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Carbonic Anhydrase IX: A New Drug Target for Designing Diagnostic Tools and Antitumor Agents

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Abstract

The tumor associated metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) isozyme CA IX is highly overexpressed in many cancer types by the hypoxia inducible factor-1a (HIF-1a) cascade. Many hypoxic tumors overexpress CA IX and show a bad response to classical chemo- and radiotherapies. CA IX significantly contributes to acidification of the tumor environment, by catalyzing the hydration of carbon dioxide to bicarbonate and protons with its extracellularly situated active site. This leads to the acquisition of metastasic phenotypes and chemoresistance with many anticancer drugs. The report of the X-ray crystal structure of CA IX, which is a dimeric protein with a quaternary structure not evidenced earlier for this family of enzymes, allows for structure-based drug design campaigns of inhibitors against this novel antitumor target. Indeed, it has been known for some time that aromatic/heterocyclic sulfonamides and sulfamates have good affinity for this isoform, but generally they do not show specificity for the inhibition of the tumor-associated isoform versus the remaining CA isozymes (CA I-VII, and XII-XV) found in mammals. Inhibition of CA IX by specific and potent sulfonamide inhibitors was shown to revert the tumor acidification processes, establishing a clear-cut role of CA IX in tumorigenesis. The development of a wide range of such inhibitors belonging to diverse chemical classes (sulfonamides, sulfamates, sulfamides), such as membrane-impermeant, fluorescent or metal-containing compounds, provides useful tools for highlighting the exact role of CA IX in hypoxic cancers, to control the pH (im)balance of tumor cells, and to develop novel diagnostic or therapeutic applications for the management of tumors. Work from several laboratories recently reported the proof-of-concept studies for the use of CA IX inhibitors as well as antibodies both in the therapy and imaging of hypoxic tumors.

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INTRODUCTION

α-Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in higher vertebrates, including humans [1,2]. 16 isozymes have been characterized up untill now, which differ in their subcellular localization, catalytic activity, and susceptibility to different classes of inhibitors. There

are cytosolic isozymes (CA I, CA II, CA III, CA VII and CA XIII), membrane bound ones (CA IV, CA IX, CA XII and CA XIV), mitochondrial (CA VA and CA VB) and secreted (CA VI) isoforms. Isoform CA XV is not expressed in human nor in other primates (where it is encoded by a pseudogene), but it is quite abundant in rodents and other higher vertebrates [1-4]. Three acatalytic forms are also known, called CA-related proteins (CARPs): CARP VIII, CARP X and CARP XI. In humans, CAs are present in a large variety of tissues such as the gastrointestinal tract (GI), the reproductive tract, the nervous system, kidneys, lungs, skin, eyes, etc [1,2].

These zinc enzymes play crucial physiological roles [1]. Most CAs are very efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), the only physiological reaction in which they are involved [1]. Table 1 shows the catalytic properties and subcellular localization for human CAs [1]. Many CA isoforms are involved in critical physiologic processes such as respiration and acid-base regulation, electrolyte secretion, bone resorption, calcification and biosynthetic reactions which require bicarbonate as a substrate (lipogenesis, gluconeogenesis, and ureagenesis) [1,2]. Recently, it has been discovered that two CA isozymes (CA IX and CA XII) are prominently associated with and overexpressed in many tumors, being involved in critical processes connected with cancer progression and response to therapy [1-4]. CA IX, the most widesprad of the two cancer-associated CAs is confined to few normal tissues, but it is ectopically induced and highly overexpressed in many tumor types mainly due to its strong transcriptional activation by hypoxia, accomplished via the hypoxia inducible factor - 1 (HIF-1) transcription factor [1-7]. CA IX was also shown to serve as a surrogate marker of hypoxia and as a prognostic indicator for many cancer types, which unfortunately, are often hypoxic [4-15].

Table 1. Kinetic parameters for CO_2 hydration reaction catalysed by the 13 vertebrate catalytically active α-CA isozymes, at 20°C and pH 7.5, and their subcellular localization [1,8].

Isozyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}.s^{-1}$)	Subcellular localization
hCA I	2.0×10^5	4.0	5.0×10^7	cytosol
hCA II	1.4×10^6	9.3	1.5×10^8	cytosol
hCA III	1.3×10^4	52.0	2.5×10^5	cytosol
hCA IV	1.1×10^6	21.5	5.1×10^7	membrane-bound
hCA VA	2.9×10^5	10.0	2.9×10^7	mitochondria
hCA VB	9.5×10^5	9.7	9.8×10^7	mitochondria
hCA VI	3.4×10^5	6.9	4.9×10^7	secreted into saliva/milk
hCA VII	9.5×10^5	11.4	8.3×10^7	cytosol
hCA IX	1.1×10^6	7.5	1.5×10^8	transmembrane
hCA XII	4.2×10^5	12.0	3.5×10^7	transmembrane
hCA XIII	1.5×10^5	13.8	1.1×10^7	cytosol
hCA XIV	3.1×10^5	7.9	3.9×10^7	transmembrane
mCA XV	4.7×10^5	14.2	3.3×10^7	membrane-bound

h = human; m = mouse enzyme.

CA IX and its role in tumor acidification

The expression of CA IX is strongly up-regulated by hypoxia and is down-regulated by the wild-type von Hippel-Lindau tumor suppressor protein (pVHL) [2-4,11,12]. The transcription factor HIF-1 is a heterodimer consisting of an inducible subunit (HIF-1α) and a constitutively expressed subunit (HIF-1β) [1,2,12]. HIF-1 activation under hypoxia is achieved by stabilization and/or expression of the α-subunit. Oxygen-dependent prolyl-4-hydroxylase domains (PHD) covalently modify a HIF-1α domain known as the oxygen-dependent degradation domain, by hydroxylating proline residues. Hypoxia attenuates proline hydroxylation due to inactivity of PHD in the absence of oxygen, resulting in HIF-1α stabilization and non-recognition by pVHL. The association of HIF-1α with the β-subunit leads to the formation of HIF-1 and expression of target genes that contain HRE (hypoxia responsive element) sites, including glucose transporters (GLUT-1 and GLUT-3), vascular endothelial growth factor (VEGF), which triggers neoangiogenesis, and, last but not least, CA

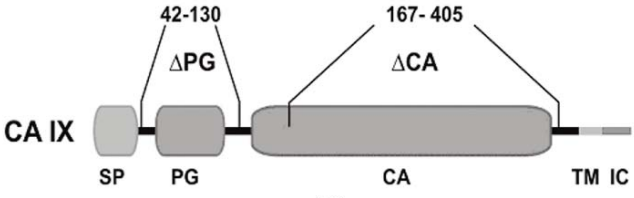
IX, which is involved in pH regulation and cell adhesion [13-15].

Expression of CA IX is strongly increased in many types of tumors, 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. In some cancer cells, the VHL gene is mutated leading to the strong upregulation of CA IX (up to 150-fold) as a consequence of constitutive HIF activation [13-15].

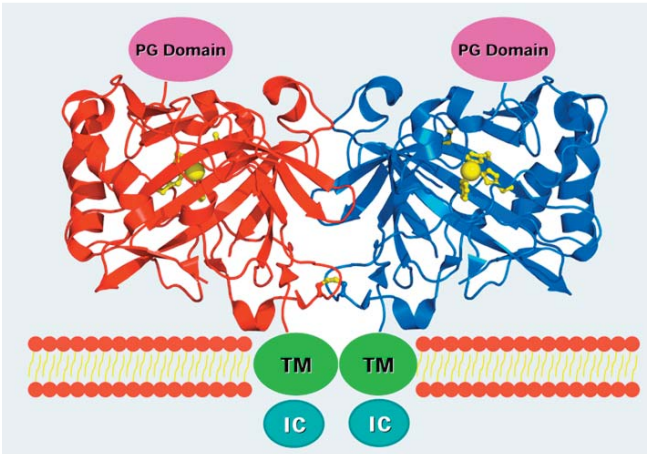
The overall consequence of the strong CA IX over-expression is the pH imbalance of the tumor tissue, with most hypoxic tumors having acidic extracellular pH (pHe) values around 6.5, in contrast to normal tissue which has characteristic pHe values around 7.4. The role played by CA IX in such acidification processes of hypoxic tumors was only recently demonstrated [16]. Using Madin-Darby canine kidney (MDCK) epithelial cells, Svastova and colleagues proved that CA IX is able to decrease the pHe of these cultivated cells. CA IX selective sulfonamide inhibitors (of type **1** and **2**, see later in the text) reduced the medium acidity by inhibiting the catalytic activity of the enzyme, and thus the generation of H^+ ions, binding specifically only to hypoxic cells expressing CA IX. Deletion of the CA active site was also shown to reduce the medium acidity, but a sulfonamide inhibitor did not bind to the active site of such mutant proteins [16]. Therefore, tumor cells decrease their pHe both by production of lactic acid (due to the high glycolysis rates), and by CO_2 hydration catalyzed by the tumor-associated CA IX, possessing an extracellular catalytic domain. Low pHe has been associated with tumorigenic transformation, chromosomal rearrangements, extracellular matrix breakdown, migration and invasion, induction of the expression of cell growth factors and protease activation [12-16]. CA IX probably also plays a role in providing bicarbonate to be used as a substrate for cell growth, whilst it is established that bicarbonate is required in the synthesis of pyrimidine nucleotides [16].

Structure and catalytic activity of CA IX

Unlike other CAs which usually possess one polypeptide chain comprising just the catalytic domain [1], CA IX is a multidomain, transmembrane protein possessing a more complex organization (Figure 1A), and consists of: i) a small intracytosolic (IC) tails whose function is unknown; ii) a short transmembrane segment (TM); iii) the extracellular catalytic (CA) domain, which shows high sequence homolgy with that of other α-CAs [1-4]; iv) a proteoglycan-like (PG) domain unique to this CA isozyme which is critical to cell adhesion processes in which this protein is involved [1-4], and v) a short signal peptide (SP). Many experiments have been performed with the PG-deleted (DPG) or catalytic domain-deleted (DCA) constructs of this protein in order to understand the role(s) of the various domains to its function and role in tumorigenesis [1-4].



(A)



(B)

Figure 1. (A) Domain organization of CA IX. (B) Dimeric structure of CA IX as recently reported by X-ray crystallography.

The crystal structure of the catalytic domain of human CA IX, was recently reported by this group [17]. The CA IX catalytic domain appeared as a compact globular domain, with an ovoid shape of 47 x 35 x 42 $Å^3$ in size. CA IX has a 3D fold characteristic of other α-CAs, for which the structure has been solved earlier [17], in which a ten-stranded antiparallel b-sheet forms the core of the molecule. An intramolecular disulfide bond, which is common to the other membrane-associated α-CAs (CA IV, CA XII and CA XIV), was observed between Cys23 and Cys203 [1,17]. This disulfide bridge has been reported to play an important role in the stabilization of the Pro201-Pro202 *cis*-peptide linkage [17], and whence in the orientation of the polypeptide loop containing Thr199; an amino acid residue critical for the catalytic cycle, as it orients opportunely the Zn^{2+}-bound nucleophile through hydrogen bond interactions involving also the residue Glu106, conserved in all α-CAs [1].

The CA IX active site is located in a large conical cavity, which spans from the surface to the center of the protein, with the zinc ion located at its bottom [17]. Two distinct regions made of hydrophobic or hydrophilic amino acids delimitate the two halves of the active site. In particular, Leu91, Val121, Val131, Leu135, Leu141, Val143, Leu198 and Pro202 define the hydrophobic region, while Arg58, Arg60, Asn62, His64, Ser65, Gln67, Thr69, and Gln92 identify the hydrophilic one. The crystallographic data confirmed the dimeric nature of the enzyme, which has been inferred from previous experiments reported by Hilvo et al. [8]. Indeed, two identical dimers, resulting from a Cys41-mediated intermolecular disulfide bond between two adjacent monomers, were observed in the asymmetric unit of the crystals. Two hydrogen bonds, involving Arg137 side chain and the carbonyl oxygen of Ala127, and numerous van der Waals interactions, contribute to the stabilization of the dimer, whose interface area extends over 1578.6 $Å^2$ [17]. The dimer assembly, by means of an intermolecular disulfide bond, is consistent with the proposed function of the enzyme in tissues where its expression has been reported, as both active sites of the dimer are clearly exposed to the extracellular medium, being thus able to efficiently hydrate CO_2. In addition, the N-terminal regions of both monomers are located on the same face of the dimer, while both the C-termini are situated on the opposite face (Figure 1B). This structural organization allows for concomitant positioning of both PG domains, at the entrance to the active site clefts, oriented toward the extracellular milieu to mediate cell interaction, and of both C-terminal transmembrane portions for proper CA IX anchoring to the cell membrane (Figure 1B). Furthermore, the position of the PG portion, at the border of the active site, suggests a further role of this domain in assisting CA domain-mediated catalysis. Indeed, as shown recently by our groups [22], the CO_2 hydrase activity of the CA IX full length has an optimum at a pH of 6.5 (typical of hypoxic solid tumors) whereas that of CA IX catalytic domain (similarly to that of CA I or CA II) has an optimum at pH around 7 [17,18]. Thus, the PG domain, which is rich in acidic amino acid residues (26 dicarboxylic amino acids, Asp and Glu, on a total of 58 amino acid residues forming the PG domain) was postulated to act as an intrinsic buffer of this enzyme, which facilitates the CO_2 hydration reaction at acidic pH values which are one of the main features of hypoxic tumors [17,18]. It should be also stressed that the dimeric structure of CA IX is strikingly different of the proposed dimeric structure of CA XII [17], as recently evidenced by some of us [17].

Many CAs present in the human body possess very high catalytic activity for the physiological reaction, i.e., hydration of carbon dioxide to bicarbonate and a proton, and CA IX is among such high activity isoforms [1,2], as seen from data of Table 1. CA IX together with CA II are highly active catalysts for the CO_2 hydration reaction being among the most

effective enzymes known in Nature, see Table 1 [8]. CA IX is also susceptible to inhibition by anions and sulfonamides/sulfamates/sulfamides, as all the other α-CAs [1], the inhibitors coordinating directly to the metal ion within the active site cavity and participating to various other favorable interactions with amino acid residues situated both in the hydrophobic and hydrophilic halves of the active site [1]. Many low nanomolar CA IX inhibitors were detected in the last several years [1,2].

CA IX inhibition in cancer therapy and diagnosis?

The involvement of CAs and of their sulfonamide inhibitors in cancer has been investigated recently, as mentioned above [1-4,17]. Many potent CA inhibitors derived from acetazolamide, ethoxzolamide and other aromatic/heterocyclic sulfonamide scaffolds were shown to inhibit the growth of several tumours cell lines *in vitro* and *in vivo* [1,19-39].

The compounds specifically designed for targeting the tumor-associated CA IX, may be classified as follows:

(i) fluorescent sulfonamides, used for imaging purposes and for determining the role of CA IX in tumor acidification;

(ii) positively or negatively-charged compounds, which cannot cross plasma membranes due to their charged character and thus inhibit selectively only extracellular CAs, such as CA IX;

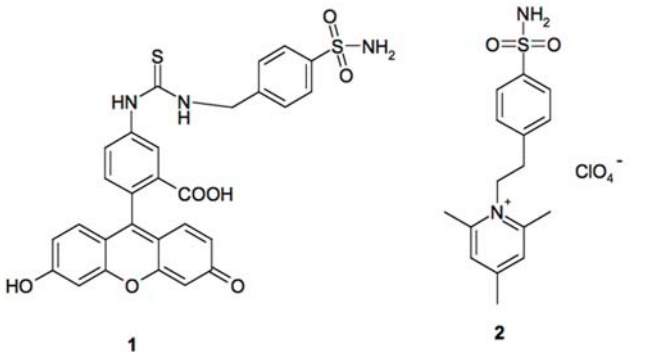
(iii) hypoxia-activatable compounds, which exploit the reducing conditions of hypoxic tumors to convert an inactive prodrug into an active CAI;

(iv) 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;

(v) diverse chemotypes than the sulfonamides and their bioisosteres, such as the phenols, coumarins and other compounds recently investigated as alternative CAIs to the classical types of inhibitors [40-42]. In the next paragraphs, some of the most notable such CAIs recently developed and investigated will be discussed.

Indeed, some of the most interesting CA IX inhibitors available at this time are the compounds investigated by Svastova et al [16] (possessing structures **1** and **2**) for their *in vivo* role in tumor acidification. These compounds present a special interest because derivative **1** is a fluorescent sulfonamide with high affinity for CA IX (K_I of 24 nM) [1,16], which was shown to be useful as a fluorescent probe for hypoxic tumors [1,16,28,30]. This inhibitor binds to CA IX only under hypoxia *in vivo*, in cell cultures or animals with transplanted tumors, see later in the text [16,26]. Although the biochemical rationale for this phenomenon is not understood at this point, these properties may be exploited for designing diagnostic tools for the imaging of hypoxic tumors [16,43]. Compound **2** on the other hand, belongs to type (ii) mentioned above, of permanently charged, membrane-impermeant derivatives, and is also a very strong CA IX inhibitor ((K_I of 14 nM) [36]. It belongs to the class of positively charged, membrane-impermeant compounds previously reported by one of our groups [1,36], which are highly attractive for targeting CA IX with its extracellular active site, since such compounds do not inhibit intracellular CAs, and may thus lead to drugs with less side effects as compared to the presently available compounds (acetazolamide is the prototypical one [1,40]), which indiscriminately inhibit all CAs [1]. The X-ray crystal structure of compound **2** in adduct with CA II has been reported recently [36]. It has been observed that the positively-charged pyridinium derivative **2** favorably binds within the enzyme active site, coordinating with the deprotonated sulfonamide moiety to the catalytically critical Zn(II) ion. It also participates in many other favorable interactions

with amino acid residues present in the active site cavity, among which a stacking between the trimethylpyridinium ring of the inhibitor **2** and the phenyl ring of Phe131, an amino acid important for the binding of inhibitors to CAs [36]. A similar binding was subsequently reported for the fluorescein derivative **1** [37]. Thus, such structures can be used for the rational drug design of more isozyme IX selective and potent CA inhibitors [1,37].

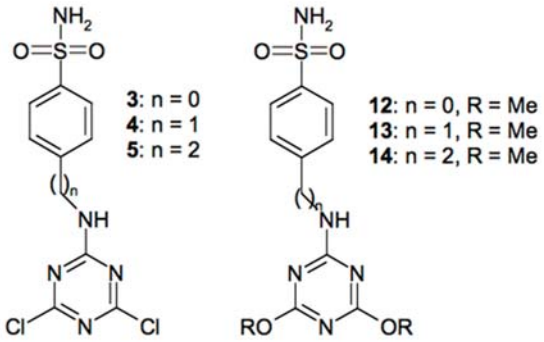


1

K_I (CA IX) = 24 nM
K_I (CA XII) = 5 nM

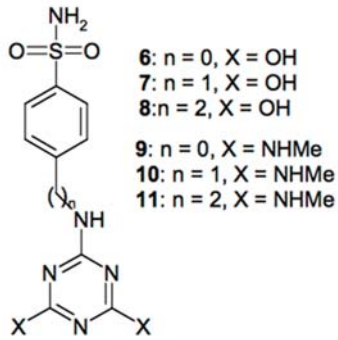
2

K_I (CA IX) = 14 nM
K_I (CA IX) = 7 nM



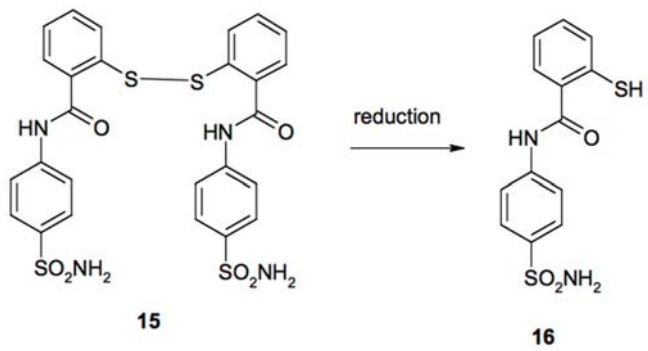
3: n = 0
4: n = 1
5: n = 2

12: n = 0, R = Me
13: n = 1, R = Me
14: n = 2, R = Me



6: n = 0, X = OH
7: n = 1, X = OH
8:n = 2, X = OH

9: n = 0, X = NHMe
10: n = 1, X = NHMe
11: n = 2, X = NHMe



15

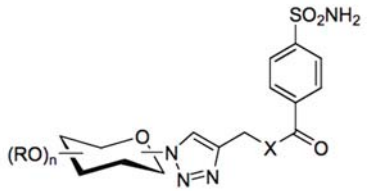
16

Among the many sulfonamide compounds developed ultimately for targeting CA IX, one should mention the 1,3,5-trazine derivatives incorporating benzenesulfonamide tails, of types **3-14**, which showed excellent selectivities for the inhibition of the tumor-associated isoform IX (low nanomolar inhibitors) over the cytosolic, ubiquitous one CA II, with selectivity ratios in the range of 166-706 [38]. Furthermore, by introducing amino acyl moieties in their molecules, some of these highly selective CA IX inhibitors also become membrane-impermeant and thus inhibit only transmembrane isoforms, such as CA IX and XII [38].

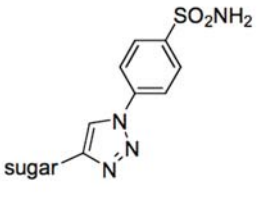
The bioreductive, disulfide-type derivative **15**, belonging to type (iii) of inhibitors mentioned above [44], which is converted to the thiol **16** by reduction under hypoxic conditions, is also of great interest due to its excellent selectivity ratio for the inhibition of the tumor-associated enzyme (CA IX) over the cytosolic ones, and for its properties of prodrug [48]. Indeed, the dimeric disulfide inhibitor **15** weakly inhibited CA IX (K_I of 650 nM), whereas the reduced, thiol **16** was a very potent CAI against the tumor-associated isoform (K_I of 9 nM) [44].

Many sulfonamides incorporating the highly polar sugar and triazole moieties have been reported ultimately by this and Poulsen's groups [29,45,46]. Among the highly effective CA IX/XII inhibitors were derivatives **17** - **19**, possessing various sugar (triazolyl)-benzenesulfonamide moieties in their molecules [29,45,46]. Most of them were low nanomolar CA IX and CA XII inhibitors but appreciably inhibited other CA isoforms too [29,45,46].

Some sulfamides possessing sugar tails, of types **20** and **21** have been also designed and showed to possess excellent CA IX and XII inhibitory activities [47]. Compound **20** is also a very weak CA II inhibitor, resolving thus the non-selectivity issue of



17: R = H, Ac; X = O, NH



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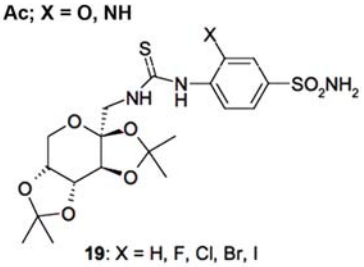
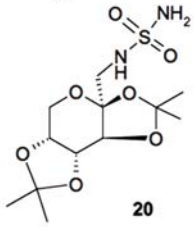


19: X = H, F, Cl, Br, I



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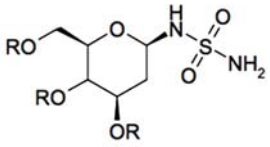
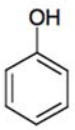


21: R = Ac, Bn

other sugar-sulfonamides mentioned above [47].



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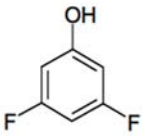

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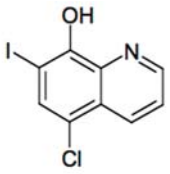

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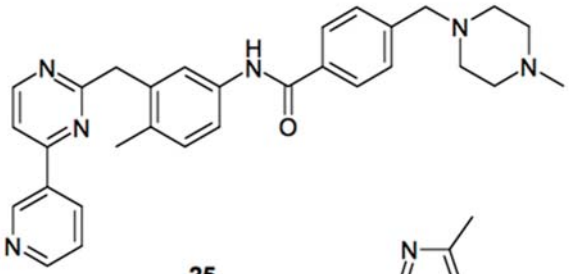

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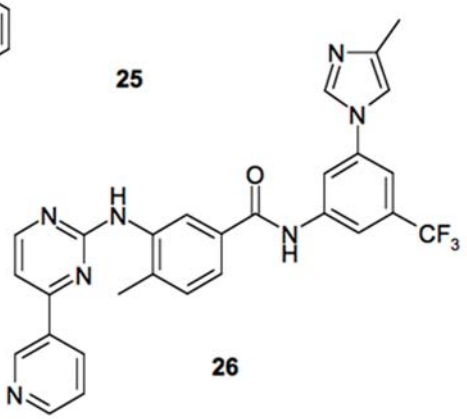


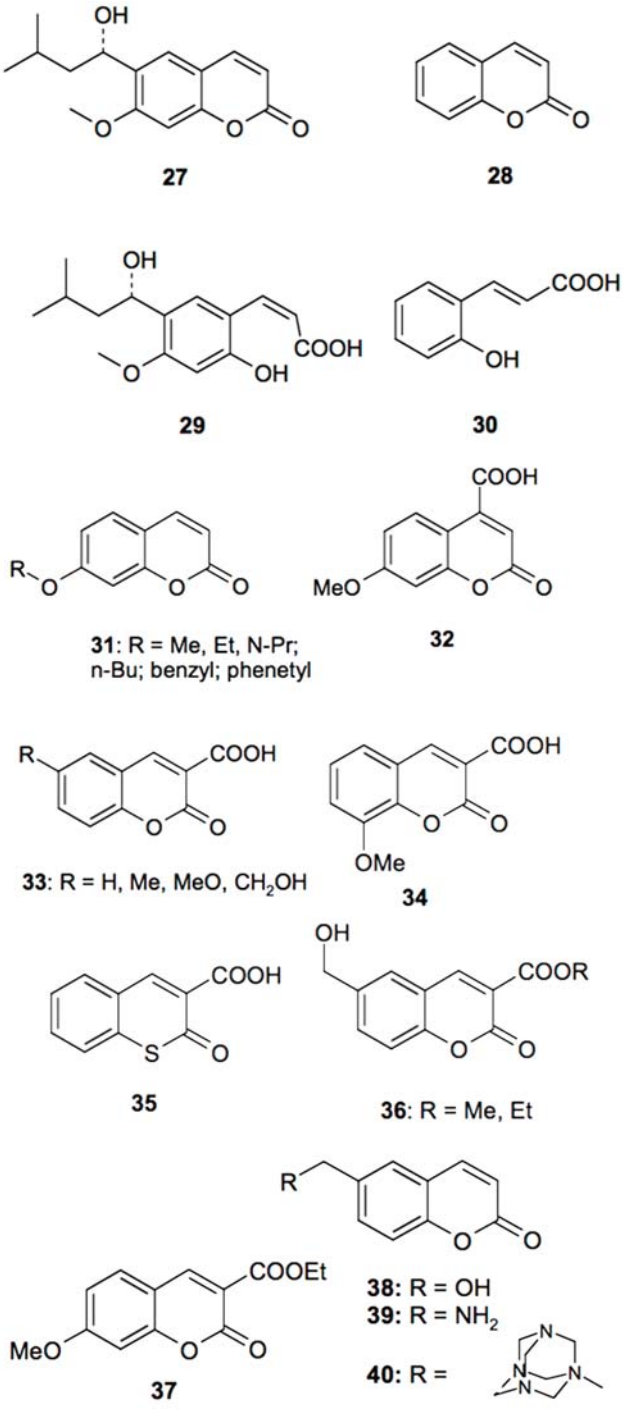
26

Phenols have been investigated only recently as inhibitors of the tumor-associated CA isozymes CA IX and XII, by one of our groups [48-50]. Phenols possess a different inhibition mechanism compared to sulfonamides and their bioisosteres, as they do not directly coordinate to the metal ion within the enzyme active site, but are anchored by means of at least two hydrogen bonds to the non-protein zinc ligand, i.e., a water molecule or hydroxide ion. Indeed, phenols are weaker inhibitors compared to sulfonamides, generally inhibiting CA IX and XII in the low micromolar (not low nanomolar) range. However, this class of relatively under-investigated CAIs deserves more studies, since several interesting leads emerged even with the few studies reported so far. Thus, simple phenol **22** and its substituted congeners **23** as well as clioquinol **24**, showed inhibitory activities of 5.6 - 9.4 mM against CA IX, and of 8.1 - 70.2 mM against CA XII, respectively [48-50]. No X-ray crystal structures are available so far for an adduct of anyone of these two isozymes with phenol derivatives.

The protein tyrosine kinase inhibitors (PTKIs) in clinical use as anticancer agents imatinib **25** and nilotinib **26** were recently shown to be nanomolar CA IX/XII inhibitors, although their inhibition mechanism is not yet understood, as no X-ray crystal structures for the adducts of any CA isozymes with the two compounds have been obtained so far. However, this is an important finding which may also explain the potent antitumor effects of the two compounds in many types of malignancies, which in addition to the PTK inhibition may be also due to the inhibition of the cancer-associated CA isoforms discussed in this review [42].

Coumarin and thiocoumarins were only recently discovered to act as CAIs, and their inhibition mechanism deciphered in detail by one of our groups [40,41]. We demonstrated recently that the natural product 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one **27** as well as the simple, unsubstituted coumarin **28** are hydrolyzed within the CA active site with formation of the 2-

hydroxy-cinnamic acids **29** and **30**, respectively, which represent the *de facto* enzyme inhibitors [40,41]. At least two other interesting facts emerged during these studies: (i) this new class of CAIs, the coumarins/thiocoumarins, binds in hydrolyzed form at the entrance of the CA active site and does not interact with the metal ion, constituting thus an entirely new category of mechanism-based inhibitors; and (ii) for the specific case of compound **27**, the formed substituted-cinnamic acid **29** was observed bound within the CA active site as the *cis* isomer, although these derivatives are stable in solution as *trans* isomers. However, for the simpler coumarin **28**, the *trans*-2-hydroxycinnamic acid **30** has been evidenced bound within the enzyme active site, by means of X-ray crystallography [40,41]. The tentative explanation for the unusual geometry of inhibitor **29** within the enzyme active site was that **29** would be too bulky as *trans*-isomer in the restricted space of the CA active site, while the unstable in solution *cis*-isomer would be stabilized when bound within the enzyme cavity. It should be also mentioned that coumarins **27** and **28** were potent inhibitors against some investigated human CA isoforms, which makes this entire class of derivatives of paramaount interest for designing novel applications for the CAIs.



In order to understand in greater detail the CA inhibition mechanism with the (thio)coumarins, which might be useful for the design of new pharmacological applications, we investigated thereafter a series of derivatives possessing various moieties substituting the (thio)coumarin ring in the 3-, 6-, 7-, 3,6-, 4,7 and 3,8- positions, of types **31-40** [41]. The most significant finding of this second study was that some coumarins are truly isoform-selective CAIs, inhibiting efficiently only one isoform of the 13 catalytically active ones found in humans. For example, thiocoumarin **35** and several coumarins (**33, 34, 36** and **37**) showed low nanomolar affinity for CA IX, with inhibition constants in the range of 45 - 98 nM [41]. These coumarins incorporate the 6-hydroxymethyl- and 3-ester moieties (**33** and **36, 37**), with no important differences of activity between the methyl and ethyl esters in this case. The monosubstituted derivatives **38** and **40** on the other hand contained either a compact (CH_2OH) or a rather bulky (hexamethylenetetramine) group in position 6 of the coumarin ring, which render our findings quite important, as it is clear that for effective CA IX inhibition, a large variations of structural motifs are

allowed in the 3- and 6-positions of the (thio)coumarin ring. Compound **40** was shown to be a low nanomolar inhibitor of only CA IX (K_I of 48 nM) whereas it inhibited in the micromolar range all other 12 CAs, a feature never evidenced before for a sulfonamide CAI [41]. Thus, this is the first CA IX-selective inhibitor ever reported up to now [41].

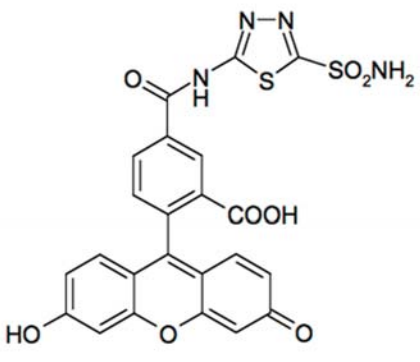
hCA XII, the other transmembrane isoform present in tumors, was poorly inhibited by **28**, whereas the natural product coumarin **27** was slightly more inhibitory (K_I of 48.6 mM) [40]. However, most of the investigated (thio)coumarins **31-40** were effective, low micromolar hCA XII inhibitors, with K_Is in the range of 3.2 - 9.0 mM. Thus, a lot of substitution patterns present in compounds **31-40** investigated here lead to effective hCA XII inhibitors, although compounds with nanomolar affinity for this isozyme were not evidenced so far [41].

***In vivo* proof-of-concept studies with CAIs as antitumor agents/diagnostic tools**

Pouyssegur's group [51] showed recently that in hypoxic LS174Tr tumor 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 tumor volume, with up-regulation of the second gene, that encoding for CA XII. Silencing of both CAIX and CAXII gave an impressive 85% reduction of tumor growth [51]. 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 [1,51].

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 [52]. By using membrane-impermeant derivatives of types **41** and **42**, based on the acetazolamide scaffold to which either fluorescein-carboxylic acid or albumin-binding moieties were attached, this group demonstrated the strong tumor retardation (in mice with xenografts of a renal clear cell carcinoma line, SK-RC-52) in animals treated for one month with these CA inhibitors [52]. These preliminary data of Pouyssegur's [51] and Neri's groups [52] show indeed the great promise of tumor growth inhibition with sulfonamides acting as CA IX/XII or related agents, making thus possible the development of alternative anticancer drugs [1].

Very recently, Neri's group [53] also published the proof-of-concept study showing that human monoclonal antibodies targeting CA IX can also be



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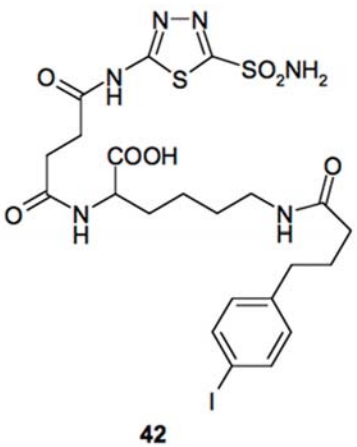

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used for imaging of hypoxic tumors. The generation of high-affinity human monoclonal antibodies (A3 and CC7) specific to hCA IX, using phage technology has been reported [53]. These antibodies were able to stain CA IX *ex vivo* and to target the cognate 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 characterised by little vascularity and low perfusion [53]. These new human anti-CA IX antibodies are expected thus to be non-immunogenic in patients with cancer and might serve as broadly applicable reagents for the non-invasive imaging of hypoxia and for pharmacodelivery applications [57]. The same conclusion has been reached by our group by using small molecule CA IX-selective inhibitors of the type **1** [43]. 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 [43]. 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 (i.e., a derivative with the same structure as compound **1** but without the SO_2NH_2 moiety). Administration of nicotinamide and carbogen, decreasing acute and chronic hypoxia, respectively, and 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 tumour reoxygenation. Such *in vivo* imaging results confirm previous *in vitro* data demonstrating that CAI binding and retention require exposure to hypoxia. Fluorescent labelled sulfonamides may thus provide a powerful tool to visualize hypoxia response in solid tumors. An important step was thus made towards clinical applicability, indicating the potential of patient selection for CA IX-directed therapies.

CONCLUSIONS

In conclusion, with its overexpression in many cancer tissues and not in their normal counterparts, CA IX constitutes an interesting target for novel approaches in the design of anticancer therapies. CA IX is crucial for tumor pH regulation contributing both to the acquisition of metastasic phenotypes and to chemoresistance. Consequently, further research needs to be done in the field of the tumor-associated CA IX in order to better understand its exact role in cancer. CA IX selective inhibitors are now available and they constitute interesting tools for studying the physiological and/or pathological effects of this enzyme. Indisulam, a sulfonamide anticancer drug, is actually in clinical trials phase II. In addition to its hypothetical action in perturbing the cell cycle, it provides a potent inhibition of CA. However, given that multiple pathways contribute to tumor growth, anti-tumor activity may be increased by agents targeting multiple pathways including CA IX, or by the combination of several agents to allow inhibition of multiple pathways. Recently, the design of CA IX selective inhibitors containing a variety of scaffolds and with interesting physico-chemical properties has also been achieved. New sulfonamides, sulfamates and sulfamides have been synthesized with some of these derivatives strongly inhibiting CA IX, with inhibition constants in the low nanomolar, and also with excellent selectivity ratios for the inhibition of the tumor-associated over the cytosolic CAs. Thus, many biochemical, physiological and pharmacological novel data point to the possible use of inhibition of the tumor associated isozyme CA IX in the management of hypoxic tumors, which do not

respond to the classical chemo- and radiotherapy. There are possibilities of developing both diagnostic tools for the non-invasive imaging of these tumors and therapeutic agents, that probably perturb the extratumoral acidification in which CA IX is involved. Much pharmacologic work is however warranted in order to understand whether a successful new class of antitumor drugs may be developed starting from these preliminary but encouraging observations.

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