Chapter 1
The Carbonic Anhydrases in Health and Disease

W. Richard Chegwidden

Abstract The zinc metalloenzyme carbonic anhydrase (CA; EC 4.2.1.1) catalyzes the reversible hydration of CO_2 and is expressed in human in 15 different isoforms—12 active isozymes and three catalytically inactive isoforms. Since the enzyme is fundamental to many physiological processes, chiefly involving ion transport, pH regulation, and substrate provision for metabolism, it has been implicated in many diseases and disorders. Consequently, carbonic anhydrase inhibitors, such as the classical sulfonamide inhibitor acetazolamide, have long been employed therapeutically. Initially developed for congestive heart failure, acetazolamide was subsequently employed for many years in the treatment of glaucoma, and other conditions such as acute mountain sickness. Over recent years, however, the number of diseases, with pathologies involving carbonic anhydrase, has expanded enormously providing potential for novel therapies through the modification of carbonic anhydrase activity. These are highlighted in the following chapter. In particular, a wealth of evidence has linked carbonic anhydrase, particularly the carbonic anhydrase IX isozyme (CA IX), with cancer. Alongside this has been a complimentary development of novel methods of drug delivery targeting CA IX.

Keywords Carbonic anhydrase · CA inhibitors · Acetazolamide · Therapeutic target · Therapeutic application · Drug delivery systems

Carbonic anhydrase (CA) catalyzes the reversible hydration of carbon dioxide to bicarbonate ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), a reaction of such profound importance that the enzyme is seemingly ubiquitous in nature, being represented by seven distinct gene families—α, β, γ, δ, ζ, η, and θ—of which only the α-family is present in human. The human family comprises 12 active isozyme members that differ in tissue distribution, sub-cellular location, catalytic power and inhibitory characteristics (see Tables 1.1 and 1.2), and three isoforms, designated CA-related proteins (CA-RPs), that are inactive owing to the absence of one or more of the histidine residues that

W. R. Chegwidden (✉)
LECOM, 1858 West Grandview Boulevard, Erie, PA 16509, USA
e-mail: wrchegwidden@lecom.edu; wrc.2417@gmail.com

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Table 1.1 Tissue distribution and sub-cellular location of human carbonic anhydrase isozymes

Isozyme	Sub-cellular location	Principal sites of tissue expression
CA I	Cytoplasmic	Red blood cell, intestine, eye
CA II	Cytoplasmic	Ubiquitous (certain cells of virtually all tissues)
CA III	Cytoplasmic	Red muscle, white and brown adipose tissue
CA IV	Membrane-bound (extracellular)	Kidney, lung, pancreas, gut, brain, eye, probably universally present in capillary endothelium
CA VA	Mitochondrial	Liver
CA VB	Mitochondrial	Widespread
CA VI	Secreted	Salivary and mammary glands
CA VII	Cytoplasmic	CNS (lower expression widespread)
CA VIII[a]	Cytoplasmic	Brain, especially Purkinje cells of cerebellum, (lower expression widespread)
CA IX	Transmembrane (extra-cellular domain)	Various tumours, gastric mucosa
CA X[a]	Cytoplasmic	Brain
CA XI[a]	Cytoplasmic	Brain (lower expression widespread)
CA XII	Transmembrane (extra-cellular domain)	Widespread, especially kidney, colon, prostate
CA XIII	Cytoplasmic	Kidney, brain, lung, gut, reproductive organs
CA XIV	Transmembrane (extra-cellular domain)	Widespread, especially kidney and heart

[a]CA VIII, CA X and CA XI lack activity because of substitution of one or more of the histidine residues required to bind the catalytically essential zinc ion. Consequently they are commonly designated CA-related proteins (CA-RP VIII, CA-RP X and CA-RP XI). Although no definitive roles have been firmly established for any of these three CA-RP molecules, recent studies suggest a role for CA-RP VIII as an allosteric modulator in pain regulation, through the regulation of neuronal cytosolic calcium levels (Zhuang et al. 2015, 2018)
N.B. CA XV is an additional catalytically active α-CA isozyme that is not expressed in human or chimpanzee (Hilvo et al. 2005)

coordinate the catalytically active zinc ion in the active site (CA-RP VIII, CA-RP X, and CA-RP XI) [for recent reviews see Supuran and Nocentini 2019; Supuran and De Simone 2015]. A thirteenth active α-CA isozyme (CA XV) is not expressed in human or chimpanzee (Hilvo et al. 2005).

The CA reaction is so fundamental that the active isozymes participate in a host of crucial physiological processes, variously involving H^+, CO_2, ion and water transport, pH regulation, and provision of bicarbonate as substrate for a range of metabolic reactions. Some of the major physiological functions of the carbonic anhydrase isozymes are summarized in Table 1.3.

Table 1.2 CO_2-hydration activity and acetazolamide inhibition of human carbonic anhydrase isozymes

Isozyme	Activity level	k_{cat}/K_M (M^{-1} s^{-1})[a]	Acetazolamide (Az) inhibition	K_I (Az) (nM)[b]
CA I	Moderate	5.0×10^7	Moderate	250
CA II	High	1.5×10^8	Strong	12
CA III	Low	2.5×10^5	Weak	2×10^5
CA IV	High	5.1×10^7	Strong	74
CA VA	Moderate	2.9×10^7	Strong	63
CA VB	High	9.8×10^7	Strong	54
CA VI	Moderate	4.9×10^7	Strong	11
CA VII	High	8.3×10^7	Strong	2.5
CA IX	High	5.4×10^7	Strong	25
CA XII	Moderate	3.5×10^7	Strong	5.7
CA XIII	Moderate	1.1×10^7	Strong	16
CA XIV	Moderate	3.9×10^7	Strong	41

[a] k_{cat}/K_M values are taken from Hilvo, Baranauskiene et al. (2008)
[b] K_I (Az) values are taken from Supuran et al. (2015) and Hilvo, Innocenti et al. (2008) (human CA XIII)

The tight evolutionary conservation of the inactive CA isoforms (CA-RPs) strongly suggests that they also possess important physiological functions (Tashian et al. 2000). In recent years, evidence has accumulated that indicates an important role for CA-RP VIII as an allosteric modulator in pain regulation through the regulation of neuronal cytosolic calcium levels (Zhuang et al. 2015, 2018).

Reflecting this wide range of functions, CA isozymes have been implicated in many diseases and disorders (Table 1.4). Consequently, CA inhibitors have, through the years, found therapeutic application in a wide and growing range of clinical conditions including acute mountain sickness (Swenson and Teppems 2007; Swenson 2014a; Lipman et al. 2019), bipolar disorder (Hayes 1994), chronic obstructive pulmonary disease (COPD) (Adamson and Swenson 2017; Van Berkel and Elefritz 2018), glaucoma (Becker 1955; Masini et al. 2013), gastric ulcer (Buzás and Supuran 2016), macular edema (Cox et al. 1988; Wolfensberger 2017), obesity (Scozzafava et al. 2013), epilepsy (Aggerwal et al. 2013; Silberstein et al. 2005), sleep apnea (Eskandari et al. 2014, 2018), and migraine (Brandes et al. 2004; Silberstein et al. 2004; Silberstein 2017). A vasodilatory effect of CA inhibitors has also been demonstrated (Swenson 2014b), raising the possibility of a role for such agents in hypertensive-related diseases. This effect, along with a contribution to the maintenance of blood gas stability may, at least in part, account for the effectiveness of acetazolamide in the treatment of obstructive sleep apnea. In recent years, much attention has been given to the potential of CA inhibitors and CA-targeted drugs to treat cancer, and most recently, evidence has also accumulated suggesting possible roles for CA inhibitors in therapy for hemorrhagic stroke (Li et al. 2016), protection

Table 1.3 Major physiological functions of the human carbonic anhydrase isozymes[a,b]

Function	CA isozymes principally involved[c]
Respiration and Acid/Base Regulation	
Hydration of CO_2 to HCO_3^- in peripheral tissues	CA II (in red cells)
Dehydration of HCO_3^- to CO_2 at lungs	CA IV (in capillary surfaces and pulmonary microvasculature)
Elimination of H^+ in kidney	CA II
Reabsorption of HCO_3^- in kidney	CA IV, CA XII
Vision	
Production of aqueous humour (ciliary body)	CA II, CA IV, CA XII
Bone Developmentand Function	
Differentiation of osteoclasts	CA II
Provision of H^+ in osteoclasts for bone resorption	CA II
Metabolic Processes	
Provision of bicarbonate for gluconeogenesis and ureogenesis	CA V
Provision of bicarbonate for pyrimidine synthesis	CA II (possibly also CAV)
Provision of bicarbonate for fatty acid synthesis	CA V (possibly also CAII)
Insulin secretion (production of HCO_3^- in pancreatic β cells)	CA V
CSF Production	
Provision of H^+ and regulation of pH (choroid plexus)	CA II, CA XII
Gustation and Olfaction	
Maintenance of appropriate CO_2 levels	CA I, CA II, and/or CA IV
Saliva Production	
Production of HCO_3^- (acinar and ductal cells)	CA II
G.L. Tract Protection	
Removal of acid from dental surfaces	CA VI
Removal of acid from gastro-oesophageal mucosa	CA II, VI
Gastric Acid Production	
Production of H^+ in stomach (parietal cells)	CA II
Bile Production	
Provision of HCO_3^- for bile (liver epithelial duct cells)	CA II
Acidification of bile (gall bladder epithelium)	CA II, CA IV

(continued)

Table 1.3 (continued)

Function	CA isozymes principally involved[c]
Pancreatic Juice Production	
Provision of HCO_3^- in pancreas (epithelial duct cells)	CA II
Reproductive System	
Regulation of pH and HCO_3^- content of seminal fluid	CA II, CA IV, CA XIII
Muscle Function	
Protection as anti-oxidant against ROS	CA III
Facilitated CO_2 diffusion	CA II, III, Membrane-bound CA
Buffering in SR (H^+ exchanged for Ca^{++})	Membrane-bound CA (CA IV?)
Oncogenesis	
Regulation of the tumor microenvironment to facilitate tumor growth and metastasis	CA IX and XII

[a]Established and putative functions
[b]This table is adapted from Chegwidden and Carter (2000)
[c]Other CA isozymes may also play a part in several of the processes listed below, in addition to those indicated as being principally involved

against diabetic brain injury (Price et al. 2017), and Alzheimer disease (Fossati et al. 2016).

The CA inhibitor acetazolamide (DIAMOX) first reached the market almost 70 years ago (Maren 1952). Initially developed as a diuretic for the treatment of congestive heart failure (Friedberg et al. 1953), it was adopted soon afterwards for the treatment of glaucoma (Breinin and Görtz 1954; Becker 1954), for which it remained central for several decades. The subsequent development of carbonic anhydrase inhibitors for topical application represented a major advance, since they obviated the undesirable systemic side effects frequently encountered with orally administered carbonic anhydrase inhibitors at the concentrations required to inhibit the enzyme activity in the ciliary processes (Talluto et al. 1997).

In ophthalmology, whilst CA inhibitors have been employed for some time in topically-administered combination therapy for glaucoma (Supuran et al. 2019), in recent years, they have also found use in the treatment of macular edema, secondary to a number of conditions such as retinitis pigmentosa and hereditary retinoschisis, and as a sequala of cataract and vitreoretinal surgery (Strong et al. 2017; Wolfensberger 1999). In addition, the topical CA inhibitors dorzolamide (TRUSOPT) and brinzolamide (AZOPT) have been demonstrated to be effective in the treatment of chronic central serous retinopathy (CSCR) (Liew et al. 2020) and infantile nystagmus syndrome (INS) (Hertle et al. 2015), respectively.

In neurology, CA inhibitors are used for epilepsy (Aggerwal et al. 2013; Silberstein et al. 2005) and for migraine (Silberstein et al. 2005; Silberstein 2017), and to decrease CSF production in pseudotumor cerebri (Thurtell and Wall 2013). There is now a

Table 1.4 Diseases and disorders associated with carbonic anhydrase isozymes

CA isozyme[a]	Associated diseases/disorders
CA I	Bipolar disorder (Hayes 1994; Song et al. 2015), Glaucoma (Maren 1987; Wistrand 2000), Retinal/cerebral edema (Gao et al. 2007),
CA II	Acute mountain sickness (Swenson and Teppems 2007; Swenson 2014a), Alzheimer disease (Jang et al. 2010; Provensi et al. 2019), COPD (Heming et al. 2012), Edema (Supuran 2008), Epilepsy (Aggerwal et al. 2013; Zavala-Tecuapetla et al. 2020), Pulmonary hypertension (Hudalla et al. 2019), Pseudotumor cerebri (Thurtell and Wall 2013), Sleep apnea (Wang et al. 2015)
CA III	Myasthenia gravis (Du et al. 2017), Oxidative stress (Zimmerman et al. 2014; Di Fiore et al. 2018)
CA IV	COPD (Heming et al. 2012), Glaucoma (Matsui et al. 1996), Retinitis pigmentosa (Datta et al. 2009), Stroke (Tang et al. 2006)
CA VA/CA VB	Alzheimer disease (Provensi et al. 2019), Obesity (Spencer et al. 1988; De Simone et al. 2008; De Simone and Supuran 2009)
CA VI	Cariogenesis (Kivelä et al. 1999)
CA VII	Epilepsy (Aggerwal et al. 2013; Zavala-Tecuapetla et al. 2020), Oxidative stress (Di Fiore et al. 2018)
CA IX	Cancer (Závada et al. 1993; Benej et al. 2014)
CA XII	Cancer (Benej et al. 2014; Pastorekova et al. 2006), Glaucoma (Liao et al. 2003)
CA XIV	Epilepsy (Aggerwal et al. 2013; Shah et al. 2005), Retinopathy (Ogilvie et al. 2007)

[a]*Note* Other CA isozymes may also be implicated in several of these diseases/disorders, in addition to those specified above

growing evidence that CA inhibitors may afford a significant neuroprotective effect following both ischemic and hemorrhagic brain injury, inhibiting cerebral edema, reducing cellular levels of ROS, and improving nerve function (Li et al. 2016). Furthermore, mitochondrial carbonic anhydrase, which is considered a major player in glucose-induced production of reactive oxygen species, has been identified as a potential target for the treatment of diabetic injury to the brain and possibly other insulin-insensitive tissues such as eye and kidney (Price et al. 2017; Salameh et al. 2016).

There is also encouraging evidence, obtained through in vitro studies employing human neuronal and glial cell cultures, and in vivo studies employing a rodent AD model, suggesting that CA inhibition may become central to a new therapeutic strategy for Alzheimer disease and related cerebral amyloidosis (Fossati et al. 2016; Solesio et al. 2018).

Possibly, the most significant advances in recent years have been in the association of carbonic anhydrase isozymes with cancer. Whilst both intracellular and extracellular isozymes have been demonstrated to play roles in tumorigenesis, the

emergence of the cell surface isozyme CA IX as an attractive diagnostic and therapeutic biomarker for targeting a wide range of hypoxic, solid malignancies has generated the most interest and activity.

The inhibition of growth of human cancer cells by direct action of specific carbonic anhydrase inhibitors was first observed by Chegwidden and Spencer (1995, 2003; Chegwidden et al. 2000) who subsequently reported similar inhibition, by carbonic anhydrase inhibitors, of solid human tumors xenografted into immunodeficient mice (Chegwidden and Linville 2007). Carbonic anhydrase inhibitors were also shown to inhibit the invasion of renal cancer cells (Parkkila et al. 2000; Chegwidden et al. 2006) from a range of human cell lines expressing different CA isozymes.

The discovery and characterization of carbonic anhydrase IX, a tumor-associated protein with a central carbonic anhydrase domain, initially designated MN protein, provided significant impetus to the investigation of the then putative role of carbonic anhydrase in cancer (Pastoreková et al. 1992; Pastorek et al. 1994). Whilst many others have made notable contributions, the Pasteroková laboratory, where MN protein was first discovered and characterized, has remained central in this field of endeavor (Benej et al. 2014; Pastoreková and Gillies 2019).

Although the expression of CA IX is almost negligible in normal tissues, where it is restricted to certain tissues of the GI tract and gall bladder epithelia, this isozyme is strongly over-expressed in numerous aggressive malignancies, such as renal, pancreatic, head and neck, ovarian, hepatocellular, lung (NSCLC), and several brain cancers, where, under the regulation of the hypoxia-induced HIF-1 transcription factor, it is a key player in the pH regulation required for cancer cell survival and growth (Pastoreková and Gillies 2019; Thiry et al. 2006; Lau et al. 2017). Thus, this isozyme both provides a therapeutic target in its own right, and also serves as a biomarker for targeting with other cytotoxic agents, thereby avoiding off-target effects.

There are now multiple reports of successful inhibition, by CA inhibitors, of the growth of cultured cells and of xenografts, both derived from a range of human tumors. Several CA inhibitors, both small molecule drug conjugates (SMDCs) and CA IX-selective biological molecules, have entered preclinical or clinical trials for cancer treatment (Lau et al. 2017; Supuran 2017). Among these, the sulfonamide inhibitor SLC-0111 has recently completed phase I clinical trials (Supuran 2017).

Furthermore, there has also been a recent proliferation of activity in the application of novel nanoparticle drug delivery systems directed at CA IX (Kazokaite et al. 2017), ranging from immuno-liposomes (Lin et al. 2017) to "prickly" nanoparticles that destroy targeted cancer cells through physical nano-piercing (Zhang et al. 2017).

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