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Published in:

Trends in Pharmacological Sciences

Publication date:

2006

Document Version

Early version, also known as pre-print

[Link to publication](#)

Citation for pulished version (HARVARD):

Thiry, A, Dogné, J-M, Masereel, B & Supuran, CT 2006, 'Targeting tumor-associated carbonic anhydrase IX in cancer therapy', *Trends in Pharmacological Sciences*, vol. 27, no. 11, pp. 566-573.

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Targeting tumor-associated carbonic anhydrase IX in cancer therapy

Anne Thiry¹, Jean-Michel Dogné¹, Bernard Masereel¹ and Claudiu T. Supuran²

¹ Drug Design and Discovery Center, University of Namur, 61 rue de Bruxelles, B-5000 Namur, Belgium

² Polo Scientifico, Laboratorio di Chimica Bioinorganica, Room 188, Università degli Studi di Firenze, Via della Lastruccia 3, 50019 Sesto Fiorentino, Florence, Italy

Carbonic anhydrase isoform IX (CA IX) is highly overexpressed in many types of cancer. Its expression, which is regulated by the HIF-1 transcription factor, is strongly induced by hypoxia and correlates with a poor response to classical chemo- and radiotherapies. CA IX contributes to acidification of the tumor environment by efficiently catalyzing the hydration of carbon dioxide to bicarbonate and protons, thereby leading to acquisition of metastatic phenotypes and chemoresistance to weakly basic anticancer drugs. Inhibition of this enzymatic activity by specific inhibitors, such as the sulfonamide indisulam, reverts these processes, establishing a clear-cut role for CA IX in tumorigenesis. Thus, selective CA IX inhibitors could prove useful for elucidating the role of CA IX in hypoxic cancers, for controlling the pH imbalance in tumor cells and for developing diagnostic or therapeutic applications for tumor management. Indeed, fluorescent inhibitors and membrane-impermeant sulfonamides have recently been used as proof-of-concept tools, demonstrating that CA IX is an interesting target for anticancer drug development.

Introduction

The α -carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in higher vertebrates including humans [1]. So far, 16 isozymes have been identified that differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. Some of these isozymes are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA VB), and one is secreted in saliva (CA VI). The recently reported CA XV isoform is not expressed in humans or in other primates (where it is encoded by a pseudogene), but it is abundant in rodents and other higher vertebrates [2]. Three acatalytic forms are also known, called CA-related proteins (CARPs): CARP VIII, CARP X and CARP XI [1]. In humans, CAs are present in various tissues such as the gastrointestinal tract, the reproductive tract, the nervous system, kidneys, lungs, skin and eyes, among others [1].

These Zn^{2+} enzymes have crucial physiological roles [1]. Most CAs are 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]. Membrane-bound CAs, such as CA IX, have an extracellular active site and can provide the H^+ or HCO_3^- ions formed during catalytic turnover for various physiological processes, among which is extracellular acidification [1]. Many CA isoforms are thus involved in essential cellular processes such as respiration and acid–base regulation, electrolyte secretion, bone resorption, calcification and biosynthetic reactions that require HCO_3^- as a substrate (e.g. lipogenesis, gluconeogenesis and ureagenesis) [1].

Recently, it has been shown that two CA isozymes (CA IX and CA XII) are prominently associated with and overexpressed in many tumors, where they are involved in crucial processes connected with cancer progression and response to therapy [3–5]. The first CA found to be associated with cancers was CA IX, as reported in 1992 by Pastoreková *et al.* [3,5]. The second one, CA XII, was subsequently shown to be coexpressed with CA IX in several tumor tissues and was also found in a wider range of normal tissues [3]. Because many CA isoforms (including CA IX) are associated with anion exchangers or sodium bicarbonate cotransporters, with which they form metabolons (protein complexes in which the two proteins are in close functional and physical contact) [6], it has been both suggested and demonstrated that they are important in the transport of ions across biological membranes and in the secretion of electrolytes in many tissues or organs [1,6].

In this review we focus on CA IX, an interesting protein that was initially reported as a ‘tumor antigen’ and has been subsequently shown to belong to the CA gene family. CA IX possesses a more complicated organization of the protein chain compared with the classical CA isoforms, such as CA I or CA II, identified originally [1,3]. Subsequent work has shown that CA IX is expressed in only a few normal tissues, but is ectopically induced and highly overexpressed in many types of tumor, mainly owing to its strong transcriptional activation by hypoxia via the transcription factor hypoxia-inducible factor (HIF-1) [7–9]. CA IX has since been shown to serve as a surrogate marker of hypoxia and as a prognostic indicator for many cancers, which unfortunately are often hypoxic [3,9,10]. CA IX has also been shown to be involved in both the pH regulation and cell-adhesion processes caused by tumor metabolism [3,9].

Corresponding author: Supuran, C.T. (claudiu.supuran@unifi.it).
Available online 25 September 2006.

Box 1. CA IX and its regulation in hypoxia

The transcription factor HIF-1 is a heterodimer consisting of an inducible subunit (HIF-1 α) and a constitutively expressed subunit (HIF-1 β) [7]. In keeping with its name, HIF-1 becomes active under conditions of hypoxia by means of stabilization and/or expression of the HIF-1 α subunit, which is unstable and almost undetectable under normal conditions. Proteins with an oxygen-dependent prolyl-4-hydroxylase domain (PHD) covalently modify a region in HIF-1 α , known as the 'oxygen-dependent degradation domain', by hydroxylating proline residues. Hydroxylated HIF-1 α subsequently forms hydrogen bonds with side chains on the von Hippel-Lindau tumor suppressor protein (pVHL), which in turn promotes polyubiquitination of HIF-1 α , followed by degradation by the 26S proteasome [7].

Under hypoxic conditions, binding of pVHL to HIF-1 α is inhibited, resulting in accumulation of HIF-1 α and its dimerization with the constitutive HIF-1 β subunit. Thus, hypoxia attenuates proline

hydroxylation owing to the inactivity of PHDs in the absence of oxygen, resulting in stabilization of HIF-1 α and its non-recognition by pVHL. Association of HIF-1 α with the HIF-1 β subunit leads to the formation of HIF-1, resulting in the expression of targets that contain hypoxia-responsive element (HRE) sites [7]. Target genes include, among others, glucose transporters (GLUT-1 and GLUT-3), which contribute to glucose metabolism; vascular endothelial growth factor, which triggers neoangiogenesis; and last but not least, CA IX, which is involved in pH regulation and cell adhesion [3,9] (Figure 1). In some cancer cells, the gene encoding pVHL is mutated, leading to the strong upregulation of CA IX (up to 150-fold) as a consequence of constitutive HIF activation [8]. In a renal carcinoma cell line, CA IX is also regulated by promoter methylation – an epigenetic mechanism involved in the control of many cancer-related genes [26].

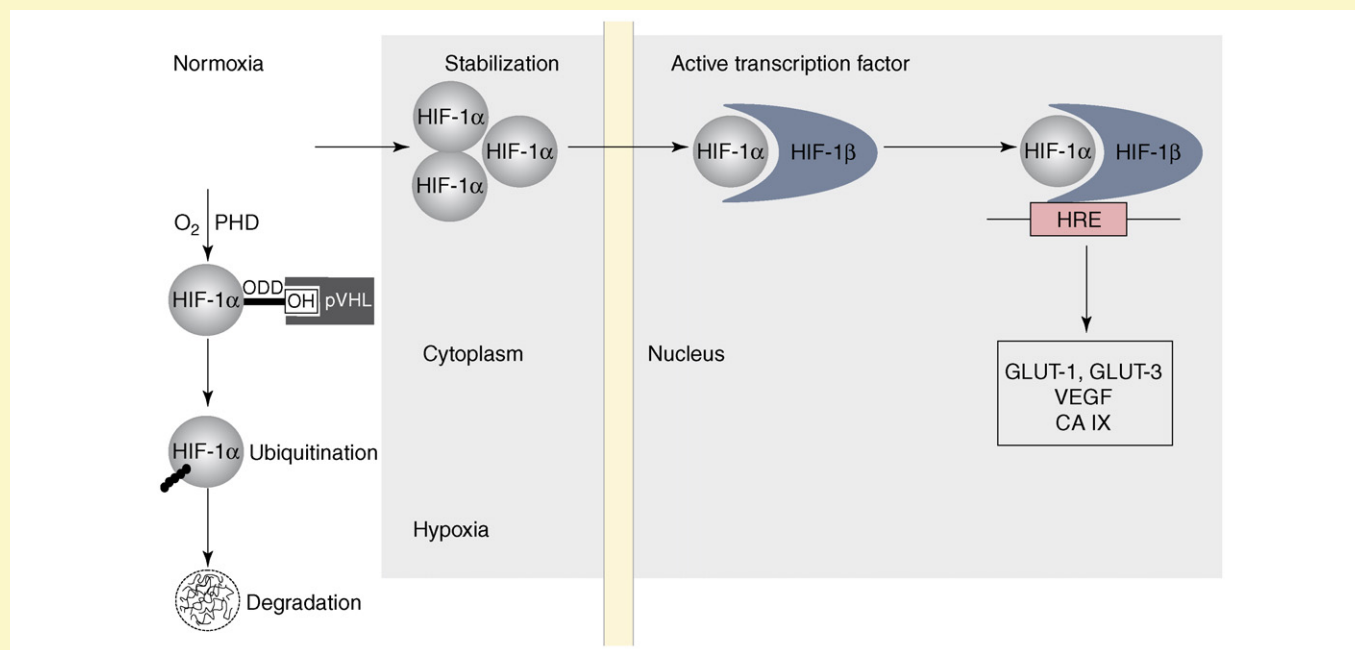


Figure 1. Regulation of hypoxia-induced gene expression mediated by the HIF-1 transcription factor. Adapted, with permission, from Refs [3,48].

Numerous potent (and sometimes selective) inhibitors of CA IX have been developed in the past few years on the basis of the idea that inhibition of tumor acidification processes and re-establishment of a more normal pH might lead to regression of the tumor, especially when used in combination with classical anticancer drugs. Indeed, preliminary results have shown that this is the case, establishing CA IX as a novel drug target for the development of both diagnostic tools and therapeutic agents.

Each of these aspects will be discussed here, with emphasis on how inhibitors of an enzyme that has been known for decades (the classical CA isoforms were discovered in the 1930s and characterized in the 1960s [1]) could lead to completely new applications owing to the revolutionary discovery of Pastoreková *et al.* [5] that a protein with this type of simple and unexpected enzymatic activity is so abundant in tumors.

Cancer-related CA IX

In tumors, hypoxia stems from an inadequate supply of oxygen, which is primarily a pathophysiological consequence of structurally and functionally disturbed microcirculation and the deterioration of O₂ diffusion processes

[7]. Solid tumor growth is limited by vascularization, which is necessary for oxygen and nutrient supply.

Tumor hypoxia usually occurs at a distance of 100–200 μ m from blood vessels [7,8] and seems to be strongly associated with tumor propagation, malignant progression and resistance to chemo- and radiotherapy [6,8]. Hypoxia can regulate the expression of several genes, including that encoding CA IX, through binding of the transcription factor HIF-1 to a hypoxia-responsive element in the gene. CA IX is thus a target of HIF-1 and functions as an intrinsic marker of hypoxia in a wide spectrum of tumors [9,10].

CA IX expression and distribution

The expression of CA IX is upregulated by hypoxia (through the HIF-1 activation cascade) and is downregulated by the wild-type von Hippel-Lindau tumor suppressor protein [8,9] (Box 1).

The distribution of CA IX in human tissues is unusual: this isoform is significantly expressed in only a few normal tissues, and this expression is either decreased or lost during carcinogenesis [3]; however, CA IX is ectopically expressed in numerous tumors, predominantly

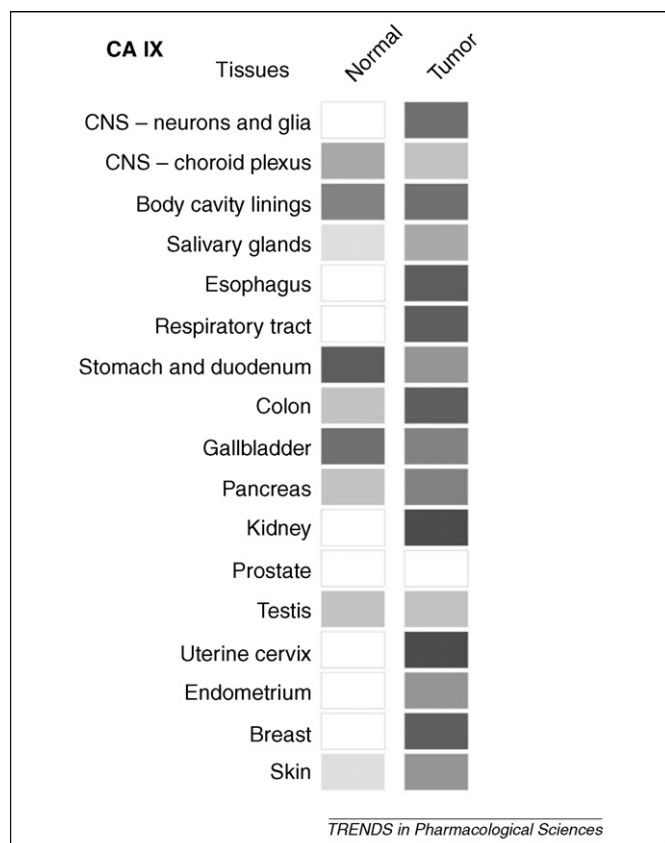


Figure 1. Distribution of CA IX in human normal and tumor tissues. Adapted, with permission, from Ref. [3].

carcinomas, that are mostly derived from tissues that do not normally express CA IX (Figure 1). This atypical differential expression pattern enables us to designate CA IX as a tumor-associated protein and to use it as a biomarker of cancer [3,11–20].

Of the normal tissues that do contain high levels of CA IX, mucosa of the glandular stomach shows the most abundant expression [3]. Therein, CA IX is localized to the basolateral surfaces of all types of epithelial cell. Its roles in this organ might involve maintaining both gastric mucosa integrity and the balance between cell differentiation and proliferation [3]. Expression of CA IX frequently declines or disappears in gastric cancers, possibly as a consequence of neoplastic changes that involve dedifferentiation [3]. Such studies suggest that CA IX has a crucial role as a differentiation factor involved in morphogenesis and homeostasis of the gastric epithelium [3]. Gallbladder epithelial cells but not hepatocytes also show high levels and basolateral localization of CA IX [3]. In biliary epithelial tumors, expression of CA IX also decreases with an increasing grade of malignancy, indicating a similar relationship of CA IX to cell differentiation as described for the gastric mucosa [3].

Furthermore, CA IX has been found in the basolateral membranes of acinar and ductal pancreatic cells: its expression is increased in hyperplastic areas and persists in some malignant pancreatic tumors [3]. CA IX is present in the intestinal epithelium, where it is confined to the deep cryptal areas characterized by high cell proliferation [3]. Expression of CA IX is increased in superficial

colorectal adenomas and in both superficial and deep parts of malignant carcinomas [3]. CA IX expression is also retained in both mesotheliomas and some germ cell tumors. The only organ that does not show any CA IX positivity, irrespective of whether it is healthy or cancerous, is the prostate gland [3].

Human tumors showing a high ectopic expression of CA IX in a significant proportion of specimens include carcinomas of the uterine cervix, kidney, esophagus, lung, breast, brain and vulva, among others [3,11–20] (Figure 1). Comparison of the cDNA sequence of CA IX expressed in HeLa cervical carcinoma cell lines with that expressed in normal stomach found no differences between the two proteins [3], indicating that mutations are not responsible for the association of CA IX with tumors and suggesting that cancer-related regulatory pathways are involved in controlling CA IX expression [3]. Furthermore, as part of the hypoxic acidification machinery, CA IX has been suggested to facilitate nucleolar sequestration of the von Hippel–Lindau tumor suppressor protein and activation of HIF-1, which together represent a recently described pH-dependent mechanism with a proposed protective role in reoxygenated cells [21].

Structure and catalytic activity of CA IX

Unlike other CA isoforms that possess one polypeptide chain comprising only the catalytic domain [1], CA IX is a multidomain, transmembrane protein with a more complex organization (Figure 2). This isoform comprises (i) a small intracytosolic tail with an unknown function; (ii) a short transmembrane segment; (iii) the extracellular catalytic domain, which shows high sequence homology to the catalytic domain of other α -CAs [3,5]; (iv) a proteoglycan-like domain unique to CA IX, which is crucial to the cell-adhesion processes in which this protein is involved [3]; and (v) a short signal peptide. Many experiments (see later) have been performed with protein constructs lacking either the proteoglycan-like domain or the catalytic domain in order to understand the relevance of the various domains to its function and role in tumorigenesis [3,5,22–24]. The X-ray crystal structure of CA IX is unknown, but polyacrylamide gel electrophoresis experiments have led to the conclusion that CA IX forms trimers linked by disulfide bonds [22–24].

Many CAs expressed in humans have high catalytic activity for their physiological reaction (i.e. hydration of CO_2 to HCO_3^- and H^+), and CA IX is among these high-activity isoforms [1,3]. Amino acid sequencing of CA IX has shown that it contains four cysteine residues, three of them in the catalytic domain and the fourth in the region proximal to the transmembrane anchor (Figure 2). Cys156 and Cys336 are in positions analogous to

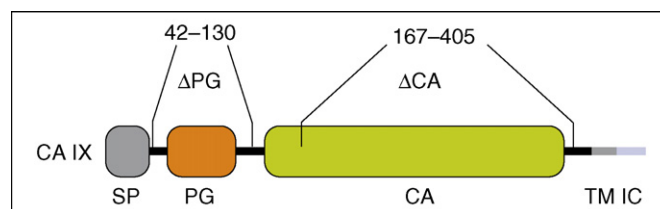


Figure 2. Domain organization of the CA IX protein [3,5].

intramolecular disulfide-bond-forming cysteines in other CA isoforms [1,3], whereas the other two, Cys174 and Cys409, probably participate in the formation of intermolecular disulfide bonds in the above-mentioned trimers. A single *N*-glycosylation site has been predicted in the catalytic domain at position 346. The cytoplasmic tail of CA IX contains three putative phosphorylation sites (Thr443, Ser448 and Tyr449), the possible regulatory roles of which remain to be investigated [3,9]. As in all other α -CA isozymes investigated so far [1], the catalytic domain in CA IX contains an essential Zn^{2+} ion coordinated by three histidine residues (His94, His96 and His119 in CA I numbering [1]) and a water molecule, which, by deprotonation assisted by the active-site residue His64 (CA I numbering for historical reasons [1]), leads to the zinc hydroxide form that functions as the nucleophilic species in the catalytic cycle [25].

As mentioned above, CA IX is a highly active human α -CA, and its catalytic properties for the CO_2 hydration reaction are comparable to those of CA II, a perfectly evolved catalyst [1], with the kinetic parameters $k_{\text{cat}} = 3.8 \times 10^5 \text{ s}^{-1}$ and $k_{\text{cat}}/K_m = 5.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (where k_{cat} is the catalytic rate constant and K_m is the Michaelis constant) [1,25]. CA IX is susceptible to inhibition by anions and sulfonamides or sulfamates, as are all of the other α -CA isoforms [1,25]: the inhibitors coordinate the Zn^{2+} ion directly in the active-site cavity (see later) and participate in various other favorable interactions with amino acids situated in both the hydrophobic and the hydrophilic halves of the active site [1,9]. Many low-nanomolar inhibitors of CA IX have been detected in the past several years [1,9,25].

Roles of CA IX in cancer biology

In tumors other than renal cell carcinomas, CA IX has a predominantly perinecrotic distribution that is typical of hypoxia-regulated proteins [3,11,14–20]. Necrosis usually develops in regions that are most distant from the functional blood vessel and suffer from long-lasting severe hypoxia (or even anoxia), which does not permit the affected cells to survive [3]. By contrast, cells that are localized nearer the functional blood vessel (in the perinecrotic regions) and are not exposed to such high stress can adapt to low-oxygen conditions via the induction of many HIF-1 targets including CA IX.

Studies investigating the relationship of CA IX to hypoxia in a large series of specimens of different tumor types have confirmed the perinecrotic pattern of CA IX expression [3]. For example, expression of CA IX highly correlates with the level of hypoxia measured in cervical tumors by needle electrodes or by incorporation of the chemical hypoxia marker pimonidazole [3], with necrosis and high tumor grade in breast and lung carcinomas [3], and with necrosis, microvascular density and advanced stage in head and neck cancer [3]. On the basis of these data, CA IX has been proposed as a reliable intrinsic marker of tumor hypoxia [3,26]. Such a marker would be extremely helpful in clinical practice because its detection by the monoclonal antibody M75 developed by Pastoreková and Pastorek [3] does not require an invasive approach (e.g. needle electrodes) or metabolic incorporation (e.g.

pimonidazole) before biopsy and can be standardized for routine use [3].

As mentioned earlier, hypoxia, via the HIF-1 cascade, leads to strong overexpression of CA IX in many tumors with the overall consequence that the imbalance in pH in the tissue is increased. Indeed, most hypoxic tumors are acidic ($\text{pH} \approx 6$), in contrast to normal tissues ($\text{pH} \approx 7.4$). The role of CA IX in the acidification processes of hypoxic tumors has been demonstrated only recently by one of our groups and by Pastoreková's group [27]. Using Madin–Darby canine kidney epithelial cells constitutively expressing human CA IX, Svastova *et al.* [27] showed that CA IX can decrease the extracellular pH (pH_e) of the cultivated cells. CA-IX-selective sulfonamide inhibitors (type 1 and 2; see later) reduced acidification of the medium, by inhibiting the catalytic activity of the enzyme and thus the generation of H^+ ions, and bound only to hypoxic cells expressing CA IX (and not to the same cells expressing a similar level of CA IX under normal conditions) [27]. In addition, deletion of the active site in the catalytic domain of CA IX was shown to reduce acidification of the medium, but the sulfonamide inhibitors did not bind to the active site of these mutant proteins [27].

It is thus clear that tumor cells decrease their pH_e both by the production of lactic acid (owing to the high glycolysis rate), and by CO_2 hydration catalyzed by the tumor-associated CA IX isoform, which possesses an extracellular catalytic domain [28] (Figure 3). Low pH_e has been associated with tumorigenic transformation, chromosomal rearrangements, extracellular matrix breakdown, migration and invasion, induction of cell growth factors, and protease activation [3,29].

In a recent study using RNA interference, Robertson *et al.* [30] found interesting evidence supporting the action of CA IX in tumor cell invasion. In normal tissues such as gastrointestinal epithelial cells, CA IX regulates morphogenesis and homeostasis by controlling cell proliferation and differentiation [25]. CA-IX-deficient mice constructed by targeted disruption of the *Ca9* gene (encoding CA IX), show gastric hyperplasia with aberrant cell lineage development. The unique proteoglycan-like domain present in CA IX has been thus implicated in cell adhesion and differentiation [22,23]. CA IX is probably also involved in providing bicarbonate used as a substrate for cell growth, because it has been established that bicarbonate is required in the synthesis of pyrimidine nucleotides [31].

Chemoresistance

The presence of an H^+ gradient across the membrane of tumor cells also has interesting implications for chemotherapy [32]. Acidification of the solid tumor milieu might decrease the uptake of weakly basic anticancer drugs, leading to chemoresistance [33]. Most anticancer drugs are transported by either active transport or passive diffusion into cells, where they frequently undergo further metabolism [29]. Because all of these processes are pH sensitive, the cytotoxic activity of anticancer drugs could depend on both intracellular pH (pH_i) and pH_e .

In particular, drugs that are weakly ionized enter cells by passive diffusion in their non-ionized form. Such drugs will tend to partition preferentially across the cell

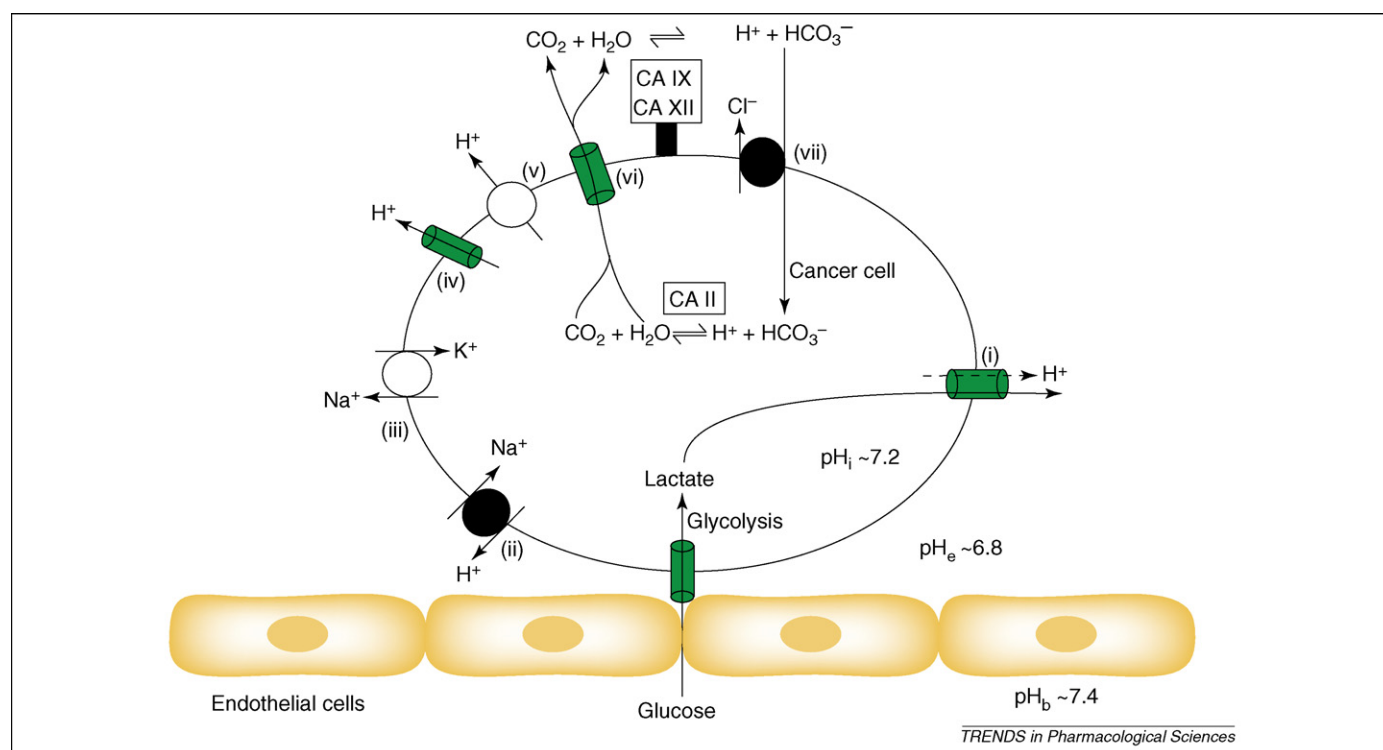


Figure 3. Molecular mechanisms involved in pH regulation and ion transport in cancer cells. Lactic acid is produced from glucose by glycolysis. The lactate and H^+ ions are exported from the extracellular fluid by the monocarboxylate carrier (i). The Na^+ - H^+ antiporter (ii), which is activated in transformed cells, exports H^+ and imports Na^+ , contributing to high levels of intracellular Na^+ , a common feature in tumor cells. Several other protein machines are used by cancer cells to maintain intracellular neutrality, including ATP-dependent Na^+ - K^+ antiporters (iii), H^+ channels (iv), and the plasma membrane proton pump H^+ -ATPase (v). H_2O and CO_2 provided by the cytosolic isozyme CA II are exported through aquaporins and used by cell-surface CAs to produce HCO_3^- (vi). Conversely, CA IX and CA XII produce HCO_3^- ions outside the cell, which are transported inside by HCO_3^- - Cl^- anion exchangers (vii) and used by the cytosolic isozyme CA II. Adapted, with permission, from Refs [3,12,29].

membrane into the compartment where their ionized form predominates [29]. For example, primary amines tend to be excluded from, and carboxylic acids tend to be accumulated by, the more alkaline intracellular compartment. In addition, the toxicity of several drugs is sensitive to variations in pH as a result of various mechanisms that are not dependent on ionization-dependent diffusion through the cell membrane [29]. For example, the toxicity of melphalan is enhanced by pH_e modification without an increase in uptake or accumulation.

Various strategies for altering pH_i and pH_e have been tried in the quest for new anticancer therapies for solid tumors. Enhancing the pH_e has been found to improve the cytotoxic efficacy of the weakly basic drugs mitoxantrone, paclitaxel and topotecan [32]. Furthermore, the delay in tumor growth induced by the weak base doxorubicin is enhanced by increasing the pH_e of tumors after chronic ingestion of a sodium bicarbonate solution [33]. CA inhibitors have been previously shown to elicit synergistic effects when used in combination with other chemotherapeutic agents in animal models [34]. Targeting CA IX with specific inhibitors or antibodies should contribute, on the one hand, to enhancing the action of weakly basic drugs and, on the other hand, to reducing the acquisition of metastatic phenotypes by controlling the pH imbalance in the tumor cells.

Targeting CA IX in cancer therapy

The involvement of some CAs and of their sulfonamide inhibitors in cancer has been investigated recently

[1,3,9,27]. Many potent CA inhibitors derived from acetazolamide, ethoxzolamide and benzenesulfonamides have been shown to inhibit the growth of several tumor cell lines *in vitro* and *in vivo* [35,36]. Many of the cell lines used are known to express either of the tumor-associated CA IX and CA XII isoforms, or both; thus, it is not impossible that the observed antiproliferative effects of sulfonamides are mediated by inhibition of these cancer-related isozymes [3,35,36].

However, CA IX and CA XII seem to be functionally linked to tumor metabolism and microenvironment *in vivo* rather than *in vitro* [9]. Xiang *et al.* [37] have demonstrated that acetazolamide can suppress tumor metastasis *in vivo*, at least in part, by both inhibiting the expression of aquaporin, a water channel protein that might be implicated in vascular permeability and interstitial fluid pressure in tumors, and strongly inhibiting several CA isozymes, among which are the tumor-associated isoforms CA IX and XII, and the ubiquitous cytosolic isoform CA II [1,9,27,35,36]. Aquaporin is also thought to be involved in CO_2 transport processes, and suppression of its expression by sulfonamide or sulfamate CA inhibitor drugs might constitute an additional mechanism (poorly understood at this moment) explaining the antitumor effects of such compounds [36–38]. Nevertheless, the antiproliferative effect of CA inhibitors might also be due to their effect on other CA isoforms such as CA II or CA V, which provide the bicarbonate substrate for cell growth in carboxylation reactions involved in lipogenesis, nucleotide biosynthesis and gluconeogenesis,

among others, thereby limiting the unrestrained proliferation of the tumor cells [1,9,31].

Indisulam and other CA IX inhibitors

Indisulam is a sulfonamide derivative (originally called E7070) discovered by Owa's group [39] through screening studies that demonstrated its powerful anticancer activity. Indisulam has been recently shown to act as a nanomolar inhibitor of CA IX [38]. Its mechanism of action is not clear, however, because the drug is involved in perturbation of the cell cycle in the G1 and/or G2 phases, in downregulation of cyclins, in reduction of CDK2 activity, in inhibition of phosphorylation of the retinoblastoma protein and in differential expression of molecules known to participate in cell adhesion, signaling and immune response, in addition to its inhibitory properties against CA IX [39,40]. Indisulam shows *in vivo* efficacy against human tumor xenografts in nude mice, where it exhibits significant antitumor effects [41], and has progressed to Phase I and Phase II clinical trials for treatment of solid tumors.

Some of the most interesting CA IX inhibitors currently available, among the many derivatives reported [42–44], are the compounds investigated by Svastova and *et al.* [27] for their *in vivo* role in tumor acidification (Figure 4, structures 1 and 2). These compounds are especially interesting because derivative 1 is a fluorescent sulfonamide with high affinity for CA IX (inhibition constant, $K_i = 24$ nM) [43] that has been shown to be useful as a fluorescent probe for hypoxic tumors [27]. This inhibitor binds to CA IX only under conditions of hypoxia *in vivo* [27]. Although the biochemical rationale for this phenomenon is not understood at present, these properties might be exploited for designing diagnostic tools for the imaging of hypoxic tumors [27,44,45].

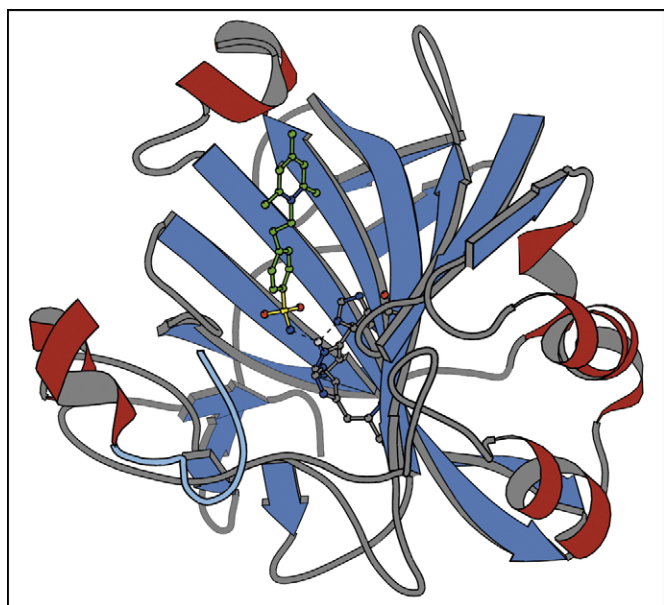


Figure 4. Sulfonamide inhibitors demonstrate the involvement of CA IX in tumor acidification processes. The sulfonamide inhibitors used include the fluorescent compound 1 and the membrane-impermeant pyridinium 2 [27,32–45]. Shown is the X-ray crystal structure of the complex between CA II and 2, revealing the inhibitor (green), the Zn^{2+} -coordinating residues His94, His96, and His119, and the Zn^{2+} ion (in ball-and-stick representation). The protein backbone is shown in ribbon representation [44].

By contrast, compound 2, which is also a strong CA IX inhibitor ($K_i = 14$ nM) [44], belongs to a class of positively charged, membrane-impermeant compounds previously reported by Scozzafava, A. *et al.* [46]. These compounds are highly attractive for targeting CA IX with its extracellular active site, because they do not inhibit intracellular CAs and thus might lead to drugs with fewer side-effects compared with those that are currently available (acetazolamide is the prototypical one), which indiscriminately inhibit all CA isoforms [25]. The X-ray crystal structure of compound 2 in adduct with CA II (which has an active site similar to that of CA IX, as shown recently by homology modeling [43,47]) has been solved [44]. The positively charged pyridinium derivative 2 favorably binds in the enzyme active site, where the deprotonated sulfonamide moiety coordinates with the catalytically essential Zn^{2+} ion (Figure 4). It also participates in many other favorable interactions with amino acids in the active site cavity, among which is a stacking between the trimethylpyridinium ring of the inhibitor 2 and the phenyl ring of Phe131, a residue that is important in the binding of inhibitors to CAs [44]. A similar mode of binding has subsequently been reported for the fluorescein derivative 1 [43].

Thus, such structures can be used for the rational drug design of more isozyme-IX-selective and potent CA inhibitors [42,43]. We should mention that, because the X-ray structure of CA IX is not yet available, most studies have used the CA II structure for modeling and designing CA IX inhibitors. We therefore stress again that positively charged compounds of which 2 is a representative might have the advantage of selectively inhibiting only CA IX *in vivo*, owing to their membrane impermeability [44].

Concluding remarks and future perspectives

With its overexpression in many cancer tissues but not in their normal counterparts, CA IX constitutes an interesting target for novel approaches to anticancer therapy. CA IX has been shown to acidify the extratumoral medium, contributing to both the acquisition of metastatic phenotypes and chemoresistance to weakly basic anticancer drugs. Consequently, further research needs to be done in the field of the tumor-associated CA IX isozyme to understand better its exact role in cancer.

CA-IX-selective inhibitors constitute interesting tools with which to study the physiological or pathological effects of CA IX. Indisulam, a sulfonamide anticancer drug, is already in Phase II clinical trials. In addition to its hypothetical action in perturbing the cell cycle, indisulam acts as a potent inhibitor against CA IX but it is difficult to quantify at present the contribution of inhibition of CA IX to the overall antitumor effects of the drug. Given that several pathways contribute to tumor growth, however, anti-tumor activity might be increased by agents that target multiple molecules, including CA IX, or by the combination of several agents to facilitate inhibition of several mechanisms.

Recently, the design of CA-IX-selective inhibitors containing boron has been proposed [42]. Sulfonamides, sulfamates and sulfamides have been synthesized by derivatization reactions of 4-carboxy-, amino- or

hydroxy-phenylboronic acid pinacol esters with amino- or isothiocyanato-substituted aromatic or heteroaromatic sulfonamides or by sulfamoylation reactions of amines or phenols with sulfamoyl chloride [42]. Many of these derivatives strongly inhibit CA IX, with K_i values in the range 7.3–89 nM [42]. Because hypoxic tumors highly over-express CA IX, the design of boron-containing inhibitors with high affinity for this tumor-associated isozyme might lead to important advances in boron neutron capture therapy that target such tumors, which are non-responsive to both classical chemo- and radiotherapy. Indeed, accumulation of the boron-containing inhibitor in the tumor might lead to the selective destruction of only the diseased part with no appreciable harm to healthy tissues.

In conclusion, many biochemical, physiological and pharmacological data point to the potential use of inhibition of tumor-associated CA isozyme IX in the management of hypoxic tumors that do not respond to classical chemo- and radiotherapy. These compounds provide the possibility of developing both diagnostic tools for the non-invasive imaging of these tumors and therapeutic agents that might perturb the extratumoral acidification process in which CA IX is involved. Many classes of highly effective *in vitro* CA IX inhibitors have been developed and the pharmacological evaluation of some of them has recently begun. Much pharmacological work is, however, warranted to understand whether a successful new class of antitumor drugs might be developed from these preliminary but encouraging observations.

Acknowledgements

We are grateful to Silvia Pastoreková and Jaromir Pastorek for discovering this fascinating protein and for many discussions on the drug design of inhibitors targeting it. A.T. thanks the Belgian 'Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture' (FRIA) for financial support. C.T.S. is grateful to the European Union for financing part of his research on CA IX (Euroxy project).

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