl/alkyl isocyanates. The ureido-substituted benzenesulfonamides showed a very interesting profile for the inhibition of hCAII and three β-CAs from pathogenic fungal or bacterial species. The *C. albicans* enzyme was inhibited with potencies in the range of 3.4–3970 nM, whereas the mycobacterial ones Rv1284 and Rv3273 with K_Is in the range of 4.8–6500 nM and of 6.4–6850 nM, respectively. The structure–activity relationship for this class of inhibitors is rather complex but the main features associated with effective inhibition of both α- and each β-CAs investigated here have been delineated. The nature of the R moiety substituting the second ureido nitrogen is the determining factor in controlling the inhibitory power, probably due to the flexibility of the ureido linker and the possibility of the R group to orientate in different subpockets of the active site cavity of these enzymes.

Acknowledgements

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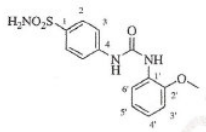
16. General procedure for the preparation of compounds **4–30**. Sulfanilamide **3** (2.90 mmols) was dissolved in acetonitrile (20–30 mL) and then treated with a stoichiometric amount of commercially available isocyanate **A1–A27**. The mixture was stirred at rt or heated at 50 °C for 2 h, until completion (TLC monitoring). The heavy precipitate formed was filtered-off, washed with diethyl ether (100 ml), dried under vacuo, and recrystallized. For example **8** was obtained by reaction of sulfanilamide **3** (0.50 g; 2.90 mmols) with 2-methoxyphenyl isocyanate (0.43 g; 2.90 mmols). The reaction was stirred at rt overnight, treated as described above, to give **8** as a white solid in 40.4% yield.



8

4-{[(2′-Methoxyphenyl)amino]carbonyl}aminobenzenesulfonamide (**8**): mp 234–236 °C; silica gel TLC R_f 0.47 (ethyl acetate/petroleum ether 33%); ν_{max} (KBr) cm^{-1}, 3362 (N–H urea), 2838 (C–H aliphatic), 1684 (C=O urea), 1592 (aromatic); δ_H (400 MHz, DMSO-d_6) 3.92 (3H, s, CH_3), 6.94 (1H, ddd, *J* 8.2 7.4 1.4, 4′-H), 7.01 (1H, ddd, *J* 8.0 7.4 1.6, 5′-H), 7.07 (1H, dd, *J* 8.2 1.2, 3′-H), 7.23 (2H, s, SO_2NH_2), 7.64 (2H, d, *J* 8.8, 2 × 2-H), 7.77 (2H, d, *J* 8.8, 2 × 3-H), 8.16 (1H, dd, *J* 8.0 1.6, 6′-H), 8.38 (1H, s, *NH*), 9.73 (1H, s, *NH*); δ_C (100 MHz, DMSO-d_6) 153.0 (C=O, urea), 148.7, 143.8, 137.7, 129.2, 127.8, 123.2, 121.5, 119.4, 118.1, 111.7, 56.7 (CH_3).
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21. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10–20 mM Hepes (pH 7.5, for α-CAs) or TRIS (pH 8.3 for β-CAs) as buffers, and 20 mM Na_2SO_4 (for α-CAs) or 10–20 mM NaCl—for β-CAs (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the *E–I* complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[14,15] and represent the mean from at least three different determinations.
22. The α- and β-CAs used in this work were recombinant enzymes obtained as reported earlier.[7,8,14,15]

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Journal of Medicinal Chemistry

ARTICLE

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Ureido-Substituted Benzenesulfonamides Potently Inhibit Carbonic Anhydrase IX and Show Antimetastatic Activity in a Model of Breast Cancer Metastasis

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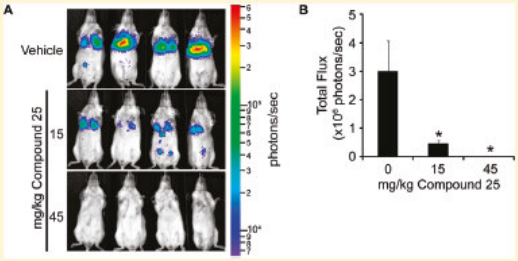
Supporting Information

ABSTRACT: A series of ureido-substituted benzenesulfonamides was prepared that showed a very interesting profile for the inhibition of several human carbonic anhydrases (hCAs, EC 4.2.1.1), such as hCAs I and II (cytosolic isoforms) and hCAs IX and XII (transmembrane, tumor-associated enzymes). Excellent inhibition of all these isoforms has been observed with various members of the series, depending on the substitution pattern of the urea moiety. Several low nanomolar CA IX/XII inhibitors also showing good selectivity for the transmembrane over the cytosolic isoforms have been discovered. One of them, 4-{[(3′-nitrophenyl)carbamoyl]amino}benzenesulfonamide, significantly inhibited the formation of metastases by the highly aggressive 4T1 mammary tumor cells at pharmacologic concentrations of 45 mg/kg, constituting an interesting candidate for the development of conceptually novel antimetastatic drugs.



INTRODUCTION

Carbonic anhydrase (CA, EC 4.2.1.1) IX (CA IX) has recently been shown to be a druggable target for imaging and treatment of hypoxic tumors overexpressing this protein.[1−6] CA IX is the most strongly overexpressed gene in response to hypoxia in human cancer cells.[2−4] This enzyme is a multidomain protein[2] with the CA subdomain situated outside the cell and possessing a very high CO_2 hydrase catalytic activity,[7] making it a key player in the regulation of tumor pH.[1−6] CA IX expression is strongly increased in many types of solid tumors, such as gliomas/ependymomas, mesotheliomas, and papillary/follicular carcinomas; carcinomas of the bladder, uterine cervix, kidneys, esophagus, lungs, head and neck, breast, brain, and vulva; and squamous/basal cell carcinomas.[8,9] Furthermore, such hypoxic tumors do not generally respond to the classic chemo- and radiotherapy.[8,9]

Pouyssegur's group showed recently[5] that in hypoxic LS174Tr tumor cells expressing either CA IX or both CA IX and CA XII isoforms, in response to a CO_2 load, both enzymes contribute to extracellular acidification and to maintaining a more alkaline resting intracellular pH (pH_i), an action that preserves ATP levels and cell survival in a range of acidic outside pH (6.0−6.8) and low bicarbonate medium. In vivo experiments showed that silencing of CA IX alone leads to a 40% reduction in xenograft tumor volume, with up-regulation of the second gene, that encoding for CA XII. Silencing of both CA IX and CA XII gave an impressive 85% reduction of tumor growth.[5] Hypoxia-induced CA IX and CA XII are major tumor prosurvival pH-regulating enzymes, and their combined inhibition held potential for the design of anticancer drugs with a novel mechanism of action. The in vivo proof-of-concept study that sulfonamide CA IX inhibitors may indeed show antitumor effects has been only very recently published by Neri's group.[6a] Membrane-impermeable derivatives based on the acetazolamide AAZ scaffold to which either fluorescein carboxylic acid or albumin-binding moieties were attached have been employed. A strong tumor growth retardation in animals treated for 1 month with these CA inhibitors (CAIs) in mice with xenografts of a renal clear cell carcinoma line, SK-RC-52, was observed.[6a] Such data show the great promise of tumor growth inhibition with sulfonamides acting as CA IX/XII inhibitors.[1] The same group reported the

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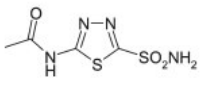
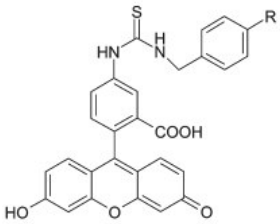
proof-of-concept study showing that human monoclonal antibodies targeting CA IX can also be used for imaging of hypoxic tumors.[6b] The generation of high-affinity human monoclonal antibodies (A3 and CC7) specific to hCA IX, using phage technology, has been recently reported.[6b] These antibodies were able to stain CA IX ex vivo and to target the cognate antigen in vivo. In one studied animal model of colorectal cancer (LS174T), CA IX imaging closely matched pimonidazole staining, with a preferential staining of tumor areas characterized by little vascularity and low perfusion.[6b] The same conclusion has been reached by our group by using small molecule CA IX selective inhibitors of the type **1**.[3] Fluorescent sulfonamides **1** with a high affinity for CA IX have been developed and shown to bind to cells only when CA IX protein was expressed and while cells were hypoxic. NMRI-nu mice subcutaneously transplanted with HT-29 colorectal tumors were treated with 7% oxygen or with nicotinamide and carbogen and were compared with control animals. Accumulation of compound **1** was monitored by noninvasive fluorescent imaging. Specific accumulation of **1** could be observed in delineated tumor areas compared with a structurally similar non-sulfonamide analogue incorporating the same scaffold **2**. Administration of nicotinamide and carbogen, decreasing acute and chronic hypoxia, respectively, prevented accumulation of **1** in the tumor. When treated with 7% oxygen breathing, a 3-fold higher accumulation of **1** was observed. Furthermore, the bound inhibitor fraction was rapidly reduced upon tumor reoxygenation.[3] Such in vivo imaging results confirm previous in vitro data demonstrating that CAI binding and retention require exposure to hypoxia. Labeled sulfonamide CAIs may provide a powerful tool to visualize hypoxia response in solid tumors. An important step was thus made toward clinical applicability, indicating the potential of patient selection for CA IX directed therapies.[3]



AAZ



1: R = SO_2NH_2
2: R = H

One main draw-back of classical sulfonamide CAIs (such as AAZ) is the lack of selectivity for inhibiting CA IX over the other CA isoforms present in humans (15 isoforms, CAs I−XIV, are present in primates and 16 isoforms, i.e., CAs I−XV, in other vertebrates).[1] Thus, it is crucial to explore chemotypes possessing a more selective inhibition profile against the target isoforms, e.g., with selectivity for the inhibition of the tumor-associated over other CA isoforms. A small series of five 4-RNHCONH-substituted benzenesulfonamide derivatives was recently investigated as inhibitors of the cytosolic isoform hCA II (h = human) by one of these groups.[10] It has been observed that their potency varied between 3.3 and 226 nM, and by means of X-ray crystallography a highly variable orientation of the R-ureido moieties was evidenced when the inhibitor was bound within the enzyme active site (Figure 1).[10] The R moieties were not disordered in

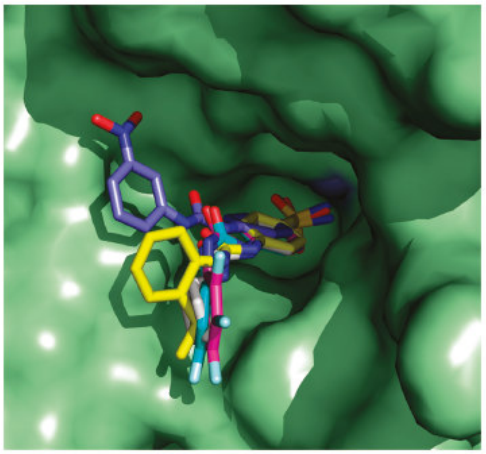


Figure 1. Binding of ureido-substituted benzenesulfonamides to the hCA II active site. The benzenesulfonamide moiety of five inhibitors is totally superposable, whereas the RNHCONH tails adopt different orientations in various subpockets of the active site (R = 3-nitrophenyl, violet; R = 2-isopropylphenyl, yellow; R = pentafluorophenyl, magenta; R = 4-fluorophenyl, sky blue; R = cyclopentyl, gray).[10] The PDB entries of these structures are 3N4B, 3N0N, 3N3J, 3N2P, and 3MZL, respectively.[10]

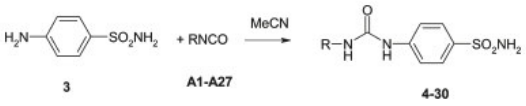
the X-ray crystal structures, but they adopted diverse orientations based on their specific chemical nature (Figure 1). Furthermore, a strong correlation has been observed between the binding pattern and R group orientation and the CA II inhibitory properties for this small library of compounds, where R was 4-fluorophenyl, pentafluorophenyl, 2-isopropylphenyl, 3-nitrophenyl, and cyclopentyl.[10]

In this paper we report the synthesis of a larger series of such ureido-substituted sulfonamide CAIs that possess strong affinity for CA IX, an acceptable selectivity profile for inhibiting the tumor-associated isoforms CAs IX and XII over the cytosolic ones CAs I and II, and excellent in vivo antimetastatic effects in a breast cancer xenograft model.

RESULTS AND DISCUSSION

Chemistry. In a recent work[10] we showed that benzenesulfonamides incorporating 4-substituted ureido moieties act as hCA II inhibitors with potencies that correlate well with the orientation of the R moiety present in the ureido tail of the compound (Figure 1). As other classes of ureido-substituted benzenesulfonamides have been reported earlier,[11,12] and we observed that they may lead to isoform-selective compounds,[12] we decided to investigate in greater detail such derivatives. We report here the synthesis of a large series of 4-ureidosubstituted-benzenesulfonamides **4**−**30** prepared by reaction of sulfanilamide **3** with aryl/alkyl isocyanates **A1**−**A27** (Scheme 1). The chemical diversity was generated by varying the nature of the starting isocyanate, **A1**−**A27** (Table 1). The CA isoforms have differing amino acid sequences in the external portion of their active sites; therefore, compounds with a better selectivity profile for the various isoforms will contain an R group moiety that can interact with this region.[13] This has been reported earlier by our group,[12] with

Scheme 1. Preparation of the 4-Ureidosubstituted Benzenesulfonamides 4−30 from Sulfanilamide 3 and Isocyanates A1−A27



3 A1-A27 4-30

the observation that some ureido-substituted sulfonamides show selective inhibition of isoform hCA I over the dominant one hCA II.

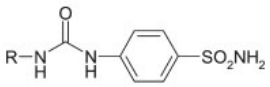
As shown in Scheme 1 and Table 1, a large variety of aromatic and aliphatic R moieties have been incorporated in the new CAIs **4**−**30** reported here, in order to have an extensive structure−activity relationship (SAR) insight regarding their interactions with various CA isoforms with medicinal chemistry relevance. The synthesis of such compounds is a one-step process, generally occurring in high yield, allowing for the preparation of a large number of derivatives. Most of the CAIs of sulfonamide type investigated earlier contained CONH or SO_2NH linkers between the sulfonamide head moiety and the tail instead of the ureido one present in **4**−**30**. These different linkers allow less flexibility for the inhibitor scaffold, and this is probably the reason why such sulfonamides bind in the cannonical hydrophobic pocket of hCA II.[13,14] They also generally do not show isoform selectivity.[10] In contrast, for derivatives incorporating the ureido linker (of type **4**−**30**), it has been shown[10] that the tails (all of them well ordered in the crystal structures; see Figure 1) are found in various hydrophobic pockets of the CA II active site. As seen from the crystallographic data (Figure 1),[10] the torsion angles between these two fragments of the scaffold were quite different for the five compounds investigated by X-ray crystallography (i.e., **7**, **13**, **16**, **25**, and **28**).[10] This probably allows the flexibility of the inhibitor to select the hydrophobic subpocket in such a way to avoid steric clashes[10,13,14] and to make as many as possible favorable interactions with amino acid residues within the enzyme cavity.

CA Inhibition. Inhibition of four physiologically relevant α-CA isoforms with compounds **4**−**30** and acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide, AZA, a clinically used drug) is presented in Table 1.[15] hCAs I and II (cytosolic, widespread enzymes) and hCAs IX and XII (transmembrane, tumor-associated CAs) have been included in this study because of their relevance as targets/offtargets when developing CAIs. Indeed, CA II for example is the drug target for developing antiglaucoma CAIs,[1] but it is an off-target when considering CA IX/XII inhibition.[1−3] In this latter case, only the transmembrane, tumor-associated isozymes (IX and XII) should be inhibited, as CA II may have the function of housekeeping enzyme and its inhibition may lead to side effects.[1−3]

The following structure−activity relationship (SAR) can be observed from data of Table 1 for the series of ureidosulfonamides **4**−**30** investigated here.

(i) The slow cytosolic isoform hCA I was inhibited by compounds **4**−**30** with a wide range of potency, with inhibition constants in the range 9.0−5530 nM. Potent hCA I inhibition has been observed with the following compounds: **11**, **16**, **19**, **22**, and **30**. These compounds incorporate 2- or 4-substuted phenyl moieties (such as 4-trifluoromethylphenyl, 2-isopropylphenyl, 4-*n*-butoxyphenyl, and 2-cyanophenyl) except **30** which

Table 1. Inhibition of hCAs I and II (Cytosolic Isoforms) and hCAs IX and XII (Transmembrane, Tumor-Associated Enzymes) with Ureidosulfonamides 4−30 and Acetazolamide AAZ as Standard[15]



4-30

		K_I (nM)			
compd	R	hCA I	hCA II	hCA IX	hCA XII
4	Ph	760	3730	575	67.3
5	$PhCH_2$	92	2200	41.4	49.5
6	Ph_2CH	83	3725	58.8	64.5
7	4-FC_6H_4	5080	96	45.1	4.5
8	Cl-FC_6H_4	2150	781	58.0	5.3
9	4-BrC_6H_4	1465	1290	69.3	7.9
10	4-IC_6H_4	5500	2634	24.5	4.3
11	4-$CF_3C_6H_4$	9.7	1150	6.2	2.3
12	3,5-$(CF_3)_2C_6H_3$	3690	75	53	39
13	C_6F_5	2395	50	5.4	5.1
14	2-$MeOC_6H_4$	92	4070	465	61.2
15	4-AcC_6H_4	388	1060	5.4	4.6
16	2-*i*-PrC_6H_4	9.0	3.3	0.5	4.2
17	4-*i*-PrC_6H_4	4330	5005	541	49.7
18	4-*n*-BuC_6H_4	5530	2485	376	28.5
19	4-*n*-$BuOC_6H_4$	11.3	2.1	0.8	2.5
20	4-*n*-octyl-C_6H_4	536	9600	47.1	52.8
21	4-NCC_6H_4	57.0	64.7	6.0	6.5
22	2-NCC_6H_4	10.9	2.4	0.3	4.6
23	4-$PhOC_6H_4$	604	85	69.1	7.1
24	2-PhC_6H_4	1170	9.7	65.7	65.1
25	3-$O_2NC_6H_4$	23.4	15	0.9	5.7
26	4-MeO-2-MeC_6H_3	89.2	3310	73.3	6.0
27	9*H*-fluoren-2-yl	1700	908	102	55.4
28	cyclopentyl	470	226	7.3	7.0
29	3,5-$Me_2C_6H_3$	6530	1765	6.9	6.2
30	indan-5-yl	9.8	8.9	7.0	2.5
AAZ		250	12	25	5.7

possesses an indane moiety. It is thus clear, as in the crystallographic work reported earlier[10] for 5 of the 27 derivatives reported here, that the nature of the R moiety present in these ureidosulfonamiodes is the main player influencing potency against all investigated CA isoforms. Indeed, a number of these derivatives, including **5**, **6**, **14**, **4**, **25**, and **26**, behaved as medium potency hCA I inhibitors with K_I in the range 23.4−92 nM. Again the nature of the R moieties present in these compounds was rather variable (benzyl, diphenylmethyl, and 2-, 3-, or 4-substituted phenyls). The remaining derivatives showed a decreased affinity for hCA I, with K_I in the range 388−5530 nM. AAZ is similar to these compounds, with an inhibition constant of 250 nM against hCA I (Table 1).

(ii) The second offtarget isoform, the cytosolic hCA II, has also been inhibited with potencies ranging from the low nanomolar to the micromolar by ureidosulfonamides **4**−**30** (Table 1). Thus, compounds **16**, **19**, **22**, **24**, and **30** were very potent hCA II inhibitors, with K_I in the range 2.1−9.7 nM. These derivatives incorporate 2- or 4-substituted phenyl moieties or the indane ring. It is interesting to note that **16** and **19** contain in their molecule the rather bulky, lipophilic i-Pr, n-BuO, or biphenyl moieties, which one could consider to be too bulky to fit well within the hCA II active site. However, as shown here and in the crystallographic, preliminary communication,[10] the *i*-Pr moiety of **16** makes hydrophobic contacts in a patch within the enzyme active site never seen earlier to accommodate inhibitors, also making a stacking with Phe131. This particular binding mode thus explains the excellent hCA II inhibitory of **16**. However, four other derivatives of the series investigated here, i.e., **7**, **13**, **25**, and **28**, exploited different binding pockets within the enzyme active site[10] and also possessed diverse inhibitory power. Another series of compounds showed medium potency hCA II inhibition, with K_I in the range 15−96 nM. These compounds (**7**, **12**, **13**, **21**, **23**, and **25**) also possess aromatic groups at the second nitrogen ureido group, such as 4-fluorophenyl, 3,5-di(trifluoromethyl)phenyl, pentafluorophenyl, 4-cyanophenyl, 4-phenoxyphenyl, or 3-nitrophenyl. The remaining derivatives showed lower hCA II inhibitory properties, with K_I in the range 226−9600 nM. The least effective hCA II inhibitor was the derivative incorporating the long 4-*n*-octylphenyl moiety (**20**).

(iii) The tumor-associated hCA IX was generally better inhibited by these compounds compared to hCAs I and II discussed above, with K_I in the range 0.3−575 nM. Compounds such as **11**, **13**, **15**, **16**, **19**, **21**, **22**, **25**, **28**, and **29** showed excellent hCA IX inhibiting properties, with K_I in the range 0.3−7.3 nM. A few weak hCA IX inhibitors were detected (e.g., **4**, **14**, **18**, and **27**), with K_I in the range 102−575 nM, whereas all other derivatives presented inhibition constants of <100 nM. Substitution patterns favorable to potent hCA IX inhibition include the presence of 4-trifluoromethylphenyl, pentafluorophenyl, 4-acetylphenyl, 2-isopropylphenyl, 4-*n*-butoxyphenyl, 2- and 4-cyanophenyl, 3-nitrophenyl, cyclopentyl, and 3,5-dimethylphenyl moieties. However, most of the other substitution patterns present in the remaining compounds lead to effective hCA IX inhibitors, with K_I in the range 24.5−73.3 nM. AAZ was an effective hCA IX inhibitor too, with a K_I of 25 nM (Table 1).

(iv) hCA XII was also effectively inhibited by sulfonamides **4**−**30**, with K_I in the range 2.3−67.3 nM. This was the isoform with the highest affinity for these compounds, as most of them were excellent inhibitors. Inhibition constants of <10 nM have been observed with a rather large number of compounds, among which are **7**−**11**, **13**, **15**, **16**, **19**, **21**, **25**, **26**, **28**−**30**, and AAZ. Thus, many substitution patterns lead to very effective hCA XII inhibitors in this congeneric series investigated here. The excellent inhibition of all CA isoforms investigated here and also the variability in inhibitory power for the various members of this congeneric series are phenomena never observed earlier for any class of CAIs investigated in detail and are probably due to the presence of the ureido linker in these molecules, which leads to the binding of the compound in multiple ways to the enzymes active sites.

(v) The selectivity ratio for inhibiting the target over the offtarget isoforms for this congeneric series of sulfonamides is rather complex because of the factors mentioned above. However, some interesting findings emerged. For example, compound **15** has a selectivity ratio of 71.8 for inhibiting CA IX over CA I and of 196.3 for inhibiting CA IX over CA II (the corresponding ratios for inhibiting CA XII over CAs I and II are even better), thus being a selective inhibitor of the tumor-associated isoforms over the cytosolic ones. Compound **25** was a stronger inhibitor of the cytosolic isoforms compared to **15** and had good selectivity ratio for inhibiting the tumor-associated isoforms over the cytosolic isoforms.

Antimetastatic Effects of CA IX Inhibitors. CA IX is highly expressed in breast malignancies,[16] and studies have demonstrated that CA IX and CA XII are variably expressed in breast cancer cell lines.[8] Furthermore, CA IX is associated with a poor prognosis for patients with breast cancer[17] and it is significantly associated with distant metastasis (Lou et al., unpublished results). Therefore, we have chosen breast cancer as a malignancy model in which to test, in vivo, the antimetastatic activity of some of the CA IX inhibitors described here. Specifically, we have used the 4T1 syngeneic mouse mammary tumor model.[18] 4T1 cells are highly tumorigenic and metastasize spontaneously to multiple sites in a manner similar to that for human breast cancer following orthotopic implantation into an immunocompetent host.[18,19] We have shown recently that 4T1 tumors overexpress CA IX[20] and that these cells induce CA IX during hypoxia in vitro (Lou et al., unpublished results). These attributes make the 4T1 mouse mammary tumor cells a good model in which to examine the effects of inhibiting CA IX activity on breast cancer metastasis.

Although the 4T1 tumor model can be used to evaluate spontaneous metastatic events subsequent to tumor implantation and growth, such studies are lengthy and require surgical removal of the primary tumor. Because we were interested in evaluating the effect of the compounds on metastasis specifically, we elected to test the inhibitors using an experimental metastasis approach. Two compounds were chosen for the in vivo studies, **15** and **25**, both of which show good CA IX and XII inhibitory activities and acceptable selectivity ratios for inhibiting the tumor-associated over the cytosolic isoforms, as discussed above. We injected 4T1 cells expressing luciferase intravenously and assessed the ability of the cells to form lung metastases after treating with compounds **15** (Figure 2) and **25** (Figure 3) using bioluminescent imaging techniques. Mice were treated with the inhibitors or equal amounts of vehicle beginning 24 h after injection of cells, and metastases were imaged the day following the final dose of inhibitor. We found that whereas treatment with **15** was not able to decrease the metastatic burden relative to the vehicle control group (Figure 2), treatment with **25** was effective in limiting colonization of the cells in the lungs in a dose-dependent fashion (Figure 3). Quantification of the bioluminescent signal revealed a statistically significant decrease in the formation of metastases in the mice administered **25** (Figure 3), while no significant differences were observed among the vehicle and **15**-treated groups (Figure 2). Importantly, administration of the inhibitors did not result in significant weight loss in any animals, indicating that the inhibitors were not generally toxic (Supporting Information Figure 1). These data show that some of the ureidosulfonamide inhibitors of CA IX reported here are effective in attenuating formation of metastases in a model of aggressive breast cancer. It is interesting to note that even if **15** and **25** showed comparable CA inhibitory activity in vitro, **15** was ineffective in vivo probably because of pharmacokinetic reasons. The pharmacokinetic evaluation for some of these derivatives is in a preliminary stage, but we suspect

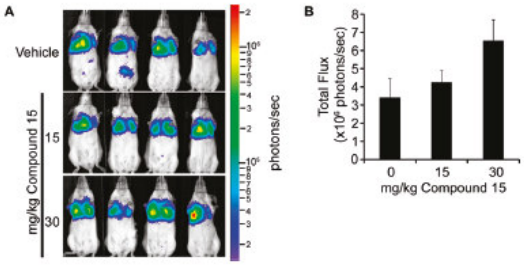


Figure 2. Ureidosulfonamide **15** did not reduce the formation of metastases by 4T1 mammary tumor cells. (A) 4T1 cells were injected directly into the tail vein of BALB/c mice. Mice then received vehicle or **15** starting 24 h after injection of cells. Animals received therapy every day, and five doses were provided. Bioluminescent images were acquired 24 h after the final dose of inhibitor. (B) Quantification of bioluminescence: $n = 6$ per group. No significant differences were observed among the vehicle inhibitor-treated groups.

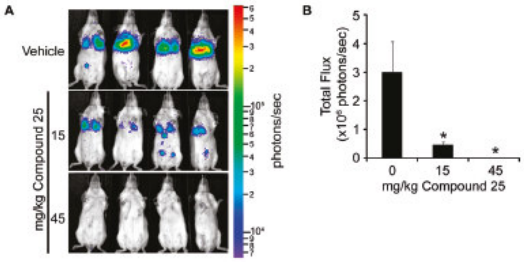


Figure 3. Ureidosulfonamide **25** inhibits the formation of metastases by 4T1 mammary tumor cells. (A) 4T1 cells were injected directly into the tail vein of BALB/c mice. Mice then received vehicle or **25** starting 24 h after injection of cells. Animals received therapy every other day, and three doses were provided. Bioluminescent images were acquired 24 h after the final dose of inhibitor. (B) Quantification of bioluminescence: $n = 4$ per group; *, $p < 0.05$.

that the acetyl moiety of **15** may undergo metabolic oxidation to the corresponding carboxylate followed by its conjugation and rapid elimination, which may explain its lack of antimetastatic effects compared to the highly efective **25** which is metabolically stable.

CONCLUSIONS

We report here a series of sulfonamides prepared by reaction of sulfanilamide with aryl/alkyl isocyanates. The ureido-substituted benzenesulfonamides showed a very interesting profile for the inhibition of hCAs I and II (cytosolic, off-target isoforms) and hCAs IX and XII (transmembrane, tumor-associated enzymes). The structure−activity relationship for this class of inhibitors was rather complex, but the main features associated with effective inhibition of all these α-CAs investigated here have been delineated in detail. The nature of the R moiety substituting the second ureido nitrogen is the main player in controlling the inhibitory power, probably because of the flexibility of the ureido linker and the possibility of the R group to orientate in different subpockets of the active site cavity of these enzymes. The compounds possessing as R moiety 4-trifluoromethylphenyl, 2-isopropylphenyl, 2-cyanophenyl, or indan-5-yl were effective hCA I inhibitors with inhibition constants in the range 9.0−10.9 nM. The best hCA II inhibitor was the 2-isopropylphenyl-substituted compound (K_I of 3.3 nM), whereas many low nanomolar and subnanomolar hCA IX/XII inhibitors were obtained (e.g., R = 4-trifluoromethylphenyl, pentafluorophenyl, 4-acetylphenyl, 4-*n*-butoxyphenyl, 2-cyanophenyl, 3-nitrophenyl, cyclopentyl, indan-5-yl, etc.). One of them, 4-{[(3′-nitrophenyl)carbamoyl]amino}benzenesulfonamide, strongly inhibited the formation of metastases by the highly aggressive 4T1 mammary tumor cells at pharmacologic concentrations of 15−45 mg/kg, constituting thus an interesting candidate for the development of conceptually novel antimetastatic drugs.

EXPERIMENTAL PROTOCOLS

Chemistry. ^{1}H, ^{13}C, and ^{19}F spectra were recorded using a Bruker Advance III 400 MHz spectrometer. The chemical shifts are reported in parts per million (ppm), and the coupling constants (J) are expressed in hertz (Hz). Infrared spectra were recorded on a Perkin-Elmer Spectrum R XI spectrometer as solids on KBr plates. Melting points (mp) were measured in open capillary tubes, unless otherwise stated, using a Büchi B-540 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} aluminum backed plates. Elution of the plates was carried out using ethyl acetate/petroleum ether as the eluting system. Visualization was achieved with UV light at 254 nm by dipping into a ninhydrin TLC stain solution and heating with a hot air gun. Flash column chromatography was carried out using silica gel (obtained from Aldrich Chemical Co., Milan, Italy). The crude product was introduced into the column as a solution in the same elution solvent system. Solvents and chemicals were used as supplied from Aldrich Chemical Co., Milan, Italy. Purity of the obtained compounds was assessed by HPLC and elemental analysis (C, H, N) and was >95% for all of the new derivatives reported here.

General Procedure for the Preparation of Compounds 4−30. Sulfanilamide **3** (2.90 mmol) was dissolved in acetonitrile (20−30 mL) and then treated with a stoichiometric amount of isocyanates **A1**−**A27**. The mixture was stirred at room temperature or heated at 50 °C for 2 h, until completion (TLC monitoring). The heavy precipitate formed was filtered off, washed with diethyl ether (100 mL), and dried in vacuo. All compounds described here were characterized in detail by spectrosopic and analytic methods (see Supporting Information).

4-{[(4′-Acetylphenyl)amino]carbonyl)amino}benzenesulfonamide (15). White solid; mp 258−260 °C; silica gel TLC R_f = 0.27 (ethyl acetate/petroleum ether 33%); ν_{max} (KBr) cm^{-1}, 3300 (N−H urea), 1659 (C═O urea), 1590 (aromatic); δ_H (400 MHz, DMSO-d_6) 2.56 (3H, s, CH_3), 7.26 (2H, s, SO_2NH_2), 7.64 (2H, d, J = 8.8, 2 × 2′-H), 7.67 (2H, d, J = 8.8, 2 × 3-H), 7.79 (2H, d, J = 8.8, 2 × 2-H), 7.96 (2H, d, J = 8.8, 2 × 3′-H), 9.22 (1H, s, N*H*), 9.25 (1H, s, N*H*); δ_C (100 MHz, DMSO-d_6) 197.3 (C═O), 152.9 (C═O urea), 144.9, 143.4, 138.2, 131.7, 130.6, 127.8, 118.7, 118.4, 27.3 (CH_3); m/z (ESI+): 344.36 $[M + H]^+$, 356.34 $[M + Na]^+$. C atom numbering is as for **4**.

4-{[(3′-Nitrophenyl)carbamoyl]amino}benzenesulfonamide (25). Yellow solid; 44.3% yield; mp 246−248 °C; silica gel TLC R_f = 0.39 (ethyl acetate/petroleum ether 33%); ν_{max} (KBr) cm^{-1}, 3370 (N−H urea), 1709 (C═O urea), 1592 (aromatic); δ_H (400 MHz, DMSO-d_6) 7.23 (2H, s, SO_2NH_2), 7.59 (1H, dd, J 8.4 8.0, 5′-H), 7.65 (2H, d, J = 9.0, 2 × 3-H), 7.73 (1H, ddd, J = 8.4 2.0, 0.8, 6′-H), 7.76 (2H, d, J = 9.0, 2 × 2-H), 7.86 (1H, ddd, J = 8.0 2.4 0.8, 4′-H), 8.58 (1H, appt, J = 2.2, 2′-H), 9.25 (1H, s, N*H*), 9.35 (1H, s, N*H*); δ_C (100 MHz,

DMSO-d_6) 153.2 (C=O urea), 149.1, 143.3, 141.6, 138.3, 131.1, 127.7, 125.5, 118.8, 117.6, 113.3; *m/z* (ESI+): 359.23 $[M + Na]^+$.

CA Inhibition. An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity.[15] Phenol red (at 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.4) and 20 mM $NaBF_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for 10−100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5−10% of the reaction were used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled−deionized water, and dilutions up to 0.01 nM were done thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay in order to allow for the formation of the E−I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes were from Lineweaver−Burk plots, as reported earlier,[3] and represent the mean from at least three different determinations.

Pharmacological Inhibitors. For in vivo studies, compound **25** was solubilized in 37.5% PEG400/12.5% ethanol/50% saline prior to injection, while compound **15** was solubilized in 37.5% PEG400/12.5% ethanol/10% Cremophor/40% saline. Drug aliquots were made fresh daily from powder. Drugs were administered by ip injection using a volume of 200 μL for a 20 g mouse. Specific dosing schedules are described in the appropriate figures.

Experimental Metastasis Assay. All animal studies and procedures were carried out in accordance with protocols approved by the Institution Animal Care Committee at the BC Cancer research Centre and the University of British Columbia (Vancouver, Canada). 4T1 cells (5×10^5/mouse) were injected directly into the tail vein of 7−9 week-old female BALB/c mice. Metastatic burden was monitored and quantified using bioluminescent imaging as previously described.[21] Briefly, mice were imaged 24 h following the final dose of a given compound. Images showing tumor burden are displayed using a log scale color range, and data were quantified using images acquired for 1 min at a binning level of 8. Data were analyzed using the contour ROI feature in the Living Image software (Xenogen). Results were subjected to statistical analysis using the Data Analysis ToolPack in Excel software. Two-tailed *p* values were calculated using Student's *t* test. Data were considered significant for $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information. Complete characterization of compounds **4**−**30** and graphs showing the variation of the mice weight after treatment with **15** and **25**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CA, carbonic anhydrase; hCA, human carbonic anhydrase; CAI, carbonic anhydrase inhibitor; SAR, structure−activity relationship

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Cancer Research

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Targeting Tumor Hypoxia: Suppression of Breast Tumor Growth and Metastasis by Novel Carbonic Anhydrase IX Inhibitors

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Running Title: Targeted inhibition of CAIX suppresses breast cancer progression

SD and CTS are founding members of Metasignal Therapeutics Inc.

Abstract

Carbonic Anhydrase IX (CAIX) is a hypoxia and HIF-1 inducible protein that regulates intra- and extracellular pH under hypoxic conditions and promotes tumor cell survival and invasion in hypoxic microenvironments. Interrogation of 3,630 human breast cancers provided definitive evidence of CAIX as an independent poor prognostic biomarker for distant metastases and survival. shRNA-mediated depletion of CAIX expression in 4T1 mouse metastatic breast cancer cells capable of inducing CAIX in hypoxia resulted in regression of orthotopic mammary tumors and inhibition of spontaneous lung metastasis formation. Stable depletion of CAIX in MDA-MB-231 human breast cancer xenografts also resulted in attenuation of primary tumor growth. CAIX depletion in the 4T1 cells led to caspase-independent cell death and reversal of extracellular acidosis under hypoxic conditions in vitro. Treatment of mice harboring CAIX-positive 4T1 mammary tumors with novel CAIX-specific small molecule inhibitors that mimicked the effects of CAIX depletion in vitro resulted in significant inhibition of tumor growth and metastasis formation in both spontaneous and experimental models of metastasis, without inhibitory effects on CAIX-negative tumors. Similar inhibitory effects on primary tumor growth were observed in mice harboring orthotopic tumors comprised of lung metatstatic MDA-MB-231 LM2-4^{Luc+} cells. Our findings demonstrate that CAIX is vital for growth and metastasis of hypoxic breast tumors and is a specific, targetable biomarker for breast cancer metastasis.

Introduction

Cancer metastasis is a complex process that results in establishment of secondary tumors in distant organs (1). There is increasing recognition that hypoxia plays an important role in cancer progression and metastasis (2, 3), including breast cancer metastasis (4, 5). Furthermore, there is now growing evidence that altered tumor metabolism and hypoxia inducible factor 1α (HIF-1α)-regulated enzymes such as carbonic anhydrase IX (CAIX) and CAXII may be vital to the process of tumor progression to metastasis (2, 6).

CAIX is a dimeric membrane-bound enzyme that efficiently catalyzes the reversible hydration of CO_2 (7, 8). CAIX is selectively expressed in hypoxic tumors, including breast malignancies (9, 10), and its presence is a poor prognostic marker for patients with breast cancer (11, 12). The tumor-specific expression of CAIX and its association with cancer progression and poor treatment outcome has led to interest in targeting this enzyme for cancer therapy (8). Studies have focused on the utilization of CAIX as a biomarker of hypoxic tumors, spurring the development of specific antibodies and sulfonamide-based small molecules for imaging CAIX in vivo (13-17). However, the relevance of CAIX function to the biology of tumors has only recently come into focus. Evidence suggests that, together with the activity of proteins such as the Na+/H+ exchanger NHE1, Na^+-HCO_3^- co-transporters and monocarboxylate transporters MCT-1 and MCT-4 (18, 19), the activity of CAIX plays an important role in the survival of tumor cells in hypoxic regions of tumors (18, 20) through the regulation of tumor pH (18, 20). The HCO_3^- produced at the extracellular surface

by CAIX is transported into the cytosol to control an intracellular pH (pHi) that is challenged by the abnormally acidic extracellular pH (pHe) produced in hypoxia (18, 20-22). The protons derived from CAIX activity further contribute to the decrease in pHe, thereby potentiating extracellular matrix breakdown and cell invasion (6, 19). Therefore, CAIX may increase metastatic potential by allowing aggressive tumor cells to survive the hostile environment imposed by hypoxia, and may further function to potentiate extracellular acidosis, facilitating growth and invasion of surviving cells (18, 20-22).

Although targeting CAIX for the treatment of cancer has garnered much scientific and clinical interest, appropriate carbonic anhydrase-relevant cell and animal models of tumor hypoxia for testing novel, CAIX-active compounds have only recently become available (23). To date, studies have focused on the role of CAIX in the regulation of primary tumor growth (22, 24). Importantly, neither the functional requirement of CAIX in breast tumor growth and metastasis in vivo, nor the benefit of therapeutic targeting this enzyme in aggressive breast cancer has been addressed.

In this study, we provide definitive evidence, using a large (>3600) cohort of human breast cancer samples, that CAIX is a poor prognostic marker for distant metastasis and survival. Furthermore, using a combination of gene depletion strategies and pharmacologic inhibition with novel small molecule inhibitors, we demonstrate a functional requirement of CAIX in the growth and metastasis of mouse and human breast tumors in several pre-clinical models.

Our findings establish CAIX as a therapeutic target for the treatment of CAIX-positive breast cancer.

Materials and Methods

Cell culture and hypoxic exposure

The acquisition, generation and culture of the luciferase expressing mouse breast cancer cell lines 4T1, 66cl4 and 67NR have been described previously (25). The MDA-MB-231 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC) and was maintained as described previously (26). The MDA-MB-231 LM2-4^{Luc+} cell line was provided by Dr. Robert Kerbel (University of Toronto, Canada) in July, 2010 and cells were cultured as described previously (27). For in vitro studies, cell lines were passaged for a maximum of 3 months, after which fresh seed stocks were thawed for experimental use. All cells were incubated at 37^{O}C with 5% CO_2 in a humidified incubator (normoxia). For culture in hypoxia, cells were maintained in 1% O_2 and 5% CO_2 balanced with N_2 at 37^{O}C in a humidified incubator in a sealed anaerobic workstation. Cell lines were evaluated routinely for morphology, hypoxia-induced CAIX expression and in vivo tumor growth.

Generation of transfected and transduced cells

shRNAmir vectors targeting mouse CAIX and a non-silencing sequence (Open Biosystems) were transfected into 4T1 cells using LipofectAMINE-PLUS (Invitrogen Life Technologies) according to the manufacturer's instructions. Transfected cells were selected using hygromycin. Stable shCAIX clones were derived by limited dilution cloning. For (re-)introduction of CAIX, human CAIX

(gift from Dr. Jacques Pouysségur, University of Nice) was transfected into 4T1 cells stably expressing mouse shCAIX and Zeocin was used for selection.

For stable depletion of human CAIX in the MDA-MB-231 cells, two different shRNAmir constructs (Open Biosystems) were transduced into cells using lentivirus as per the manufacturer's instructions. Tranduced cells were selected using puromycin.

All transfected and transduced cell lines were selected, propogated and frozen as seed stocks at early passage. For in vivo studies, cells were thawed from frozen stocks, passaged 1-2 times to expand the culture and implanted in mice. Cell lines were tested for mycoplasma contamination by a commercial testing facility prior to implantation in mice.

Measurement of extracellular pH

Changes in pHe were assessed using procedures published previously (28-30). In brief, cells were plated and allowed to recover overnight. A standard volume of 3 ml of fresh media/dish was then added and cells were incubated in normoxia or hypoxia for 72 h. Care was taken to ensure that cultures grown in normoxia and hypoxia were subconfluent and contained similar cell numbers. Media was collected and pH was measured immediately using a digital pH meter.

Pharmacological Inhibitors

The chemical properties of the sulfonamide, CAI17, have been described previously (8, 30). For in vitro studies, CAI17 was dissolved in DMSO, stored at -80^{O}C and diluted into culture medium prior to application. Subconfluent cells were incubated with CAI17 for 72 hours, washed 3x in PBS and imaged using a

Zeiss Axioplan epifluorescence microscope. For in vivo studies, CAI17, ureido-sulfonamide U-104, and glycosylcoumarins GC-204 and GC-205 were solubilized in 37.5 % PEG400/12.5% ethanol/50% saline prior to injection. Drug aliquots were made fresh daily or were prepared, frozen at -80^{O}C in single-use aliquots and thawed prior to administration. Drugs were administered by i.p. injection, except for CAI17 where the first 2 doses were administered by i.v. injection, followed by i.p. injection of the remaining doses. Specific dosing schedules are described in the appropriate figures.

Analysis of protein expression

Cells or flash-frozen tumor tissues were lysed as described previously (26). Equal amounts of protein were loaded on SDS-PAGE gels. Western blots were performed as described previously (26) using mouse CAIX (1:500), human CAIX (1:1000) (R&D Systems), caspase 3 (1:1000; Cell Signaling), PARP-1 (1:1000; Cell Signaling) and β-actin (1:10,000, Sigma) antibodies.

Mouse tumor models

All animal studies and procedures were done in accordance with protocols approved by the Institution Animal Care Committee at the BC Cancer research Centre and the University of British Columbia (Vancouver, BC, Canada).

Syngeneic orthotopic tumors and spontaneous metastasis

4T1 cells (1 x 10^6) or 67NR cells (2 x 10^6) were orthotopically implanted into the fourth mammary fat pad of 7-9 week-old female BALB/c mice as described previously (25). Primary tumor growth rates were calculated from caliper measurements using the modified ellipsoid formula $(LxW^2)/2$. Tumor formation

and metastasis progression was monitored and quantified using bioluminescent imaging as previously described (25, 27).

Experimental metastasis assays

For studies involving genetic depletion of CAIX, 4T1 or 67NR cells ($5x10^5$) were injected directly into the tail vein of 7-9 week-old female BALB/c mice. Mice were imaged once per week to follow the growth of metastases. Mice were euthanized 20 days post-injection and lungs were resected for further analysis. Tumor burden in the lung was quantified by manually counting nodules visible on the lung surface. For studies using U-104, 4T1 cells ($1x10^5$) were injected as described above, while $2x10^5$ cells were used for studies with GC-204 and GC-205.

Human xenograft tumors

For studies involving CAIX depletion, 1 x 10^7 MDA-MB-231 cells suspended in a 50% Matrigel/PBS solution were implanted subcutaneously in 6-8 week-old female NOD.CB17-prkdc^{scid}/J mice. For primary breast tumor xenografts using the MDA-MB-231 LM2-4^{Luc+} variant, cells were implanted orthotopically in mice as described above. Therapy was initiated when the tumors reached 200 mm^3. For both models, tumor growth was monitored by caliper measurement.

Immunohistochemistry

2 h before tumor excision mice were injected i.p. with a saline solution containing 1500 mg/kg BrdUrd (Sigma) and 60 mg/kg Pimonidazole (Chemicon), and i.v. 5 min before with $DiOC_7(3)$ (70 µl, 0.6 mg/ml; Molecular Probes). Tumors were

then harvested and analyzed for vasculature, perfusion, hypoxia, apoptosis, proliferation and necrosis as described previously (31, 32). Paraffin embedded tumor sections were also stained for CAIX (1:50 for lung metastases, Santa Cruz Biotechnology) as previously described (25).

Apoptosis assay

TUNEL labeling (Roche Applied Science) was employed for analysis of apoptosis according to the manufacturer's instructions. Briefly, subconfluent cells grown on coverslips were incubated for 48 h in normoxia or hypoxia in 1% serum, fixed and analyzed for TUNEL-positive cells. Quantification was achieved by counting the number of TUNEL-positive cells in 5 random fields/cell line at 20x magnification.

Clinical Analysis

The methods used to create the TMAs have been described (33). 3,630 cases had adequate tumor and staining results for assessment of all biomarkers. Immunohistochemistry for ER, PR, HER2, CK 5/6, EGFR and Ki67 was performed concurrently on serial sections and scored as described previously (33). CAIX expression was assessed using a murine monoclonal antibody (M75; 1:50) (34). Scoring of CAIX expression was either 0: no staining or 1: any staining and performed independently and blindly by 2 pathologists. This scoring system has been developed and validated for this very large TMA, and has been used previously to demonstrate prognostic significance for breast cancer biomarkers such as HER-2 and Ki67 (33, 35). The stained images of the complete tissue core set are available at the publicly accessible website

http://bliss.gpec.ubc.ca. Prior approval of the study was obtained from the Ethics Committee of the University of British Columbia.

Statistical Analysis

Results were subjected to statistical analysis using the Data Analysis ToolPack in Excel software. Two-tailed p values were calculated using Student's t-test. Data were considered significant for $p<0.05$. Statistical analysis for the clinical outcomes was performed using SPSS 13.0 (Chicago, IL), S-Plus 6.2 (Seattle, WA) and R 2.1.1 (http://www.r-project.org). In univariate analysis, BCSS (date of diagnosis of primary breast cancer to date of death with breast cancer as the primary or underlying cause) and RFS (date of diagnosis of primary breast cancer to the date of a local, regional or distant recurrence) and distant RFS (date of diagnosis of primary breast cancer to the date of a distant recurrence) were estimated by Kaplan-Meier curves. Log-rank test was used to estimate the survival differences. For multivariate analysis, a Cox proportional hazards model was used to estimate the adjusted hazard ratios and significance. To assess the violations of proportional hazard models, smoothed plots of weighted Schoenfeld residuals were used.

Results and Discussion

CAIX is a poor prognostic marker in a large cohort of breast cancer patients

Although previous studies have reported that CAIX expression in several types of cancer, including breast cancer, correlates with poor patient prognosis (11, 34, 36, 37), the sample sizes have been relatively small and adjuvant

treatments not uniform. To provide definitive evidence for CAIX as an important breast cancer prognostic marker, we analyzed the expression of CAIX in a primary breast tumor tissue microarray (TMA) containing more than 3600 patient samples subjected to standardized treatment with a median follow-up of 10.5 years (Table S1). Previously, we demonstrated prognostic significance of CAIX in a cohort of 103 breast cancers in which CAIX expression was examined both as a continuous and a categorical variable (11). We found that even 1% staining was significantly prognostic. For the large cohort of patients examined here, a simplified scoring system (present vs absent) allows for less analytical variability, an issue which plagues immunohistochemical testing for hormone receptors and the HER-2 receptor in breast cancer. CAIX expression was seen in 15.6% of assessable tumors and CAIX was differentially expressed among the biological subtypes, with the highest correlation in the basal breast cancers (51%) and the lowest proportion in the luminal A subtype (8%) (Table S2).

In Kaplan-Meier analyses, CAIX expression was significantly associated with worse relapse free survival (Figure 1A), distant relapse free survival (Figure 1B) and breast cancer specific survival (Figure 1C), achieving very high levels of statistical significance. The 10 year distant relapse free survival and breast cancer specific survival rates in the CAIX positive versus CAIX negative groups were 57% compared to 73%, and 62% compared to 78%, respectively. In multivariate analyses, including all standard prognostic variables and biological subtypes, CAIX expression remained a strong independent poor prognostic factor with a hazard ratio of 1.4 (Table S3). These data confirm and extend the

results of previous studies, and demonstrate a clear link between CAIX expression and a higher rate of distant metastasis for breast cancer. Our data substantiate previous studies that have also shown CAIX as a poor prognostic marker of breast cancer (11, 12). In addition, other studies have shown a clear correlation between the expression of CAIX, hypoxia-induced HIF-1α and altered metabolic proteins such as GLUT-1 (4, 9, 38), althoughHIF-1α is a more labile protein, and thus more susceptible to pre-analytical variables.

Preclinical models for interrogating the requirement of hypoxia-induced CAIX expression in breast cancer

Tumor hypoxia is linked both to the expression of CAIX and to the selection of tumor cells that are better able to metastasize. However, while a few studies have investigated the role of CAIX in the regulation of primary tumor growth (22, 24), the relationship between CAIX expression and metastatic potential has not been investigated. Therefore, to interrogate the functional role of CAIX in metastatic breast cancer, we were interested in selecting tumor models that exhibit both hypoxic microenvironments and the ability to metastasize. We have shown previously that the highly metastatic 4T1 mouse mammary tumor (39) overexpresses CAIX (25). Further characterization of this model revealed that it is poorly vascularized and contains large regions of hypoxia and necrosis (Figure 2A). Indeed, tumors formed by 4T1 cells have significantly fewer blood vessels, and significantly higher amounts of hypoxia, necrosis and apoptosis compared with tumors formed by isogenic, non-metastatic (25) 67NR cells (Figure 2A and 2B). Furthermore, bioinformatic

analysis of differential gene expression data (25) identified several hypoxia-regulated genes, including CAIX, that are expressed at higher levels in the 4T1 tumors relative to the 67NR tumors (Figure 2C). These attributes make the 4T1 mouse mammary tumor a robust model for examining the effects of manipulating CAIX expression and activity on the progression of breast cancer.

In addition to this syngeneic mouse model, we selected the MDA-MB-231 human breast tumor cell line as previous studies have shown this model to be hypoxic and to have hypoxia-inducible levels of CAIX (40, 41). We confirmed the effect of hypoxia on CAIX expression in these cell lines to validate them as appropriate models for subsequent in vivo studies. In keeping with previously published findings (25, 41), we found that both cell lines induced CAIX expression in hypoxia (Figure 2D, Supplemental Figure 1A), in contrast to the absence of hypoxia-induced CAIX in the 67NR cells (Figure 2D, Supplemental Figure 1A). These models reflect the clinical findings shown in Figure 1 and in other studies (11, 12) of hypoxia-induced upregulation of CAIX expression.

Depletion of CAIX in 4T1 cells inhibits cell survival and alters pHe

Next, we silenced CAIX gene expression in the 4T1 cells (Figure 3A) and the MDA-MB-231 cells (Figure 3B) by stably expressing CAIX shRNA constructs. 4T1 cells expressing a non-silencing control shRNA (shNS) upregulated expression of CAIX in hypoxia as expected, whereas hypoxia-induced CAIX expression was markedly attenuated in 2 independent clones expressing a single, identical shRNA targeting mouse CAIX (shCAIX; Figure 3A, Supplemental Figure 1B). Similarly for the MDA-MB-231 cell line, two distinct shRNA

sequences targeting human CAIX were transduced and hypoxia-induced CAIX expression was analyzed. Only one of the transduced shRNA constructs was found to effectively deplete CAIX expression (Figure 3B, Supplemental Figure 1C). The cell line expressing the "non-silencing" CAIX shRNA sequence was used as a control in subsequent in vivo experiments.

Recent data suggest that the regulation of pH by CAIX may be important for cell survival in conditions of hypoxic stress (22, 42) and previous studies have demonstrated a reduction in clonogenic survival in hypoxia of MDA-MB-231 cells treated with siRNA to CAIX (41). To examine whether depletion of CAIX may be influencing cell survival in the 4T1 system, we cultured control and CAIX-depleted 4T1 cells in hypoxia and assessed the amount of cell death by using a TUNEL assay. 4T1 shCAIX cells showed a significant increase in cell death compared shNS cells (Figure 3C). To determine whether the increase in TUNEL-positive cells was due to an increase in apoptosis, we analyzed the levels of active caspase-3 and PARP. No clear evidence of increased caspase-3 cleavage (Supplemental Figure 2A) or PARP cleavage (Supplemental Figure 2B) was observed, suggesting that cell death in the CAIX-depleted cells may be occurring by a caspase-independent mechanism, possibly related to depletion of the intracellular ATP concentration (Supplemental Figure 2C). While the decrease in ATP concentration was modest, it was statistically significant and similar in magnitude to data reported previously (43).

CAIX is functionally linked to the control of tumor pH (18, 22, 42), and hypoxia-induced, extracellular acidosis is a measure of the biological activity of

CAIX in cultured cells (30). Therefore, we examined the effect of CAIX depletion on pHe in hypoxia. Acidification of the extracellular medium in hypoxia was inhibited in the shCAIX-expressing 4T1 clones relative to the parental and shNS-expressing 4T1 cells (Figure 3D), suggesting that silencing CAIX gene expression induces functional inhibition of pHe regulation in this cell line.

Depletion of CAIX expression results in regression or growth inhibition of mouse and human breast tumors

Having evaluated the biological response of 4T1 cells to CAIX depletion in vitro, we next tested the impact of silencing CAIX expression on the growth of these tumors in vivo. We observed that whereas control 4T1 cells formed tumors that grew steadily over 30 days, tumors established from CAIX-depleted cells regressed significantly after initial tumor growth (Figure 4A). The regression of the tumors appeared to be stable, as there were only two mice with primary tumor recurrence appearing towards the end of the study (Table S4). Examination of the primary tumors confirmed downregulation of CAIX expression in the tumors (Supplemental Figure 3). The reduction of CAIX expression had a dramatic effect on the overall survival of the mice (Figure 4B). While the animals bearing tumors that express CAIX did not survive, the survival rate of animals inoculated with CAIX-depleted 4T1 cells remained at 100% over the course of the study.

To validate the observed in vivo effects of CAIX depletion on primary breast tumor growth, we performed similar experiments using the MDA-MB-231 cells expressing human shCAIX (see Figure 3B). Depletion of CAIX significantly

attenuated tumor growth of MDA-MB-231 xenografts (Figure 4C). Importantly, both the parental cells and the cells expressing the non-silencing construct (shCAIX 1 NS) formed tumors at a similar rate (Figure 4C), demonstrating the specificity of the effect of CAIX depletion on tumor growth. These data provide strong evidence for an important functional role of CAIX in the growth of hypoxic primary breast tumors.

To demonstrate that the tumor regression seen with the 4T1 shCAIX cells was dependent specifically on CAIX, we attempted to rescue the tumor growth by introducing human CAIX (resistant to mouse CAIX shRNA) into 4T1 cells expressing mouse-specific shCAIX. Human CAIX expression was readily detectable on the cell surface, together with stable expression of mouse shCAIX (Figure 4D). Cell cultures expressing human CAIX in tandem with shRNA to mouse CAIX showed low numbers of TUNEL-positive cells (Supplemental Figure 4), similar to control 4T1 cells (see Figure 3C). Furthermore, the mammary tumors established from these cells grew at rates similar to those of the parental 4T1 tumors (Figure 4D), confirming the specificity of CAIX-targeting in the regression of the 4T1 tumors.

Previous work in a xenograft model of colon cancer reported a requirement of hypoxia-induced CAIX and CAXII in the regulation of tumor growth (22). In this model, shRNA-mediated depletion of CAIX expression resulted in a partial reduction in tumor growth and compensatory upregulation of CAXII, while depletion of expression of both proteins resulted in greater inhibition of tumor growth (22). Our current findings suggest that, in breast cancer, CAIX

alone is important for the growth of breast tumors. Our data demonstrating that overriding CAIX depletion by constitutive expression of human CAIX can rescue tumor growth strongly implicates the dependency of these metastatic tumors on CAIX function. In addition, CAIX depletion in MDA-MB-231 human breast cancer cells, a cell line which is deficient in CAXII expression (44), also significantly reduced tumor growth. The reasons for the lack of CAXII dependence in these models are not known, but it is possible that the relative expression of CAIX versus CAXII in different cell types may be an important factor.

Inhibition of CAIX inhibits metastasis of 4T1 breast tumors

While a reduction in colon tumor growth in response to CAIX depletion has been demonstrated previously (22) and CAIX has been shown to influence cell migration in vitro (45), CAIX has not been previously linked to metastasis in vivo. Given the metastatic potential of the 4T1 cells, in particular, we were interested in determining whether inhibition of CAIX expression or activity also inhibited breast tumor metastasis. To investigate the effects of CAIX depletion on spontaneous metastasis of primary breast tumors in vivo, we employed bioluminescent imaging to detect metastases arising from control (parental and shNS) and CAIX-depleted 4T1 tumors (Figure 5A). Whereas the mice implanted with control cells showed clear evidence of metastasis to several organs, IVIS-detectable metastases were not observed in mice that had been implanted with the CAIX-depleted cells.

To demonstrate that the observed effects on the formation of metastases were not simply due to primary tumor regression, we injected the various 4T1

tumor cell lines intravenously and assessed the ability of the cells to form lung metastases (Figure 5B). We found that, whereas the shNS cells formed robust lung metastases, cells depleted of CAIX showed almost no visible metastasis to the lungs. 67NR cells also showed little evidence of experimental metastasis (Figure 5B). Quantification of bioluminescent signal at 2 weeks and 3 weeks post-injection demonstrated a significant increase in signal in the shNS group, but not in the shCAIX and 67NR groups (Figure 5B). Examination of the gross lungs revealed that animals injected with shNS cells exhibited large numbers of lung surface nodules, while nodules were largely absent in mice injected with shCAIX cells or 67NR cells (Figure 5C). Quantification of surface nodules showed that mice harboring shNS cells had significantly higher counts than mice harboring shCAIX or 67NR cells (Figure 5C). Membrane-localized CAIX expression was also evident in some of the metatstatic foci in histologic sections taken from control animals, but not from mice inoculated with CAIX-depleted cells (Figure 5D). Taken together, these data suggest that CAIX may be required for colonization and growth of metastatic cells at secondary sites.

Pharmacologic inhibition of CAIX reduces the growth and metastasis of mouse and human tumors.

Our data showing the requirement of CAIX expression for survival and eventual metastasis of primary breast tumor cells suggested to us that targeting the activity of CAIX with specific pharmacologic inhibitors may be useful for inhibiting disease progression. In previous studies, treatment of renal clear cell carcinoma xenografts with fluorescein- and albumin-based membrane-

impermeant derivatives of acetazolamide, a general carbonic anhydrase inhibitor, resulted in inhibition of tumor growth compared to vehicle-treated controls (24). These data have provided initial proof of principle for sulfonamide-based antitumor effects, but acetazolamide lacks specificity for CAIX. First, we investigated the ability of the metastatic and non-metastatic cells to bind to a previously described, highly selective sulfonamide-based inhibitor of CAIX (CAI17) with a Ki in vitro of 24 nM (8). This fluorescent inhibitor (Figure 6A) interacts only with active CAIX in hypoxic conditions (8) and has been used successfully to image hypoxic xenografts (15). Similar to findings in MDCK cells expressing CAIX (30), we observed that the inhibitor was not able to bind appreciably 4T1 or 67NR cells in normoxia (Figure 6A). In contrast, CAI17 bound to the cell surfaces of metastatic, CAIX-expressing 4T1 cells cultured in hypoxia, but not the non-metastatic, CAIX-negative 67NR cells cultured in similar conditions (Figure 6A). We next tested the effect of CAI17 on hypoxia-induced changes in pHe in these two cell types. In the absence of the inhibitor, the pHe of cultured 4T1 cells decreased significantly in hypoxia, but remained unchanged in the 67NR cultures (Figure 6A). Treatment of the 4T1 cells with CAI17 reversed the hypoxia-induced decline of pHe, indicating functional inhibition of CAIX activity (Figure 6A).

To evaluate the effect of pharmacologic inhibition of CAIX activity in vivo, we treated mice harboring established 4T1 tumors with CAI17. We observed significant inhibition of tumor growth in mice treated with the inhibitor, compared to vehicle controls (Figure 6B). To test for the possibility of non-specific cytotoxic

activity of CAI17, we took advantage of the 67NR tumor model as a negative control. We treated 67NR cell-derived tumors using a dosing schedule and concentrations of CAI17 identical to those used for the 4T1 model (Figure 6C). There was no effect of the CAI17 compound relative to the vehicle control, even at the highest concentration of the inhibitor (Figure 6C). The inhibitor concentrations and the dosing schedule were well-tolerated, and no significant weight reduction was noted in any of the treated mice (Supplemental Figure 5A and 5B). In addition to CAI17, we tested the effect of a novel ureido-sulfonamide inhibitor of CAIX, U-104 (Figure 6D, Table 1), on primary breast tumor growth using a highly metastatic variant of the MDA-MB-231 cell line (27). These cells were observed to induce robustly CAIX in hypoxia (Figure 6D, inset). Tumor volume measurements showed significant inhibition of primary tumor growth in the mice treated with the U-104 compound compared to vehicle controls (Figure 6D). Taken together, these data suggest the ability of sulfonamide-based CAIX inhibitors to specfically target CAIX-expressing tumors.

Having demonstrated that selective sulfonamide-based compounds inhibit the growth of primary breast tumors, we next tested U-104 for its ability to inhibit metastasis formation in the 4T1 experimental metastasis model. Intravenous injection of 4T1 cells into mice and subsequent daily treatment of these animals beginning 24 hours post-injection with U-104 resulted in inhibition of metastases formation (Figure 7A). Quantification of the bioluminescent signal revealed a statistically significant decrease in the formation of metastases in the treated mice (Figure 7A), suggesting that CAIX-specific inhibition may be useful in

treating metastatic disease in breast cancer. Further structure/function analyses for the ureido-sulfonamide compounds will be described elsewhere (46).

Finally, we also tested the ability of 2 additional selective inhibitors of CAIX, GC-204 and GC-205 (Figure 7B, Table 1), to inhibit metastasis in the same model. These two novel compounds are glycosylcoumarins and are representatives of the coumarin class of CAIX inhibitors. GC-204 and GC-205 were effective in limiting colonization in the lungs (Figure 7B). Quantification of the bioluminescent signal revealed a statistically significant decrease in the formation of metastases in the treated mice (Figure 7C), with GC-205 being particularly efficacious at 15 mg/kg (Figure 7C). Collectively, these pharmacologic studies provide strong "proof of principle" data for the therapeutic inhibition of CAIX activity for breast tumor growth and metastasis formation. Moreover, our results suggest that perturbation of CAIX function reduces metastasis both by inhibiting cell survival in hypoxia and perhaps also by preventing migration and invasion, as inhibition of CAIX reduced the metastatic burden in models of experimental metastasis.

In conclusion, our results not only solidify CAIX as a poor prognostic biomarker for human breast cancer, but also show it to be a promising therapeutic target for breast tumor growth and metastasis. Our data demonstrate that CAIX is an essential factor in the survival of tumor cells in hypoxic regions of breast tumors and, in addition, its activity contributes to metastasis in breast cancer. Its use would allow for the identification and selection of patients whose tumors are likely to metastasize, and for treatment with CAIX inhibitors to prevent

this deadly process. Although CAIX expression is elevated in about 16% of breast cancer patients, this percentage falls in with the frequency of upregulation of Her-2 and of basal breast cancers which have the highest expression of CAIX (Table S2). Since these subgroups of breast cancers are the most difficult to treat and are also the most aggressive in terms of metastatic potential, we suggest that CAIX inhibitors, such as those described here, should be used to treat hypoxic breast tumors with elevated CAIX expression. The development of small molecule inhibitors of CAIX activity (8), anti-CAIX neutralizing antibodies, and CAIX imaging agents, should accelerate clinical translation of our findings. Finally, our findings are likely to be applicable to other CAIX-expressing tumors.

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Table 1. K_I values for ureido-sulfonamide and glycosylcoumarin inhibitors of CAIX

Compound	K_I (nM)*			
	CA I	CA II	CA IX	CA XII
U-104	5080	9640	45.1	4.5
GC-204	>100 000	>100 000	9.2	43
GC-205	>100 000	>100 000	201	184

*KI values were derived using in vitro assays for CA activity as described in (47).

Figure Legends

Figure 1

CAIX expression is an independent prognostic factor in a large cohort of breast cancer patients. The Kaplan-Meier plots show cumulative survival (Cum Survival) as a function of time to event cut off at 10 years post-diagnosis. CAIX expression was significantly associated with poor relapse-free survival (RFS) **(A)**, distant RFS **(B)**, and disease specific (breast cancer) survival **(C)**. DSS = distant site survival. $P<10^{-17}$, $P<10^{-16}$, $P<10^{-13}$ for A, B, and C, respectively. CAIX expression on the TMA was binarized as 0 and 1 for analysis.

Figure 2

The metastatic 4T1 primary tumor is a valid preclinical model of hypoxia-induced CAIX expression. **(A)** Representative composite, pseudocolored images of 4T1 and 67NR tumor tissue sections showing the distribution of the indicated parameters. Scale bar = 150 μm. **(B)** Quantification of the parameters outlined in (A) using whole tumor tissue sections. n = 10 animals/group. *$p \leq 0.02$, **$P<10^{-5}$. **(C)** Differential gene expression data derived from tumor tissue from each cell model (25) was analyzed bioinformatically for expression of hypoxia-induced genes (high expression, red; low expression, green). n = 3/group. **(D)** The indicated cell lines were cultured in normoxia or hypoxia and levels of CAIX expression were analyzed by Western blot. β-actin served as a loading control.

Figure 3

Silencing CAIX expression in metastatic 4T1 cells inhibits cell survival and alters pHe in hypoxia. **(A)** Cells expressing non-silencing shRNA (shNS) or shRNA targeting mouse CAIX (shCAIX) were cultured in normoxia (N) or hypoxia (H) and analyzed for CAIX expression. Two independent clones (C2, C5) expressing a single, identical shCAIX construct were tested. **(B)** MDA-MB-231 cells expressing either of 2 different shRNA constructs targeting human CAIX (shCAIX 1 and shCAIX 2) were cultured as above and analyzed for CAIX expression. **(C)** 4T1 cells expressing shNS or shCAIX were cultured in hypoxia and the amount of cell death was analyzed. Left panel, representative images of TUNEL-positive cells (arrows). Scale bar = 100 μm. Right panel, quantification of the TUNEL-positive cells. Data are expressed as fold change in TUNEL-positive cells, compared to control cells cultured in normoxia. n = 5. *P<0.01, compared to the controls. **(D)** Cells were cultured in normoxia or hypoxia and pHe was measured. n = 3. Data show the mean change in pH ± s.e.m. Changes in the pHe in hypoxia are assessed relative to the baseline pHe values in normoxia. *P<0.01, compared to the 4T1 cells expressing shNS in hypoxia.

Figure 4

Growth of primary breast tumors characterized by hypoxia requires the expression of CAIX. **(A)** Parental 4T1 cells, or 4T1 cells expressing shNS or shCAIX were implanted orthotopically into BALB/c mice and tumor volume was monitored over time. n = 10 for each group. Arrows denote changes in the n due to surgery and revised values are indicated. * denotes completion of primary tumor excision from the control groups. Results are expressed as mean ± s.e.m

for each group. ***$P<10^{-11}$, compared to the shNS group. **(B)** The groups of mice described in (A) were monitored for the percentage of mice surviving (pooled control and shCAIX groups) over time. n=18/group. **(C)** Parental MDA-MB-231 cells or cells expressing shCAIX 1 or shCAIX 2 were implanted subcutaneously into the flank of NOD/SCID mice and tumor growth was monitored. Note that shCAIX 1 does not silence CAIX expression (see Figure 3B) and is used as a non-silencing (NS) control. n=7 for each group. *P<0.01, compared to NS control tumors. **(D)** 4T1 cells expressing mouse shCAIX were transfected with human CAIX (hCAIX) and immunocytochemistry was performed to assess levels of human CAIX expression (red, arrowheads) in these cells (shCAIX + hCAIX). Cells are simultaneously positive for GFP (mouse shCAIX) and are counterstained with DAPI (blue). Cells were implanted orthotopically in BALB/c mice and monitored for tumor growth. n=9 for each group. *P<0.04, compared to the shCAIX group.

Figure 5

Inhibition of CAIX expression or activity attenuates metastasis of 4T1 mouse mammary tumors. (A) Using the experimental design detailed in Figure 4A, mice were examined for evidence of spontaneous metastasis. Primary tumors in the control arm were removed by day 30 post implantation, while primary tumors in the CAIX-depleted groups either regressed or were removed in a similar fashion to control tumors. Representative bioluminescent images are shown as heat maps (blue, least intense; red, most intense;) overlaid on gray-scale body images. **(B)** 4T1 cells expressing shNS or shCAIX, or 67NR cells

were injected directly into the tail vein of BALB/c mice. Representative images of tumor cell bioluminescence at 20 days post inoculation are shown. The graph shows quantification of bioluminescence at week 2 and week 3 post injection of tumor cells. n = 8/group. *P<0.02. **(C)** Comparison of surface nodules on gross lungs resected from animals at 3 weeks post-injection. The graph shows quantification of the number of metastatic foci (nodules) on the lung surface. n = 8. *P=0.0001. **(D)** Representative images of lung metastases stained immunohistochemically for CAIX (arrowheads). Scale bar = 50 μm.

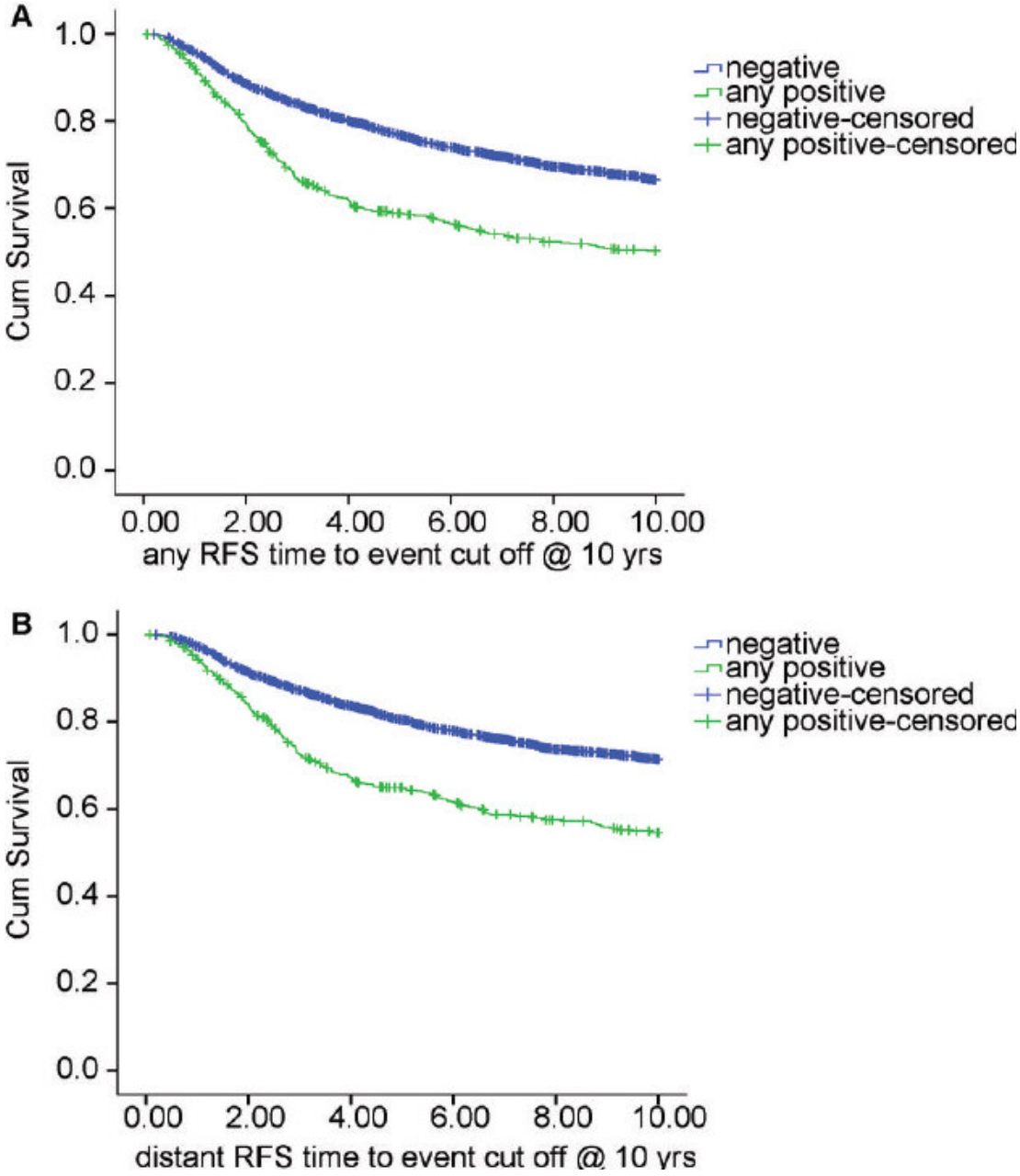
Figure 6

Targeting CAIX activity with selective small molecule inhibitors of CAIX attenuates the growth of mouse and human breast tumors. **(A)** Left panel, chemical structure of sulfonamide-based CAIX inhibitor CAI17 (formally called CAI17 (8). Center panel, cells were cultured with 10 μM CAI17. Shown are representative images of the FITC-tagged inhibitor (green) bound to cells in normoxia and hypoxia. Right panel, cells were cultured with or without 400 μM CAI17. The mean changes in pHe ± s.e.m. are shown. Changes in pHe in hypoxia were assessed relative to the baseline pHe values measured in parallel cultures grown in normoxia. n = 3. *P<0.001. **(B)** BALB/c mice were inoculated orthotopically with 4T1 cells and tumors were grown for 14 days. Animals then received CAI17 3 times per week for 2 weeks and tumor growth was monitored. Treatment initiation and termination are indicated. Vehicle-treated and untreated animals served as controls. n = 6 to 8. *P<0.02, **P<0.01, compared to vehicle

controls. **(C)** Animals were implanted with 67NR cells, treated as described in (B) and monitored for tumor growth. n = 5 to 6. **(D)** Top, chemical structure of U-104. Bottom, MDA-MB-231 LM2-4^{Luc+} cells were implanted orthotopically into NOD/SCID mice. When tumors reached an average of 200 mm^3, animals received the indicated doses of U-104 daily and tumor growth was monitored. n = 8/group. *P<0.03, **P<0.001. Inset, hypoxia-induced CAIX expression by LM2-4^{Luc+} cells.

Figure 7

Novel selective small molecule inhibitors of CAIX inhibit metastasis formation by 4T1 mammary tumor cells. **(A)** 4T1 cells were injected directly into the tail vein of BALB/c mice. Daily treatment for 5 days with vehicle or U-104 was initiated 24 hours post inoculation of cells and mice were imaged 24 hours following the final dose. The graph shows quantification of bioluminescence. n = 6 per group. *P<0.01. **(B)** Left panel, chemical structures for the 2 glycosylcoumarins, GC-204 and GC-205. Right panel, using the experimental design outlined in A, mice were treated daily for 6 days with GC-204 or GC-205. Mice were imaged 24 hours following the final dose. **(C)** Quantification of bioluminescence shown in (B). Data are reported as the mean ± s.e.m. n = 8/group. *P<0.02.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any RFS time to event cut off @ 10 yrs
negative
any positive
negative-censored
any positive-censored
B
1.0
0.8
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0.4
0.2
0.0
Cum Survival
0.00
2.00
4.00
6.00
8.00
10.00
distant RFS time to event cut off @ 10 yrs
negative
any positive
negative-censored
any positive-censored

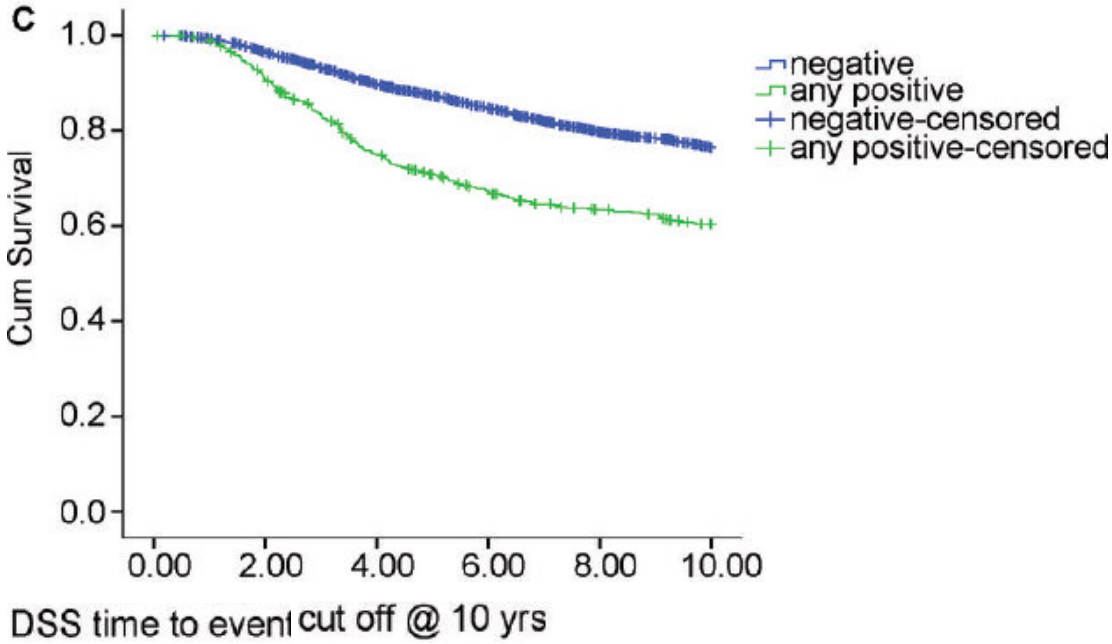
C
1.0
0.8
0.6
0.4
0.2
0.0
Cum Survival
0.00
2.00
4.00
6.00
8.00
10.00
DSS time to event
cut off @ 10 yrs
negative
any positive
negative-censored
any positive-censored

Figure 1

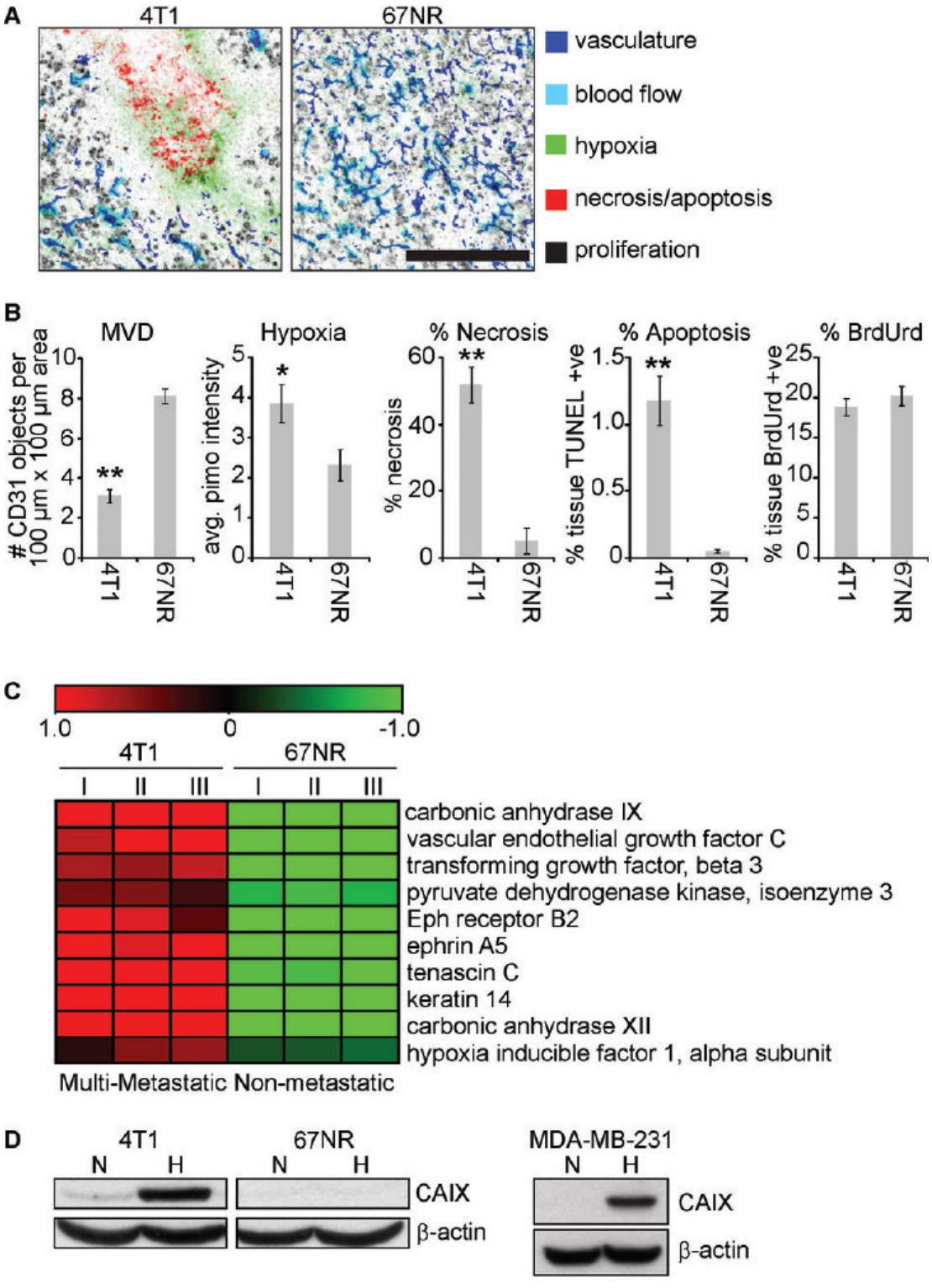
A
4T1
67NR
vasculature
blood flow
hypoxia
necrosis/apoptosis
proliferation
B
MVD
CD31 objects per 100 µm x 100 µm area
Hypoxia
avg. pimo intensity
% Necrosis
% necrosis
% Apoptosis
% tissue TUNEL +ve
% BrdUrd
% tissue BrdUrd +ve
4T1
67NR
C
1.0
0
-1.0
I
II
III
carbonic anhydrase IX
vascular endothelial growth factor C
transforming growth factor, beta 3
pyruvate dehydrogenase kinase, isoenzyme 3
Eph receptor B2
ephrin A5
tenascin C
keratin 14
carbonic anhydrase XII
hypoxia inducible factor 1, alpha subunit
Multi-Metastatic Non-metastatic
D
4T1
67NR
MDA-MB-231
N
H
CAIX
β-actin

Figure 2

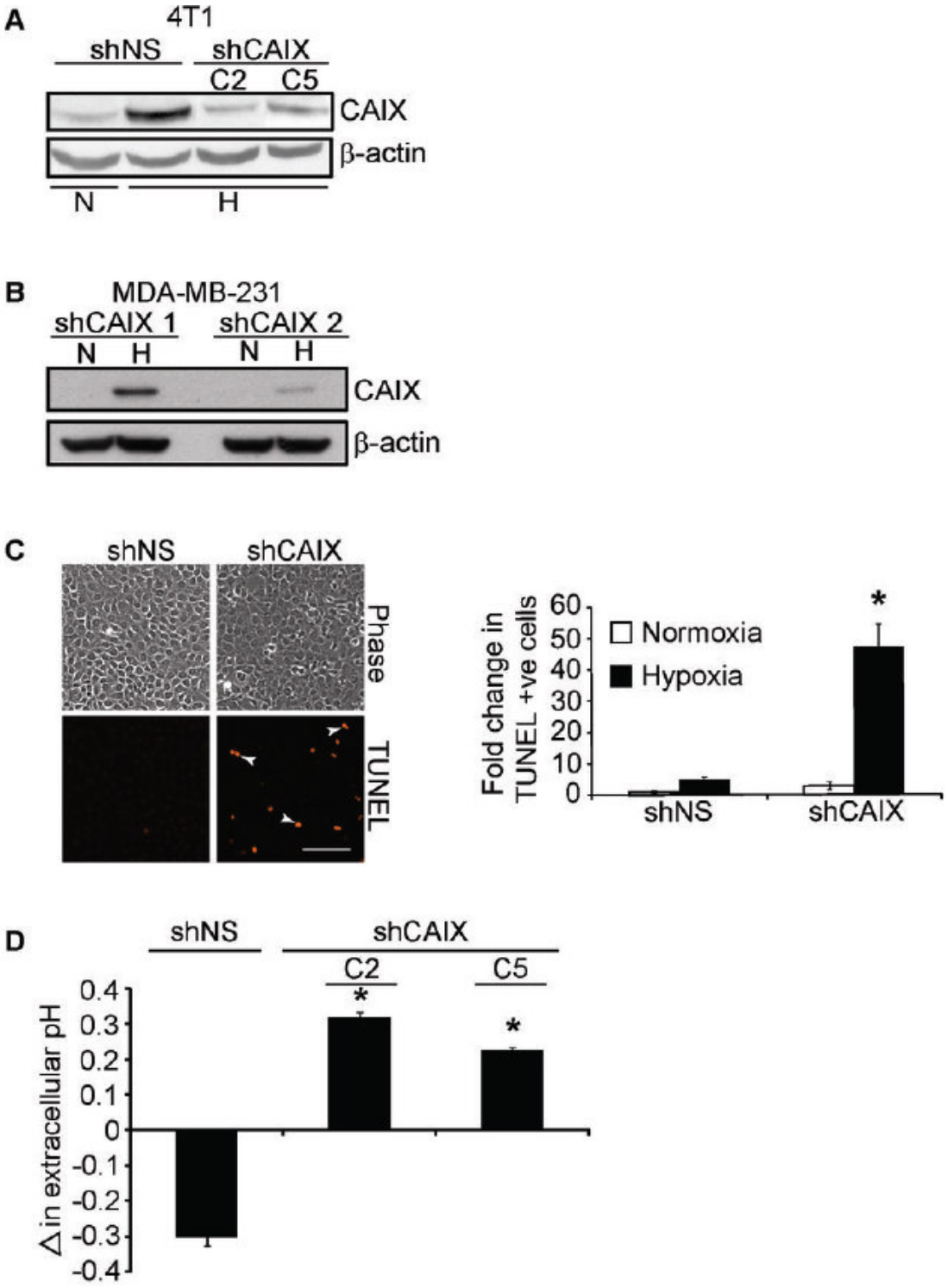
A
4T1
shNS
shCAIX
C2
C5
CAIX
β-actin
N
H
B
MDA-MB-231
shCAIX 1
shCAIX 2
N
H
N
H
CAIX
β-actin
C
shNS
shCAIX
Phase
TUNEL
Fold change in TUNEL +ve cells
60
50
40
30
20
10
0
Normoxia
Hypoxia
*
shNS
shCAIX
D
shNS
shCAIX
C2
C5
*
*
Δ in extracellular pH
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4

Figure 3

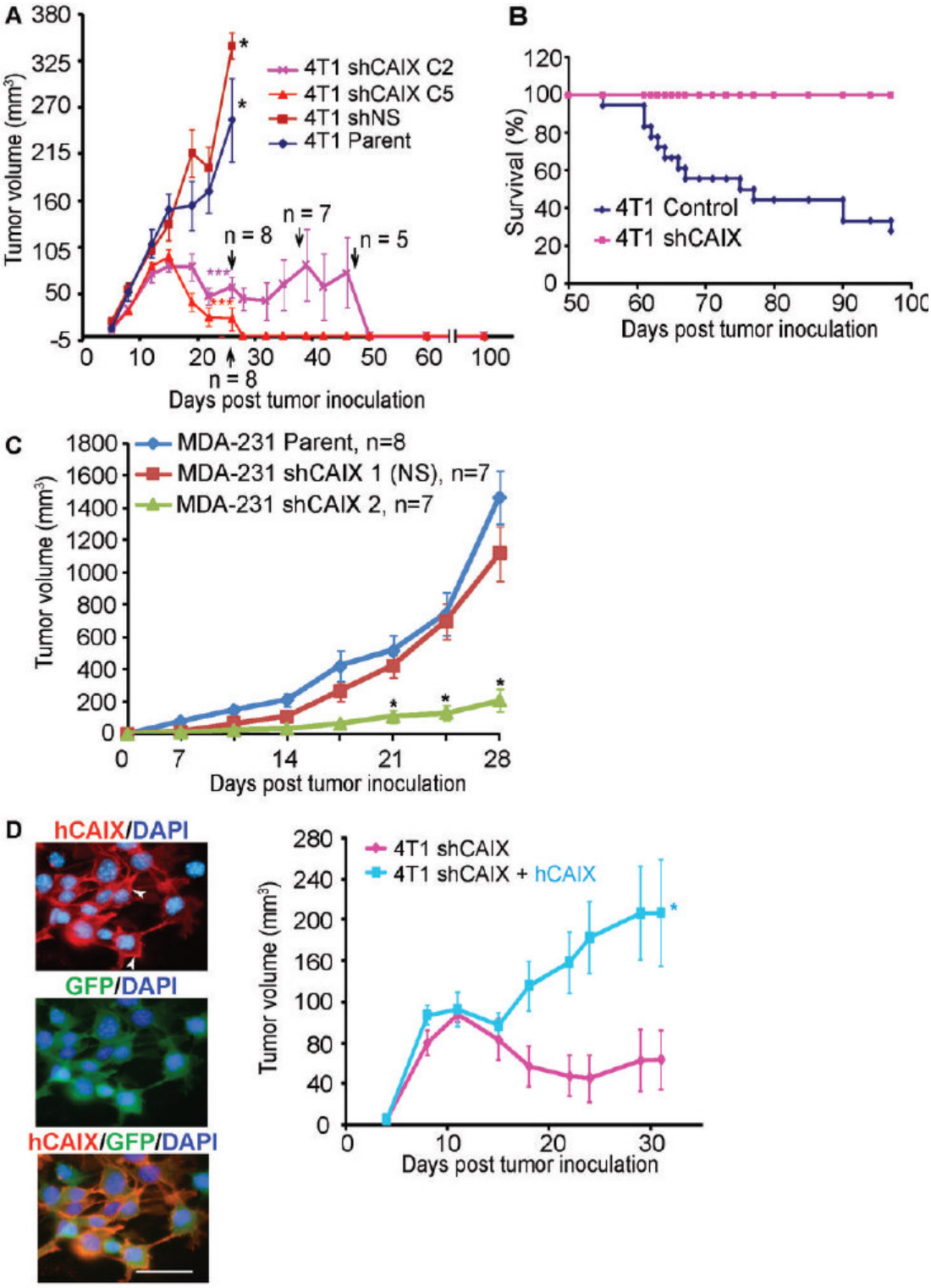
A
Tumor volume (mm3)
4T1 shCAIX C2
4T1 shCAIX C5
4T1 shNS
4T1 Parent
n = 8
n = 7
n = 5
n = 8
Days post tumor inoculation
B
Survival (%)
4T1 Control
4T1 shCAIX
Days post tumor inoculation
C
Tumor volume (mm3)
MDA-231 Parent, n=8
MDA-231 shCAIX 1 (NS), n=7
MDA-231 shCAIX 2, n=7
Days post tumor inoculation
D
hCAIX/DAPI
GFP/DAPI
hCAIX/GFP/DAPI
Tumor volume (mm3)
4T1 shCAIX
4T1 shCAIX + hCAIX
Days post tumor inoculation

Figure 4

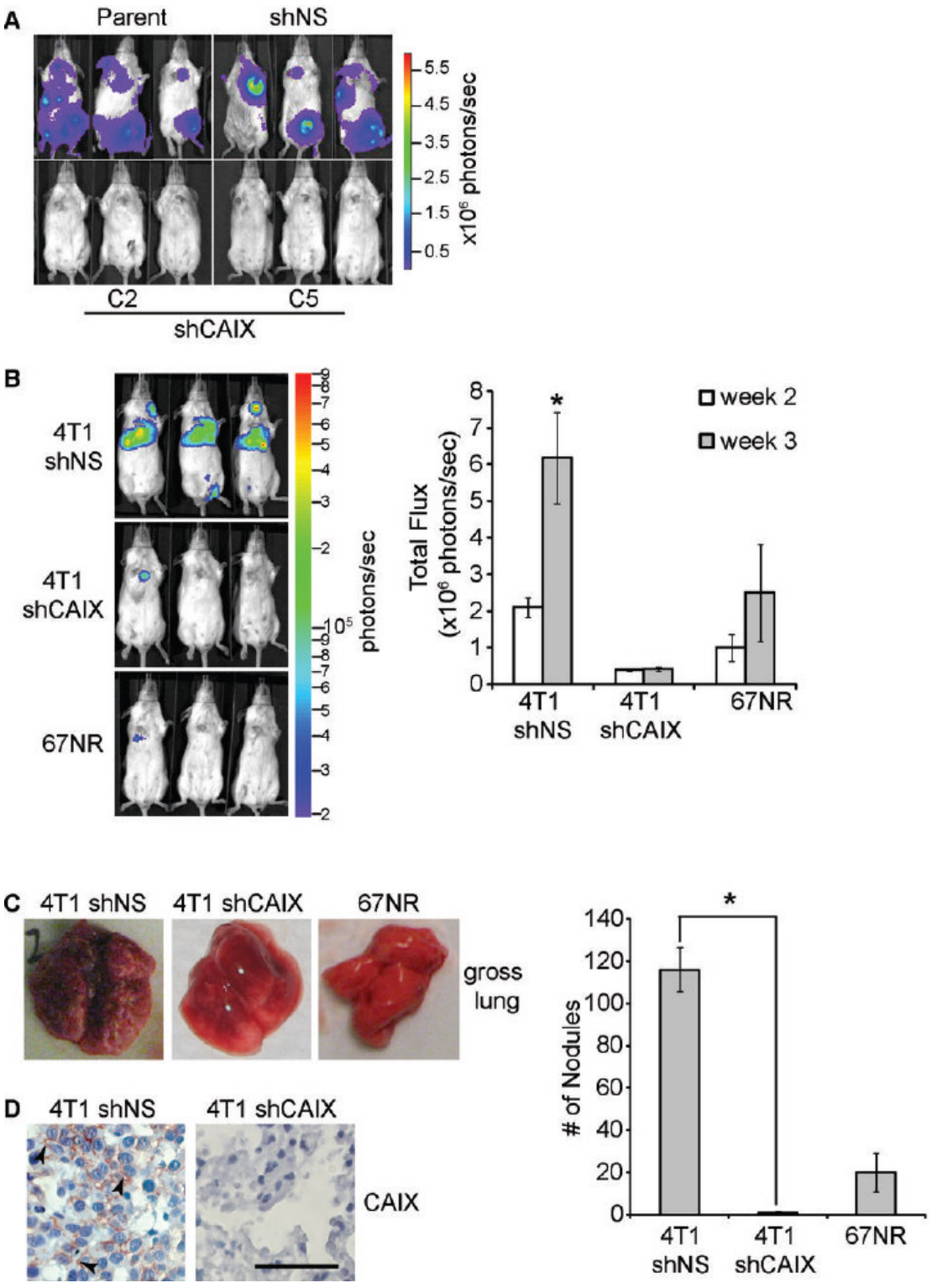
A
Parent
shNS
5.5
4.5
3.5
2.5
1.5
0.5
x10⁶ photons/sec
C2
C5
shCAIX
B
4T1 shNS
4T1 shCAIX
67NR
photons/sec
Total Flux (x10⁶ photons/sec)
week 2
week 3
*
4T1 shNS
4T1 shCAIX
67NR
C
4T1 shNS
4T1 shCAIX
67NR
gross lung
*
# of Nodules
4T1 shNS
4T1 shCAIX
67NR
D
4T1 shNS
4T1 shCAIX
CAIX

Figure 5

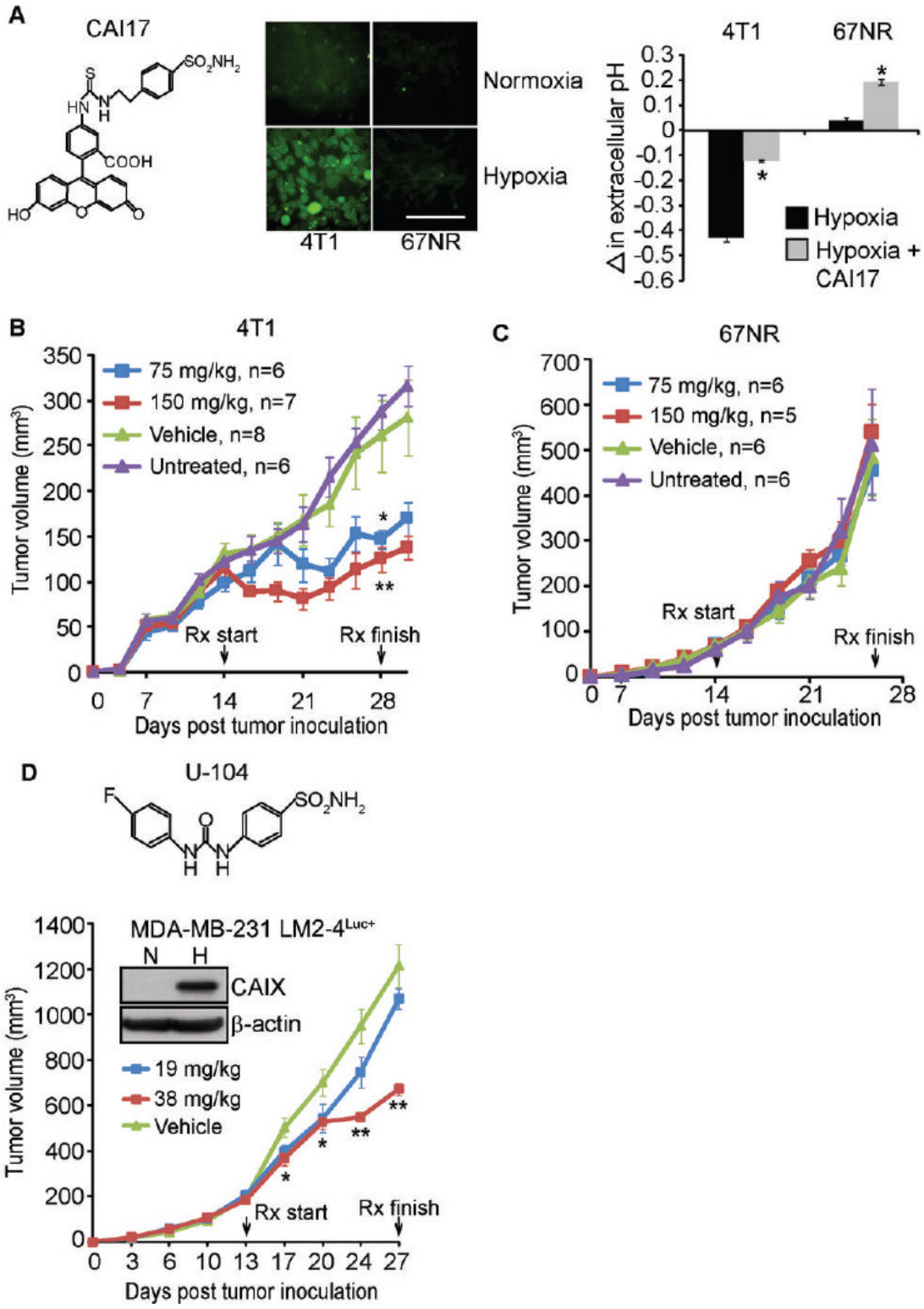
A
CAI17
Normoxia
Hypoxia
4T1
67NR
Δ in extracellular pH
Hypoxia
Hypoxia + CAI17
B
4T1
75 mg/kg, n=6
150 mg/kg, n=7
Vehicle, n=8
Untreated, n=6
Tumor volume (mm³)
Rx start
Rx finish
Days post tumor inoculation
C
67NR
75 mg/kg, n=6
150 mg/kg, n=5
Vehicle, n=6
Untreated, n=6
Tumor volume (mm³)
Rx start
Rx finish
Days post tumor inoculation
D
U-104
MDA-MB-231 LM2-4Luc+
N H
CAIX
β-actin
19 mg/kg
38 mg/kg
Vehicle
Tumor volume (mm³)
Rx start
Rx finish
Days post tumor inoculation

Figure 6

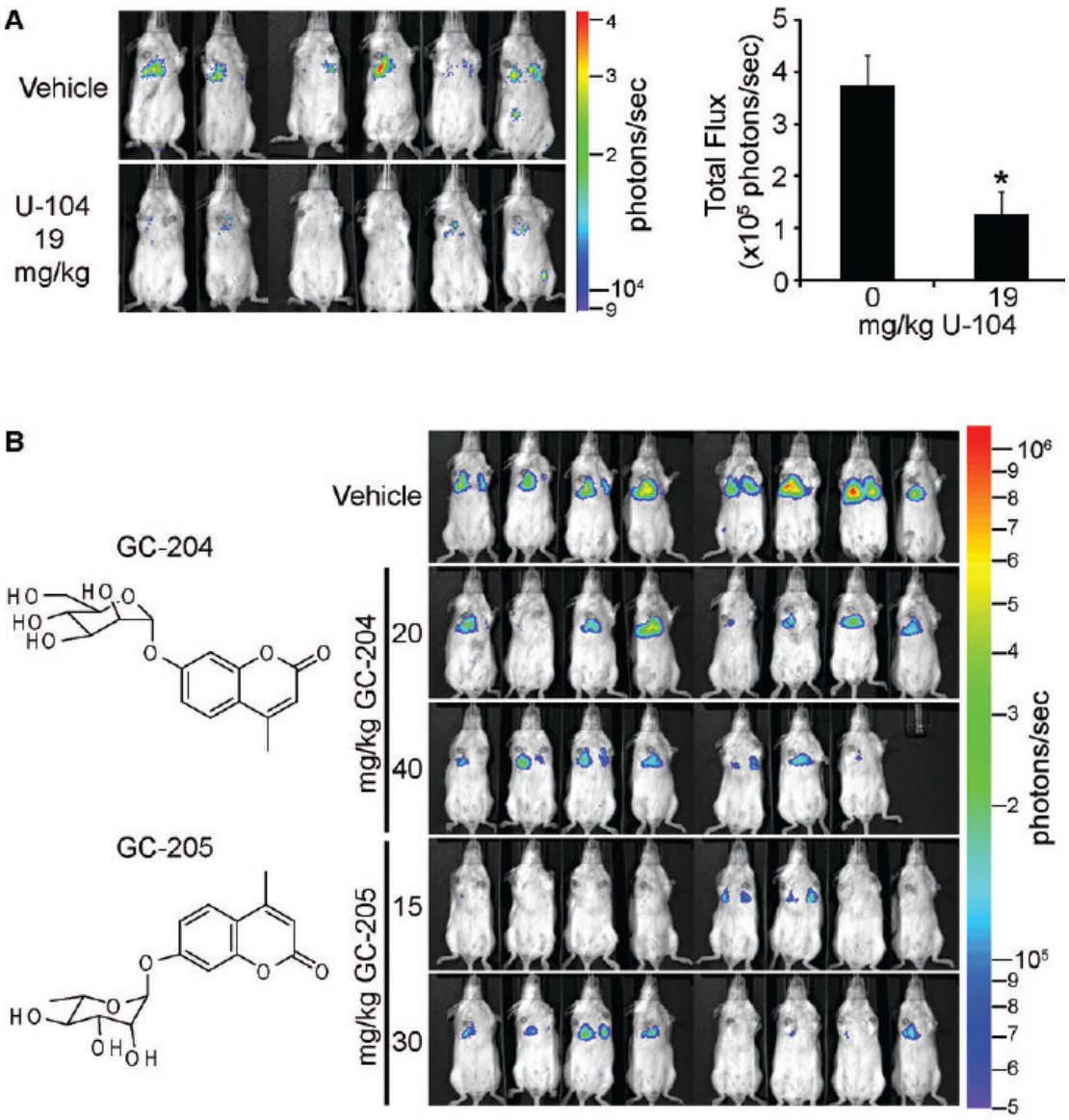
A
Vehicle
U-104
19
mg/kg
photons/sec
Total Flux
(x10⁵ photons/sec)
mg/kg U-104
B
Vehicle
GC-204
mg/kg GC-204
20
40
GC-205
mg/kg GC-205
15
30
photons/sec
C
Total Flux
(x10⁶ photons/sec)
mg/kg GC-204
mg/kg GC-205

Figure 7