

Carbonic Anhydrase IX as Prognostic Marker for Tumor Progression and as a Target for Novel Antitumor Drugs

[Yeni Antitümör İlaç Hedefi ve Prognostik Belirteç Olarak Karbonik Anhidraz IX]

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ABSTRACT

One of the genes highly upregulated by hypoxia is that encoding isozyme IX of the metalloenzyme carbonic anhydrase [CA, EC 4.2.1.1], CA IX. CA IX is a high activity tumor-associated membrane enzyme predominantly found in hypoxic tumor tissues being absent from most normal tissues except for a low level of expression in the gastrointestinal tract. CA IX was demonstrated to be a druggable target for the development of novel anticancer therapies and as a tumor progression marker. Inhibition of this tumor associated membrane enzymatic activity by specific inhibitors, such as fluorescent- and membrane-impermeant sulfonamides, was shown to lead to changes in the tumor pH, with a reversal of the acidification towards more normal values by 0.5 – 0.7 pH units. For this reason CA IX is an interesting target for anticancer drug development whereas more selective and powerful CA IX inhibitors could prove useful for elucidating the role of this protein in hypoxic cancers, for controlling the pH imbalance in tumor cells and for developing diagnostic or therapeutic applications for the management of hypoxic tumors, generally non-responsive to classical chemo-and radiotherapy.

Key Words: Carbonic anhydrase, CA IX, CA XII, tumorigenesis, hypoxia, sulfonamides, enzyme selective inhibition

ÖZET

Metaloenzim karbonik anhidrazın [CA, EC 4.2.1.1] izoenzimi IX -CA IX- hipokside yüksek düzeyde ifade edilen genlerden birisidir. CA IX özellikle hipoksik tümör dokularında bulunup gastrointestinal yoldaki düşük düzeydeki ekspresyonu haricinde birçok normal dokuda bulunmayan yüksek aktiviteli ve tümörle ilişkili bir membran enzimidir. CA IX'un yeni antikanser tedavileri için ilaçlara hedef oluşturabileceği ve bir tümör ilerleme belirteci olduğu gösterilmiştir. Floresan ve membran geçirgenliğini etkileyen sülfanamidler gibi özgül inhibitörler kullanılarak bu tümörle ilişkili membran enzim aktivitesinin engellenmesinin tümör pH'sını değiştirip asiditeyi azalttığı ve normal pH değerlerine doğru 0.5-0.7 pH ünitesi kadar yaklaştırdığı gösterilmiştir. Bu nedenle, CA IX antikanser ilaç geliştirilmesi ve daha seçici ve etkin CA IX inhibitörlerinin kullanımıyla hipoksik kanserlerde pH dengesizliğinin kontrol edilmesi ve genellikle klasik kemo ve radyo terapiye cevap vermeyen bu tümörlerin tanı ve tedavisinde yararlı olabilecektir.

Anahtar Kelimeler: Karbonik anhidraz, CA IX, CA XII, tümörleşme, hipoksi, sülfonamidler, enzim seçici inhibisyon

INTRODUCTION

Hypoxia constitutes a challenging clinical problem, being common in many cancer types which are inaccessible to radio- and chemotherapy. Acidic extracellular pH is also a typical attribute of the hypoxic tumor microenvironment, with a strong impact on cancer progression and treatment outcome [1,2].

In 1994, Pastorek's group discovered a carbonic anhydrase [CA, EC 4.2.1.1] isozyme, later denominated CA IX, as being present in many types of tumors [3]. Over the past decades, many studies have been made to find the role of this CA isozyme and that of CA XII, the second cancer-associated CA isoform [4] in tumor progression, either as a biomarker or a tumor-associated protein. The expression of CA I and CA II has been most frequently investigated in a variety of tumor cells, cell lines and some carcinoma patients [3-6], but it has been difficult to find a clear-cut relationship between the expression of such CA isozymes in normal and malignant cells. However, no evidence of a direct relationship between malignant transformation and CA expression has been presented for CA isoforms I - VII. It appears that only the expression of the above-mentioned isoforms CA IX and CA XII is strongly associated with tumorigenesis [7].

Carbonic anhydrase IX expression is dramatically increased in a variety of human tumours, whilst its expression in normal tissues is low [8]. Investigation of the involvement of specific CA IX extracellular domains in the pH control had showed the elimination of the catalytic active domain perturbed the acidification capacity of CA IX, but still produced similar levels of lactic acid compared with the intact domain [9]. According to these findings, the excessive pH decrease observed upon hypoxia could be explained by CA IX catalytic activity and not only by the production of lactic acid explained by the group of Pastorekova [10]. CA IX, a candidate protein of possible marker for tumor therapies that was initially reported as a 'tumor antigen' of belonging 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 [11,12].

Many CAs present in humans have high catalytic activity for the physiological reaction [i.e. hydration of CO_2 to HCO_3^- and H^+], and CA IX is among them [11]. CA IX is a transmembrane protein with several domains: an extracellular CA catalytic domain with high catalytic activity, a proteoglycan-like segment [PG], mediating cell-cell adhesion. Both CA and PG domains were shown to play a role in tumorigenesis, as it will be shown shortly in this review. Thus, as a consequence of the CO_2 hydration/dehydration reaction catalyzed by various CA isozymes present within cells, the cytosolic pH becomes more alkaline because of the increased intracellular HCO_3^- . Within this reaction, H^+ ions are transferred out of the cell and cause acidification of the extracellular milieu, which may facilitate tumor invasion by the activation of

proteolytic enzymes in an acidic extracellular pH [13]. In accordance with this hypothesis, in vitro studies using a Matrigel invasion assay [BD Biosciences] showed that inhibition of CA activity leads to a reduced invasion rate of renal cancer cells [14].

Hypoxia and CA IX

The regulation of pH homeostasis by the CA enzymatic activity also facilitates biosynthetic processes which involve an early carboxylation step requiring bicarbonate. This physiological reaction [i.e., CO_2 hydration to bicarbonate and a proton] is critical for respiration and transport of CO_2 between metabolizing tissues and excretion sites, secretion of electrolytes in a variety of tissues and organs, pH regulation and homeostasis [15-17].

CA IX is a tumor-associated transmembrane isoform with a high enzyme activity and functional involvement in the pH regulation and cell adhesion which has been linked to oncogenesis, and its overexpression has been observed in malignant tumour cells was originally detected in a human carcinoma cell line HeLa as a cell density regulated membrane antigen named MN [18]. In a short time it was recognized that expression of the MN antigen correlates with tumorigenic phenotype of somatic cell hybrids of HeLa and normal human fibroblasts [19]. 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 [20-22].

In figure 1 the relation between pH regulation by the hypoxia inducible factor HIF-1 α -regulated gene products is presented. This pathway plays pivotal roles in tumor progression, aggressiveness, and metabolic adaptation, and probably contributes to increased resistance of hypoxic tumors [23].

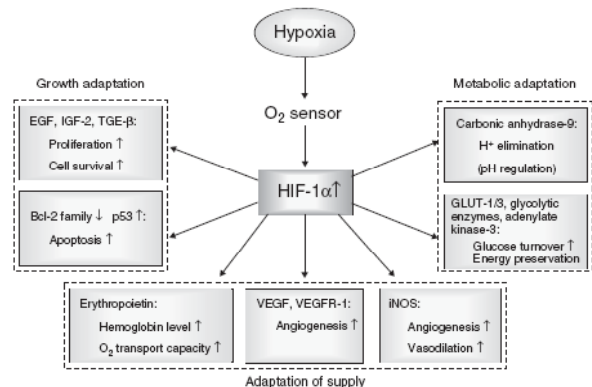


Figure 1. Schematic representation of the role of hypoxia induced accumulation of HIF-1 in human cancers Adapted from Vaupel et al. [23].

The dehydration reaction leads intracellular protons and might help neutralize the cell interior, whose pH is regulated by a very complex interplay of proton extrusion pathways [Figure 2]. The acidification of the extracellular milieu of malignant tumours increases the invasive behavior of cancer cells. The acidic pH can also influence the uptake of anticancer drugs exhibiting weakly

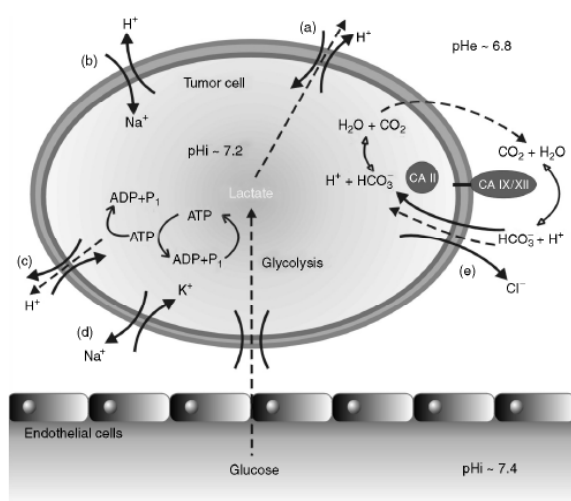


Figure 2: Mechanisms of pH regulation and ion transport in tumors. Adapted from Pastorekova S and Pastorek J [24].

electrolytic properties and impose varying effects on the response of tumor cells to conventional anticancer therapy. Trying to understand this milieu, several studies are present in the literature for the inhibition of CA IX with possible sulfonamides and other moiety of this type of compounds as well to solve the relation with pH regulation with CA IX for a possible contribution to the management of tumor-specific acid load and provide a therapeutic target.

Ca IX Inhibition with Sulfonamides

CA inhibitors could prevent the acidification of intracellular milieu that is why numerous sulfonamide inhibitors of CA IX have been developed in the past few years [25-27]. Parkkila S et al. [28] investigated the functional role of CA activity in cancer cells by analyzing the effect of acetazolamide, a potent CA inhibitor, on the invasive capacity of renal carcinoma cell lines. The results clearly showed that acetazolamide alone reduced invasiveness of these cancer cells in vitro and suggests that the CAs expressed in these renal cancer cells contribute to invasiveness. CA inhibitors may also reduce invasiveness in other tumours that overexpress one or more CA's. These compounds may reduce the provision of bicarbonate for the synthesis of nucleotides and other cell components. E7070, a member of recently reported class of antitumor sulfonamides, blocks cell cycle progression in the G1 phase. It has been suggested that E7070, possessing a free SO_2NH_2 moiety, probably acts as a strong CA inhibitor. This compound demonstrates significant antitumor activity both in vitro and in vivo against different human tumors, e.g. human colon carcinoma. E7070 produces not only growth suppression but also reduction in tumour size. Presently, E7070 is in Phase II clinical trials [29]. A fluorescent sulfonamide with high affinity for CA IX [inhibition constant, $K_i = 24 \text{ nM}$, has been shown to inhibit hypoxia-mediated tumor acidification [30]. Aromatic sulfonamide compounds have been shown to reverse the effect of tumor acidification; to inhibit the

growth of cancer cells and to suppress tumor invasion mediated by these CA's [31-34]. Thus, the data from these many physiological studies appear to have identified a CA mediated, hypoxic tumor-specific pathway. This provides firm grounds for exploring the effects of this class of compounds as a novel approach to discriminate between healthy cells and cancerous cells, specifically targeting hypoxic tissues—an attractive attribute that is lacking in many existing cancer therapies [27,28].

A study deals with the drug design, synthesis, and biological investigation of a group of thioureidosulfonamides, which have been obtained by reaction of isothiocyanate-substituted aromatic sulfonamides with amines was reported by Supuran's group [34]. These compounds have potent inhibitory properties against CA IX with K_i values in the range of 10–37 nM and Papp values $> 0.34 \cdot 10^{-6} \text{ cm/s}$ for the absorptive transepithelial transport in Caco-2 cells. In Caco-2 cells, one of these compounds [4-[3-[2-Dimethylaminoethyl] thioureido]-benzenesulfonamide, A6] was shown to be a substrate for efflux transporters such as P-glycoprotein [P-gp]. P-gp activity is not likely to be rate-limiting for intestinal absorption, but might be useful when targeting hypoxic tumors expressing both P-gp and CA IX. Another study was reported by Ozensoy et al. with the inhibition of the two transmembrane, tumor-associated isozymes hCA IX and XII with a library of aromatic and heteroaromatic sulfonamides and ureas/thioureas incorporating 4-aminoethyl-benzenesulfonamide and metanilamide moieties had determined as the best hCA IX inhibitors [35]. A library of glycoconjugate benzenesulfonamides that contain diverse carbohydrate-triazole tails were investigated for their ability to inhibit the enzymatic activity of the three human transmembrane carbonic anhydrase [CA] isozymes hCA IX, hCA XII and hCA XIV. The most potent hCA IX inhibitor was the glucuronic acid derivative 20 [$K_i = 23 \text{ nM}$]. This compound was uniquely hCAIX selective from all other isozymes [16.4-16.8- and 4.6-fold selective against hCA II, XII, and XIV, respectively] [36].

Consequently, acidification of the extracellular milieu of malignant tumors has been reported to increase the invasive behavior of cancer cells [37-40]. In normal tissues, production of acid is catalyzed by carbonic anhydrases [CAs], some of which are known to be overexpressed in certain cancers. To investigate the functional role of CA activity in such cancer cells will lead to better understanding for designing novel drugs.

Ca XII Inhibition With Sulfonamides

CA XII is another transmembrane, tumor-associated CA isozyme with a more diffused expression in some normal tissues thus the expression level of isozymes hCA IX and XII is elevated in response to hypoxia and research on the involvement of these isozymes in cancer [11]. Aromatic sulfonamide compounds have been shown to reverse the effect of tumor acidification; to inhibit the growth of cancer cells and to suppress tumor invasion mediated by these CAs [41].

In addition to a potential role in cancer, it was recently

determined that hCA XII is highly expressed in the eyes of glaucoma patients [42]. Past studies had showed the current antiglaucoma drugs were thought to target hCA II and IV [43], but hCA XII may in part be responsible for the intraocular pressure effects of clinically used sulfonamides and further research on the role of isozyme XII in glaucoma therapies is necessary to verify. With the results of Supuran's group hCA XII has a good affinity for fluoride and bicarbonate but is not inhibited by heavier halides, perchlorate, nitrate, and nitrite. The best hCA XII inhibitors were cyanide [KI of 1 μ M] and azide [KI of 80 μ M] [44].

The extracellular location of the CA isozymes, it is possible to design membrane-impermeant CAIs, which in this way become specific inhibitors for the membrane-associated CA's.

Ca Ix/xii as Tumor Marker

CA IX and CA XII are transmembrane proteins with catalytic domain on the cell exterior, suggesting that they might attend in acid-base regulation of the extracellular space. There is substantial evidence that extracellular pH of human tumours is generally more acidic than that of normal tissues [41] and that this acidic pH may enhance both the migration and the invasive behaviour of some tumour types. Thus, use of an endogenous marker of hypoxia would be a convenient alternative to current methods that measure tumor oxygenation, provided the marker could be shown to reliably identify viable, radiation-resistant, hypoxic cells. Carbonic anhydrase 9 [CA9] is a transmembrane protein overexpressed in a wide variety of tumor types and induced by hypoxia. Tumors lacking or low in oxygen are often less curable not only by radiotherapy but also by surgery [45]. Since the presence of hypoxic tumor cells is likely to indicate a poor outcome after therapy, it would be useful to identify hypoxic tumors at the start of treatment and then modify treatment accordingly. Methods used to detect hypoxic cells are rather difficult or need administration of chemicals to mark hypoxic cells which are also quite difficult as well.

Membrane-bound CA's with an extracellular active site, CA IX and XII represent key enzymes in the maintenance of an appropriate pH in the extracellular milieu thus as an endogenous marker of hypoxia that could be identified in conventional formalin-fixed tumor sections would be an important step that is why we strongly defend CA IX could possibly serve as a target for therapy.

Future Aspects of the Cancer Therapies with CA IX/XII inhibitors

The conclusion is that CA IX and its inhibitors are indeed remarkable; after many years of intense research in this field, that continue to offer interesting opportunities for the development of novel drugs, new diagnostic tools, or for understanding in greater depth of the fundamental processes of the life sciences.

CA IX acidifies pH of the culture medium in hypoxia but not in normoxia, independent of the lactic acid production. Sulfonamide CA IX-selective inhibitors belonging to various classes were observed to bind only to hypoxic cells containing CA IX, and to reverse the tumour acidification processes mediated by the enzyme. Since it was previously shown that many sulfonamides possess appreciable tumor cell growth inhibitory properties in vitro and in vivo [45] such findings constituted the proof-of-concept that anticancer therapies based on tumour-associated CA isozyme inhibition can be developed, but also offer interesting tools for investigating hypoxic tumours as well as for their imaging [46,47].

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