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Review Article

Carbonic anhydrase IX: An atypical target for innovative therapies in cancer

Roberto Ronca [a,b,*], Claudiu T. Supuran [c]

[a] Department of Molecular and Translational Medicine, University of Brescia, Viale Europa 11, 25123 Brescia, Italy
[b] Consorzio Interuniversitario per le Biotecnologie (CIB), Italy
[c] NEUROFARBA Department, Sezione di Scienze Farmaceutiche e Nutraceutiche, University of Florence, Florence 50019, Italy

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ABSTRACT

Carbonic anhydrases (CAs), are metallo-enzymes implicated in several pathophysiological processes where tissue pH regulation is required. CA IX is a tumor-associated CA isoform induced by hypoxia and involved in the adaptation of tumor cells to acidosis. Indeed, several tumor-driving pathways can induce CA IX expression, and this in turn has been associated to cancer cells invasion and metastatic features as well as to induction of stem-like features, drug resistance and recurrence. After its functional and structural characterization CA IX targeting approaches have been developed to inhibit its activity in neoplastic tissues, and to date this field has seen an incredible acceleration in terms of therapeutic options and biological readouts. Small molecules inhibitors, hybrid/dual targeting drugs, targeting antibodies and adoptive (CAR-T based) cell therapy have been developed at preclinical level, whereas a sulfonamide CA IX inhibitor and an antibody entered Phase Ib/II clinical trials for the treatment and imaging of different solid tumors. Here recent advances on CA IX biology and pharmacology in cancer, and its therapeutic targeting will be discussed.

1. Introduction

Acid/base homoeostasis is a main feature of tumor metabolism and exerts a key role in tumor growth, metastasis, resistance to therapies and the conditioning of tumor microenvironment. The regulation of pH requires the concerted interplay between various acid/base/ions transporters (Na^+/H^+ exchangers, bicarbonate transporters, chloride/bicarbonate exchangers, V-ATP-ase, monocarboxylate transporters, etc.) and several carbonic anhydrase (CA) isoforms. Human CAs comprise 15 isoforms, 12 of which are catalytically active and with different subcellular localization. In particular, carbonic anhydrase IX (CA IX) is a tumor-associated, cell-surface glycoprotein induced by hypoxia, involved in adaptation to acidosis and widely described as implicated in cancer progression via its catalytic activity and/or non-catalytic functions. In addition to being located in chronically hypoxic tumor regions, CA IX is also found in mild hypoxic or even normoxic regions, its expression being activated by various oncogenic pathways including the mitogen-activated protein kinase (MAPK) pathway [1,2].

Since the discovery of the first CA inhibitors in the '40s, the possibility to target this key enzyme in acid/base imbalances opened various clinical applications, as CA inhibitors were and are still used as diuretics, antiepileptics, antiglaucoma and antiobesity agents [3]. New perspectives in the treatment of solid tumors by targeting CA IX started to be considered only in the last decade, but to date this field has seen an incredible acceleration in terms of therapeutic options and biological readouts [4,5], mainly because CA IX-selective small molecule inhibitors belonging to various chemical classes started to be available together with several mAbs [6–9].

Here we will discuss recent advances on the biology of CA IX in cancer with a main focus on the therapeutic application of targeting CA IX in tumors. The following aspects will be discussed: i) the biological relevance of CA IX in cancer progression, metastasis and resistance to therapies; ii) old and new approaches to target CA IX (small molecules, hybrid molecules, antibodies, immune-conjugates); iii) therapeutics perspectives of CA IX targeting (combination therapies, new delivery systems, combination with immune-therapeutics); iv) imaging hypoxic tumors with CA IX inhibitors.

2. Carbonic anhydrase IX

2.1. Transcriptional regulation

Carbonic anhydrase IX (CA IX) is a transmembrane enzyme, and represents one of the 14 CA isoforms found in humans. The human CA IX

* Corresponding author at: Roberto Ronca, Department of Molecular and Translational Medicine, University of Brescia, Viale Europa 11, 25123 Brescia, Italy.
E-mail addresses: roberto.ronca@unibs.it (R. Ronca), claudiu.supuran@unifi.it (C.T. Supuran).

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coding gene is located on the p12-p13 region of chromosome 9, codified by 11 exons and its transcription results into a single 1.5 kb mRNA. The CA IX promoter has a very low basal activity, but it is readily induced by different factors, thus creating a strict control on CA IX expression. In particular, the GC-rich promoter is composed of 6 functionally characterized *cis*-acting elements, 5 with a positive influence on the promoter activity (HBS, PR1, PR2, PR3 and PR5) and one with a repressing influence (PR4). Among these, the most important activation region is the hypoxia inducible factor (HIF)-binding site (HBS) located upstream the transcription start and representing the conserved core of the *cis*-acting hypoxia-responsive elements (HREs). Importantly, HBS activation by HIF alone is not sufficient for the activation of the hypoxia-mediated pathway, and requires the cooperation of other transcription factors like p300/CBP [10].

HIF-1 is a heterodimeric complex consisting of two subunits, HIF-1α and HIF-1β and represents the major regulator of CA IX expression. The α subunit exists in three different isoforms (HIF-1α, HIF-2α and HIF-3α), but only HIF-1α is able to induce CA IX expression.

Both HIF-α and HIF-β are ubiquitously expressed in normal and neoplastic tissues in normoxic and hypoxic conditions [11], but HIF-1α levels and stability are controlled by two enzymes, the prolyl hydroxylase domain proteins (PHDs) and the hydroxylase HIF-1 inhibiting factor (FIH-1), which are sensitive to O_2-dependent molecular switches [12]. As a result, PHDs and FIH-1 inhibition in hypoxia leads to HIF-1α stabilization and transcriptional activation, its binding to HIF-1β in the cytosol and the consequent translocation to the nucleus where the transcription of >800 genes, including CA IX, can by activated after binding the HRE elements [11,13].

Interestingly, not only hypoxia, but also other microenvironment conditions, signalling pathways, soluble factors and genome mutations can modulate HIF-1 pathway, thus regulating CA IX expression [14].

The hydroxylase activity of PHDs and FIH-1 dependents on several factors like O_2, Fe^{2+} and α-ketoglutarate, then all the conditions able to modulate these elements can influence the activity of these enzymes and consequently CA IX expression [10,12]. PHDs activity can be modulated by reactive oxygen species (ROS). Indeed, low ROS levels increase PHDs activity, whereas high ROS levels inactivate PHDs stabilizing HIF-α, as demonstrated by the capacity of the antioxidant ascorbic acid to regulate CA IX expression [12]. High cell density can lead to the activation of signalling pathways like PI-3 K [10] and MAPK pathways [15] which can contribute to CA IX upregulation probably mediating the activation of the *cis*-acting element of the CA IX promoter [16].

Beyond high cell density, also acidosis is implicated in the regulation of CA IX expression. First, acidosis can trigger the nucleolar sequestration of the von Hippel-Lindau factor (VHL) impairing its function and resulting in HIF-1α accumulation. Moreover, acidosis can modulate the activity of PI-3 K and MAPK pathways probably because low pH is sensed by ion transporter and metabolons which, in turn, trigger the aforementioned pathways through their cytoplasmic domains [17].

A strict connection between CA IX expression and tumor suppressors were established for VHL and p53. Regarding VHL, epigenetic silencing or inactivating mutations lead to constitutive overexpression of CA IX by disrupting the HIF-1α degradative pathway and resulting in the accumulation of transcriptionally active HIF-1α [10]. For p53, its activation by genotoxic stress increases proteasome-dependent degradation of HIF-1α [18] and competes for the binding of trasncriptional co-activators, leading to CA IX down-regulation [19]. Oncogenic pathways like PI-3 K and ERK are also implicated in CA IX expression regulation by targeting *cis*-acting elements of the CA IX promoter [10].

2.2. Structure and function

The X-ray crystal structure of the catalytic domain of human CA IX has been reported in 2009 [20] for the enzyme in complex with acetazolamide, a sulfonamide inhibitor, and several years later also for the non-complexed enzyme [21]. Such data were essential for the drug design of effective and isoform-selective CA IX inhibitors [3–6].

The active site of CA IX is shown in Fig. 1, as determined by X-ray crystallography [20,21]. Similar to all other catalytically active CA isoforms [22], a zinc ion at the bottom of a rather spacious active site cleft is essential for catalysis and binding of inhibitors. It is coordinated by three His residues (His94, 96 and 119) and a water molecule/hydroxide ion, which is nucleophilically activated upon binding to the metal ion, and efficiently transforms CO_2 into bicarbonate, with k_{cat}/K_M values of $>10^8\ M^{-1}.s^{-1}$ and k_{cat} values of $>10^6\ s^{-1}$, being thus one of the best catalysts known in Nature [23]. The catalytic cycle consists in two half reactions: (i) the conversion of CO_2 into bicarbonate, through the nucleophilic attack promoted by the zinc coordinated hydroxide ion, and (ii) the formation of the nucleophilic zinc hydroxide species of the enzyme from the acidic species, i.e., the one with water coordinated to zinc (Fig. 1), which consists in a proton transfer reaction, mediated by the His64 proton shuttle residue, situated in the middle of the active site cavity, which is also the rate-determining step of the entire catalytic cycle [20–23]. This is probably the reason why half of the CA IX active site is mainly aligned with hydrophilic amino acid residues (Thr199, Thr200, His64, Pro201, Pro202, etc), whereas the opposite site is hydrophobic (Val121, Val143, Val131, Leu198) – see Fig. 1. This particular active site architecture is essential for the design of tight-binding inhibitors, also considering the fact that among the 12 catalytically active human CAs, the active site amino acid residues are rather conserved [20,22]. There are however differences with other isoforms, especially with the cytosolic ones (such as CA I and II, highly abundant proteins in many tissues/cells [22]), affording thus the possibility to obtain CA IX-selective compounds [3–5]. These differences principally regard the residue in position 131, which is Val in CA IX and Phe in CA I and II (and many other isoforms [22]), as well as the residues at the entrance of the

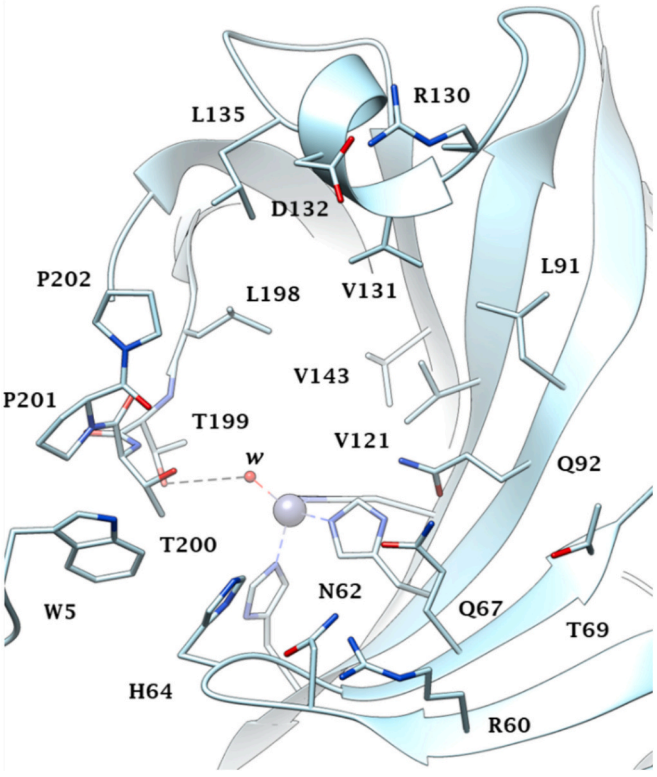


Fig. 1. Representation of hCA IX active site (PDB 5FL4) with the zinc ion (grey sphere), its three His ligands (His94, 96 and 119), and the water (w) molecule (red sphere) bound to the zinc and other amino acid residues involved in the catalytic cycle or in the interaction with inhibitors. H-bonds are depicted as black dashed lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

active site cavity, among which those in positions 130, 132, 135, etc. [24].

It should be also mentioned that CA IX, unlike other human isoforms, has been observed as a homodimer in the crystal structure [20], whereas most other mammalian isoforms are monomers [22,24]. In addition, CA IX is a multidomain protein, which apart for the extracellular CA active site, incorporates a short cytosolic tail, a transmembrane domain, and after the enzyme active site, a proteoglycan (PG) domain, unique to this isoform, which although not crystallized yet, seems to possess an important physiological function, being able to open or close the active site of the enzyme, depending on physiological conditions, e.g., presence or lack of hypoxia [25,26]. Recent studies suggest that the PG domain is an intrinsically disordered protein [27].

Apart the efficient CO_2 hydration, CA IX, similar to other isoforms such as CA I, II and XIII [28] also shows esterase activity with 4-nitrophenyl acetate as substrate [29]. It is not known whether catalysis of other reactions than CO_2 hydration has physiological functions for CA IX or other CA isoforms studied so far [22,28].

3. Carbonic anhydrase IX in cancer biology

While in normal tissues CA IX expression is quite restricted (and mainly in the gastrointestinal tract), in cancer its expression increases and has been described at high levels in various range of neoplastic tissues that include, among others, breast, kidney, lung, pancreas, colon/rectum, oral cavity (head/neck), uterine cervix, gallbladder, brain (high-grade), liver and gastric epithelium (Fig. 2). At molecular level, no differences have been reported between the sequence and structure of CA IX isolated from normal and tumor tissues, thus suggesting a similar biological/physiological function in these contexts [11]. In addition, the expression of CA IX has been correlated with poor prognosis and tumor aggressiveness in breast, lung, ovarian and bladder cancer patients, indicating an important role as a potential prognostic biomarker [30]. For these and other reasons, CA IX is now considered a key mediator in the tumor milieu being implicated in numerous features that contribute to tumor growth and aggressiveness, metastasis and trigger resistance to conventional therapies [31] as summarized in Fig. 3.

3.1. CA IX in tumor growth

A proper regulation of pH is essential for cell survival and becomes even more important and difficult to obtain in cancer cells due to their tendency to shift their glucose metabolism towards the so called Warburg effect [32]. In this condition tumor cells choose a glycolytic phenotype for their energy needs even in abundance of O_2, a faster mechanism of energy production conferring several advantages including the support of biosynthetic requirements for uncontrolled proliferation.

Moreover, the hypoxic conditions of the tumor and the aberrant activation of various oncogenic pathways and components of the tumor microenvironment can further trigger this metabolic switch [33,34]. In this context, of fast glycolysis, increased amount of lactate and H^+ are generated, together with CO_2 resulting from the residual aerobic respiration events and from the pentose phosphate pathway (essential for biosynthetic and detoxification reactions). These metabolic acids must be removed from the cell to avoid intracellular acidosis. Mechanistically, CO_2 is exported out of the cell by passive diffusion through the plasma membrane or by gas channels like aquaporins, while lactate can leave the cell only by means of monocarboxylate transporters (MCTs) in cotransport with H^+ (1:1 stoichiometry), or by passive diffusion through gap junctions. Finally, H^+ can be extruded from the cells by different protein exchangers, the most important being represented by the Na^+/H^+ exchanger (NHE), the H^+-ATPase, and the H^+-lactate co-transporters like MCTs [31,35].

Despite all, due to the exacerbated metabolic fitness of tumor cells and to the restricted perfusion between extracellular and intracellular spaces, these extrusion mechanisms are not sufficient to prevent the extracellular acidification first and then the intracellular lowering of the pH. Therefore, the maintenance of pH homeostasis in cancer cells is

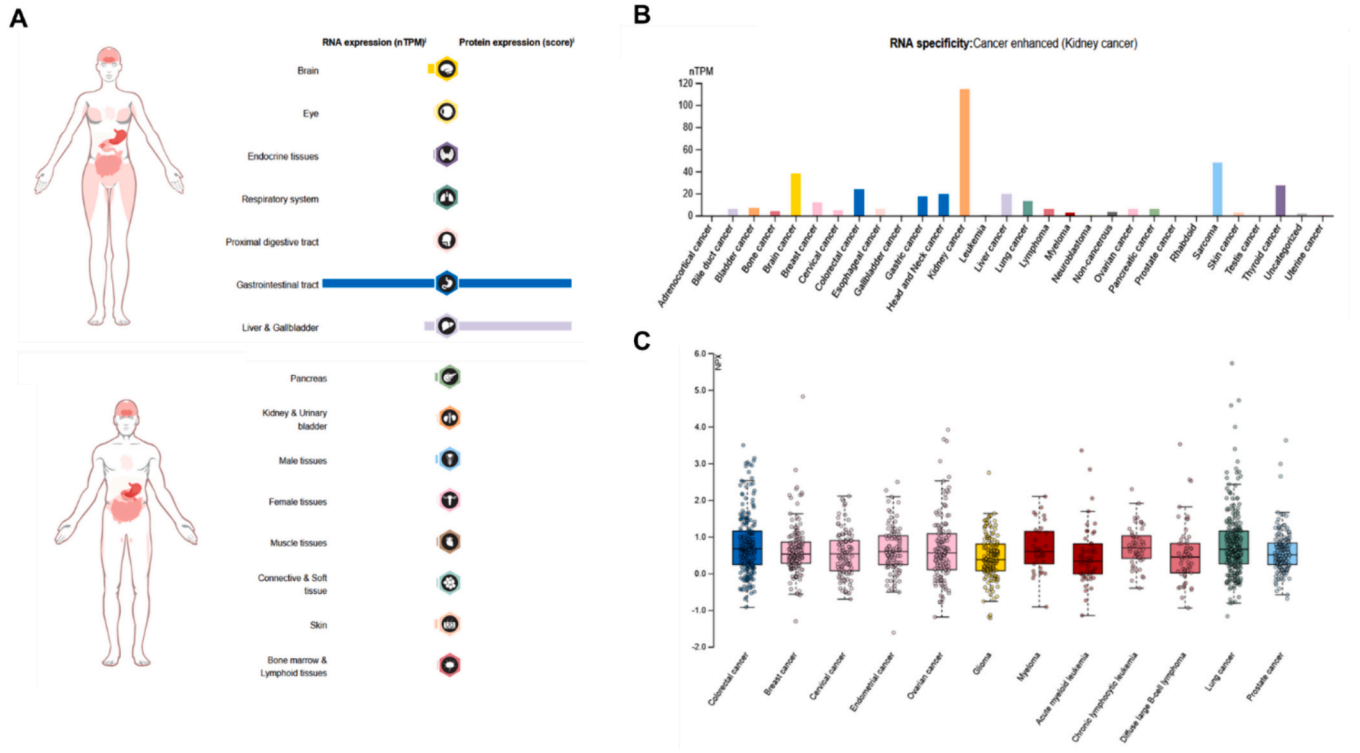


Fig. 2. Protein Atlas database reports that CA IX is poorly expressed, both at RNA and protein level, in normal tissues (panel A), while its expression is significantly increased in cancer cell lines (RNA levels, panel B) and in tumor tissues (protein level, panel C). Data are obtained from www.proteinatlas.org

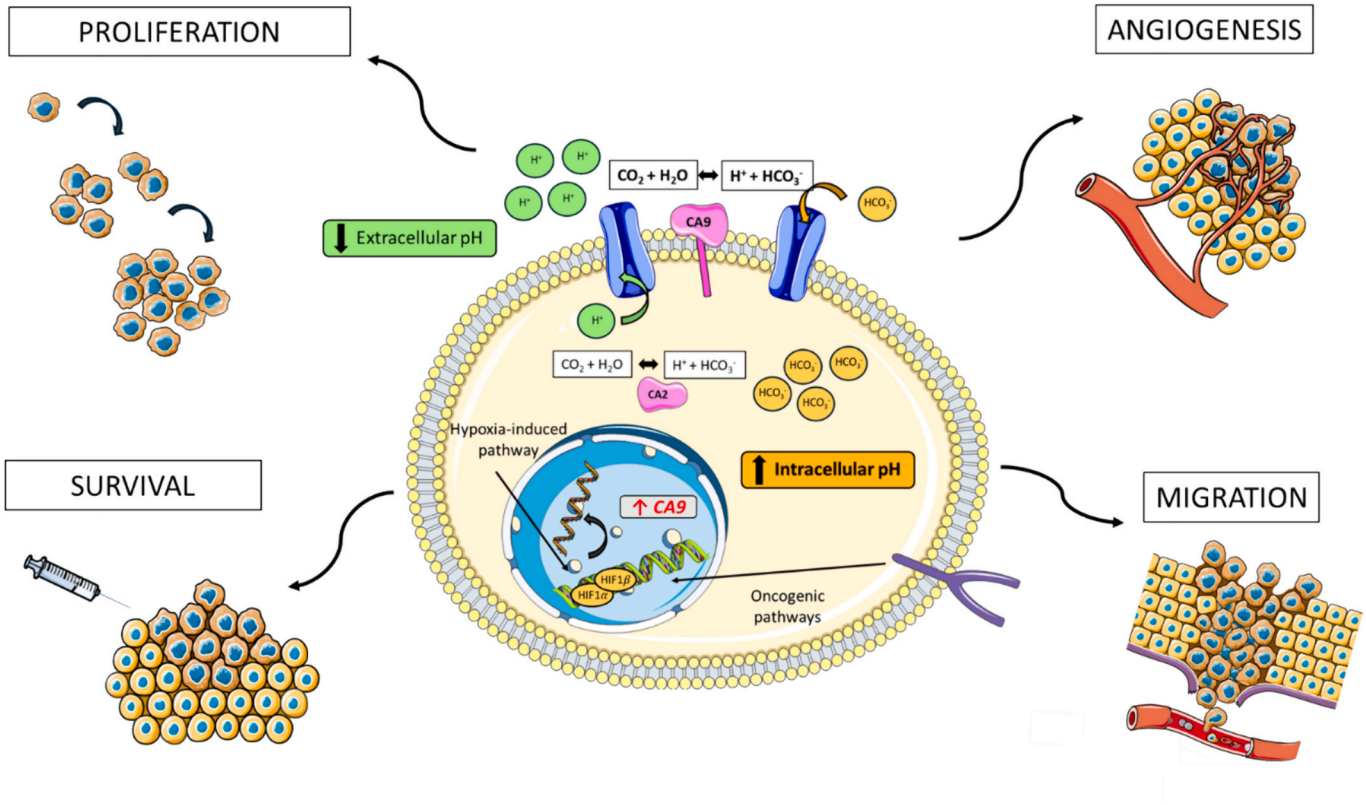


Fig. 3. Schematic representation of the biological features activated in cancer cells and microenvironment by the upregulation CA9 gene by hypoxia or oncogenic pathways.

achieved through several complex mechanisms, involving H^+ and HCO_3^- transport, in which CA IX plays a key role acting as a "catalytic converter" to create a close loop with other membrane transporters in order to release toxic metabolic acids from cells [14]. Indeed, CO_2 is exported on the extracellular side of the plasma membrane where it can be converted by CA IX into H^+ and HCO_3^-, and this rapid mechanism of CO_2 removal allows the maintenance of an outward-directed CO_2 gradient across the plasma membrane, thereby facilitating CO_2 excretion especially in poorly perfused but metabolically active tissues, where the distance from blood capillaries limits the disposal of metabolic waste products. Then, the H^+ remain in the extracellular space, the HCO_3^- can be re-imported into the cell by Na^+-HCO_3^- co-transporters (NBCs) and Cl^-- HCO_3^- exchangers, where cytosolic CAs, like CA II, convert HCO_3^- and H^+ to CO_2, which finally diffuses through the membrane closing the transport loop and contributing to the additional extrusion of H^+ from the cell [35]. The net balance allows the establishment of an acidic extracellular pH ($pH_e \approx 6.5–6.8$) and a moderately alkaline intracellular pH ($pH_i \approx 7.2–7.4$) that are responsible of typical cancer features [14]. This rheostat pH regulation between pH_e and pH_i is further guaranteed by the fact that CA IX results most active at $pH > 6.8$, similar to tumor pH_e, thus increasing the rate of the hydration reaction and acidifying the extracellular space, while at $pH < 6.8$ CA IX its dehydration activity increases to counteract further extracellular acidification.

It has been demonstrated that protein kinase A (PKA) phosphorylates the Thr443 residue in the intracellular domain of CA IX, and this is a critical step for CA IX activation in hypoxic cells. Notably, PKA is dependent of cAMP, which is elevated by hypoxia, and this further explains the activation and functionality of CA IX in regulating pH in the hypoxic tumor microenvironment. Also, the full activation of CA IX also requires dephosphorylation of Ser448 residue [36].

As the final result, in this acidic pH_e cancer cells, due to their extreme adaptability and compensatory mechanisms are spared and can survive, while normal cells suffer and undergo a p53- and caspase3-mediated necrosis and apoptosis. Indeed, using a genome-wide synthetic lethal CRISPR screening in hypoxic triple-negative breast cancer cells, it has been demonstrated that CA IX plays a fundamental role in the regulation of intracellular redox homeostasis and ferroptosis. In this highly proliferating and metabolically active cancer model the suppression of CA IX increased intracellular reactive oxygen species (ROS) and the vulnerability to iron levels, thus confirming the importance of CA IX in maintaining an alkaline pHi to suppress ferroptosis [37].

Another recently reported role of CA IX in cancer growth resides in its involvement in nutrients supply and its interplay with amino acids transporters. Cancer cells experience nutrients restriction due to their rapid proliferation and the distance from vasculature. For these reasons they undergo continuous adaptation of the metabolism to maintain their biological functions.

Amino acids are a fundamental source of carbon and nitrogen required for biosynthesis of macromolecules and their transport across the plasma membrane is critical for cancer cell survival.

In this context, several cancer types rely on glutamine (Gln) that is exploited for fatty acid synthesis through reductive carboxylation of α-ketoglutarate (α-KG) to citrate, which then enters the lipid synthesis route. Indeed, Gln transport into the cells is fundamental and it is mediated by several amino acid transporters (AATs) such as the Alanine Serine Cysteine Transporter 2 (ASCT2), the Sodium Coupled Neutral Amino Acid Transporter 2 (SNAT2) and the L-type Amino Acid Transporter (LAT1), all of which can be influenced by pH gradients created by CA IX. For instance, extracellular protons compete with sodium ions for SNAT2 transport inhibiting its activity. On the contrary, pH does not modulate Gln transport by ASCT2, but it regulates glucose transport by ASCT2. In addition, ASCT2 expression is upregulated by acidosis through HIF-2α [38]. Altogether this demonstrate that CA IX can modulate ATTs activity and set the basis for deeper studies in this new field of research. Santi et al. on the other hand demonstrated that even bicarbonate formed from CO_2 by hydration in the presence of CA IX (or

XII), may supply cancer cells with Krebs cycle intermediates which sustain the high proliferation rate, through transformation in metabolites such as pyruvate, succinate, or even fatty acids [39], demonstrating thus also a metabolic role of this enzyme in cancer cells, in addition to that of pH regulation.

3.2. CA IX in cancer invasiveness

Despite its main function as "pH regulator", CA IX activity in tumor cells has been widely associated with key aspects of cancer dissemination such as adhesion, migration and invasion. Indeed, numerous studies reported that CA IX inhibition results in reduced motility and invasion in vitro and in vivo in different cancer types [40–44].

Cell-cell adherens junctions interconnect cells through a complex glycoprotein system comprising cadherins, catenins (α, β and p120), actin filaments and other intracellular proteins. In particular, E-cadherin is extremely important to form cell surface homophilic complexes stabilized by these cytoplasmic protein complexes associated with actin cytoskeleton [45].

To this regard, CA IX was localized to cell-cell contacts, its distribution in lateral membranes overlapping with E-cadherin. Indeed, it has been observed that disruption of cell-cell contacts by calcium treatment results in the in relocalization of both CA IX and E-cadherin to cytoplasm and back to plasma membrane, similarly to what was observed in hypoxia and reoxygenation experiments. Also, it has been reported that CA IX is able to interact with β-catenin, disrupting cadherin-actin connection, attenuating cell-cell adhesion and promoting cell migration [46].

In line with these observations, CA IX has been involved in the promotion of focal adhesion turnover, a key aspect in the multistep process of cell migration and motility. Focal adhesions represent condensed molecular assemblies anchoring actin filaments to the extracellular matrix (ECM) through integrin receptors. It has been reported that in human cervical carcinoma cells the overexpression of CA IX resulted in cytoskeletal remodelling, focal adhesion disassembly, and increased motility. This effect was associated with the inhibition of the Rho/Rock pathway, a key regulator in the epithelial-mesenchymal transition (EMT) driving the invasive and metastatic phenotype of cancer cells [47]. Mechanistically, the N-terminal domain of CA IX interacts with the Val60-Tyr168 site of the dickkopf-1 (DKK-1) protein, a negative regulator of the Wnt pathway that is implicated in focal adhesion formation, thus preventing its activation. Indeed, this interaction leads to paxillin phosphorylation and the consequent activation of the paxillin-mediated signalling promoting focal adhesion turnover and cell migration [48].

In the invasive process, a relevant role is played by the ECM-tumor cell interactions and by the capacity of cancer cells to degrade and remodel the ECM. In this context, it has been shown that CA IX can directly interact with hyaluronan and collagens composing the ECM though its PG domain thus promoting cell adhesion [31].

CA IX is also involved in ECM remodelling by promoting the overexpression and activation of several metalloproteinases (MMPs), that facilitate tumor cell motility and invasion. Indeed, in models of oral cancer cells it has been reported that CA IX overexpression promotes the expression of MMP-9 though the activation of FAK and Src signalling that drives NF-κB activation and the activity of the transcription factors c-Jun and c-Fos. As a result, MMP-9 expression, drives invasion and metastasis of oral cancer cells [49].

Furthermore, it has been recently reported that CA IX can associate with MMP-14 on the plasma cell membrane of the invadopodia and enhance its catalytic activity. Indeed, it has been suggested that the three phosphorylation sites in the intracellular tail of the CA IX can act as regulators of CA IX-MMP-14 association. Moreover, CO_2 hydration catalysed by CA IX provides protons necessary for MMP-14 activity [50]. Finally, the lower pH_e triggered by CA IX creates an extracellular environment which facilitates MMPs activation [31].

3.3. CA IX in cancer stemness and resistance to therapy

Beyond pH regulation, adhesion and migration, CA IX has been involved in mechanisms of cancer stemness and resistance to radiation and chemotherapy.

Cancer stem cells (CSCs) are a subset of tumor cells inside the tumor mass endowed with the ability to self-renew and differentiate into different cancer cell lineages, thus contributing to cancer heterogeneity and resistance to therapies [51]. Experimental evidence show that CSCs reside in and prefer a hypoxic environment. Indeed, hypoxia triggers the activation of signalling pathways promoting CSC survival and self-renewal, and adaptation of cancer cells to a hypoxia involves the upregulation of HIFs. Notably, it has been reported that hypoxia can induce the expression of the so called "Yamanaka factors" that can lead to cell reprogramming into a pluripotent state [52]. In line with this, CA IX has been activity has been linked with the acquisition an maintenance of stemness features in cancer cells [53].

It has been shown that CA IX regulates cancer cell stemness in breast cancer cells though the mTORC1 axis. This CA IX-mediated activity is a requirement to support the activity transcription of EMT and "stemness drivers" such as Notch1 and Jagged1 [54]. Accordingly, pharmacological inhibition of CA IX resulted in cancer stem cells depletion of breast cancer, tumor growth delay and suppression of lung metastases [43,54].

In thyroid cancer cell lines, and increased expression of CA IX was observed in tumor spheroid culture conditions when compared to monolayer cultures, and this and was related with increased expression of stemness markers. Indeed, CA IX inhibition by genetic silencing or drug treatment significantly reduced cell proliferation and the tumor initiating potential of stem-like thyroid cancer cells [55].

Along with differentiation features, hypoxia favours resistance to the conventional anti-cancer approaches mainly represented by radiation and chemo-therapies. The role of hypoxia in radioresistance has been widely described and mainly resides in the key role of O_2 in generating reactive oxygen species (ROS) and increase DNA and cell damage [56]. Hypoxic cells are then two to threefold more resistant to the same radiation dose due to a lower double DNA strand break effect [57]. In addition, increased acidification lowers the efficacy of radiation since acidic pHe reduces the efficacy of radiation-induced DNA damage [58].

Furthermore, increased lactate levels in the extracellular space, in which CA IX is involved, have been linked to radioresistance, and this can be explained perhaps by the antioxidant effects of lactate [53]. Also, the rapid removal of CO_2 from the extracellular milieu mediated by CA IX can contribute to radiation resistance reducing free-oxygen species formation [31].

Radiation treatment has been associated with the activation of EGF-EGFR signalling in different cancer, including clear cell renal carcinoma, leading to PI3K/AKT pathway activation and the consequent inhibition of the apoptotic cell death. In this context the intracellular domain of CA IX can be phosphorylated in an EGF/EGFR-dependent manner, allowing its interaction with the p85 regulatory subunit of PI3K, the activation of survival pathways, and suggesting a positive feedback loop at the basis of the progression of clear cell renal carcinoma after radiation treatment [59].

Other possible mode of interaction between CA IX and radiation responses can be explained by the interaction between CA IX and β1 integrin in tumor cells [50]. β1 integrin expression is increased in many cancer cells that can be sensitized to radiotherapy by targeting β1 integrin [60,61]. Also, CA IX can interact with the NF-κB signalling pathway via a mechanism involving β1-integrin. Indeed, CA IX is required for NF-κB activation in hypoxic conditions and can trigger the production of G-CSF (granulocyte colony stimulating factor), an important secreted factor promoting linked with protection from radiation damage [62].

Finally, the signal transducer and activator of transcription 3 (STAT3) has been found to increase radiation sensitivity in cancer cells [63] and to be involved in the expression of CA IX [64]. Notably, IL-6 is

an NF-κB responsive gene promoting tumor growth and invasion through STAT3, and is strictly linked to radio-resistance [65]. To this regard, the presence and involvement of an IL-6-STAT3-NF-κB signalling axis involving CA IX in radiation resistance is predictable [66]. All these findings are corroborated by the fact that CA IX inhibition widely results in cancer cell sensitization to radiation [67,68].

Along with that, CA IX has been implicated in resistance to chemotherapy a key problem for patients' treatment since tumors initially respond to therapy while resistant clones in the tumor mass emerge with new, aggressive and drug-resistant features. At molecular level this is in agreement with the stem-like phenotype triggered by hypoxia and CA IX. Moreover, CA IX, proton exchangers and transporters are involved in this mechanism leading to an acidic tumor microenvironment which protonates pharmacological compounds interfering with their passage through the plasma membrane and their entrance into cancer cells [31].

As an example, CA IX-positive breast cancer patients belonging to any phenotype, have a significantly worse prognosis when treated with chemotherapy if compared with CA IX-negative patients [69]. This is true for other tumor types, so that targeting CA IX, protons exchangers and transporters results in more efficient chemotherapy in patients [31,70]. In line with this, a recent study reported that gastric cancer patients not responding to perioperative chemotherapy expressed increased levels of CA IX compared to the responders, and treatment with the clinical grade CA IX inhibitor SLC0111 significantly improved the therapy response re-sensitizing gastric cancer cells to perioperative chemotherapy [71].

4. Targeting of CA IX for therapy and diagnosis

CA IX inhibition was shown to interfere with pH regulation in cells transfected with CA IX in hypoxic conditions already in 2004 [25] by using fluorescent sulfonamide inhibitors [25,26], providing thus the rationale for developing agents that may be useful both for the treatment and imaging of hypoxic tumors [3–5,72,73]. The in vivo proof-of-concept that CA IX inhibition is indeed a powerful anticancer strategy, in animal models with orthotopic tumors, was on the other hand provided in work from Dedhar's group [50,54,74]. As the early stages of research aimed to targeting CA IX for obtaining antitumor agents were reviewed in detail [3–5], we will discuss here only the most promising and recent developments in the field (also summarized in Table 2).

4.1. Small molecule inhibitors

Sulfonamides and their isosteres, such as sulfamates and sulfamides, are among the most effective classes of CA inhibitors (CAIs) [3,22], acting through coordination to the zinc ion from the enzyme active site (Fig. 1), in deprotonated form at the nitrogen form the SO_2NH_2 functionality, as shown extensively by many X-ray crystallographic studies [75,76]. In fact, many such derivatives, among which acetazolamide **1** (Fig. 4) are used clinically as diuretics, antiglaucoma, antiepileptic, or antiobesity agents for decades [3,22]. They also led to the development of second/third generation agents, such as benzolamide **2** (an orphan drug), SLC-0111 **3** (an antitumor agent in Phase Ib/II clinical trials [77]) and some of its congeners of types [4]. Table 1 reports some of the most recent anticancer clinical studies on solid tumors in which some of these CAI drugs are being investigated.

However, a major limitation of the first and second generation CAIs such as acetazolamide **1** and benzolamide **2**, is the fact that they effectively inhibit most human CA isoforms [22], thus leading to a multitude of side effects associated with the inhibition of off-target CAs present in the GI tract, kidneys, brain, etc. [3]. Thus, the tail approach (and its variants, the two- and three-tails approaches) have been developed in the past years in order to obtain isoform-selective (in this specific case, CA IX-selective) CAIs [78–80]. As summarized in Fig. 5, these approaches exploit the fact that although the bottom and the middle parts

Table 1
Clinical trials involving sulfonamide CAIs for anticancer/antimetastatic action in combination with other antitumor agents.

CAI	Combination drug	Phase	ClinicalTrials.gov Identifier
Acetazolamide **1**	Temozolamide	I	NCT03011671 (malignant glioma)
Acetazolamide **1**	Etoposide + Platinum derivatives	I	NCT03467360 (small cells lung cancer)
Benzolamide **2**	Temozolamide	II	NCT04209790 (glioblastoma)
SLC-0111 **3**	Safety trial	I (completed)	NCT02215850 (advanced solid tumors)
SLC-0111 **3**	Gemcitabine	Ib/II	NCT03450018 (pancreas, metastatic)

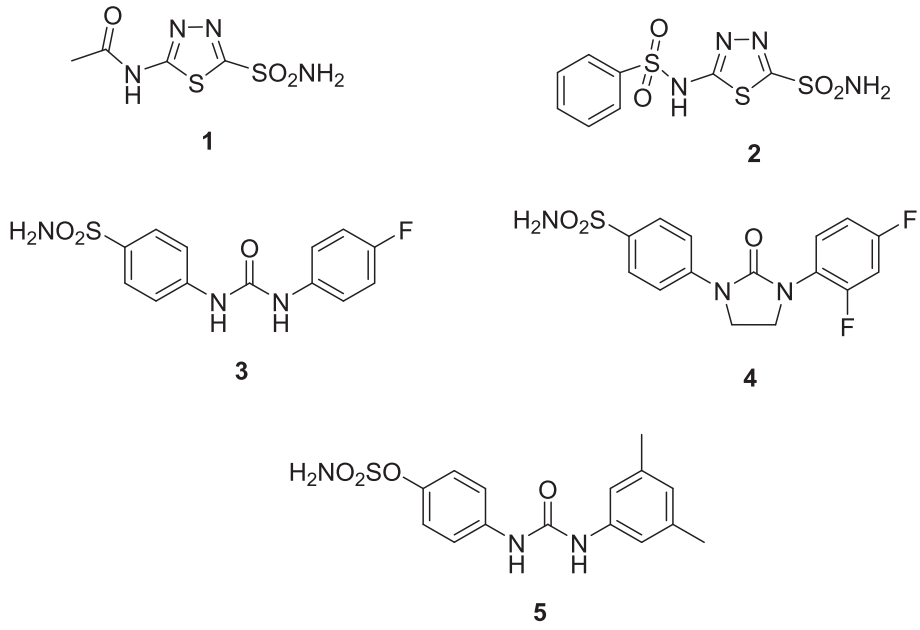


Fig. 4. Classical sulfonamide CAIs acetazolamide **1**, benzolamide **2**, and new compounds based on SLC-0111 (**3**) structure, such as derivatives **4** and **5**.

Table 2
The most relevant CA IX targeting approaches described in Chapter 4 are summarized.

Therapeutic group	Targeting agent	Additional moiety	Ref
Sulfonamides	acetazolamide benzolamide SLC-0111		[3,22,77]
Hybrid molecules	sulfonamide or coumarin based	Auristatin E	[6]
		tubulysin B	[82]
		maytansinoids	[83]
		artemisinin	[84]
		EGFR antagonists	[85]
		15-lipoxygenase-cyclooxygenase 2	[86]
		–	[87]
Antibodies	Girentuximab	INF-α2a	[89]
		Lutetium177, Zirconium89	[93]
		–	[96]
	M75	Nanomaterials for photoactivation	[99,100]
ADCs	BAY 79–4620	3ee9 mAb + auristatin E	[102]
	IL2-XE114-TNFmut	XE114 mAb + IL2 + TNF	[103]
	Girentuximab	–	[106]
CAR-T	G36 scFv	–	[110]
	G36 scFv	G36 scFv +anti-PD-L1 mAb	[111]
CAR-NK	G250 mAb		[115]

of the CA isoform active sites are quite conserved, the amino acid residues at the entrance of the cavities are rather variable [22,78–80]. Thus, introducing functionalities ("tails") that can interact with those parts of the enzyme active site may lead to effective but also isoform-selective inhibitors, which has been confirmed by a multitude of kinetic and crystallographic studies of all human CAs [22,75,76]. Successful examples of tail approach applications for obtaining CA IX-selective and potent CAIs are for example compounds **3–5** (Fig. 4), which are low nanomolar CA IX inhibitors whereas their inhibition of other physiologically relevant isoforms (CA I, II, etc.) is not that effective [80,81].

A multitude of sulfonamide and other such highly effective zinc-binding CA IX inhibitors have been reported over the last years, as recently reviewed in ref. [80]. The most advanced derivative (Phase IB/II clinical trials) is SLC-0111 (compound **3** in Fig. 4), which has been also the lead for obtaining many new such derivatives.

4.1.1. *Hybrid small molecules*

In order to enhance the antitumor effects of the CA IX inhibitors alone, many hybrid drug approaches have been developed (Fig. 6), by which a second chemotype with cytotoxic activity has been incorporated in the original CAi molecule. Such hybrids which incorporate sulphonamide CA IX inhibitors as "warhead" and cytotoxins such as monomethyl auristatin (**6** in Fig. 6) [6], tubulysin B (**7** in Fig. 6) [82], the maytansinoids **8** and **9** [83], as well as mertansine **26** [83] (Fig. 6). These hybrid agents showed an effective capacity to kill various tumor cells, being more effective than the toxin alone or the CA IX inhibitor alone in many tumor animal models [5]. Other approaches proposed the use of artemisinin (derivatives **11–13** in Fig. 6) [84], EGFR antagonists (derivatives **14** in Fig. 6) [85] as well as dual inhibitors of 15-lipoxygenase-cyclooxygenase 2 (derivatives **15** and **16** in Fig. 6) [86]. Again, more effective antitumor effects have been registered compared to the administration of the single agents, in many animal models of cancer [5].

4.2. *Antibodies and antibody–drug conjugates (ADCs)*

Girentuximab, initially named G250, is a monoclonal antibody

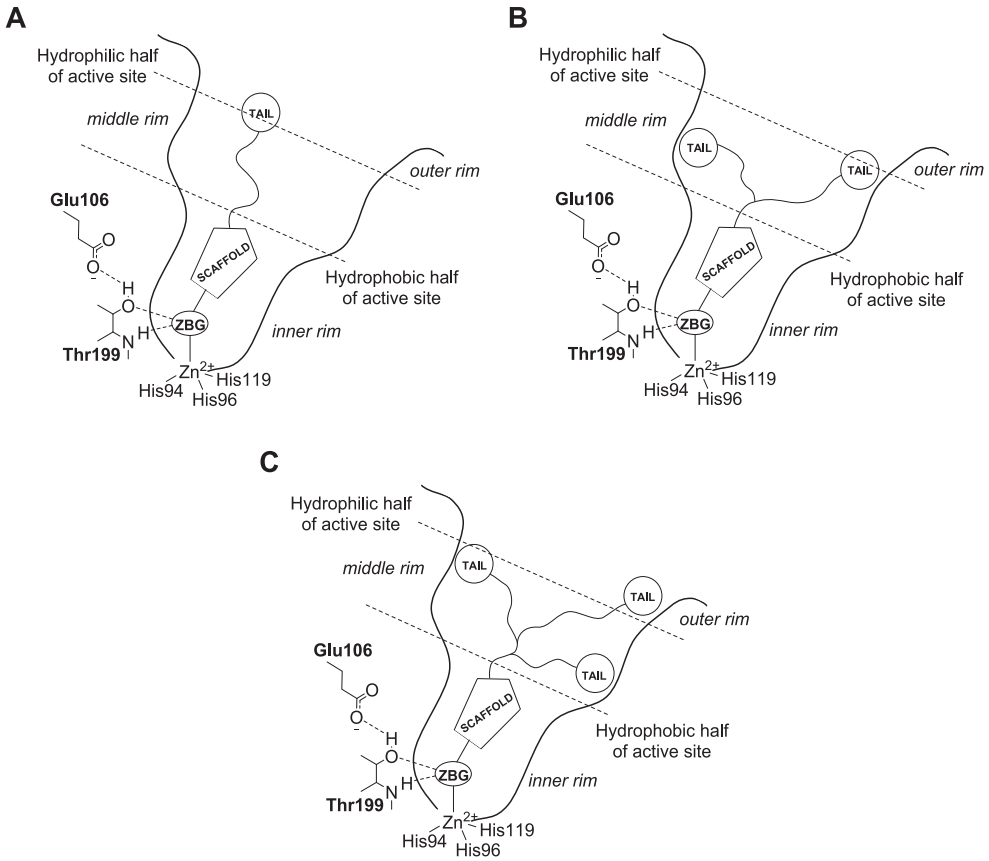


Fig. 5. The tail approach (A) and its variants with two (B) and three (C) tails attached to the scaffold of the inhibitors.

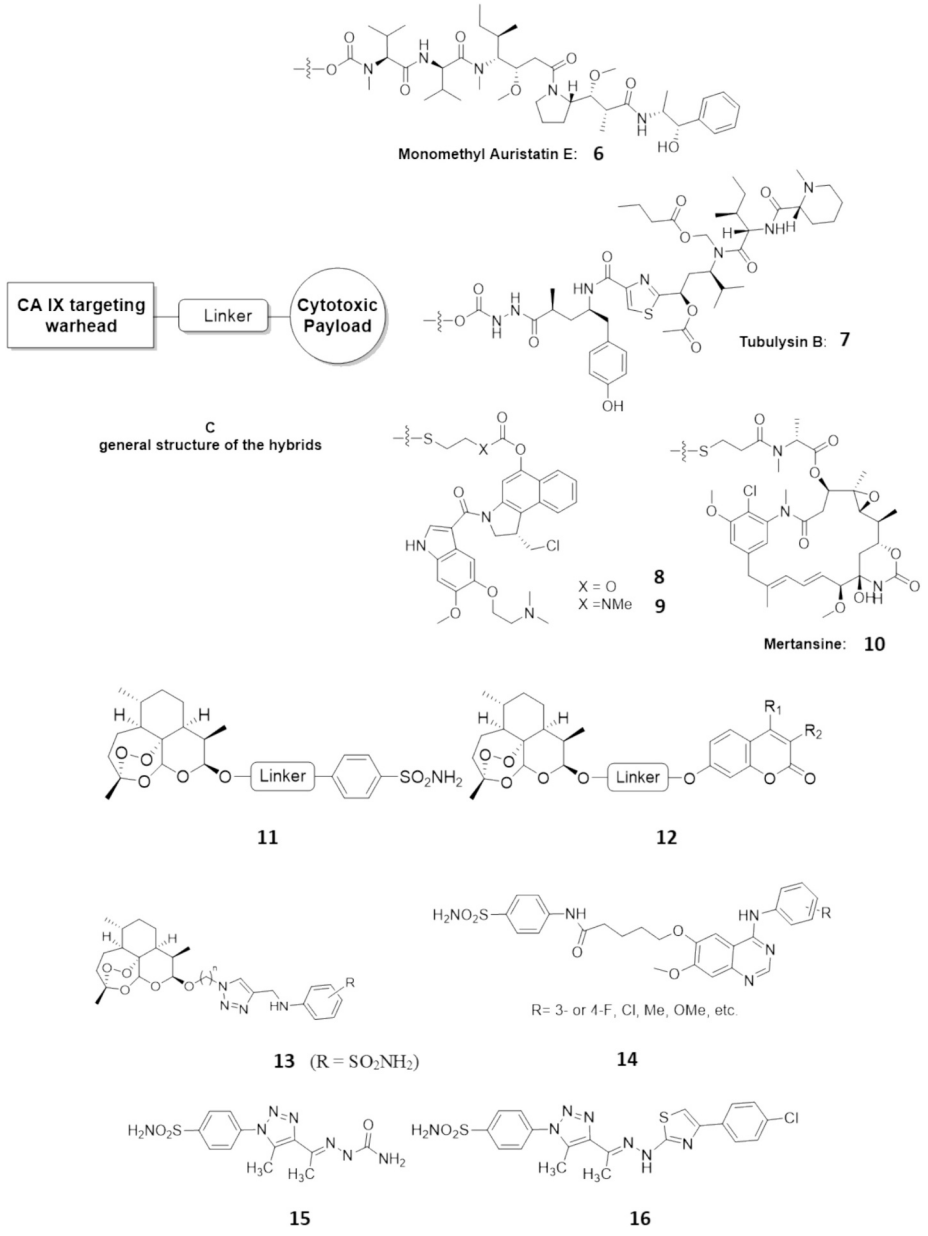


Fig. 6. Hybrid compounds **6–16** incorporating CA IX inhibitors of the sulfonamide or coumarin type and other anticancer agents, such as cytotoxic agents, artemisinin, EGFR antagonists and inhibitors of 15-lipoxygenase-cyclooxygenase 2.

(mAb) known to bind to renal cancers since the late '90s [87], but few years later it has been shown to target CA IX [88,89], overexpressed in many renal cancers both due to mutations in the von Hippel Lindau protein or due to hypoxia [89–91]. Among the various other mAbs targeting this enzyme, in various phases of clinical development, Girentuximab is the most advanced one and it has been shown to possess both antitumor effects alone or in combination with other drugs [89,92,93] as well as the potential to image CA IX–positive tumors, especially when radiolabeled with Lutetium177, Zirconium89 or other nuclides [93–95].

M75, is a mAb developed in Pastorek's laboratory [96], highly specific to CA IX and has been extensively used in immunohistochemistry for detecting the enzyme in hypoxic tissues/tumors, with very high affinity for the enzyme, binding to its PG region [97,98]. Ultimately, Pastorekova's group demonstrated the possibility to bioconjugate M75 on various nanomaterials (graphene, molybdenum oxides, etc.) and the potent antitumor effects obtained through photothermal activation of these nanoconjugates [99,100].

Dedhar's group also isolated tight-binding mAbs targeting CA IX [9], which have been shown to bind to the catalytic domain of CA IX.

Only few work evaluated the possibility to obtain Antibody–Drug Conjugates (ADCs) targeting CA IX and delivering small molecule inhibitors of the sulphonamide and coumarin type [101]. In both cases the obtained ADCs (7 of them were reported) showed picomolar affinity for CA IX, with no inhibition of any other CA isoforms, such as CA I, II or XII [101]. BAY 79–4620 is a selective CAIX antibody-drug conjugate developed from the 3ee9 mAb conjugated to monomethyl auristatin E through a cleavable linker, and displayed significant anticancer effect in preclinical human xenograft models [102].

Finally, the realization and preclinical evaluation of immune-conjugated constituted by fusion proteins of a CA IX-targeting mAb (XE114) linked to IL2 and a mutated form/low-potency TNF has been reported. The therapeutic effect exerted by this dual cytokine IL2-XE114-TNF^{mut}, resulted in the significant reduction of tumor growth in a murine colon adenocarcinoma [103].

4.3. Adoptive cell therapy

Chimeric antigen receptor (CAR) T-cell therapy is now a reality for the adoptive immunotherapy where autologous T cells are engineered to express chimeric receptors combining the antigen-specific binging region of an antibody (usually a single-chain variable fragment, scFv) and various costimulatory molecules [104]. The resulting CAR-T cells are administered to patients and retain the capacity to traffic to and recognize cancer cells in an HLA-independent manner. In the clinical practice this approach revealed striking results and found its application in the treatment of hematological tumors, mainly CD-19–expressing B-cell acute lymphocytic leukemia [105]. Solid tumors remain an issue for this adoptive cell therapy, nevertheless new approaches are now emerging, also considering CA IX as a specific target.

In preclinical models, Weijtens et al. designed a first-generation CAR-modified T cells directed against CA IX, based on the G250 monoclonal antibody and demonstrated a consistent cytokine production and cytotoxic activity of these first-generation CA IX–directed CAR-T cells against renal carcinoma cells (RCC) [106]. Later on, Lamers et al. performed a first clinical study including three patients where hepatotoxicity was described due to the recognition of CA IX expressing epithelial cells from the biliary duct [107]. Then, these same CAR-T were tested for safety in a proof-of-concept phase I/II trial in patients with CA IX-expressing metastatic renal cell carcinoma (mRCC). Twelve patients were treated, circulating CAR T-cells were transiently detectable in all patients, and blood cytokine profiles reflected CAR T-cell presence and in vivo activity. Nevertheless, no objective responses were reported for any of the 12 patients, and several recommendations emerged for future trials [108,109].

Second generation CAR-T cells were created expressing the anti-CA IX human scFv G36 antibody as a targeting moiety, tested in preclinical settings, and proved to be more efficient in treating RCC in comparison with first-generation G36-expressing CAR-T cells [110]. These cells were further engineered in another study to simultaneously express and secrete the human anti-programmed death ligand 1 (PD-L1) antibody at the tumor site. In this preclinical trial the expression of anti-PD-L1 diminished 5 times tumor growth in a humanized mouse model of clear cell RCC (ccRCC) when compared with the CAR-T cells solely targeting CA IX [111].

Another second-generation CAR-T approach targeting CA IX was carried out against RCC showing strong cytokine releasing and tumor cell killing activity. This effect was synergistically implemented by combination with sunitinib and significantly reduced lung metastasis in a murine model of RCC [112]. Moreover, CAR-T cells bearing the anti-CA IX G36 scFv demonstrated complete remission in an orthotopic model of ccRCC in NSG-SGM3 mice, supporting the rationale for their clinical exploitation for adoptive cell therapy in ccRCC [113]. The effect of another CA IX-targeting CAR-T cell approach revealed strong anti-tumor activity in vitro and the capacity to eradicate glioblastoma in an orthotopic preclinical model [114].

Finally, in the field of adoptive cell therapies natural killer (NK) cells represent a new exploratory field and CAR-NK are a reality in the preclinical and clinical immunotherapy. A CA IX-targeting third-generation CAR (carrying the G250 antibody) was expressed into NK92 cells to obtain CAR-NK92 able to recognize CA IX expressing RCC cells. The therapeutic potential of this adoptive cell therapy approach was evaluated first in vitro and then in vivo alone and in combination with bortezomib in a mouse model with human RCC xenografts. In vitro, CA IX-specific CAR-NK92 cells specifically recognized target cells displaying a specific cytotoxicity effect and releasing cytokines. In vivo, the combination regimen had significant impact on tumor growth, more powerful than the treatment with CAR-NK92 cells or bortezomib alone. These data suggest that bortezomib can improve the effect of the CA IX-targeting CAR-NK based therapy for the treatment of RCC [115].

4.4. Imaging hypoxic tumors with CA IX inhibitors

The labelling of CA IX inhibitors by introducing positron-emitting radionuclides in their molecules was shown to lead to the possibility to image hypoxic tumors in which these compounds accumulate due to their high affinity for the enzyme, usually in the nanomolar range [116–118]. Many such derivatives incorporate ^{18}F, one of the mostly used positron-emitting radionuclides and their structures are shown in Fig. 7. They incorporate aromatic or heterocyclic sulfonamides as warhead (**17–20**, Fig. 7), sulfamates (**32**) or belong to the coumarin (**21**) class of CAIs [119], one of them (compound **20**) being the positron emitting variant of SLC-0111.

Other radiolabelled CA IX inhibitors described so far incorporate other nuclides, such as ^{64}Cu, ^{68}Ga and ^{111}In (Fig. 8) and are again based on aromatic/heterocyclic sulfonamides to which metal complexing moieties have been appended, as shown in Fig. 8.

These and many other similar derivatives (recently reviewed in [116,117]) were demonstrated to effectively accumulate only in the hypoxic regions of the tumors, rich in CA IX, being thus of great clinical value [120].

4.5. Targeting CA IX in combination therapy

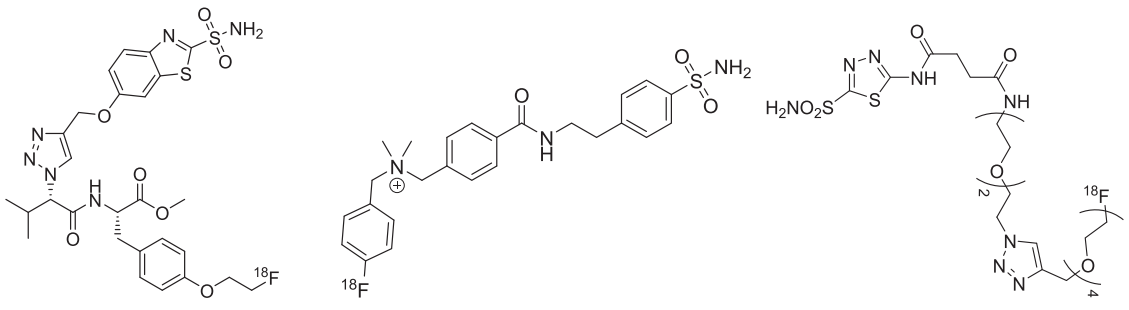
It has been widely described that CA IX inhibitors can influence several tumor features including pHi, drug availability, redox homeostasis cell cycle. On the other hand, inhibition of growth factors/cytokine pathways, angiogenesis and other therapeutic approaches result in the increase of CA IX expression inside the tumor in specific areas driving resistance [121]. On this bases it not surprising that several combination regimens have been explored for CA inhibitors and represent the most applicable clinical perspective.

One of the pioneering attempts to employ small molecules CA IX inhibitors in combination with conventional cytostatic agents was performed using acetazolamide and doxorubicin on human colon carcinoma and melanoma cells. The treatment with acetazolamide significantly increased the toxicity of doxorubicin and the authors concluded that the effect of acetazolamide was related to the blocking of CA IX, able to hamper the membrane transport of weakly basic drugs, including doxorubicin. This was further demonstrated for the weakly acidic drug melphalan under the same conditions [122]. Acetazolamide enhanced the cellular uptake of the weakly basic tyrosine kinase inhibitor imatinib and an increase of drug efficacy in their combination was reported both in vitro and in vivo [123]. Similarly, acetazolamide combined with the DNA-alkylating drug cisplatin significantly reduced cell viability of Hep-2 laryngeal carcinoma cells without affecting normal human umbilical vein endothelial cells (HUVEC) [124].

Another combination explored was the association acetazolamide *plus* the small-molecule histone deacetylase (HDAC) inhibitor MS-275, in vitro and in vivo on neuroblastoma cells. Intriguingly, the combination resulted in reduced tumor growth, vascularization and invasion, as well as in a significant decrease in the expression of stemness markers by tumor cells [125].

The mechanistic target of rapamycin (mTOR) inhibitor rapamycin has been described as a good monotherapeutic agent in preclinical models, but resulted in limited efficacy and recurrence in patients. Indeed, in vivo experiments on human and murine colorectal carcinoma models revealed that mTORC1 triggers tumor-cell proliferation in normoxia, its activity being abrogated in hypoxic areas [126]. On this bases, in vivo experiments demonstrated that combination of acetazolamide and rapamycin significantly reduced tumor growth in both hypoxic and non-hypoxic compartments with a long lasting effect, while the use of the single agents alone displayed a reduced effect [126].

Acetazolamide was used in combination with the anti-VEGF mAb bevacizumab to treat an in vivo model of cholangiocarcinoma. In this settings bevacizumab effectively reduced tumor growth, but CA inhibition triggered a superior reduction in tumor growth in response to the



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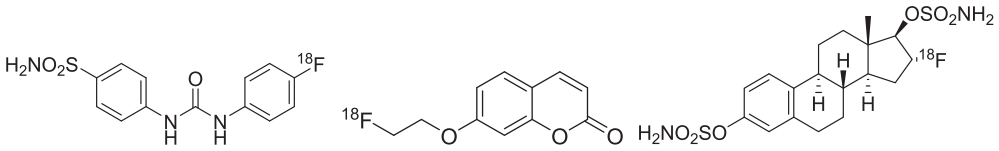
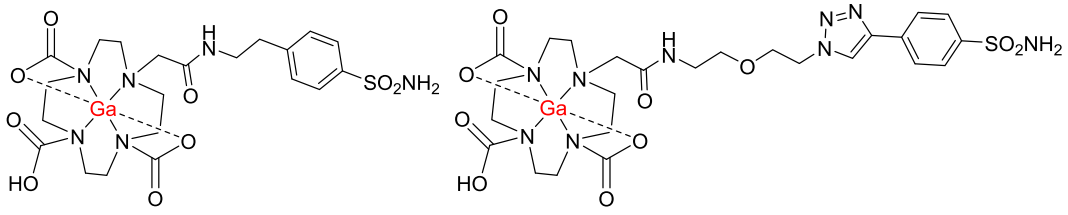


Fig. 7. ^{18}F-labbeled CA IX inhibitors of the sulphonamide, coumarin or sulfamate type **17–22**.



23 **24**

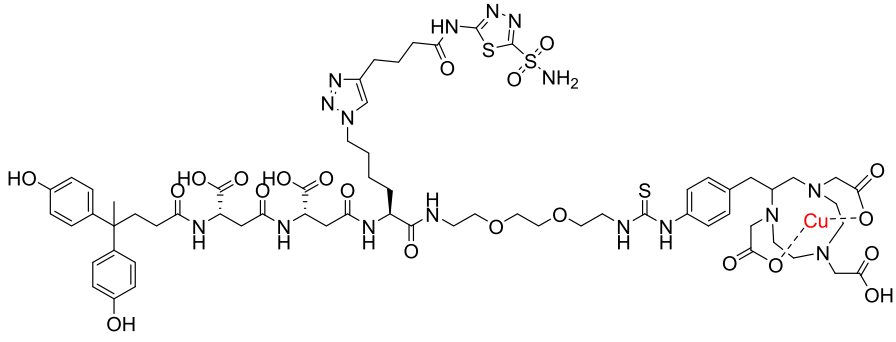


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Fig. 8. ^{68}Ga and ^{64}Cu labelled CA IX inhibitors **23–25**. Variants of **23** and **24** with ^{111}In are also described.

increase of HIF1α and CA IX after VEGF abrogation [127].

Recently, Mehes and colleagues reported the use of acetazolamide as adjuvant agent with a chemotherapy combination (CHOP, i.e. cyclophosphamide, doxorubicin, vincristine and prednisone) commonly used to treat non-Hodgkin lymphoma in a murine model of aggressive lymphoma. In this model the combined treatment resulted in increased therapeutic profile, due to increased delivery of cytotoxic drugs and to enhanced immune T-cell infiltration at the tumor site [128].

The clinical grade CA IX selective inhibitor SLC-0111 significantly sensitizes tumor cells to chemotherapy increasing tumor cell death in combination with various anti-cancer agents such as dacarbazin, temozolomide, doxorubicin and 5-fluorouracil [129]. For instance, SLC-0111 enhanced the therapeutic profile of temozolomide in vitro and in vivo in glioblastoma, with a significant impact on the so called "brain tumor initiating cells" [130].

SLC-0111 was shown to improve in vitro and in vivo the therapeutic effect of gemcitabine in a KRAS-driven pancreatic ductal adenocarcinomas model. In these cancer cells CA IX was identified as a pharmacologically targetable vulnerability and SLC-0111 *plus* gemcitabine resulted in a dramatic increase of survival in the KRAS-mutant pancreatic cancer patient-derived xenografts [131].

In vitro, in a 3D patient-derived pancreatic tumor spheroid model, the inhibitor APX3330 (targeting a multifunctional protein functioning in the DNA base excision repair) combined with SLC-0111 was strongly potentiated. Indeed, in these spheroids cancer cells were killed even in the protective environment of the fibroblasts [132,133]. Also, in treatment with the anti-angiogenic tyrosine kinase inhibitor sunitinib, SLC-0111 strongly reduced tumor growth, angiogenesis and mostly the

metastases of a highly metastatic human triple-negative breast cancer cell line [134].

In a model of head and neck squamous cell carcinoma, SLC-0111 sensitized tumor cells to cisplatin in vitro (in a 2D and 3B model), and caused reduction of tumor growth and metastatic spread in vivo [135].

Recently, a synergic increase of tumor cell death was described by forcing ferroptosis thanks to the co-administration of SLC-0111 and erastin or sulfasalazine [37].

Finally, with regard to immune modulation, in vitro tests with co-culture of murine skin melanoma B16F10 cells and activated T-cells, showed that the use of SLC-0111 increased T-cell antitumor response. This activation of T cells was confirmed in vivo in murine breast (4 T1) and melanoma (B16F10) models where the combination of SLC-0111 and anti-PD1/anti-CTLA4 increased survival of tumor bearing mice. Intriguingly, this therapeutic effect was strictly connected with the reduction of T regulatory and T-helper 17 cells, and increase of T-helper 1, and cytotoxic CD8+ T-lymphocytes [136].

5. Concluding remarks and perspectives

Since its discovery in the early 1990s the relevant role of CA IX in tumor biology has been thoroughly investigated in many publications. The overexpression of CA IX in many solid cancers and metastases compared to normal tissue counterparts from which it is generally lacking, has been used as a marker of hypoxia and frequently associated with poor prognosis. Nevertheless, it has become clear that, despite its prevalent expression mediated by HIF and hypoxia-related pathways, CA IX expression can be driven in non-hypoxic areas by various oncogenic pathways as well as by growth factors and cytokines. For this reason, the pH regulatory function of CA IX in low O_2 concentration is implemented by other roles that directly involve CA IX in tumor cells invasiveness, tumor metabolism, immune modulation, the acquisition of stem-like features and recurrence in various solid cancers. Also, CA IX represents a key regulator in the cellular mechanisms driving the resistance of tumor cells to treatments with weakly basic anticancer drugs and radiation therapy. All these features made CA IX a promising target for anticancer therapy as well as for targeted diagnostic imaging.

The therapeutic approaches realized so far and hereby described basically reside in the validation of small molecule inhibitors and specific antibodies. Starting from these classes various drug derivatives, double-targeting hybrid molecule as well as antibody drug conjugates have been reported and validated in cellular and animal models of various tumor types. This led to successful preclinical studies and in the planning and realization of clinical trials to evaluate the safety profile of CAIs/Abs and of their therapeutic profile in combination with chemotherapy agents. In the meanwhile, the use of CA IX targeting agents has been explored also for the imaging of tumors, and on the other hand CA IX targeting moieties have been translated into adoptive cell therapies (CAR-T and CAR-NK) that will represent the new frontier for the immunotherapy of hypoxic or CA IX expressing cancers.

Nevertheless, what emerged till now is that CAIs may represent adjuvant agents able to increase the efficacy and the durability of other conventional or new therapeutic approaches. Indeed, in the complex tumor microenvironment where various pro-tumor cells reside, and heterogeneous areas exist with fluctuating levels of vascularization, pH, O_2, nutrients, etc. Disrupting the protective role of CA IX uncovers vulnerabilities that can better tackle tumor cells leading to apoptosis, ferroptosis or necrosis. In this frame new studies are required to find better drug and schedules combinations in order to maximize anticancer activity of CAIs/targeting agents. A recent example is represented by a palmitoyl ascorbate (PA)-liposome (PL)-based nanoplatform incorporating also SLC-0111, which shows a multiphasic enhancement of the immunotherapeutic effects of CA IX inhibition and achieved outstanding immunotherapeutic effects in eradicating tumor and preventing tumor metastasis [137].

On the other side, an intriguing challenge is represented by the possibility to extend the understanding of CA IX biology/role and its targeting to hematological malignancies that remain an open and poorly explored field [138] for this kind of research.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Roberto Ronca reports financial support was provided by Italian Association for Cancer Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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