

# An update on anticancer drug development and delivery targeting carbonic anhydrase IX

Justina Kazokaite<sup>1,\*</sup>, Ashok Aspatwar<sup>2,3,\*</sup>, Seppo Parkkila<sup>2,3</sup> and Daumantas Matulis<sup>1</sup>

<sup>1</sup> Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Vilnius University, Vilnius, Lithuania

<sup>2</sup> Faculty of Medicine and Life sciences, University of Tampere, Tampere, Finland

<sup>3</sup> Fimlab Ltd, Tampere, Finland

\* These authors contributed equally to this work.

## ABSTRACT

The expression of carbonic anhydrase (CA) IX is up-regulated in many types of solid tumors in humans under hypoxic and acidic microenvironment. Inhibition of CA IX enzymatic activity with selective inhibitors, antibodies or labeled probes has been shown to reverse the acidic environment of solid tumors and reduce the tumor growth establishing the significant role of CA IX in tumorigenesis. Thus, the development of potent antitumor drugs targeting CA IX with minimal toxic effects is important for the target-specific tumor therapy. Recently, several promising antitumor agents against CA IX have been developed to treat certain types of cancers in combination with radiation and chemotherapy. Here we review the inhibition of CA IX by small molecule compounds and monoclonal antibodies. The methods of enzymatic assays, biophysical methods, animal models including zebrafish and *Xenopus* oocytes, and techniques of diagnostic imaging to detect hypoxic tumors using CA IX-targeted conjugates are discussed with the aim to overview the recent progress related to novel therapeutic agents that target CA IX in hypoxic tumors.

Submitted 2 August 2017

Accepted 30 October 2017

Published 23 November 2017

Corresponding author

Daumantas Matulis, matulis@ibt.lt,  
daumantas.matulis@bti.vu.lt

Academic editor

Camillo Rosano

Additional Information and  
Declarations can be found on  
page 15

DOI 10.7717/peerj.4068

© Copyright

2017 Kazokaite et al.

Distributed under

Creative Commons CC-BY 4.0

OPEN ACCESS

**Subjects** Biochemistry, Biophysics, Drugs and Devices

**Keywords** CA IX monoclonal antibodies, Hypoxic tumors, CA IX antitumor agents, Carbonic anhydrase IX, Drug development, Conjugated probes

## Introduction

Recent advances in cancer therapy show that hypoxia is the major contributor to tumor development (*Semenza, 2014; Hanahan & Weinberg, 2011*). The poor and chaotic tumor angiogenesis leads to the insufficient oxygen and nutrient supply which drastically affects the cellular metabolism (*Welti et al., 2013*). Due to the up-regulated glycolysis, tumor cells produce increased amounts of lactate and protons. As a consequence of mTORC1&2 mediated functional and transcriptional activation of c-Myc, tumor cells tend to metabolize glucose preferably via glycolysis rather than oxidative phosphorylation despite sufficient levels of oxygen. This phenomenon is known as Warburg effect (*Warburg, 1956; Vander Heiden, Cantley & Thompson, 2009*). The resultant hypoxic and acidic extracellular milieu

significantly increases the resistance of cancer cells to chemotherapy and radiotherapy as well as promotes invasiveness and metastasis ([Wojtkowiak et al., 2011](#); [Good & Harrington, 2013](#)).

Hypoxia stimulates crucial pathways, one of which is implemented by the activation of the heterodimeric hypoxia-inducible factor (HIF) ([Denko, 2008](#)). This hypoxia-induced transcriptional program is important for tumor cells to survive harsh conditions. There are many downstream-target genes of HIF, which encode proteins, such as adhesion molecules ([Ryu et al., 2010](#)), matrix metalloproteinases ([O'Toole et al., 2008](#)), chemokine receptors ([Li et al., 2009a](#)), growth factors ([Kotch et al., 1999](#)), differentiation proteins ([Takubo et al., 2010](#)), glycolytic enzymes ([Obach et al., 2004](#)), lactate transporters ([Ullah, Davies & Halestrap, 2006](#)), and ion transporters ([Parks, Chiche & Pouyssegur, 2013](#)). Some HIF-regulated proteins have been shown to be hypoxia-related anticancer targets and possess therapeutic applications ([Wilson & Hay, 2011](#)). Thus, HIF is critically essential for cancer cells to survive and metastasize in the hostile tumor environment due to the HIF-dependent activation of oncogenes and inactivation of tumor suppressor genes.

As a consequence of HIF-mediated transcriptional response to tumor hypoxia, the intracellular and extracellular pH is unbalanced. Normal cells differ from cancer cells by the mechanisms of pH regulation, which create the reversed pH gradient in tumors. Physiologically the intracellular pH ( $\text{pH}_i$ ) is lower than the extracellular pH ( $\text{pH}_e$ ), which is  $\sim 7.4$ . Pathologically  $\text{pH}_i$  is higher than  $\text{pH}_e$ , which is 6.7–7.1 ([Hashim et al., 2011](#); [Mazzio, Smith & Soliman, 2010](#)). This phenomenon of extracellular acidification under hypoxic conditions is created by HIF-dependent induction of proteins, such as transmembrane enzymes, ion pumps, and transporters. They export lactate and protons and import bicarbonate ions to optimize the tumor progression. Key pH-regulators are V-ATPase,  $\text{Na}^+/\text{H}^+$  exchanger (NHE), monocarboxylate transporters (MCTs) and carbonic anhydrase (CA) IX.

There are seven evolutionarily distinct CA gene families:  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -,  $\zeta$ -  $\eta$ -, and  $\theta$ -CAs ([Prete et al., 2014](#); [Supuran & Capasso, 2015](#); [Krishnamurthy et al., 2008](#); [Kikutani et al., 2016](#); [Aggarwal et al., 2013](#); [Capasso & Supuran, 2015](#)). In humans, there are 15  $\alpha$ -CA isoforms, of which 12 are catalytically active and exhibit diverse enzymatic activity, various cellular distribution and physiological functions ([Frost, 2014](#)). Being a member of  $\alpha$ -CA isoforms in human body, CA IX is a transmembrane homodimer, which catalyzes the reversible hydration of carbon dioxide to bicarbonate and proton outside the cell. The intracellular pH of cancer cells is regulated by the export of lactate and protons and on the import of bicarbonate ions generated by the hydration of  $\text{CO}_2$ . The acidic metabolites accumulate pericellularly because of the ineffective tumor vasculature and extracellular acidosis. To reduce changes of intracellular pH, the bicarbonate is transported into the cell through the bicarbonate transport metabolon composed of CA IX and bicarbonate transporters. Thereby CA IX is important for cancer cell proliferation because of the participation in both processes: the extracellular acidification and the intracellular alkalization ([Aggarwal et al., 2013](#); [Alterio et al., 2009](#)).

CA IX is relevant not only for the cancer cell survival, but also to several other biological processes, such as the maintenance of cancer stem cell (CSC) function, migration, and

invasion. Cell migration depends on the formation of lamellipodia, which have been shown to be partially produced by activation of CA IX and its interaction with bicarbonate transporters (Svastova *et al.*, 2012). In addition, acidosis under hypoxic conditions activates proteolytic enzymes, which degrade the extracellular matrix and promote metastasis formation. Thus, CA IX targeting compounds have shown to significantly diminish the cancer stem cell population, inhibit the growth of primary tumors, and reduce metastatic burden (Swietach *et al.*, 2010; Pastorek & Pastorekova, 2015; Sedlakova *et al.*, 2014; Lock *et al.*, 2013; McDonald *et al.*, 2010).

In normal tissues, the expression of CA IX is negligible with the exception of the stomach and gallbladder epithelia (Pastorekova *et al.*, 1997). There is a broad spectrum of aggressive malignancies, where CA IX is predominantly overexpressed, namely, neuroblastoma (Ameis *et al.*, 2016), breast tumor (Betof *et al.*, 2012), head and neck tumors (Yang *et al.*, 2014), ovarian tumor (Choschzick *et al.*, 2011), pancreatic tumor (Couvelard *et al.*, 2005), hepatocellular carcinoma (Huang *et al.*, 2015), etc. In addition, there are several reviews, which summarize the significance of CA IX as a promising biomarker for the tumor development (Van Kuijk *et al.*, 2016). Thus, CA IX has emerged as the clinically relevant biomarker and a potential anticancer-drug target.

At the core of  $\alpha$ -CA active site, the metal ion, Zn (II), is tetrahedrally coordinated to three imidazole rings from His94, 96, and 119 (numbering according to CA II) and a water/hydroxide anion (Fisher *et al.*, 2007). The catalytic site is located at approximately 15 Å depth conical cavity which consists of hydrophobic (Val121, Val143, Leu198, Val207, Trp209) as well as hydrophilic (Tyr7, Asn62, His64, Asn67, Thr199, Thr200) regions and provides the accessibility to the solvent (Krishnamurthy *et al.*, 2008; Eriksson, Jones & Liljas, 1988; Pocker & Sarkanen, 1978).

A high conservation of amino acids in the active site and surrounding faces has been found among the 12 catalytically active human CA isoforms (Aggarwal *et al.*, 2013; Pinard *et al.*, 2015). Thus, the design of CA isoform-selective inhibitors has been the challenging goal for many researchers. In 1954, acetazolamide was approved in clinic as the first CA-targeting antiglaucoma drug (Supuran, 2012). In the next decades, a vast collection of CA inhibitors with various affinities and selectivities has been designed and has been extensively reviewed (Lomelino & McKenna, 2016; Supuran, 2016; Supuran, 2017; Alterio *et al.*, 2012; Monti, Supuran & De Simone, 2013).

It is a challenging task to design inhibitors that would be not only highly selective to CA IX, but also safe for use in humans for the treatment and diagnosis of hypoxic tumors. Many aspects need to be considered to achieve the final goal of developing the promising drugs, that could selectively inhibit CA IX in hypoxic tumors. The knowledge about the active site structure of the protein and permeability of the inhibitor across the cell membrane is essential for designing the CA IX specific inhibitors. An inhibitor may be selective for CA IX, but it may need to be attached to a conjugate to make it impermeable through the membrane.

Similarly, the potential inhibitors need to go through the physical and biochemical screening and various modifications to develop as CA IX isoform specific compounds. The most promising CA IX inhibitors have to be screened for safety and toxicity *in vivo* using

animal models, such as zebrafish, before subjecting them to preclinical characterization. In addition to chemical compounds, CA IX-selective biological molecules, such as monoclonal antibodies (mAbs), are at various stages of preclinical and clinical trials as potential anticancer agents targeting CA IX in hypoxic tumors. In addition, the anticancer agents based on CA IX selective inhibitors can be conjugated with various probes for the diagnosis of hypoxic tumors.

## SURVEY METHODOLOGY

A wide variety of chemical compounds have been described in the literature that target tumor-associated CA IX. In this review, we selectively describe only aromatic sulfonamides that have been demonstrated to bind and inhibit the catalytic domain of recombinant human CA IX by at least two experimental approaches, such as inhibition of enzymatic activity and biophysical assays including the fluorescent thermal shift assay (FTSA), isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR). We emphasize the use of non-mammalian animal models, such as zebrafish and *Xenopus* oocytes for the toxicity, affinity, and selectivity studies of CA IX targeting sulfonamides. Published in 2016–2017, these studies suggest possibilities that could help in the development of antitumor agents prior to preclinical characterization in mice models.

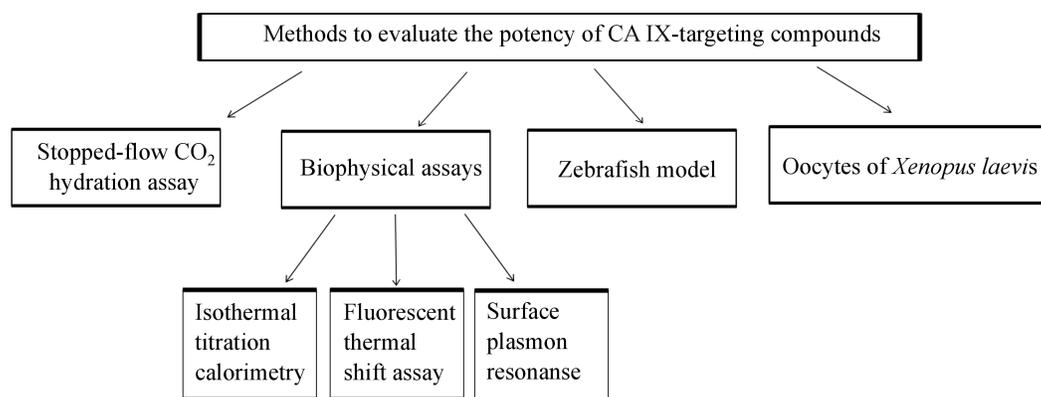
For reviewing the information, we identified the articles containing information about different biological and chemical antitumor agents that target CA IX in hypoxic tumors. The literature search was performed using the relevant keywords in PubMed. For example, the antibody section was compiled with all available articles published since 1986 up to 2017, in which the use of antibodies for the detection of CA IX in patients was described. Publications were retained if they contained relevant information about the promising agents that target CA IX in humans and also during the development of these agents in human cell lines and mice models. Priority was given to the antitumor agents that have been developed either for the treatment or imaging of the tumors using novel strategies.

The focus of this review is also to present recent developments in the treatment and diagnosis of solid tumors under hypoxic conditions that express CA IX. We present the recent achievements on the 8 diagnostic tools including chemical and biological antitumor agents targeting CA IX that are at various stages of preclinical and clinical trials for treating the hypoxic tumors. This review combines the information about animal models, enzymatic, biophysical methods used in CA field, as summarized in Fig. 1, with the latest references of novel anticancer agents that are currently applied to target CA IX for the diagnosis and treatment.

## CA INHIBITOR ASSAYS

### CA enzymatic activity inhibition assay

To evaluate the potency of CA-targeting inhibitor, the stopped-flow CO<sub>2</sub> hydration assay (SFA) has been widely applied for more than five decades since the discovery of the method to measure CA catalyzed CO<sub>2</sub> hydration rate by Gibbons and Edsall and by Khalifah (*Gibbons & Edsall, 1963; Gibbons & Edsall, 1964; Khalifah, 1971*). This approach



**Figure 1** Methods which might be applied for developing CA IX-targeting compounds before pre-clinical characterization in tumor cells and mice.

Full-size DOI: [10.7717/peerj.4068/fig-1](https://doi.org/10.7717/peerj.4068/fig-1)

is based on the monitoring of the changes in absorbance of pH sensitive indicator upon CA catalyzed CO<sub>2</sub> hydration reaction. The half-maximal inhibitory concentration, IC<sub>50</sub>, is determined by fitting the compound dose curve according to the Hill model or Morrison equation (Morrison, 1969). The inhibition constant, K<sub>i</sub>, can be obtained from IC<sub>50</sub> value by Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Supuran and co-authors have developed a large library of CA inhibitors by SFA and divided them into five groups according to CA inhibition mechanisms: (1) the zinc binders (sulfonamides and their isosteres, dithiocarbamates and their isosteres, hydroxamates, etc.) (Supuran, 2012; Alterio et al., 2012; Carta et al., 2013; Innocenti, Scozzafava & Supuran, 2010; Carta et al., 2012; Supuran, 2013); (2) compounds that anchor to the zinc-coordinated water molecule/hydroxide ion (phenols, polyamines, sulfocoumarins, etc.) (Nocentini et al., 2016; Davis et al., 2014; Carta et al., 2010; Innocenti et al., 2008; Santos et al., 2007); (3) inhibitors which occlude the entrance to the CA active site (coumarins and their isosteres) (Nocentini et al., 2015; Bozdog et al., 2017; Tars et al., 2013); (4) compounds which bind out of the active site (carboxylic acid derivatives) (D'Ambrosio et al., 2015); (5) inhibitors which bind in an unknown way (secondary/tertiary sulfonamides, imatinib, nilotinib, etc.) (Parkkila et al., 2009; Supuran, 2016; Métayer et al., 2013). Since these various compounds have been subject of numerous recent reviews, here we concentrate only on aromatic sulfonamides as CA inhibitors. Supuran's group also measured the affinity of monoclonal antibodies to target CA isoforms using SFA (Dekaminavičūtė et al., 2014). In addition to other previously synthesized compounds containing fluorine, our group has identified a series of fluorinated benzenesulfonamides as strong CA IX inhibitors by SFA and have shown a correlation between parameters obtained by enzymatic and biophysical assays (Dudutienė et al., 2014).

Importantly, CA isoforms share not only hydratase, but also esterase activity which was discovered in early 1960s (Tashian, Douglas & Yu, 1964). Both reactions occur in the same catalytic pocket suggesting similarities in their mechanisms. The method to determine

esterase activity is a high-throughput colorimetric assay with various applications, such as screening chemical molecules or antibodies against CA isozymes ([Akıncioğlu et al., 2013](#); [Uda et al., 2015](#)).

### **Biophysical assays of inhibitor binding to CAs**

Advantages and limitations of enzymatic inhibition versus biophysical assays of inhibitor binding have been assessed and are compared in our recent manuscript ([Smirnovienė, Smirnovas & Matulis, 2017](#)). Biophysical methods not only determine the thermodynamic parameters of ligand binding to CAs, but also provide insight into numerous significant factors, which influence the binding: local water structure, hydrogen bonding, hydrophobic interactions, and desolvation. The thermodynamic profiles of drug candidate binding to CA have been widely used. Here we will focus on biophysical techniques, such as fluorescent thermal shift assay (FTSA), isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR), which have been applied in the rational drug design of isoform-selective CA inhibitors.

#### ***Isothermal titration calorimetry***

Since the invention of first analog of an isothermal titration calorimeter in 1966 ([Izatt et al., 1966](#); [Christensen et al., 1966](#)) and its modifications for biological applications in 1980s ([Ramsay, Prabhu & Freire, 1986](#); [Schön & Freire, 1989](#)), ITC has become the method of choice to study protein target-ligand interactions. During the experiment, in the current commercial titration calorimeters, the inhibitor solution from the syringe is injected at constant temperature into the protein solution preloaded to the calorimeter cell until all binding sites of the protein become occupied by the ligand. Importantly, ITC does not require the inhibitor or protein to be labeled or immobilized and allows the determination of the affinity, the binding enthalpy and the stoichiometry in a single titration experiment ([Klebe, 2015](#); [Krimmer & Klebe, 2015](#); [Geschwindner, Ulander & Johansson, 2015](#); [Falconer, 2016](#)).

Numerous studies of interactions between diverse ligands and target CA isoforms have been performed by ITC ([Krishnamurthy et al., 2008](#); [DiTusa et al., 2001](#); [Khalifah et al., 1993](#)). The binding of anions to CA II was evaluated using ITC, X-ray crystallography, and molecular dynamics simulations by Whitesides group ([Fox et al., 2015](#)). For the deeper understanding of structure–activity relationships, the analysis of buffer ionization effects was performed by ITC upon an inhibitor binding to recombinant human CA isoforms, including CA I ([Morkūnaitė et al., 2015](#)), CA II ([Morkūnaitė et al., 2015](#)), CA VB ([Kasiliauskaitė et al., 2015](#)), CA VI ([Kazokaitė et al., 2015](#)), CA VII ([Pilipuitytė & Matulis, 2015](#)), CA IX ([Linkuvienė et al., 2016](#)), CA XII ([Jogaitė et al., 2013](#)), and CA XIII ([Baranauskienė & Matulis, 2012](#)). In addition, ITC standard and displacement titrations were combined with the X-ray crystallographic structures to determine the intrinsic, buffer-independent affinity of *para* substituted tetrafluorobenzenesulfonamides binding to several human CA isoforms ([Zubrienė et al., 2015](#)).

#### ***Fluorescent thermal shift assay***

FTSA, also called differential scanning fluorimetry and, in high-throughput format, ThermoFluor<sup>®</sup>, has been widely applied by numerous researchers and companies, such as

Johnson & Johnson, New Brunswick, United States. It is a rapid screening method in the drug discovery to measure the binding affinities of chemical compounds to targets (*Kranz & Schalk-Hihi, 2011; Lo et al., 2004; Pantoliano et al., 2001; Niesen, Berglund & Vedadi, 2007*). FTSA monitors the equilibrium of a protein between its folded and unfolded states by detecting the fluorescence of solvatochromic probes, such as 1,8-anilinonaphthalene sulfonate or SYPRO<sup>®</sup> orange, while the temperature is steadily increased. This method determines the protein melting temperature which can be highly affected by the affinity of ligand and its concentration (*Cimmperman & Matulis, 2011; Cimmperman et al., 2008*). In addition, FTSA is a convenient technique to characterize protein thermal stabilities at various conditions including diverse buffers, excipients, etc (*Mezzasalma et al., 2007; Cummings, Farnum & Nelen, 2006*).

FTSA has been widely applied in the search of CA inhibitors. The binding of sulfamate and sulfamide derivatives to human CA II was investigated using FTSA by *Klinger et al. (2006)*. FTSA was also applied by our group to investigate the interactions between human CA isoforms and various series of inhibitors, including tri- and tetrafluorobenzenesulfonamides (*Dudutienė et al., 2013; Dudutienė et al., 2015*), benzenesulfonamide derivatives with pyrimidine moieties (*Čapkauskaitė et al., 2013*), saccharin sulfonamides (*Morkūnaitė et al., 2014*), benzenesulfonamides with benzimidazole moieties (*Zubrienė et al., 2014*), 4-amino-substituted benzenesulfonamides (*Rutkauskas et al., 2014*). In addition, the profiles of thermal stabilities of recombinant human CA VB (*Kasiliauskaitė et al., 2015*), CA VI (*Kazokaitė et al., 2015*), CA IX (*Linkuvienė et al., 2016*), and CA XII (*Jogaitė et al., 2013*) was described using FTSA.

### **Surface plasmon resonance**

SPR was first demonstrated for the monitoring of biomolecular interactions by Lundstrom et al. in 1983 (*Liedberg, Nylander & Lunström, 1983*) and the first commercial SPR instrument was launched by Pharmacia Biosensors AB in 1991 (*Jönsson et al., 1991*). During the last decades, SPR biosensors have become the state-of-the-art technology in diagnostics and biomedical research to determine a real-time kinetics and binding affinities of ligand-protein interactions. To screen lead compounds, one of the binding partners, usually the target protein, is immobilized on a metal surface and the ligand flows over that surface by microfluidic system. SPR is a label-free optical method, which measures the changes in refractive index at the metal surface upon the binding reaction.

Studies of SPR application in CA research used recombinant human CA I (*Jecklin et al., 2009*) or mostly CA II (*Myszka, 2004; Navratilova & Hopkins, 2010; Papalia et al., 2006*) isoform as a model for the screening of numerous inhibitors. In contrast, Talibov et al. immobilized six human recombinant CA isoforms (full-length CA I, CA II, CA VII, CA XIII, catalytic domains of CA IX and CA XII) and analyzed their interactions with 17 benzenesulfonamide ligands by SPR. Interestingly, results revealed one compound from investigated series to be as a tight binder to recombinant CA IX with the dissociation rates too slow to be determined by SPR (*Talibov et al., 2016*).

## Zebrafish model for compound toxicity

Phenotype-based screening using zebrafish has become a promising high-throughput assay for the drug discovery. This approach revealed that 62% of drugs approved from 1999 till 2008, were discovered by phenotype-based screens despite that they represented only a small fraction of all screens (MacRae & Peterson, 2015). Phenotypic screens possess many significant advantages over target-based screens including the identification of drugs without a validated target or the characterization of the therapeutic profile of the compound, which affects several targets simultaneously. Zebrafish has emerged as a powerful model system for phenotypic screens of drug-candidates *in vivo* because of many advantages that include high homology between zebrafish and mammalian CAs, low cost, and avoidance of most ethical issues associated with the use of other animals. However, zebrafish lack lung, prostate, and mammary glands, heart septation, limbs, and it is necessary to grow zebrafish at 30 °C, while compounds against mammalian targets are usually optimized for 37 °C (Lin, Chiang & Tsai, 2016; Rennekamp & Peterson, 2015).

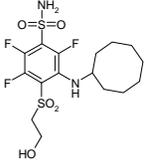
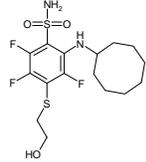
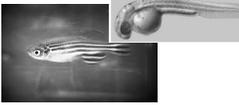
Zebrafish can be particularly useful to carry out toxicological studies of CA inhibitors. Toxic effects of two fluorinated benzenesulfonamides as CA IX inhibitors were investigated on zebrafish development (Kazokaitè et al., 2016b).  $LC_{50}$  values showed that one compound exhibited 10-fold lower toxicity than ethoxzolamide (EZA), a compound used as a drug in humans. In addition, light-field microscopy and histological analysis revealed that EZA induced side effects such as pericardial edema, unutilized yolk sac and abnormal body shape of zebrafish. In contrast, developmental abnormalities were not detected in embryos treated with the fluorinated benzenesulfonamides (Table 1). Thus, this study showed that CA IX inhibitors did not have adverse effects on phenotype and morphology of zebrafish larvae. Such toxicological screenings of the compounds using zebrafish could provide information on the safety of lead molecule that could be useful for further development into a drug.

## Oocyte system for heterologous expression of CAs to determine compound affinity and selectivity

Since 1960s, the *Xenopus laevis* has been widely used as a convenient animal model in various biomedical fields including molecular and physiological research. The *Xenopus* oocytes have many advantages including a large number of offspring, easy manipulations because of their big size (1.1–1.3 mm) and easy maintenance. Furthermore, oocytes feature highly efficient translation of heterologous RNA into protein.

Native *Xenopus* oocytes do not possess any CA activity and thus have become a convenient *in vivo* model system to investigate CA inhibitors. The enzymatic activity of CA can be evaluated with microelectrodes while monitoring the intracellular and extracellular acidification. Results can be confirmed by mass spectrometric gas analysis of lysed or intact oocytes (Becker, 2014). The transfection of *Xenopus* oocytes with cRNA of CA isozymes has been published by Deitmer's group (Klier et al., 2016; Schneider et al., 2013). They showed the complete inhibition of CA IX enzymatic activity with 30  $\mu$ M EZA according to the rates of cytosolic pH changes and amplitudes of pH changes at the outer membrane side (Klier et al., 2016). The same effect was found in CA IX expressing

**Table 1 Biological model systems for the investigation of CA IX inhibitors.** The compounds did not show any significant toxicity on zebrafish and possessed nanomolar  $IC_{50}$  for heterologous CA IX expressed in *Xenopus* oocytes. In addition, the selectivity of compounds toward CA isoforms was evaluated according to the effect of compounds on the reduction of extracellular (CA IX, CA IV, and CA XII) and intracellular (CA II) CA-induced acidification in oocytes (Kazokaitė et al., 2016a; Kazokaitė et al., 2016b).

Inhibitor	VD11-4-2	VD12-09
Type of study		
Toxicology	$LC_{50} = 120 \mu\text{M}$	$LC_{50} = 13 \mu\text{M}$
	Methods: 1. light-field microscopy 2. histological analysis	
Affinity and selectivity	CA IX: $IC_{50} = 25 \text{ nM}$ CA II: <5.0% effect on pH at $10 \mu\text{M}$ CA IV: 57.8% effect on pH at $10 \mu\text{M}$ CA XII: 28.0% effect on pH at $50 \text{ nM}$	CA IX: 25.5% effect on pH at $10 \mu\text{M}$ CA II: <5.0% effect on pH at $10 \mu\text{M}$
	Methods: 1. pH monitoring with microelectrodes 2. mass spectrometric gas analysis	

$IC_{50}$  - the concentration causing 50% inhibition of target activity,  $LC_{50}$  - 50% lethal concentration.

oocytes treated with  $1 \mu\text{M}$  fluorinated benzenesulfonamide targeting CA IX (Kazokaitė et al., 2016a). The  $IC_{50}$  was found to be in the range of 15–25 nM for both intracellularly and extracellularly expressed CA IX. Moreover, the compound exhibited strong selectivity over CA II, CA IV or CA XII in oocytes expressing a particular CA isoform (Table 1). This novel *in vivo* approach allows the identification of the affinity and selectivity of CA IX inhibitors in the living eukaryotic cell with fully matured target CA isozyme.

## CA IX-TARGETED STRATEGIES

Targeting CA IX enzyme is a promising approach for the development of new therapeutics against hypoxic tumors. There are several agents that can selectively target CA IX by using different strategies. Here, we present therapeutic agents that have been used against CA IX for diagnosis and treatment of hypoxic tumors in humans (Table 2).

### Monoclonal antibodies for CA IX-targeted therapy

M75 and chimeric G250 (cG250) are two widely-applied monoclonal antibodies (mAbs) recognizing human CA IX. These mAbs have been used for clinical detection or therapy (Oosterwijk et al., 1986; Závada et al., 1993). The M75 targets the PG-domain of CA IX and is used for the detection of CA IX in human tissues (Chrastina, Pastoreková & Pastorek, 2003; Chrastina et al., 2003; Zatovicova et al., 2010). cG250 has been successfully developed

**Table 2** Anti-tumor agents for targeting hypoxia-induced CA IX for therapy and diagnosis.

Anti-tumor agents	Therapy stage	Diagnosis	References
SLC-0111	Phase I trial	Solid tumors	<i>Wellichem Biotech Inc &amp; Ozmosis Research Inc (2014)</i>
U-104	Preclinical trials	Xenograft tumor model (pancreatic ductal adenocarcinoma cell line Pt45.P1/asTF+)	<i>Pacchiano et al. (2011), Lou et al. (2011), Ramchandani et al. (2016)</i>
G250 (girentuximab)	Phase III clinical trial	ccRCC diagnosis	<i>Willex (2004)</i>
<sup>177</sup> Lu-labelled girentuximab	Phase II clinical trials	ccRCC diagnosis	<i>Pal &amp; Agarwal (2016)</i>
Indisulam	Phase I clinical trials	Solid tumors	<i>Dittrich et al. (2007), Eisai Limited (2005)</i>
NIR fluorescent derivative of the acetazolamide	Preclinical trials	Xenograft tumor model	<i>Tafreshi et al. (2012)</i>
<sup>99m</sup> Tc-(HE)3-ZCAIX:2	Preclinical trials	Disseminated cancer	<i>Garousi et al. (2016)</i>
<sup>125</sup> I-ZCAIX:4	Preclinical trials	Primary renal cell carcinoma	<i>Garousi et al. (2016)</i>

for anticancer immunotherapy (*Cardone, Casavola & Reshkin, 2005*) due to its ability to elicit antibody-dependent cellular cytotoxicity (*Surfus et al., 1996*). The clinical trials showed that cG250 is safe, and has effect on the disease burden, when applied alone or together with interferon- $\alpha$  (*Davis et al., 2007; Siebels et al., 2011*). This mAb is marketed by WILEX AG using RENCAREX<sup>®</sup> as a trade name and has been used for renal cell carcinoma patients (RCC) who are at high risk of relapse (*McDonald et al., 2012*). In the recent past, this mAb under the name of girentuximab, has been assessed as an adjuvant in Phase III ARISER trial in RCC patients and showed that the patients expressing CA IX benefited more than ones without or minimal expression of CA IX (*Willex, 2004*). In a phase II study, the mAb labeled with lutetium (<sup>177</sup>Lu-girentuximab) demonstrated the significantly positive impact on the progressive metastatic ccRCC patients (*Pal & Agarwal, 2016*). In addition, REDECTANE<sup>®</sup> (<sup>124</sup>I-girentuximab) has been in clinical development targeting ccRCC (*Willex, 2017*). Furthermore, A3 and CC7 have been developed as CA IX-selective mAbs by the phage display method. They showed promising results in animal models of colorectal cancer and may be useful for the drug delivery (*Oosterwijk et al., 1986*). These studies clearly showed that mAbs and their modified versions are potential candidates for the development as anticancer agents targeting tumors that express CA IX.

Several monoclonal antibodies have been developed that influence the catalytic activity of CA IX (*Zat'ovicová et al., 2003*). Pastorekova's group has demonstrated that the mAb VII/20 binds to the catalytic domain of CA IX, causing the receptor-mediated internalization of the antibody-protein complex. Authors have shown that this process is important for the immunotherapy because significant anticancer effects of VII/20 were found in mouse xenograft model of colorectal carcinoma (*Zatovicova et al., 2010*). Thus, the application of CA IX-targeting antibodies might be significantly beneficial immunotherapeutic strategy.

Furthermore, mAbs have been considered as the ligands of choice for the design of antibody-drug conjugates (ADCs). In current clinical development, there are 65 ADCs mostly targeting various proteins at cell surface (Xu, 2015; Beck et al., 2017). Since antibodies might cause problems related with the penetration or immunogenicity, there is a demand for smaller agents, such as peptides or chemical derivatives, for the drug delivery. Recently, Neri with co-authors has described CA IX-targeting small-molecule drug conjugates. Monovalent and divalent conjugates of acetazolamide with the cytotoxic maytansinoid DM1 exhibited promising anticancer activity in SKRC52 renal cell carcinoma *in vivo* (Krall, Pretto & Neri, 2014; Krall et al., 2014).

### Chemical compounds targeting CA IX for therapy

A wide range of CA IX selective inhibitors has been designed with the help of X-ray crystallography and computational analysis. Among them, a group of sulfonamides show potential for developing as anticancer agents. A sulfonamide compound, indisulam, has shown a significant antitumor activity in preclinical cancer models (Dittrich et al., 2007). Phase II clinical trials were conducted to determine the efficacy, safety and tolerability of indisulam in combination with irinotecan in patients with metastatic colorectal cancer who were previously treated with 5-fluorouracil/leucovorin and oxaliplatin (Eisai Limited, 2005) but no further information is available about the outcome of the trial. Similarly, bis-sulfonamides have shown promising results *in vitro* in tumor sections and target tumors *in vivo* (Buller et al., 2011). Preclinical studies using ureidosulfonamide inhibitor of CA IX, named as U-104 or SLC-0111 (SignalChem Lifesciences Corp, Richmond, BC, CA), showed positive effects with the negligible toxicity for the treatment of various tumors (Pacchiano et al., 2011; Lou et al., 2011). Recently, U-104 has been demonstrated to be effective *in vitro* and *in vivo* models of the pancreatic ductal adenocarcinoma (Pt45.P1/asTF +). U-104 significantly decreased the growth of pancreatic cells in hypoxia but not in normoxia and reduced the tumor growth in mice emphasizing the potential of the compound as a therapeutic agent against CA IX (Ramchandani et al., 2016).

Small molecule-drug conjugates (SMDCs) have been used for the selective delivery of therapeutic agents to tumor sites. The series of stable and therapeutically active SMDCs were generated by attaching acetazolamide to monomethyl auristatin E using dipeptide linkers. They showed a promising antitumor activity in mice bearing SKRC-52 renal tumors. Since CA IX is a transmembrane protein, the findings of this study is significantly important for the targeted drug delivery in kidney cancer patients (Corso & Neri, 2017). Similarly, PEGylated bis-sulfonamide CA inhibitors were synthesized from aminosulfonamide pharmacophores conjugated with either ethyleneglycol oligomeric or polymeric diamines. These compounds efficiently controlled the growth of several CA IX-expressing cancer cell lines including colon HT-29, breast MDA-MB-23, and ovarian SKOV-3 (Akocak et al., 2016).

To demonstrate the antitumor effect of CA IX inhibition *in vivo*, the vast library of conjugates against CA IX has been designed. Dual targeting bioreductive nitroimidazole-based sulfamide drug, named as DH348, was used to evaluate the impact on the extracellular acidification and radiosensitivity in HT-29 colorectal cancer cells and mouse xenograft

models. By using nontoxic doses of DH348, the hypoxia-induced extracellular acidification was significantly reduced and the tumor growth was decreased. DH348 also sensitized the tumor to irradiation and the effect was CA IX-dependent (*Dubois et al., 2013*). In addition, the combination of SLC-0111 and APX3330 has been reported in patient-derived 3D pancreatic cancer models. Results of dual treatment showed a greater decrease in the intracellular pH and 3D tumor spheroid growth than treatment with either inhibitor alone (*Logsdon et al., 2016*). Recently, phase I clinical trial of SLC-0111 has been finished and the compound was scheduled to enter Phase II trials (*Welichem Biotech Inc & Ozmosis Research Inc, 2014*). Since results of phase I trials have not been published, the characterization of pharmacodynamics and pharmacokinetics of SLC-0111 is not available yet.

### Targeting CA IX using nanoparticles

Gold nanoparticles coated with chemical inhibitors are a relatively new to the field of the development of agents targeting CA IX. The gold nanoparticles modified with CA IX inhibitors cannot pass through the membrane. Thus, they show a great potency to be effective in targeting and inhibiting the extracellular active site of CA IX.

The nanoparticles, which were modified with thiols and benzenesulfonamide groups, selectively inhibited CA IX ( $K_i$  32 nM) but their affinities toward CA I and CA II were more than 10-folds lower ( $K_i$  451 nM). In addition, these nanoparticles possessed a greater affinity toward CA IX than acetazolamide and may be suitable candidates for imaging and treatment of hypoxic tumors (*Stiti et al., 2008*). Recently, gold nanoparticles were used to target CA IX for photoacoustic imaging and optical hyperthermia (*Supuran & Winum, 2015*). In addition, derivatives of benzenesulfonamides combined with nanorods showed a significant impact on the reduction of the extracellular acidification in hypoxic human mammary and colorectal carcinomas (*Ratto et al., 2015*). These studies suggest that the use of nanoparticles can be used to efficiently target extracellular part of CA IX in hypoxic tumors.

To improve the potency and selectivity of novel inhibitors, recently multivalent nanoconstructs have been developed (*Touissni et al., 2015; Kanfar et al., 2015*). These nanoconstructs showed excellent inhibitory effects with  $K_i$  values of 6.2–0.67 nM against tested CA isozymes. They contain multiple copies of a ligand, which are displayed closely on the same derivative. Thus, a weak mM binder can be changed to nM binder and the biomolecular recognition can be enhanced (*Kanfar et al., 2017*). Even though the use of multivalent nanoconstructs in the field of CA IX inhibition is quite recent, there is a great potential to develop CA IX inhibitors with high affinity and selectivity properties using this multivalent strategy.

## IMAGING METHODS

Detection of hypoxic regions of solid tumors is an important step for cancer treatment (*Bertout, Patel & Simon, 2008*). The application of selective ligands against CA IX in diagnostic imaging has been widely investigated. They could help to decide which patients can benefit from the adjunctive therapy (*Höckel et al., 1996*). Both antibodies and

small molecular weight compounds have been used for non-invasive imaging of CA IX in a number of aggressive and late stage types of tumors and metastases ([McDonald et al., 2012](#)).

### Imaging of tumors using CA IX-specific mAbs

CA IX is a useful biomarker for clear cell renal cell carcinoma (ccRCC) because CA IX is absent in normal kidney tissues. The CA IX-specific cG250, radiolabeled with iodine-124 or zirconium-89, has been used for the diagnosis of ccRCC ([Stillebroer et al., 2007](#)). High parameters of sensitivity and specificity were determined by positron emission tomography/computed tomography (PET/CT) when cG250 labeled with iodine-124 was applied for the imaging of ccRCC ([Divgi et al., 2007](#)). This study suggests a great potential to monitor ccRCC in patients and allows the differentiation of ccRCC versus non-ccRCC.

An iodine-125 radiolabelled M75, CA IX-selective mAb, has been developed for pre-clinical imaging of CA IX in hypoxic tumors in mouse xenograft models ([Chrastina, Pastoreková & Pastorek, 2003](#); [Chrastina et al., 2003](#)). In addition, human A3 and CC7 mini-antibodies have been designed. Their small size enables them to distribute faster compared to full sized antibodies. These antibodies do not inhibit the catalytic activity of CA IX and are selective for the extracellular domain of human CA IX ([Ahlskog et al., 2009b](#)). By using mAbs coated with near-infrared fluorescent (NIRF) molecules, molecular imaging probes have been developed and applied for the non-invasive detection of breast cancer axillary lymph node (ALN) metastases. The high selectivity of these probes have been confirmed *in vitro* and *in vivo* using models of preclinical breast cancer metastasis ([Tafreshi et al., 2012](#)).

### Affibody molecules for imaging of CA IX expression

The affibodies are specially engineered small proteins that can bind to target proteins with a high affinity similarly to mAbs. These molecules can be used as novel anticancer drugs and/or for radionuclide imaging of tumors. In a recent study, several *in vitro* and *in vivo* properties of affibodies labeled with  $^{99m}\text{Tc}$  and  $^{125}\text{I}$  were characterized. Tested affibodies were highly specific to CA IX in SK-RC-52 cells and selectively accumulated in SK-RC-52 xenografts ([Garousi et al., 2016](#)). The study suggests the usefulness of CA IX-binding affibodies for cancer detection and therapy.

### Imaging of CA IX expression with small molecular chemical probes

Chemical probes can be applied for labeling and detection of biomolecules in order to study molecular processes occurring within living cells. The sulfonamide-based CA inhibitors efficiently bind to CA IX in hypoxic tumors as the active site of the enzymes is only available upon hypoxic conditions ([Svastová et al., 2004](#)). Unlike CA IX-specific mAbs, sulfonamides can recognize cells that are in hypoxic conditions. Thus, CA IX inhibitors and mAbs can give the different information about imaging and prognosis ([Pastorekova, Ratcliffe & Pastorek, 2008](#)). To prevent the sulfonamide-based inhibitors from passing through the membrane, inhibitors can be conjugated with fluorescent dye (FITC), albumin or hydrophilic sugar moieties that would prevent their entry into the cell ([Li et al., 2009b](#)). Among them, sulfonamides attached to FITC were shown to be membrane-impermeable with a high affinity to CA IX. This imaging agent was able to bind

to CA IX, expressed in cells under hypoxic but not normoxic conditions (Svastová *et al.*, 2004). Similarly, acetazolamide-based derivatives bearing many types of NIRF dyes were designed as promising probes for the imaging of hypoxia-induced CA IX in tumor cells. Compounds were characterized to be up to 50-fold selective to CA IX compared to CA II. In preclinical studies using mice with HT-29 tumors, the significant impact of CA IX inhibitors with NIRF group on the non-invasive quantification of CA IX was determined (Groves *et al.*, 2012). Moreover, fluorescent sulfonamides containing a charged fluorophore have been used *in vivo* and have shown a great efficiency in detecting CA IX in HT-29 and SK-RC-52 tumor xenografts (Cecchi *et al.*, 2005; Ahlskog *et al.*, 2009a).

### Imaging hypoxic tumor areas with nonpeptidic ligand conjugates

Recently, nonpeptidic ligand conjugates have been evaluated for single-photon emission computed tomography (SPECT) imaging of hypoxic cancers that express CA IX (Lv, Putt & Low, 2016). For a better clinical care, a broader knowledge about the level of hypoxia is needed. CA IX-targeting ligand was synthesized with the aim to deliver the attached  $^{99m}\text{Tc}$ -chelating agent to hypoxic regions. The studies of binding characterization *in vitro* and imaging of the biodistribution *in vivo* were carried out. Results showed that several such conjugates can selectively bind to CA IX in tumors. This study revealed the significantly important applications of nonpeptidic ligand conjugates to evaluate the level of hypoxia in tumors (Lv, Putt & Low, 2016).

In summary, the mAbs G250 and M75 have the advantages of binding to CA IX selectively on the surface of cancer cells, and thus they are able to detect cancer cells that overexpress CA IX. This is because the mAbs are raised against specific epitopes of CA IX, and they are unable to pass through the cell membranes due to the high molecular weight. However, the mAbs (G250 and M75) bind to the PG domain, and therefore they cannot affect its catalytic activity. In contrast, chemical inhibitors recognize the active site and can inhibit the enzymatic activity of CA IX, but they might possess several disadvantages including the low selectivity because of similarity of the  $\alpha$ -CAs active sites, and the permeation through the plasma membrane. Thus, they might have off-target effects because of affinity to both intracellular and extracellular CAs. If the chemical inhibitors are conjugated with bulky molecules to avoid the internalization, they may still bind to other membrane CAs, such as CA XII. Thus, the properties of mAbs and chemical inhibitors need to be taken into consideration for using them as anticancer agents or as probes for the imaging of solid tumors.

## CONCLUSION

The critical role of CA IX in the tumor progression and aggressiveness has been shown and CA IX has been proposed as a promising therapeutic drug target and a clinically useful biomarker of the broad range of hypoxic tumors. Our review described efforts in the development of selective agents against CA IX. It is a challenging task to develop a compound of high affinity and selectivity towards only one CA isoform due to the high homology between twelve catalytically active CA isoforms in human body. Deeper insight in the structural analysis and interactions of proteins involved in pH regulatory mechanisms

of tumor cell could provide the relevant new strategies for rational drug design of CA IX-selective compounds for the therapy and diagnostic imaging.

#### List of abbreviations

<b>ADCs</b>	Antibody-drug conjugates
<b>ALN</b>	Axillary lymph node
<b>EZA</b>	Ethoxzolamide
<b>IC<sub>50</sub></b>	The concentration causing 50% inhibition of target activity
<b>CA</b>	Carbonic anhydrase
<b>ccRCC</b>	Clear cell renal cell carcinoma
<b>FITC</b>	Fluorescent dye
<b>FTSA</b>	Fluorescent thermal shift assay
<b>HIF</b>	Hypoxia-inducible factor
<b>ITC</b>	Isothermal titration calorimetry
<b>mAbs</b>	Monoclonal antibodies
<b>NIRF</b>	Near-infrared fluorescent
<b>PET/CT</b>	Positron emission tomography/computed tomography
<b>SFA</b>	Stopped-flow CO <sub>2</sub> hydration assay
<b>SLC</b>	SignalChem Lifesciences Corp
<b>SMDCs</b>	Small molecule-drug conjugates
<b>SPECT</b>	Single-photon emission computed tomography
<b>SPR</b>	Surface plasmon resonance
<b>U-104</b>	Ureidosulfonamide inhibitor of CA IX

## ADDITIONAL INFORMATION AND DECLARATIONS

### Funding

The work was supported by grants from the Research Council of Lithuania (Daumantas Matulis, grant number S-MIP-17-87), Jane & Aatos Erkkö Foundation (Seppo Parkkila), Sigrid Jusélius foundation (Seppo Parkkila), and Academy of Finland (Sepö Parkkila). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Grant Disclosures

The following grant information was disclosed by the authors:

Research Council of Lithuania: S-MIP-17-87.

Jane & Aatos Erkkö Foundation.

Sigrid Jusélius foundation.

Academy of Finland.

### Competing Interests

Daumantas Matulis declares that he has patents and patent applications for CA inhibiting compounds. Ashok Aspatwar and Seppo Parkkila are currently employed by Fimlab Ltd. Other authors confirm that this article content has no conflicts of interest.

## Author Contributions

- Justina Kazokaitė and Ashok Aspatwar conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Seppo Parkkila and Daumantas Matulis conceived and designed the experiments, analyzed the data, wrote the paper, reviewed drafts of the paper.

## Data Availability

The following information was supplied regarding data availability:

This is a review article that did not generate any additional previously unpublished raw data.

## REFERENCES

- Aggarwal M, Boone CD, Kondeti B, McKenna R. 2013.** Structural annotation of human carbonic anhydrases. *Journal of Enzyme Inhibition and Medicinal Chemistry* **28**:267–277 DOI [10.3109/14756366.2012.737323](https://doi.org/10.3109/14756366.2012.737323).
- Ahlskog JKJ, Dumelin CE, Trüssel S, Mårilind J, Neri D. 2009a.** *In vivo* targeting of tumor-associated carbonic anhydrases using acetazolamide derivatives. *Bioorganic & Medicinal Chemistry Letters* **19**:4851–4856 DOI [10.1016/j.bmcl.2009.06.022](https://doi.org/10.1016/j.bmcl.2009.06.022).
- Ahlskog JKJ, Schliemann C, Mårilind J, Qureshi U, Ammar A, Pedley RB, Neri D. 2009b.** Human monoclonal antibodies targeting carbonic anhydrase IX for the molecular imaging of hypoxic regions in solid tumours. *British Journal of Cancer* **101**:645–657 DOI [10.1038/sj.bjc.6605200](https://doi.org/10.1038/sj.bjc.6605200).
- Akıncioğlu A, Akbaba Y, Göçer H, Göksu S, Gülçin İ, Supuran CT. 2013.** Novel sulfamides as potential carbonic anhydrase isoenzymes inhibitors. *Bioorganic & Medicinal Chemistry* **21**:1379–1385 DOI [10.1016/j.bmc.2013.01.019](https://doi.org/10.1016/j.bmc.2013.01.019).
- Akokac S, Alam MR, Shabana AM, Sanku RKK, Vullo D, Thompson H, Swenson ER, Supuran CT, Ilies MA. 2016.** PEGylated bis-sulfonamide carbonic anhydrase inhibitors can efficiently control the growth of several carbonic anhydrase IX-expressing carcinomas. *Journal of Medicinal Chemistry* **59**:5077–5088 DOI [10.1021/acs.jmedchem.6b00492](https://doi.org/10.1021/acs.jmedchem.6b00492).
- Alterio V, Di Fiore A, D'Ambrosio K, Supuran CT, De Simone G. 2012.** Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? *Chemical Reviews* **112**:4421–4468 DOI [10.1021/cr200176r](https://doi.org/10.1021/cr200176r).
- Alterio V, Hilvo M, Fiore AD, Supuran CT, Pan P, Parkkila S, Scaloni A, Pastorek J, Pastorekova S, Pedone C, Scozzafava A, Monti SM, Simone GD. 2009.** Crystal structure of the catalytic domain of the tumor-associated human carbonic anhydrase IX. *Proceedings of the National Academy of Sciences of the United States of America* **106**:16233–16238 DOI [10.1073/pnas.0908301106](https://doi.org/10.1073/pnas.0908301106).
- Ameis HM, Drenckhan A, Freytag M, Izbicki JR, Supuran CT, Reinshagen K, Holland-Cunz S, Gros SJ. 2016.** Carbonic anhydrase IX correlates with survival and is a potential therapeutic target for neuroblastoma. *Journal of Enzyme Inhibition and Medicinal Chemistry* **31**:404–409 DOI [10.3109/14756366.2015.1029471](https://doi.org/10.3109/14756366.2015.1029471).

- Baranauskienė L, Matulis D. 2012.** Intrinsic thermodynamics of ethoxzolamide inhibitor binding to human carbonic anhydrase XIII. *BMC Biophysics* 5:12 DOI 10.1186/2046-1682-5-12.
- Beck A, Goetsch L, Dumontet C, Corvaia N. 2017.** Strategies and challenges for the next generation of antibody-drug conjugates. *Nature Reviews Drug Discovery* 16:315–337 DOI 10.1038/nrd.2016.268.
- Becker H. 2014.** Transport of lactate: characterization of the transporters involved in transport at the plasma membrane by heterologous protein expression in *Xenopus* oocytes. In: Hirrlinger J, Waagepetersen HS, eds. *Brain energy metabolism*. New York: Springer, 25–43.
- Bertout JA, Patel SA, Simon MC. 2008.** The impact of O<sub>2</sub> availability on human cancer. *Nature Reviews. Cancer* 8:967–975 DOI 10.1038/nrc2540.
- Betof AS, Rabbani ZN, Hardee ME, Kim SJ, Broadwater G, Bentley RC, Snyder SA, Vujaskovic Z, Oosterwijk E, Harris LN, Horton JK, Dewhirst MW, Blackwell KL. 2012.** Carbonic anhydrase IX is a predictive marker of doxorubicin resistance in early-stage breast cancer independent of HER2 and TOP2A amplification. *British Journal of Cancer* 106:916–922 DOI 10.1038/bjc.2012.32.
- Bozdog M, Alafeefy AM, Altamimi AM, Vullo D, Carta F, Supuran CT. 2017.** Coumarins and other fused bicyclic heterocycles with selective tumor-associated carbonic anhydrase isoforms inhibitory activity. *Bioorganic & Medicinal Chemistry* 25:677–683 DOI 10.1016/j.bmc.2016.11.039.
- Buller F, Steiner M, Frey K, Mircsof D, Scheuermann J, Kalisch M, Buhlmann P, Supuran CT, Neri D. 2011.** Selection of carbonic anhydrase IX inhibitors from one million DNA-encoded compounds. *ACS Chemical Biology* 6:336–344 DOI 10.1021/cb1003477.
- Capasso C, Supuran CT. 2015.** An overview of the alpha-, beta- and gamma-carbonic anhydrases from bacteria: can bacterial carbonic anhydrases shed new light on evolution of bacteria? *Journal of Enzyme Inhibition and Medicinal Chemistry* 30:325–332 DOI 10.3109/14756366.2014.910202.
- Čapkauskaitė E, Zubrienė A, Smirnov A, Torresan J, Kišonaitė M, Kazokaitė J, Gyltė J, Michailovienė V, Jogaitė V, Manakova E, Gražulis S, Tumkevičius S, Matulis D. 2013.** Benzenesulfonamides with pyrimidine moiety as inhibitors of human carbonic anhydrases I, II, VI, VII, XII, and XIII. *Bioorganic & Medicinal Chemistry* 21:6937–6947 DOI 10.1016/j.bmc.2013.09.029.
- Cardone RA, Casavola V, Reshkin SJ. 2005.** The role of disturbed pH dynamics and the Na<sup>+</sup>/H<sup>+</sup> exchanger in metastasis. *Nature Reviews. Cancer* 5:786–795 DOI 10.1038/nrc1713.
- Carta F, Aggarwal M, Maresca A, Scozzafava A, McKenna R, Masini E, Supuran CT. 2012.** Dithiocarbamates strongly inhibit carbonic anhydrases and show antiglaucoma action *in vivo*. *Journal of Medicinal Chemistry* 55:1721–1730 DOI 10.1021/jm300031j.

- Carta F, Akdemir A, Scozzafava A, Masini E, Supuran CT. 2013.** Xanthates and trithio-carbonates strongly inhibit carbonic anhydrases and show antiglaucoma effects *in vivo*. *Journal of Medicinal Chemistry* 56:4691–4700 DOI 10.1021/jm400414j.
- Carta F, Temperini C, Innocenti A, Scozzafava A, Kaila K, Supuran CT. 2010.** Polyamines inhibit carbonic anhydrases by anchoring to the zinc-coordinated water molecule. *Journal of Medicinal Chemistry* 53:5511–5522 DOI 10.1021/jm1003667.
- Cecchi A, Hulikova A, Pastorek J, Pastoreková S, Scozzafava A, Winum J-Y, Montero J-L, Supuran CT. 2005.** Carbonic anhydrase inhibitors. Design of fluorescent sulfonamides as probes of tumor-associated carbonic anhydrase IX that inhibit isozyme IX-mediated acidification of hypoxic tumors. *Journal of Medicinal Chemistry* 48:4834–4841 DOI 10.1021/jm0501073.
- Cheng Y, Prusoff WH. 1973.** Relationship between the inhibition constant ( $K_1$ ) and the concentration of inhibitor which causes 50 per cent inhibition ( $I_{50}$ ) of an enzymatic reaction. *Biochemical Pharmacology* 22:3099–3108 DOI 10.1016/0006-2952(73)90196-2.
- Choschzick M, Oosterwijk E, Müller V, Woelber L, Simon R, Moch H, Tennstedt P. 2011.** Overexpression of Carbonic Anhydrase IX (CAIX) is an independent unfavorable prognostic marker in endometrioid ovarian cancer. *Virchows Archiv* 459:193–200 DOI 10.1007/s00428-011-1105-y.
- Chrastina A, Pastoreková S, Pastorek J. 2003.** Immunotargeting of human cervical carcinoma xenograft expressing CA IX tumor-associated antigen by 125I-labeled M75 monoclonal antibody. *Neoplasma* 50:13–21.
- Chrastina A, Závada J, Parkkila S, Kaluz S, Kaluzová M, Raj cáni J, Pastorek J, Pastoreková S. 2003.** Biodistribution and pharmacokinetics of 125I-labeled monoclonal antibody M75 specific for carbonic anhydrase IX, an intrinsic marker of hypoxia, in nude mice xenografted with human colorectal carcinoma. *International Journal of Cancer* 105:873–881 DOI 10.1002/ijc.11142.
- Christensen JJ, Izatt RM, Hansen LD, Partridge JA. 1966.** Entropy titration. A calorimetric method for the determination of  $\Delta G$ ,  $\Delta H$ , and  $\Delta S$  from a single thermometric titration 1a,B. *The Journal of Physical Chemistry* 70:2003–2010 DOI 10.1021/j100878a049.
- Cimpmperman P, Baranauskienė L, Jachimovičiūtė S, Jachno J, Torresan J, Michailovienė V, Matulienė J, Sereikaitė J, Bumelis V, Matulis D. 2008.** A quantitative model of thermal stabilization and destabilization of proteins by ligands. *Biophysical Journal* 95:3222–3231 DOI 10.1529/biophysj.108.134973.
- Cimpmperman P, Matulis D. 2011.** Chapter 8: protein thermal denaturation measurements via a fluorescent dye. In: *Chapter 8: protein thermal denaturation measurements via a fluorescent dye*. 247–274.
- Corso AD, Neri D. 2017.** Linker stability influences the anti-tumor activity of acetazolamide-drug conjugates for the therapy of renal cell carcinoma. *Journal of Controlled Release* 246:39–45 DOI 10.1016/j.jconrel.2016.11.023.

- Couvelard A, O'Toole D, Turley H, Leek R, Sauvanet A, Degott C, Ruzzniewski P, Belghiti J, Harris AL, Gatter K, Pezzella F. 2005. Microvascular density and hypoxia-inducible factor pathway in pancreatic endocrine tumours: negative correlation of microvascular density and VEGF expression with tumour progression. *British Journal of Cancer* 92:94–101 DOI 10.1038/sj.bjc.6602245.
- Cummings MD, Farnum MA, Nelen MI. 2006. Universal screening methods and applications of thermofluor. *Journal of Biomolecular Screening* 11:854–863 DOI 10.1177/1087057106292746.
- D'Ambrosio K, Carradori S, Monti SM, Buonanno M, Secci D, Vullo D, Supuran CT, De Simone G. 2015. Out of the active site binding pocket for carbonic anhydrase inhibitors. *Chemical Communications* 51:302–305 DOI 10.1039/C4CC07320G.
- Davis ID, Wiseman GA, Lee F-T, Gansen DN, Hopkins W, Papenfuss AT, Liu Z, Moynihan TJ, Croghan GA, Adjei AA, Hoffman EW, Ingle JN, Old LJ, Scott AM. 2007. A phase I multiple dose, dose escalation study of cG250 monoclonal antibody in patients with advanced renal cell carcinoma. *Cancer Immunity* 7:13–21.
- Davis RA, Vullo D, Supuran CT, Poulsen S-A. 2014. Natural product polyamines that inhibit human carbonic anhydrases. *BioMed Research International* 2014:e374079 DOI 10.1155/2014/374079.
- Dekaminavičiūtė D, Kairys V, Zilnytė M, Petrikaitė V, Jogaitė V, Matulienė J, Gudlevičienė Z, Vullo D, Supuran CT, Žvirblienė A. 2014. Monoclonal antibodies raised against 167–180 aa sequence of human carbonic anhydrase XII inhibit its enzymatic activity. *Journal of Enzyme Inhibition and Medicinal Chemistry* 29:804–810 DOI 10.3109/14756366.2013.856424.
- Denko NC. 2008. Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nature Reviews Cancer* 8:705–713 DOI 10.1038/nrc2468.
- Dittrich C, Zandvliet AS, Gneist M, Huitema ADR, King AAJ, Wanders J. 2007. A phase I and pharmacokinetic study of indisulam in combination with carboplatin. *British Journal of Cancer* 96:559–566 DOI 10.1038/sj.bjc.6603606.
- DiTusa CA, Christensen T, McCall KA, Fierke CA, Toone EJ. 2001. Thermodynamics of metal ion binding. 1. Metal ion binding by wild-type carbonic anhydrase. *Biochemistry* 40:5338–5344 DOI 10.1021/bi001731e.
- Divgi CR, Pandit-Taskar N, Jungbluth AA, Reuter VE, Gönen M, Ruan S, Pierre C, Nagel A, Pryma DA, Humm J, Larson SM, Old LJ, Russo P. 2007. Preoperative characterisation of clear-cell renal carcinoma using iodine-124-labelled antibody chimeric G250 (124I-cG250) and PET in patients with renal masses: a phase I trial. *The Lancet. Oncology* 8:304–310 DOI 10.1016/S1470-2045(07)70044-X.
- Dubois L, Peeters SGJA, Van Kuijk SJA, Yaromina A, Lieuwes NG, Saraya R, Biemans R, Rami M, Parvathaneni NK, Vullo D, Vooijs M, Supuran CT, Winum J-Y, Lambin P. 2013. Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: a new concept of dual targeting drugs. *Radiotherapy and Oncology: Journal of the European Society for Therapeutic Radiology and Oncology* 108:523–528 DOI 10.1016/j.radonc.2013.06.018.

- Dudutienė V, Matulienė J, Smirnov A, Timm DD, Zubrienė A, Baranauskienė L, Morkūnaitė V, Smirnovienė J, Michailovienė V, Juozapaitienė V, Mickevičiūtė A, Kazokaitė J, Bakšytė S, Kasiliauskaitė A, Jachno J, Revuckienė J, Kišonaitė M, Pilipuitytė V, Ivanauskaitė E, Milinavičiūtė G, Smirnovas V, Petrikaitė V, Kairys V, Petrauskas V, Norvaišas P, Lingė D, Gibieža P, Čapkauskaitė E, Zakšauskas A, Kazlauskas E, Manakova E, Gražulis S, Ladbury JE, Matulis D. 2014. Discovery and characterization of novel selective inhibitors of carbonic anhydrase IX. *Journal of Medicinal Chemistry* 57:9435–9446 DOI 10.1021/jm501003k.
- Dudutienė V, Zubrienė A, Smirnov A, Gyltė J, Timm D, Manakova E, Gražulis S, Matulis D. 2013. 4-substituted-2,3,5,6-tetrafluorobenzenesulfonamides as inhibitors of carbonic anhydrases I, II, VII, XII, and XIII. *Bioorganic & Medicinal Chemistry* 21:2093–2106 DOI 10.1016/j.bmc.2013.01.008.
- Dudutienė V, Zubrienė A, Smirnov A, Timm DD, Smirnovienė J, Kazokaitė J, Michailovienė V, Zakšauskas A, Manakova E, Gražulis S, Matulis D. 2015. Functionalization of fluorinated benzenesulfonamides and their inhibitory properties toward carbonic anhydrases. *ChemMedChem* 10:662–687 DOI 10.1002/cmdc.201402490.
- Eisai Limited. 2005. An open label phase II study of indisulam in combination with irinotecan in patients with metastatic colorectal cancer who have been previously treated with 5-fluorouracil/leucovorin and oxaliplatin. Available at <https://clinicaltrials.gov/ct2/show/NCT00165867> (accessed on 17 October 2017).
- Eriksson AE, Jones TA, Liljas A. 1988. Refined structure of human carbonic anhydrase II at 2.0 Å resolution. *Proteins* 4:274–282 DOI 10.1002/prot.340040406.
- Falconer RJ. 2016. Applications of isothermal titration calorimetry—the research and technical developments from 2011 to 2015. *Journal of Molecular Recognition* 29(10):504–515 DOI 10.1002/jmr.2550.
- Fisher SZ, Maupin CM, Budayova-Spano M, Govindasamy L, Tu C, Agbandje-McKenna M, Silverman DN, Voth GA, McKenna R. 2007. Atomic crystal and molecular dynamics simulation structures of human carbonic anhydrase II: insights into the proton transfer mechanism. *Biochemistry* 46:2930–2937 DOI 10.1021/bi062066y.
- Fox JM, Kang K, Sherman W, Héroux A, Sastry GM, Baghbanzadeh M, Lockett MR, Whitesides GM. 2015. Interactions between Hofmeister anions and the binding pocket of a protein. *Journal of the American Chemical Society* 137:3859–3866 DOI 10.1021/jacs.5b00187.
- Frost SC. 2014. Physiological functions of the alpha class of carbonic anhydrases. *Subcellular Biochemistry* 75:9–30 DOI 10.1007/978-94-007-7359-2\_2.
- Garousi J, Honarvar H, Andersson KG, Mitran B, Orlova A, Buijs J, Löfblom J, Frejd FY, Tolmachev V. 2016. Comparative evaluation of affibody molecules for radionuclide imaging of *in vivo* expression of carbonic anhydrase IX. *Molecular Pharmaceutics* 13:3676–3687 DOI 10.1021/acs.molpharmaceut.6b00502.

- Geschwindner S, Ulander J, Johansson P. 2015.** Ligand binding thermodynamics in drug discovery: still a hot tip? *Journal of Medicinal Chemistry* **58**:6321–6335 DOI [10.1021/jm501511f](https://doi.org/10.1021/jm501511f).
- Gibbons BH, Edsall JT. 1963.** Rate of hydration of carbon dioxide and dehydration of carbonic acid at 25 degrees. *The Journal of Biological Chemistry* **238**:3502–3507.
- Gibbons BH, Edsall JT. 1964.** Kinetic studies of human carbonic anhydrases b and c. *The Journal of Biological Chemistry* **239**:2539–2544.
- Good JS, Harrington KJ. 2013.** The hallmarks of cancer and the radiation oncologist: updating the 5Rs of radiobiology. *Clinical Oncology (Royal College of Radiologists (Great Britain))* **25**:569–577 DOI [10.1016/j.clon.2013.06.009](https://doi.org/10.1016/j.clon.2013.06.009).
- Groves K, Bao B, Zhang J, Handy E, Kennedy P, Cuneo G, Supuran CT, Yared W, Peterson JD, Rajopadhye M. 2012.** Synthesis and evaluation of near-infrared fluorescent sulfonamide derivatives for imaging of hypoxia-induced carbonic anhydrase ix expression in tumors. *Bioorganic & Medicinal Chemistry Letters* **22**:653–657 DOI [10.1016/j.bmcl.2011.10.058](https://doi.org/10.1016/j.bmcl.2011.10.058).
- Hanahan D, Weinberg RA. 2011.** Hallmarks of cancer: the next generation. *Cell* **144**:646–674 DOI [10.1016/j.cell.2011.02.013](https://doi.org/10.1016/j.cell.2011.02.013).
- Hashim AI, Zhang X, Wojtkowiakk JW, Gillies RJ. 2011.** Imaging pH and metastasis. *NMR in Biomedicine* **24**:582–591 DOI [10.1002/nbm.1644](https://doi.org/10.1002/nbm.1644).
- Höckel M, Schlenger K, Mitze M, Schäffer U, Vaupel P. 1996.** Hypoxia and radiation response in human tumors. *Seminars in Radiation Oncology* **6**:3–9 DOI [10.1016/S1053-4296\(96\)80031-2](https://doi.org/10.1016/S1053-4296(96)80031-2).
- Huang W-J, Jeng Y-M, Lai H-S, Fong I-U, Sheu F-YB, Lai P-L, Yuan R-H. 2015.** Expression of hypoxic marker carbonic anhydrase IX predicts poor prognosis in resectable hepatocellular carcinoma. *PLOS ONE* **10**(3):e0119181 DOI [10.1371/journal.pone.0119181](https://doi.org/10.1371/journal.pone.0119181).
- Innocenti A, Scozzafava A, Supuran CT. 2010.** Carbonic anhydrase inhibitors. Inhibition of transmembrane isoforms IX, XII, and XIV with less investigated anions including trithiocarbonate and dithiocarbamate. *Bioorganic & Medicinal Chemistry Letters* **20**:1548–1550 DOI [10.1016/j.bmcl.2010.01.081](https://doi.org/10.1016/j.bmcl.2010.01.081).
- Innocenti A, Vullo D, Scozzafava A, Supuran CT. 2008.** Carbonic Anhydrase Inhibitors: interactions of Phenols with the 12 catalytically active mammalian isoforms (CA I–XIV). *Bioorganic & Medicinal Chemistry Letters* **18**:1583–1587 DOI [10.1016/j.bmcl.2008.01.077](https://doi.org/10.1016/j.bmcl.2008.01.077).
- Izatt RM, Rytting JH, Hansen LD, Christensen JJ. 1966.** Thermodynamics of proton dissociation in dilute aqueous solution. V. An entropy titration study of adenosine, pentoses, hexoses, and related compounds 1a, B. *Journal of the American Chemical Society* **88**:2641–2645 DOI [10.1021/ja00964a003](https://doi.org/10.1021/ja00964a003).
- Jecklin MC, Schauer S, Dumelin CE, Zenobi R. 2009.** Label-free determination of protein-ligand binding constants using mass spectrometry and validation using surface plasmon resonance and isothermal titration calorimetry. *Journal of Molecular Recognition* **22**:319–329 DOI [10.1002/jmr.951](https://doi.org/10.1002/jmr.951).

- Jogaitė V, Zubrienė A, Michailovienė V, Gylytė J, Morkūnaitė V, Matulis D. 2013.** Characterization of human carbonic anhydrase XII stability and inhibitor binding. *Bioorganic & Medicinal Chemistry* 21:1431–1436 DOI [10.1016/j.bmc.2012.10.016](https://doi.org/10.1016/j.bmc.2012.10.016).
- Jönsson U, Fägerstam L, Ivarsson B, Johnsson B, Karlsson R, Lundh K, Löfås S, Persson B, Roos H, Rönnberg I. 1991.** Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *BioTechniques* 11:620–627.
- Kanfar N, Bartolami E, Zelli R, Marra A, Winum J-Y, Ulrich S, Dumy P. 2015.** Emerging trends in enzyme inhibition by multivalent nanoconstructs. *Organic & Biomolecular Chemistry* 13:9894–9906 DOI [10.1039/C5OB01405K](https://doi.org/10.1039/C5OB01405K).
- Kanfar N, Tanc M, Dumy P, Supuran C, Ulrich S, Winum J-Y. 2017.** Effective access to multivalent inhibitors of carbonic anhydrases promoted by peptide bioconjugation. *Chemistry* 23(28):6788–6794 DOI [10.1002/chem.201700241](https://doi.org/10.1002/chem.201700241).
- Kasiliauskaitė A, Casaitė V, Juozapaitienė V, Zubrienė A, Michailovienė V, Revuckienė J, Baranauskienė L, Meškys R, Matulis D. 2015.** Thermodynamic characterization of human carbonic anhydrase VB stability and intrinsic binding of compounds. *Journal of Thermal Analysis and Calorimetry* 123:2191–2200.
- Kazokaitė J, Ames S, Becker HM, Deitmer JW, Matulis D. 2016a.** Selective inhibition of human carbonic anhydrase IX in *Xenopus* oocytes and MDA-MB-231 breast cancer cells. *Journal of Enzyme Inhibition and Medicinal Chemistry* 1–7.
- Kazokaitė J, Aspatwar A, Kairys V, Parkkila S, Matulis D. 2016b.** Fluorinated benzenesulfonamide anticancer inhibitors of carbonic anhydrase IX exhibit lower toxic effects on zebrafish embryonic development than ethoxzolamide. *Drug and Chemical Toxicology* 1–11.
- Kazokaitė J, Milinavičiūtė G, Smirnovienė J, Matulienė J, Matulis D. 2015.** Intrinsic binding of 4-substituted-2,3,5,6-tetrafluorobenzenesulfonamides to native and recombinant human carbonic anhydrase VI. *The FEBS Journal* 282:972–983 DOI [10.1111/febs.13196](https://doi.org/10.1111/febs.13196).
- Khalifah RG. 1971.** The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human enzymes B and C. *Journal of Biological Chemistry* 246:2561–2573.
- Khalifah RG, Zhang F, Parr JS, Rowe ES. 1993.** Thermodynamics of binding of the CO<sub>2</sub>-competitive inhibitor imidazole and related compounds to human carbonic anhydrase I: an isothermal titration calorimetry approach to studying weak binding by displacement with strong inhibitors. *Biochemistry* 32:3058–3066 DOI [10.1021/bi00063a017](https://doi.org/10.1021/bi00063a017).
- Kikutani S, Nakajima K, Nagasato C, Tsuji Y, Miyatake A, Matsuda Y. 2016.** Thylakoid luminal theta-carbonic anhydrase critical for growth and photosynthesis in the marine diatom *Phaeodactylum tricorutum*. *Proceedings of the National Academy of Sciences of the United States of America* 113:9828–9833 DOI [10.1073/pnas.1603112113](https://doi.org/10.1073/pnas.1603112113).
- Klebe G. 2015.** Applying thermodynamic profiling in lead finding and optimization. *Nature Reviews Drug Discovery* 14:95–110 DOI [10.1038/nrd4486](https://doi.org/10.1038/nrd4486).

- Klier M, Jamali S, Ames S, Schneider H-P, Becker HM, Deitmer JW. 2016.** Catalytic activity of human carbonic anhydrase isoform IX is displayed both extra- and intracellularly. *FEBS Journal* **283**:191–200 DOI [10.1111/febs.13562](https://doi.org/10.1111/febs.13562).
- Klinger AL, McComsey DF, Smith-Swintosky V, Shank RP, Maryanoff BE. 2006.** Inhibition of carbonic anhydrase-II by sulfamate and sulfamide groups: an investigation involving direct thermodynamic binding measurements. *Journal of Medicinal Chemistry* **49**:3496–3500 DOI [10.1021/jm058279n](https://doi.org/10.1021/jm058279n).
- Kotch LE, Iyer NV, Laughner E, Semenza GL. 1999.** Defective vascularization of HIF-1alpha-Null embryos is not associated with VEGF deficiency but with mesenchymal cell death. *Developmental Biology* **209**:254–267 DOI [10.1006/dbio.1999.9253](https://doi.org/10.1006/dbio.1999.9253).
- Krall N, Pretto F, Decurtins W, Bernardes G, Calo JL, Supuran CT, Neri D. 2014.** A small-molecule drug conjugate for the treatment of carbonic anhydrase IX expressing tumors. *Angewandte Chemie (International Edition in English)* **53**:4231–4235 DOI [10.1002/anie.201310709](https://doi.org/10.1002/anie.201310709).
- Krall N, Pretto F, Neri D. 2014.** A bivalent small molecule-drug conjugate directed against carbonic anhydrase IX can elicit complete tumour regression in mice. *Chemical Science* **5**:3640–3644 DOI [10.1039/C4SC00685B](https://doi.org/10.1039/C4SC00685B).
- Kranz JK, Schalk-Hihi C. 2011.** Protein thermal shifts to identify low molecular weight fragments. *Methods in Enzymology* **493**:277–298 DOI [10.1016/B978-0-12-381274-2.00011-X](https://doi.org/10.1016/B978-0-12-381274-2.00011-X).
- Krimmer SG, Klebe G. 2015.** Thermodynamics of protein–ligand interactions as a reference for computational analysis: how to assess accuracy, reliability and relevance of experimental data. *Journal of Computer-Aided Molecular Design* **29**:867–883 DOI [10.1007/s10822-015-9867-y](https://doi.org/10.1007/s10822-015-9867-y).
- Krishnamurthy VM, Kaufman GK, Urbach AR, Gitlin I, Gudiksen KL, Weibel DB, Whitesides GM. 2008.** Carbonic anhydrase as a model for biophysical and physical-organic studies of proteins and protein-ligand binding. *Chemical Reviews* **108**:946–1051 DOI [10.1021/cr050262p](https://doi.org/10.1021/cr050262p).
- Li Y, Qiu X, Zhang S, Zhang Q, Wang E. 2009a.** Hypoxia induced CCR7 expression via HIF-1alpha and HIF-2alpha correlates with migration and invasion in lung cancer cells. *Cancer Biology & Therapy* **8**:322–330 DOI [10.4161/cbt.8.4.7332](https://doi.org/10.4161/cbt.8.4.7332).
- Li Y, Wang H, Oosterwijk E, Selman Y, Mira JC, Medrano T, Shiverick KT, Frost SC. 2009b.** Antibody-specific detection of CAIX in breast and prostate cancers. *Biochemical and Biophysical Research Communications* **386**:488–492 DOI [10.1016/j.bbrc.2009.06.064](https://doi.org/10.1016/j.bbrc.2009.06.064).
- Liedberg B, Nylander C, Lunström I. 1983.** Surface plasmon resonance for gas detection and biosensing. *Sensors and Actuators* **4**:299–304 DOI [10.1016/0250-6874\(83\)85036-7](https://doi.org/10.1016/0250-6874(83)85036-7).
- Lin C-Y, Chiang C-Y, Tsai H-J. 2016.** Zebrafish and medaka: new model organisms for modern biomedical research. *Journal of Biomedical Science* **23**:19–30 DOI [10.1186/s12929-016-0236-5](https://doi.org/10.1186/s12929-016-0236-5).

- Linkuvienė V, Matulienė J, Juozapaitienė V, Michailovienė V, Jachno J, Matulis D. 2016.** Intrinsic thermodynamics of inhibitor binding to human carbonic anhydrase IX. *Biochimica et Biophysica Acta (BBA)—General Subjects* **1860**:708–718 DOI [10.1016/j.bbagen.2016.01.007](https://doi.org/10.1016/j.bbagen.2016.01.007).
- Lo M-C, Aulabaugh A, Jin G, Cowling R, Bard J, Malamas M, Ellestad G. 2004.** Evaluation of fluorescence-based thermal shift assays for hit identification in drug discovery. *Analytical Biochemistry* **332**:153–159 DOI [10.1016/j.ab.2004.04.031](https://doi.org/10.1016/j.ab.2004.04.031).
- Lock FE, McDonald PC, Lou Y, Serrano I, Chafe SC, Ostlund C, Aparicio S, Winum J-Y, Supuran CT, Dedhar S. 2013.** Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. *Oncogene* **32**:5210–5219 DOI [10.1038/onc.2012.550](https://doi.org/10.1038/onc.2012.550).
- Logsdon DP, Grimard M, Luo M, Shahda S, Jiang Y, Tong Y, Yu Z, Zyromski N, Schipani E, Carta F, Supuran CT, Korc M, Ivan M, Kelley MR, Fishel ML. 2016.** Regulation of HIF1 $\alpha$  under hypoxia by APE1/Ref-1 impacts CA9 expression: dual targeting in patient-derived 3D pancreatic cancer models. *Molecular Cancer Therapeutics* **15**:2722–2732 DOI [10.1158/1535-7163.MCT-16-0253](https://doi.org/10.1158/1535-7163.MCT-16-0253).
- Lomelino C, McKenna R. 2016.** Carbonic anhydrase inhibitors: a review on the progress of patent literature (2011–2016). *Expert Opinion on Therapeutic Patents* **26**:947–956 DOI [10.1080/13543776.2016.1203904](https://doi.org/10.1080/13543776.2016.1203904).
- Lou Y, McDonald PC, Oloumi A, Chia S, Ostlund C, Ahmadi A, Kyle A, Auf dem Keller U, Leung S, Huntsman D, Clarke B, Sutherland BW, Waterhouse D, Bally M, Roskelley C, Overall CM, Minchinton A, Pacchiano F, Carta F, Scozzafava A, Touisni N, Winum J-Y, Supuran CT, Dedhar S. 2011.** Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Research* **71**:3364–3376 DOI [10.1158/0008-5472.CAN-10-4261](https://doi.org/10.1158/0008-5472.CAN-10-4261).
- Lv P-C, Putt KS, Low PS. 2016.** Evaluation of nonpeptidic ligand conjugates for SPECT imaging of hypoxic and carbonic anhydrase IX-expressing cancers. *Bioconjugate Chemistry* **27**:1762–1769 DOI [10.1021/acs.bioconjchem.6b00271](https://doi.org/10.1021/acs.bioconjchem.6b00271).
- MacRae CA, Peterson RT. 2015.** Zebrafish as tools for drug discovery. *Nature Reviews Drug Discovery* **14**:721–731 DOI [10.1038/nrd4627](https://doi.org/10.1038/nrd4627).
- Mazzio EA, Smith B, Soliman KFA. 2010.** Evaluation of endogenous acidic metabolic products associated with carbohydrate metabolism in tumor cells. *Cell Biology and Toxicology* **26**:177–188 DOI [10.1007/s10565-009-9138-6](https://doi.org/10.1007/s10565-009-9138-6).
- McDonald PC, Winum J-Y, Supuran CT, Dedhar S. 2010.** Recent developments in targeting carbonic anhydrase IX for cancer therapeutics. *Oncotarget* **1**:84–97 DOI [10.18632/oncotarget.112](https://doi.org/10.18632/oncotarget.112).
- McDonald PC, Winum J-Y, Supuran CT, Dedhar S. 2012.** Recent developments in targeting carbonic anhydrase IX for cancer therapeutics. *Oncotarget* **3**:84–97 DOI [10.18632/oncotarget.422](https://doi.org/10.18632/oncotarget.422).
- Métayer B, Martin-Mingot A, Vullo D, Supuran CT, Thibaudeau S. 2013.** Superacid synthesized tertiary benzenesulfonamides and benzofused sultams act as selective hCA IX inhibitors: toward understanding a new mode of inhibition by tertiary

- sulfonamides. *Organic & Biomolecular Chemistry* **11**:7540–7549  
DOI 10.1039/c3ob41538d.
- Mezzasalma TM, Kranz JK, Chan W, Struble GT, Schalk-Hihi C, Deckman IC, Springer BA, Todd MJ. 2007.** Enhancing recombinant protein quality and yield by protein stability profiling. *Journal of Biomolecular Screening* **12**:418–428  
DOI 10.1177/1087057106297984.
- Monti SM, Supuran CT, De Simone G. 2013.** Anticancer carbonic anhydrase inhibitors: a patent review (2008–2013). *Expert Opinion on Therapeutic Patents* **23**:737–749  
DOI 10.1517/13543776.2013.798648.
- Morkūnaitė V, Baranauskienė L, Zubrienė A, Kairys V, Ivanova J, Trapencieris P, Matulis D. 2014.** Saccharin sulfonamides as inhibitors of carbonic anhydrases I, II, VII, XII, and XIII, saccharin sulfonamides as inhibitors of carbonic anhydrases I, II, VII, XII, and XIII. *BioMed Research International* **2014**:e638902.
- Morkūnaitė V, Gylytė J, Zubrienė A, Baranauskienė L, Kišonaitė M, Michailovienė V, Juozapaitienė V, Todd MJ, Matulis D. 2015.** Intrinsic thermodynamics of sulfonamide inhibitor binding to human carbonic anhydrases I and II. *Journal of Enzyme Inhibition and Medicinal Chemistry* **30**:204–211 DOI 10.3109/14756366.2014.908291.
- Morrison JF. 1969.** Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. *Biochimica Et Biophysica Acta* **185**:269–286  
DOI 10.1016/0005-2744(69)90420-3.
- Myszka DG. 2004.** Analysis of small-molecule interactions using biacore S51 technology. *Analytical Biochemistry* **329**:316–323 DOI 10.1016/j.ab.2004.03.028.
- Navratilova I, Hopkins AL. 2010.** Fragment screening by surface plasmon resonance. *ACS Medicinal Chemistry Letters* **1**:44–48 DOI 10.1021/ml900002k.
- Niesen FH, Berglund H, Vedadi M. 2007.** The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nature Protocols* **2**:2212–2221 DOI 10.1038/nprot.2007.321.
- Nocentini A, Carta F, Ceruso M, Bartolucci G, Supuran CT. 2015.** Click-tailed coumarins with potent and selective inhibitory action against the tumor-associated carbonic anhydrases IX and XII. *Bioorganic & Medicinal Chemistry* **23**:6955–6966  
DOI 10.1016/j.bmc.2015.09.041.
- Nocentini A, Ceruso M, Carta F, Supuran CT. 2016.** 7-aryl-triazolyl-substituted sulfocoumarins are potent, selective inhibitors of the tumor-associated carbonic anhydrase IX and XII. *Journal of Enzyme Inhibition and Medicinal Chemistry* **31**:1226–1233 DOI 10.3109/14756366.2015.1115401.
- Obach M, Navarro-Sabaté A, Caro J, Kong X, Duran J, Gómez M, Perales JC, Ventura F, Rosa JL, Bartrons R. 2004.** 6-phosphofructo-2-kinase (Pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. *The Journal of Biological Chemistry* **279**:53562–53570  
DOI 10.1074/jbc.M406096200.
- Oosterwijk E, Ruiter DJ, Hoedemaeker PJ, Pauwels EK, Jonas U, Zwartendijk J, Warnaar SO. 1986.** Monoclonal antibody G 250 recognizes a determinant present in

- renal-cell carcinoma and absent from normal kidney. *International Journal of Cancer* 38:489–494 DOI 10.1002/ijc.2910380406.
- O'Toole EA, Van Koningsveld R, Chen M, Woodley DT. 2008. Hypoxia induces epidermal keratinocyte matrix metalloproteinase-9 secretion via the protein kinase C pathway. *Journal of Cellular Physiology* 214:47–55 DOI 10.1002/jcp.21160.
- Pacchiano F, Carta F, McDonald PC, Lou Y, Vullo D, Scozzafava A, Dedhar S, Supuran CT. 2011. Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis. *Journal of Medicinal Chemistry* 54:1896–1902 DOI 10.1021/jm101541x.
- Pal SK, Agarwal N. 2016. Kidney cancer: finding a niche for girentuximab in metastatic renal cell carcinoma. *Nature Reviews Urology* 13:442–443 DOI 10.1038/nrurol.2016.115.
- Pantoliano MW, Petrella EC, Kwasnoski JD, Lobanov VS, Myslik J, Graf E, Carver T, Asel E, Springer BA, Lane P, Salemme FR. 2001. High-density miniaturized thermal shift assays as a general strategy for drug discovery. *Journal of Biomolecular Screening* 6:429–440 DOI 10.1177/108705710100600609.
- Papalia GA, Leavitt S, Bynum MA, Katsamba PS, Wilton R, Qiu H, Steukers M, Wang S, Bindu L, Phogat S, Giannetti AM, Ryan TE, Pudlak VA, Matusiewicz K, Michelson KM, Nowakowski A, Pham-Baginski A, Brooks J, Tieman BC, Bruce BD, Vaughn M, Baksh M, Cho YH, Wit MD, Smets A, Vandersmissen J, Michiels L, Myszka DG. 2006. Comparative analysis of 10 small molecules binding to carbonic anhydrase II by different investigators using biacore technology. *Analytical Biochemistry* 359:94–105 DOI 10.1016/j.ab.2006.08.021.
- Parkkila S, Innocenti A, Kallio H, Hilvo M, Scozzafava A, Supuran CT. 2009. The protein tyrosine kinase inhibitors imatinib and nilotinib strongly inhibit several mammalian alpha-carbonic anhydrase isoforms. *Bioorganic & Medicinal Chemistry Letters* 19:4102–4106 DOI 10.1016/j.bmcl.2009.06.002.
- Parks SK, Chiche J, Pouysségur J. 2013. Disrupting proton dynamics and energy metabolism for cancer therapy. *Nature Reviews Cancer* 13:611–623 DOI 10.1038/nrc3579.
- Pastorek J, Pastorekova S. 2015. Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: from biology to clinical use. *Seminars in Cancer Biology* 31:52–64 DOI 10.1016/j.semcancer.2014.08.002.
- Pastorekova S, Parkkila S, Parkkila A, Opavsky R, Zelnik V, Saarnio J, Pastorek J. 1997. Carbonic anhydrase IX, MN/CA IX: analysis of stomach complementary DNA sequence and expression in human and rat alimentary tracts. *Gastroenterology* 112:398–408 DOI 10.1053/gast.1997.v112.pm9024293.
- Pastorekova S, Ratcliffe PJ, Pastorek J. 2008. Molecular mechanisms of carbonic anhydrase IX-mediated pH regulation under hypoxia. *BJU International* 101(Suppl 4):8–15 DOI 10.1111/j.1464-410X.2008.07642.x.
- Pilipuitytė V, Matulis D. 2015. Intrinsic thermodynamics of trifluoromethanesulfonamide and ethoxzolamide binding to human carbonic anhydrase VII. *Journal of Molecular Recognition* 28:166–172 DOI 10.1002/jmr.2404.

- Pinard MA, Mahon B, McKenna R, Pinard MA, Mahon B, McKenna R. 2015.** Probing the surface of human carbonic anhydrase for clues towards the design of isoform specific inhibitors, probing the surface of human carbonic anhydrase for clues towards the design of isoform specific inhibitors. *BioMed Research International* 2015:e453543.
- Pocker Y, Sarkanen S. 1978.** Carbonic anhydrase: structure catalytic versatility, and inhibition. *Advances in Enzymology and Related Areas of Molecular Biology* 47:149–274 DOI 10.1002/9780470122921.ch3.
- Prete SD, Vullo D, Luca VD, Supuran CT, Capasso C. 2014.** Biochemical characterization of the  $\delta$ -carbonic anhydrase from the marine diatom thalassiosira weissflogii, TweCA. *Journal of Enzyme Inhibition and Medicinal Chemistry* 29:906–911 DOI 10.3109/14756366.2013.868599.
- Ramchandani D, Unruh D, Lewis CS, Bogdanov VY, Weber GF. 2016.** Activation of carbonic anhydrase IX by alternatively spliced tissue factor under late-stage tumor conditions. *Laboratory Investigation; a Journal of Technical Methods and Pathology* 96:1234–1245 DOI 10.1038/labinvest.2016.103.
- Ramsay G, Prabhu R, Freire E. 1986.** Direct measurement of the energetics of association between myelin basic protein and phosphatidylserine vesicles. *Biochemistry* 25:2265–2270 DOI 10.1021/bi00356a062.
- Ratto F, Witort E, Tatini F, Centi S, Lazzeri L, Carta F, Lulli M, Vullo D, Fusi F, Supuran CT, Scozzafava A, Capaccioli S, Pini R. 2015.** Plasmonic particles that hit hypoxic cells. *Advanced Functional Materials* 25:316–323 DOI 10.1002/adfm.201402118.
- Rennekamp AJ, Peterson RT. 2015.** 15 years of zebrafish chemical screening. *Current Opinion in Chemical Biology* 24:58–70 DOI 10.1016/j.cbpa.2014.10.025.
- Rutkauskas K, Zubrienė A, Tumosienė I, Kantminienė K, Kažemėkaitė M, Smirnov A, Kazokaitė J, Morkūnaitė V, Čapkauskaitė E, Manakova E, Gražulis S, Beresnevičius ZJ, Matulis D. 2014.** 4-amino-substituted benzenesulfonamides as inhibitors of human carbonic anhydrases. *Molecules* 19:17356–17380 DOI 10.3390/molecules191117356.
- Ryu MH, Park HM, Chung J, Lee CH, Park HR. 2010.** Hypoxia-inducible factor-1 $\alpha$  mediates oral squamous cell carcinoma invasion via upregulation of  $\alpha$ 5 integrin and fibronectin. *Biochemical and Biophysical Research Communications* 393:11–15 DOI 10.1016/j.bbrc.2010.01.060.
- Santos MA, Marques S, Vullo D, Innocenti A, Scozzafava A, Supuran CT. 2007.** Carbonic anhydrase inhibitors: inhibition of cytosolic/tumor-associated isoforms I, II, and IX with iminodiacetic carboxylates/hydroxamates also incorporating benzenesulfonamide moieties. *Bioorganic & Medicinal Chemistry Letters* 17:1538–1543 DOI 10.1016/j.bmcl.2006.12.107.
- Schneider H-P, Alt MD, Klier M, Spiess A, Andes FT, Waheed A, Sly WS, Becker HM, Deitmer JW. 2013.** GPI-anchored carbonic anhydrase IV displays both intra- and extracellular activity in cRNA-injected oocytes and in mouse neurons. *Proceedings*

- of the National Academy of Sciences of the United States of America **110**:1494–1499  
DOI [10.1073/pnas.1221213110](https://doi.org/10.1073/pnas.1221213110).
- Schön A, Freire E. 1989.** Thermodynamics of intersubunit interactions in cholera toxin upon binding to the oligosaccharide portion of its cell surface receptor, ganglioside GM1. *Biochemistry* **28**:5019–5024 DOI [10.1021/bi00438a017](https://doi.org/10.1021/bi00438a017).
- Sedlakova O, Svastova E, Takacova M, Kopacek J, Pastorek J, Pastorekova S. 2014.** Carbonic anhydrase IX, a hypoxia-induced catalytic component of the pH regulating machinery in tumors. *Frontiers in Physiology* **4**:400–414 DOI [10.3389/fphys.2013.00400](https://doi.org/10.3389/fphys.2013.00400).
- Semenza GL. 2014.** Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annual Review of Pathology* **9**:47–71 DOI [10.1146/annurev-pathol-012513-104720](https://doi.org/10.1146/annurev-pathol-012513-104720).
- Siebels M, Rohrmann K, Oberneder R, Stahler M, Haseke N, Beck J, Hofmann R, Kindler M, Kloepfer P, Stief C. 2011.** A clinical phase I/II trial with the monoclonal antibody cG250 (RENCAREX<sup>®</sup>) and interferon-alpha-2a in metastatic renal cell carcinoma patients. *World Journal of Urology* **29**:121–126 DOI [10.1007/s00345-010-0570-2](https://doi.org/10.1007/s00345-010-0570-2).
- Smirnovienė J, Smirnovas V, Matulis D. 2017.** Picomolar inhibitors of carbonic anhydrase: importance of inhibition and binding assays. *Analytical Biochemistry* **522**:61–72 DOI [10.1016/j.ab.2017.01.022](https://doi.org/10.1016/j.ab.2017.01.022).
- Stillebroer AB, Oosterwijk E, Oyen WJG, Mulders PFA, Boerman OC. 2007.** Radiolabeled antibodies in renal cell carcinoma. *Cancer* **7**:179–188 DOI [10.1102/1470-7330.2007.0025](https://doi.org/10.1102/1470-7330.2007.0025).
- Stiti M, Cecchi A, Rami M, Abdaoui M, Barragan-Montero V, Scozzafava A, Guari Y, Winum J-Y, Supuran CT. 2008.** Carbonic anhydrase inhibitor coated gold nanoparticles selectively inhibit the tumor-associated isoform IX over the cytosolic isozymes I and II. *Journal of the American Chemical Society* **130**:16130–16131 DOI [10.1021/ja805558k](https://doi.org/10.1021/ja805558k).
- Supuran CT. 2012.** Structure-based drug discovery of carbonic anhydrase inhibitors. *Journal of Enzyme Inhibition and Medicinal Chemistry* **27**:759–772 DOI [10.3109/14756366.2012.672983](https://doi.org/10.3109/14756366.2012.672983).
- Supuran CT. 2013.** Carbonic anhydrase inhibitors: an editorial. *Expert Opinion on Therapeutic Patents* **23**:677–679 DOI [10.1517/13543776.2013.778246](https://doi.org/10.1517/13543776.2013.778246).
- Supuran CT. 2016.** How many carbonic anhydrase inhibition mechanisms exist? *Journal of Enzyme Inhibition and Medicinal Chemistry* **31**:345–360 DOI [10.3109/14756366.2015.1122001](https://doi.org/10.3109/14756366.2015.1122001).
- Supuran CT. 2017.** Advances in structure-based drug discovery of carbonic anhydrase inhibitors. *Expert Opinion on Drug Discovery* **12**:61–88 DOI [10.1080/17460441.2017.1253677](https://doi.org/10.1080/17460441.2017.1253677).
- Supuran CT, Capasso C. 2015.** The η-class carbonic anhydrases as drug targets for antimalarial agents. *Expert Opinion on Therapeutic Targets* **19**:551–563 DOI [10.1517/14728222.2014.991312](https://doi.org/10.1517/14728222.2014.991312).

- Supuran CT, Winum J-Y. 2015.** Carbonic anhydrase IX inhibitors in cancer therapy: an update. *Future Medicinal Chemistry* 7:1407–1414 DOI [10.4155/fmc.15.71](https://doi.org/10.4155/fmc.15.71).
- Surfus JE, Hank JA, Oosterwijk E, Welt S, Lindstrom MJ, Albertini MR, Schiller JH, Sondel PM. 1996.** Anti-renal-cell carcinoma chimeric antibody G250 facilitates antibody-dependent cellular cytotoxicity with in vitro and *in vivo* interleukin-2-activated effectors. *Journal of Immunotherapy with Emphasis on Tumor Immunology* 19:184–191 DOI [10.1097/00002371-199605000-00003](https://doi.org/10.1097/00002371-199605000-00003).
- Svastová E, Hulíková A, Rafajová M, Zát'ovicová M, Gibadulinová A, Casini A, Cecchi A, Scozzafava A, Supuran CT, Pastorek J, Pastoreková S. 2004.** Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. *FEBS Letters* 577:439–445 DOI [10.1016/j.febslet.2004.10.043](https://doi.org/10.1016/j.febslet.2004.10.043).
- Svastova E, WitarSKI W, Csaderova L, Kosik I, Skvarkova L, Hulikova A, Zatovicova M, Barathova M, Kopacek J, Pastorek J, Pastorekova S. 2012.** Carbonic anhydrase IX interacts with bicarbonate transporters in lamellipodia and increases cell migration via its catalytic domain. *The Journal of Biological Chemistry* 287:3392–3402 DOI [10.1074/jbc.M111.286062](https://doi.org/10.1074/jbc.M111.286062).
- Swietach P, Hulikova A, Vaughan-Jones RD, Harris AL. 2010.** New insights into the physiological role of carbonic anhydrase IX in tumour pH regulation. *Oncogene* 29:6509–6521 DOI [10.1038/onc.2010.455](https://doi.org/10.1038/onc.2010.455).
- Tafreshi NK, Bui MM, Bishop K, Lloyd MC, Enkemann SA, Lopez AS, Abrahams D, Carter BW, Vagner J, Grobmyer SR, Gobmyer SR, Gillies RJ, Morse DL. 2012.** Noninvasive detection of breast cancer lymph node metastasis using carbonic anhydrases IX and XII targeted imaging probes. *Clinical Cancer Research* 18:207–219 DOI [10.1158/1078-0432.CCR-11-0238](https://doi.org/10.1158/1078-0432.CCR-11-0238).
- Takubo K, Goda N, Yamada W, Iriuchishima H, Ikeda E, Kubota Y, Shima H, Johnson RS, Hirao A, Suematsu M, Suda T. 2010.** Regulation of the HIF-1 $\alpha$  level is essential for hematopoietic stem cells. *Cell Stem Cell* 7:391–402 DOI [10.1016/j.stem.2010.06.020](https://doi.org/10.1016/j.stem.2010.06.020).
- Talibov VO, Linkuvienė V, Matulis D, Danielson UH. 2016.** Kinetically selective inhibitors of human carbonic anhydrase isozymes I, II, VII, IX, XII, and XIII. *Journal of Medicinal Chemistry* 59(5):2083–2093 DOI [10.1021/acs.jmedchem.5b01723](https://doi.org/10.1021/acs.jmedchem.5b01723).
- Tars K, Vullo D, Kazaks A, Leitans J, Lends A, Grandane A, Zalubovskis R, Scozzafava A, Supuran CT. 2013.** Sulfocoumarins (1,2-benzoxathiine-2,2-dioxides): a class of potent and isoform-selective inhibitors of tumor-associated carbonic anhydrases. *Journal of Medicinal Chemistry* 56:293–300 DOI [10.1021/jm301625s](https://doi.org/10.1021/jm301625s).
- Tashian RE, Douglas DP, Yu YS. 1964.** Esterase and hydrase activity of carbonic anhydrase. I. From primate erythrocytes. *Biochemical and Biophysical Research Communications* 14:256–261 DOI [10.1016/0006-291X\(64\)90445-0](https://doi.org/10.1016/0006-291X(64)90445-0).
- Touisni N, Kanfar N, Ulrich S, Dumy P, Supuran CT, Mehdi A, Winum J-Y. 2015.** Fluorescent silica nanoparticles with multivalent inhibitory effects towards carbonic anhydrases. *Chemistry – A European Journal* 21:10249–10249 DOI [10.1002/chem.201501917](https://doi.org/10.1002/chem.201501917).

- Uda NR, Seibert V, Stenner-Liewen F, Müller P, Herzig P, Gondi G, Zeidler R, Van Dijk M, Zippelius A, Renner C. 2015. Esterase activity of carbonic anhydrases serves as surrogate for selecting antibodies blocking hydratase activity. *Journal of Enzyme Inhibition and Medicinal Chemistry* 30:955–960 DOI 10.3109/14756366.2014.1001754.
- Ullah MS, Davies AJ, Halestrap AP. 2006. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1 $\alpha$ -dependent mechanism. *Journal of Biological Chemistry* 281:9030–9037 DOI 10.1074/jbc.M511397200.
- Van Kuijk SJA, Yaromina A, Houben R, Niemans R, Lambin P, Dubois LJ. 2016. Prognostic significance of carbonic anhydrase IX expression in cancer patients: a meta-analysis. *Frontiers in Oncology* 6:Article 69 DOI 10.3389/fonc.2016.00069.
- Vander Heiden MG, Cantley LC, Thompson CB. 2009. Understanding the warburg effect: the metabolic requirements of cell proliferation. *Science* 324:1029–1033 DOI 10.1126/science.1160809.
- Warburg O. 1956. On respiratory impairment in cancer cells. *Science* 124:269–270.
- Welichem Biotech Inc, Ozmosis Research Inc. 2014. Safety study of SLC-0111 in subjects with advanced solid tumours. Available at <https://clinicaltrials.gov/ct2/show/NCT02215850> (accessed on 17 October 2017).
- Welti J, Loges S, Dimmeler S, Carmeliet P. 2013. Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. *The Journal of Clinical Investigation* 123:3190–3200 DOI 10.1172/JCI70212.
- Wilex. 2004. A randomized, double blind phase III study to evaluate adjuvant cG250 treatment versus Placebo in patents with clear cell RCC and High Risk of Recurrence (ARISER). Available at <https://clinicaltrials.gov/ct2/show/NCT00087022?term=girentuximab&rank=6> (accessed on 17 October 2017).
- Wilex. 2017. Focused cancer therapies. Pipeline of proprietary and partnered programs, half-yearly report 2017. 1–24. Available at [http://www.wilex.de/wp-content/uploads/2015/01/20170713\\_WILEX\\_Press\\_Analyst\\_Presentation\\_6M-2017\\_final.pdf](http://www.wilex.de/wp-content/uploads/2015/01/20170713_WILEX_Press_Analyst_Presentation_6M-2017_final.pdf) (accessed on 17 October 2017).
- Wilson WR, Hay MP. 2011. Targeting hypoxia in cancer therapy. *Nature Reviews. Cancer* 11:393–410 DOI 10.1038/nrc3064.
- Wojtkowiak JW, Verduzco D, Schramm KJ, Gillies RJ. 2011. Drug resistance and cellular adaptation to tumor acidic pH microenvironment. *Molecular Pharmaceutics* 8:2032–2038 DOI 10.1021/mp200292c.
- Xu S. 2015. Internalization, trafficking, intracellular processing and actions of antibody-drug conjugates. *Pharmaceutical Research* 32:3577–3583 DOI 10.1007/s11095-015-1729-8.
- Yang J-S, Chen M-K, Yang S-F, Chang Y-C, Su S-C, Chiou H-L, Chien M-H, Lin C-W. 2014. Increased expression of carbonic anhydrase IX in oral submucous fibrosis and oral squamous cell carcinoma. *Clinical Chemistry and Laboratory Medicine* 52:1367–1377.
- Zatovicova M, Jelenska L, Hulikova A, Csaderova L, Ditte Z, Ditte P, Goliasova T, Pastorek J, Pastorekova S. 2010. Carbonic anhydrase IX as an anticancer therapy

target: preclinical evaluation of internalizing monoclonal antibody directed to catalytic domain. *Current Pharmaceutical Design* **16**:3255–3263

DOI [10.2174/138161210793429832](https://doi.org/10.2174/138161210793429832).

- Zat'ovicová M, Tarábková K, Svastová E, Gibadulinová A, Mucha V, Jakubicková L, Biesová Z, Rafajová M, Ortova Gut M, Parkkila S, Parkkila AK, Waheed A, Sly WS, Horak I, Pastorek J, Pastoreková S. 2003.** Monoclonal antibodies generated in carbonic anhydrase IX-deficient mice recognize different domains of tumour-associated hypoxia-induced carbonic anhydrase IX. *Journal of Immunological Methods* **282**:117–134 DOI [10.1016/j.jim.2003.08.011](https://doi.org/10.1016/j.jim.2003.08.011).
- Závada J, Zavadová Z, Pastoreková S, Ciampor F, Pastorek J, Zelník V. 1993.** Expression of MaTu-MN protein in human tumor cultures and in clinical specimens. *International Journal of Cancer* **54**:268–274 DOI [10.1002/ijc.2910540218](https://doi.org/10.1002/ijc.2910540218).
- Zubrienė A, Smirnovienė J, Smirnovė A, Morkūnaitė V, Michailovienė V, Jachno J, Juozapaitienė V, Norvaišas P, Manakova E, Gražulis S, Matulis D. 2015.** Intrinsic thermodynamics of 4-substituted-2,3,5,6-tetrafluorobenzenesulfonamide binding to carbonic anhydrases by isothermal titration calorimetry. *Biophysical Chemistry* **205**:51–65 DOI [10.1016/j.bpc.2015.05.009](https://doi.org/10.1016/j.bpc.2015.05.009).
- Zubrienė A, Čapkauskaitė E, Gyltė J, Kišonaitė M, Tumkevičius S, Matulis D. 2014.** Benzenesulfonamides with benzimidazole moieties as inhibitors of carbonic anhydrases I, II, VII, XII and XIII. *Journal of Enzyme Inhibition and Medicinal Chemistry* **29**:124–131 DOI [10.3109/14756366.2012.757223](https://doi.org/10.3109/14756366.2012.757223).