The Role of pH Regulation in Cancer Progression

Alan McIntyre and Adrian L. Harris

Abstract

Frequently observed phenotypes of tumours include high metabolic activity, hypoxia and poor perfusion; these act to produce an acidic microenvironment. Cellular function depends on pH homoeostasis, and thus, tumours become dependent on pH regulatory mechanisms. Many of the proteins involved in pH regulation are highly expressed in tumours, and their expression is often of prognostic significance. The more acidic tumour microenvironment also has important implications with regard to chemotherapeutic and radiotherapeutic interventions. In addition, we review pH-sensing mechanisms, the role of pH regulation in tumour phenotype and the use of pH regulatory mechanisms as therapeutic targets.

Keywords

pH regulation · Hypoxia · Tumour · Carbonic anhydrase 9 · acidic microenvironment

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1 Introduction

1.1 Measurements of Intracellular and Extracellular Tumour pH

For many years, it was rightly assumed that the increased conversion of glucose to lactic acid (the Warburg effect) seen in tumours would result in more acidic tumour microenvironment. It was also assumed that this would result in a more acidic intracellular pH. Indeed, initial measurements of tumour pH using pH electrodes inserted into tumours showed a more acidic pH (range 5.6–7.6) than normal tissues (range 6.9–7.6) (Griffiths 1991). However, the pH probes used in these studies were often large compared to single tumour cells and are now understood to have mostly measured extracellular pH (Griffiths 1991). Tumour pH has been measured by a number of imaging techniques including positron emission tomography (PET), magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) (Zhang et al. 2010). Measurement of pH by $^{31}$P-MRS tends to reflect the intracellular pH of tumours which is much more neutral than the extracellular pH in tumour cells (range 6.8–7.4) (Griffiths 1991; Zhang et al. 2010; Gerweck and Seetharaman 1996). In normal tissue, the intracellular pH tends to be more similar to, or more acidic than, the extracellular pH (range 6.8–7.3) (Griffiths 1991; Gerweck and Seetharaman 1996). In addition, the intracellular pH can be more alkaline in tumour cells compared to normal cells; for example, assessment of pH in malignant gliomas revealed a more alkaline steady-state intracellular pH (7.31–7.48) than was identified in normal astrocytes (6.98) (McLean et al. 2000). Therefore, there is a shift in the intracellular/extracellular balance of pH in tumour cells compared to normal cells, producing at least a reduced gradient and in many cases reversal in pH gradients for cancer cells. This has also been shown experimentally in animal studies (Stubbs et al. 1994).

More recently, research has identified higher resolution, less toxic and more clinically useful approaches for in vivo measurement of pH. These will give a greater idea of the pH of the tumour microenvironment in individual patients and enable a more tailored treatment regimen. These include the use of hyperpolarized $^{13}$C-labelled bicarbonate imaging by MRS to measure the interstitial pH of tumours, $^{99m}$Technetium-labelled pH low-insertion peptide (pHLIP) to measure extracellular pH by CT/SPECT imaging (Macholl et al. 2012; Fendos and Engelman 2012) and diamagnetic and paramagnetic chemical exchange saturation transfer (CEST)-MRI pH-responsive contrast agents to assess pH measurements in vivo (Longo et al. 2012; Liu et al. 2012; Sheth et al. 2012; Aime et al. 2002).
2 Why Are Tumours Acidic?

It is clear that tumours produce a more acidic environment and the Warburg effect first described in the 1920s identified increased glycolysis, increased glucose uptake and lactate production even in normoxic conditions seen in most cancer cells, termed aerobic glycolysis (Warburg 1956; Hanahan and Weinberg 2011). The conversion of pyruvate to lactic acid provides a source of increased H⁺ production in cancer cells. However, in addition to this, acidity can arise from other steps in the metabolic processes. For example in the pentose phosphate pathway, conversion of 1 glucose-6-phosphate to 1 fructose 6-phosphate produces 3CO₂ and 6H⁺ (6 NADPH + H⁺). The pentose phosphate pathway is up-regulated in cancer, and stabilization of HIF1α increases expression of genes involved in the pentose phosphate pathway (Riganti et al. 2012). The Krebs cycle, which still takes place in tumours, produces 6CO₂ for every molecule of glucose metabolized. Fatty acid synthesis of 1 molecule of palmitate from citrate produces 7 CO₂ and 7H⁺ and requires 7 molecules of HCO₃⁻. An additional 2H⁺ (which are NADPH + H⁺) are used in the process (Salway 2000). Fatty acid synthesis is increased in many tumour types and is associated with malignant transformation and HIF1 stabilization (Kuhajda et al. 1994; Kuhajda 2000; Yang et al. 2002; Menendez and Lupu 2007).

CO₂ forms a weak acid, carbonic acid (H₂CO₃) in solution. It can also be hydrated to form HCO₃⁻ and an H⁺, a reaction that is catalysed by members of the family of carbonic anhydrases (Supuran 2008). HCO₃⁻ is important in regulation of both intracellular and extracellular pH through its titration of H⁺ (Hulikova et al. 2012a, b); therefore, HCO₃⁻ use in fatty acid synthesis will increase intracellular acidification.

In addition, the lack of adequate functional vascularization, also a common feature of tumours, affects the acidification of tumours (Vaupel et al. 1989). The lack of vasculature reduces oxygen supply and removal of waste products including acid resulting in accumulation of H⁺ in the poorly perfused microenvironment (Vaupel et al. 1989). Therefore, the increased CO₂ and H⁺ production from up-regulated metabolic processes coupled with poor perfusion result in a more acidic tumour microenvironment.

2.1 pH Sensing

Despite the increased production of acidity, tumour cells maintain a relatively more alkali intracellular pH than normal cells, suggesting an increase in activity of mechanisms to regulate pH and therefore sense the pH changes occurring. The transcription factors such as HIF1α and HIF2α, which are stabilized in many types of tumours and which affect many metabolic pathways (Schulze and Harris 2012), are stabilized by acidic extracellular pH, in addition to the well-recognized low oxygen levels (Mekhail et al. 2004). Regulation of HIF1α and HIF2α stabilization under acidic extracellular conditions is due to nucleolar sequestration of VHL (which degrades HIF under normoxic conditions) (Mekhail et al. 2004). Further to
this, a later study identified increased normoxic expression of carbonic anhydrase IX (CAIX) in response to extracellular acidosis (Ihnatko et al. 2006). CA9 is a HIF1α target, which regulates intracellular and extracellular pH (this is discussed in detail later) (Swietach et al. 2009). Increased expression of CA9 transcription in response to extracellular acidity is regulated both by HIF and also independently of HIF (Ihnatko et al. 2006). Further investigation revealed that inhibition of the MAPK and PI3K pathways resulted in complete suppression of CAIX induction by acidosis (Ihnatko et al. 2006).

Proton-sensing G-protein-coupled receptors sense extracellular pH (Ludwig et al. 2003). Ovarian cancer G-protein-coupled receptor 1 (ORG1) was identified to be completely activated at pH 6.8 but was inactive at pH 7.8 (Ludwig et al. 2003). This study identified, by mutation studies, that the histidines in the extracellular domain of ORG1 were involved in pH sensing (Ludwig et al. 2003). ORG1 stimulated inositol phosphate formation in response to acidic extracellular pH (Ludwig et al. 2003). GPR4 and TDAG8, two additional G-protein coupled receptors, increased cyclic AMP formation in response to pH changes (Ludwig et al. 2003; Wang et al. 2004; Ishii et al. 2005). Inositol phosphate formation resulted in increased phospholipase IP3 signalling, which can induce Ca2+ release from intracellular stores (Huang et al. 2008; Rahman 2012). Extracellular acidification resulted in phospholipase C activation, IP3 formation and Ca2+ release, leading to increased phosphorylation of ERK (Huang et al. 2008). ERK forms part of the MAPK cascade, which is required for acid-induced CAIX expression described above (Ihnatko et al. 2006). Differentially localized Ca2+ concentrations activate many different processes (Petersen and Tepikin 2008). Further to this membrane potential changes induced by Ca2+ release have many critical roles including regulation of cell cycle, proliferation and intracellular pH (Kunzelmann 2005). ORG1 is strongly expressed in medulloblastoma patient samples (Huang et al. 2008).

Four acid-sensing ion channel genes (ASIC1, ASIC2, ASIC3 and ASIC4) have been identified, which open in response to extracellular H+ (Bassler et al. 2001; Glitsch 2011). These allow non-selective transport of cations (K+, Na+, Ca2+, etc.) across the membrane (Glitsch 2011). ASIC1 is more highly expressed in gliomas than in normal astrocytes (Kapoor et al. 2009). ASIC1 knockdown reduced proliferation and cell migration of glioblastoma cells in two separate studies (Kapoor et al. 2009; Rooj et al. 2012), identifying a role for ASICs in tumour cells.

In addition to the mechanisms described above, H+ can inhibit or potentiate additional ion channels and functions through allosteric binding including K+ channels and ATP-gated ion channels (Glitsch 2011).

### 2.2 Mechanisms of pH Regulation

Regulation of cellular cytoplasmic pH is mediated through 5 main families of proteins (a diagram of these is shown in Fig. 1). Three of these directly transport H+ across the membrane, namely the monocarboxylate transporters, the sodium hydrogen ion exchangers and the vacuolar-type H+-adenosine triphosphatases.
Another mechanism of pH regulation is through the uptake of bicarbonate, which is used to titrate intracellular H⁺. The bicarbonate transporters of the SLC4 and SLC26 family of genes facilitate this. Finally, there is the family of carbonic anhydrases, which regulate pH through their ability to catalyse the reversible hydration of CO₂. Each of these is covered in more detail below, including their mechanism of action, expression and prognostic associations in cancer. This information and additional information, covered later in this chapter, regarding the role for each of these pH-regulating proteins on tumour growth/proliferation and metastasis, have been tabulated for quick reference in Table 1.
### Table 1 pH regulators

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Expression in tumours</th>
<th>Prognostic tumour marker</th>
<th>Effect on tumour growth/proliferation</th>
<th>Effect on migration/invasion</th>
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<tbody>
<tr>
<td><strong>Monocarboxylate transporters</strong></td>
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<tr>
<td>MCT1</td>
<td>1/3 studies</td>
<td>High levels in GISTs, colorectal and breast cancer. Associated with the basal-like subtype of breast cancer</td>
<td>–</td>
<td>Knockdown or inhibition reduces xenograft growth rate</td>
<td>Knockdown reduced invasion in vitro</td>
<td>Pinheiro et al. (2008), Pinheiro et al. (2010b), Izumi et al. (2011), Le Floch et al. (2011), Boidot et al. (2012), de Oliveira et al. (2012)</td>
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<tr>
<td>MCT2</td>
<td>Proton-coupled monocarboxylate transporters (lactate, pyruvate and ketone)</td>
<td>–</td>
<td>Increased expression in colorectal and prostate</td>
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<td></td>
<td>Pinheiro et al. (2008), Pertega-Gomes et al. (2011)</td>
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**Table 1** (continued)

<table>
<thead>
<tr>
<th>Protein</th>
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<tr>
<td><strong>Sodium hydrogen ion exchangers</strong></td>
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<tr>
<td>NHE1</td>
<td>Electroneutral exchange of Na(^+) and H(^+)</td>
<td>Yes</td>
<td>Expressed in breast cancer DCIS and a colon cancer cell line</td>
<td>–</td>
<td>Knockdown in a small cell lung cancer cell line reduced proliferation and increased the number of cells in vitro. EIPA, a NHE inhibitor, reduced proliferation in a gastric cancer cell line in vitro and induced G1 arrest</td>
<td>Overexpression increased invasion of a breast cancer cell line in vitro. EIPA inhibition of NHE reduced hypoxia-induced migration of HEPG2 cells in vitro</td>
<td>Rios et al. (2005), Gatenby et al. (2007), Beltran et al. (2008), Li et al. (2009), Hosogi et al. (2012), Onishi et al. (2012)</td>
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<tr>
<td>NHE2</td>
<td>Expressed in a colon cancer cell line</td>
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<td>Beltran et al. (2008)</td>
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<tr>
<td>NHE4</td>
<td>Expressed in a colon cancer cell line</td>
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<td>Beltran et al. (2008)</td>
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<tr>
<td><strong>Vacuolar-type H(^+) ATPase</strong></td>
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<tr>
<td>V-ATPase</td>
<td>Actively transport H(^+) across membranes, 2H(^+) for every ATP consumed</td>
<td></td>
<td>Expression of the a4 subunit in 11/32 gangliomas. Expression in 42/46 invasive ductal pancreatic carcinomas, where increased intensity is associated with increasing pancreatic cancer stage</td>
<td></td>
<td>Knockdown of subunits a3 or a4 reduced invasion in vitro in a breast cancer cell line. Inhibition with an a3 subunit-specific inhibitor reduced bone metastasis of B16 melanoma cells in vivo</td>
<td></td>
<td>Ohta et al. (1996), Hinton et al. (2009), Chung et al. (2011), Glieze et al. (2012)</td>
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<tr>
<td><strong>Extracellular membrane-tethered carbonic anhydrases</strong></td>
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<tr>
<td>CAIX</td>
<td>Catalyses the extracellular reversible hydration of CO₂ to form HCO₃⁻ and H⁺</td>
<td>yes</td>
<td>Increased expression in many tumour types including ccRCC, colon and breast cancer</td>
<td>Expression is associated with poor prognosis in most tumour types including breast and colorectal cancer</td>
<td>Knockdown reduced spheroid growth rate in vitro in 2 colon cancer cell lines. Knockdown or inhibition reduces xenograft growth rate in vivo in 1 glioblastoma, 2 colon and 2 breast cancer models</td>
<td>Inhibition reduced migration of HeLa cells in vitro. Knockdown or inhibition reduced metastasis in a mouse breast cancer model in vivo</td>
<td>Liao et al. (1997), Wykoff et al. (2000), Chia et al. (2001), Wykoff et al. (2001), Kivela et al. (2005), Cleven et al. (2008), Chiche et al. (2009), Lou et al. (2011), McIntyre et al. (2012), Svastova et al. (2012)</td>
</tr>
<tr>
<td>CAXII</td>
<td></td>
<td>yes</td>
<td>Expressed in breast cancer where it is associated with estrogen receptor-positive tumours</td>
<td>Expression is a marker of good prognosis in breast cancer</td>
<td>Knockdown in combination with CAIX knockdown further reduced xenograft growth rates in vivo in 1 colon cancer model</td>
<td></td>
<td>Wykoff et al. (2000), Watson et al. (2003), Barnett et al. (2008), Chiche et al. (2009)</td>
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<tr>
<td><strong>Bicarbonate transporters</strong></td>
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<tr>
<td>AE1-3, CLD, Pendri, PAT1, SLC26A7, SLC26A9</td>
<td>Chloride–bicarbonate anion exchanger</td>
<td></td>
<td>AE2 is expressed in 68% of hepatocellular carcinomas</td>
<td></td>
<td>Inhibition with DIDS inhibited migration in vitro</td>
<td>Wu et al. (2006), Klein et al. (2000)</td>
<td>(continued)</td>
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<tbody>
<tr>
<td>NBCe1, NBCe2, NBCn1, AE4, NBCn2</td>
<td>Sodium-dependent bicarbonate cotransporters</td>
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<td></td>
<td>Inhibition with DIDS inhibited migration in vitro</td>
<td>Klein et al. (2000)</td>
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<tr>
<td>NDCBE</td>
<td>Sodium-dependent chloride–bicarbonate anion exchanger</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Inhibition with DIDS inhibited migration in vitro</td>
<td>Klein et al. (2000)</td>
</tr>
</tbody>
</table>

A table summarizing the published data on pH regulators in cancer, including function, evidence of hypoxic regulation, expression, prognostic significance in tumours and effects on tumour growth, proliferation, migration and invasion. Gastrointestinal stromal tumours (GISTs), ductal carcinoma in situ (DCIS), clear cell renal cell carcinoma (ccRCC), ethyl-isopropyl amiloride (EIPA) and 4,4′-diisothiocyanate-stilbene-2,2′-disulfonic acid (DIDS). ARPE-19 is a retinal pigment epithelia cell line. Madin–Darby canine kidney (MDCK). MDA-MB-231 is a triple receptor negative breast cancer cell line. HEPG2 is a hepatocellular carcinoma cell line. HeLa is a cervical cancer cell line—denotes no significant published data
3 Lactate Acid Secretion (Monocarboxylate Transporters 1-4)

Monocarboxylate transporters (MCT) 1–4 are members of the solute carrier (SLC) 16A family (Halestrap and Meredith 2004). There are 14 SLC16A family members (Halestrap and Meredith 2004). MCT1–MCT4 are proton-coupled monocarboxylate (lactate, pyruvate and ketone) transporters (Halestrap and Wilson 2012). These are electroneutral 1:1 monocarboxylate:H⁺ (Halestrap and Wilson 2012). MCT1 has the ability for both lactate and H⁺ efflux or influx, the direction of which is controlled by lactate and H⁺ gradients (Boidot et al. 2012). Whilst studies on MCT4 have characterized it as facilitating lactate and H⁺ efflux (Halestrap and Wilson 2012; Manning Fox et al. 2000), MCT4 expression increases the gradient between intracellular and extracellular pH in RAS-transformed hamster fibroblast deficient in NHE-1 (Na⁺/H⁺ exchanger-1), grown as xenografts (Chiche et al. 2012). This work highlights the important role for MCT4 in regulation of intracellular pH (Chiche et al. 2012). Further to this, siRNA knockdown of MCT4 produced intracellular acidosis and Warburg effect reversion (Gerlinger et al. 2012).

MCT4 (SLC16A3) is hypoxia regulated in a HIF1α-dependent manner (Ullah et al. 2006), and this initial study did not identify up-regulation of MCT1 in response to hypoxia (Ullah et al. 2006). However, a further study identified increased expression of MCT1 in response to hypoxia, which was p53 dependent and NFκB regulated (Boidot et al. 2012). A separate study investigating the expression of MCT1 and 4 showed that MCT4 but not MCT1 staining had a hypoxic pattern of expression in head and neck cancer biopsies (Rademakers et al. 2011). A symbiosis between hypoxic cells and normoxic cells has been identified with regard to the role of lactate. Cancer cells switch to a much greater role of glycolysis in the hypoxic microenvironment. Lactate is extruded by MCT4 from cells in these regions of tumours and is then taken up by oxygenated cancer cells and used as a fuel in respiration (Sonveaux et al. 2008). This symbiosis would also act to remove H⁺ from poorly perfused regions of tumour.

MCT1 is expressed ubiquitously in normal tissues with high expression in the heart and red muscle. MCT4 is also widely expressed (Halestrap and Wilson 2012; Halestrap and Price 1999). CD147/Basigin is a chaperone protein whose expression correlates with MCT1 and MCT4 (Kennedy and Dewhirst 2010). CD147 is required for proper folding and membrane expression of MCT1 and MCT4 (Kirk et al. 2000). High expression of MCT4 was associated with poorer relapse-free survival in ccRCC patients (Gerlinger et al. 2012). MCT1, MCT2 and MCT4 have high levels of expression in gastrointestinal stromal tumours (GISTs) (de Oliveira et al. 2012). Co-expression of MCT1 and CD147 was associated with lower GIST patient survival (de Oliveira et al. 2012). MCT2 and MCT4 had higher expression in prostate cancer samples compared with normal prostate material (Pertega-Gomes et al. 2011). MCT4 and CD147 expression correlated with poorer prognosis in
prostate cancer (Pertega-Gomes et al. 2011). Increased expression of MCT1, MCT2 and MCT4 was identified in a large study of 126 colorectal cancer patient samples by immunohistochemistry (Pinheiro et al. 2008). However, the increased expression of MCT2 was also associated with a decrease in membrane-associated expression of the protein in cancer cells (Pinheiro et al. 2008). Another study of colorectal cancer identified MCT4 expression in 50% of colorectal cancer patients where it was significantly associated with poorer prognosis (Nakayama et al. 2012). High levels of MCT1 and MCT4 were identified in breast carcinoma compared with normal tissue (Pinheiro et al. 2010a). A further study identified that MCT1 and CD147 expressions were associated with variables associated with poorer prognosis including higher-grade tumours and the basal-like subtype (Pinheiro et al. 2010b). A high level of MCT4 in addition to high GLUT1 levels was associated with poor survival in non-small cell lung adenocarcinomas (Meijer et al. 2012).

4 Sodium Hydrogen Ion Exchangers (NHE)

There are 9 Na⁺/H⁺ exchangers (NHE1–NHE9) (Slepkov et al. 2007). These are members of the solute carrier (SLC) 9A family, member 1–9 (Slepkov et al. 2007). These have heterogeneous patterns of expression (Slepkov et al. 2007). NHE proteins facilitate pH regulation via electroneutral, 1:1, exchange of Na⁺ and H⁺ along their respective gradients (Slepkov et al. 2007; Orlowski and Grinstein 2004). Establishing Na⁺ gradients achieved by Na⁺/K⁺-ATPase pumps can result in excess extrusion of H⁺ as a method of removal of metabolically derived acid (Orlowski and Grinstein 2004).

The role of NHE in pH regulation of tumour cells has been investigated. These studies include analysis of the effect of NHE1 inhibition, in the absence of CO₂/HCO₃⁻ buffering solution. This resulted in no effect on normal astrocytes whilst the malignant gliomas investigated displayed significant intracellular acidification (McLean et al. 2000). NHE1 antisense treatment of a small cell lung cancer cell line acidified the intracellular pH of these cells (Li et al. 2009). Expression of NHE1, NHE2 and NHE4 regulated pH in a colon cancer cell line (T84) (Beltran et al. 2008). Interestingly, NHE7 can shuttle between the plasma membrane, endosomes and the trans-Golgi network, regulating the intracellular pH of these organelles and cytoplasm (Orlowski and Grinstein 2004; Onishi et al. 2012).

The expression of NHE has been investigated in tumours in a small number of studies detailed below. NHE1 is the most studied and understood of the NHE family members, and is ubiquitously expressed in normal tissues (Slepkov et al. 2007). Chronic hypoxia increased NHE1 expression and NHE activity in isolated mouse pulmonary arterial smooth muscle cells (Rios et al. 2005). In addition, NHE1 expression increased in response to increases in intracellular superoxide (O₂⁻) concentrations (Akram et al. 2006). NHE1 expression in breast cancer ductal
carcinoma in situ (DCIS) had a pattern indicating hypoxic regulation (Gatenby et al. 2007). NHE1, NHE2 and NHE4 expressions were all identified in a colon cancer cell line (T84) (Beltran et al. 2008). Interestingly, inhibition of NHE reduced VEGF expression levels although the mechanism for this was unclear (He et al. 2010).

5 Vacuolar-Type H\(^+\) ATPase

Vacuolar-type H\(^+\)-adenosine triphosphatases (V-ATPases) actively transport H\(^+\) across membranes (Beyenbach and Wieczorek 2006; Sennoune et al. 2004a; Toei et al. 2010; Perez-Sayans et al. 2009). Figure 2 shows the structure and subunits of the V-ATPase. The ratio of H\(^+\) transported to ATP consumed is 2:1, respectively (Tomasek and Brusilow 2000). The functions associated with the V-ATPase include the acidification of intracellular compartments including lysosomes, regulation of intracellular pH and uptake of cations (Na\(^+\), Ca\(^{2+}\), etc.) (Beyenbach and Wieczorek 2006; Dietz et al. 2001). The V-ATPase is a multi-subunit protein containing up to 14 subunits, which make up two separate complexes (Fig. 2) (Beyenbach and Wieczorek 2006; Sennoune et al. 2004a; Perez-Sayans et al. 2009). The first is the V\(_1\) complex, which is situated on the cytoplasmic side of the membrane and interacts with ATP and is between 400 and 600 kDa in size.

Fig. 2 Structure of V-ATPase. Hydrolysis of ATP by the V\(_1\) domain (shown in yellow) drives proton transport through the membrane integral V\(_0\) domain (shown in green). The V\(_1\) domain comprises of 8 subunits (A–H). Subunit A contains the catalytic site for ATP hydrolysis. The V\(_0\) domain comprises of 6 subunits. Subunits a, c, c' and c'' make up the H\(^+\) transport site. Reprinted by permission from Macmillan Publishers Ltd: (Nature reviews molecular cell biology) (Nishi and Forgac 2002). © 2002 www.nature.com/reviews/molcellbio
The second is the V₀ complex, which is transmembrane and pumps the H⁺ across the membranes and is between 150 and 350 kDa. The variation in size depends on the specific subunit isoforms (Beyenbach and Wieczorek 2006; Sennoune et al. 2004a; Perez-Sayans et al. 2009).

The functional importance of V-ATPases was analyzed in human tumour cell lines using Baﬁlomycin, a V-ATPase inhibitor. This identiﬁed a role for V-ATPase in pH regulation on the plasma membrane of 5/13 of the cell lines tested (Martinez-Zaguilan et al. 1993). Cytoplasmic pH was acidified in the breast cancer cell line MDA-MB-231 by isoform-speciﬁc knockdown of V-ATPase subunit a3 (Hinton et al. 2009).


6 Extracellular Membrane-tethered Carbonic Anhydrases (CAIX/CAXII)

Carbonic anhydrases are zinc metalloproteins that catalyse the reversible hydration of CO₂ to form HCO₃⁻ and H⁺ (Sly and Hu 1995; Swietach et al. 2008). The carbonic anhydrase family has 14 members that can be categorized by cellular localization. There are the mitochondrial CAs (CAV A, CAV B), the cytoplasmic CAs (CAI, CAII, CAIII, CAVII, CAX, CAXI and CAXIII), the membrane-bound CA (CAIV), the transmembrane CAs (CAIX, CAXII and CAIV) and ﬁnally the secreted CA (CAVI) (Supuran 2008; Pastorek et al. 1994). CAIX and CAXII are both transmembrane carbonic anhydrase with an extracellular catalytic domain (Opavsky et al. 1996; Tureci et al. 1998). Both CAIX and CAXII are regulated by hypoxia in a HIF1α-dependent manner (Wykoff et al. 2000).

CAIX is a key enzyme in pH regulation; this was identiﬁed in work done in three-dimensional spheroid cultures. Spheroids mimic the three-dimensional gradients achieved within tumours for oxygen, lactate, ATP (Hirschhaeuser et al. 2010), etc. Analysis of pH gradients using spheroids also reveals clear intracellular and extracellular gradients (Swietach et al. 2009). These gradients are regulated by CAIX.
expression. In experiments comparing a colon cancer cell line in which CAIX had been overexpressed compared to empty vector controls, CAIX expression resulted in more alkali intracellular pH (\( \sim 6.6 \) compared to \( \sim 6.2 \) empty vector controls) and more acidic extracellular pH (\( \sim 6.6 \) compared to \( \sim 6.9 \) empty vector controls)(the pH maps of spheroids from these experiments can be seen in Fig. 3). CO\(_2\) forms carbonic acid, a weak acid in solution. It is therefore preferential for the cell to extrude it. In hydrating CO\(_2\) to form HCO\(_3^-\) and H\(^+\), CAIX acts to maintain an efflux gradient of CO\(_2\). This enables continued passive diffusion of CO\(_2\) across the membrane despite an inability to remove the CO\(_2\) from the hypoxic milieu (Hulikova et al. 2011, 2012b; Swietach et al. 2009). The crystal structure of CAIX has been resolved, and this work suggests that CAIX functions as a dimer (Alterio et al. 2009).

CAIX expression is up-regulated in many cancer types including breast (Wykoff et al. 2001), colon (Kivela et al. 2005), renal cell cancer (ccRCC) (Liao et al. 1997; Murakami et al. 1999), etc. In most tumour types, CAIX expression is associated with poor prognosis including breast (Chia et al. 2001), non-small cell lung cancer (Giatromanolaki et al. 2001), colon (Cleven et al. 2008), carcinoma of the cervix

**Fig. 3** CAIX expression maintains a more neutral intracellular pH in the hypoxic spheroid core whilst producing a more acidic extracellular pH. a A map of spheroid intracellular pH, measured using carboxy SNARF-1. b A map of spheroid extracellular pH, measured using Fluorescein-5- (and-6-) sulfonic acid. pH maps are shown for spheroids of HCT116 (empty vector) which has low levels of hypoxia-induced CAIX and HCT116 with constitutive CAIX expression (CA9 expressor) \((n = 20\), spheroid radius of \(222.0 \pm 10.6 \mu m\) (panel i) or of \(299.0 \pm 2.7 \mu m\)(panel ii)). This figure is modified from Fig. 3a and b of Swietach et al. (2009). Modified with permission © 2009 The Journal of Biological Chemistry
Loncaster et al. 2001) and soft tissue sarcoma (Maseide et al. 2004). CAIX expression is associated with better prognosis in ccRCC (Phuoc et al. 2008; Muriel Lopez et al. 2012) in contrast to other tumour types; however, non-papillary or clear cell RCC has von Hippel–Lindau (VHL) mutations, promoter hyper-methylation or loss of gene in nearly all cases (Gossage and Eisen 2010). The function of VHL protein is to regulate stability of HIF1α and HIF2α based on O2 tension. Under normoxic conditions, HIF1α and HIF2α are hydroxylated on conserved proline residues by the prolyl hydroxylases (PHDs) 1-3. This modification enables VHL to bind HIF1α and HIF2α with the E3 ubiquitin ligase complex which polyubiquiti-nates and targets them for degradation by the proteasome (Gossage and Eisen 2010). One explanation for the association of CAIX with good prognosis in the VHL mutant ccRCC could be that the CAIX expression needs to be more tightly regulated in response to O2 levels. High constitutive CAIX expression increased the levels of histological necrosis and also apoptosis in a colon cancer xenograft study (McIntyre et al. 2012). Also, HIF2 expression is associated with a more aggressive phenotype and does not regulate CAIX, so a switch to a HIF2-driven cancer would indirectly be associated with poor prognosis (Holmquist-Mengelbier et al. 2006).

This evidence suggests that the loss of CAIX expression regulation in response to O2 levels may have a negative impact on tumour survival. Interestingly, a CAIX knockout mouse was functionally normal apart from gastric hyperplasia, although this knockout has not been confirmed and may target another gene (Gut et al. 2002; Leppilampi et al. 2005).

A study of 103 breast cancer samples identified CAXII expression as a marker for good prognosis and positive estrogen receptor expression (Watson et al. 2003). The correlation between CAXII expression and estrogen receptor alpha expression was the focus of another study in breast cancer, which showed that CAXII expression was regulated by estrogen in breast cancer (Barnett et al. 2008). A study in renal cell carcinoma identified a correlation between lower CAIX and CAXII expression and worse prognosis (Kim et al. 2005). High CAXII expression was associated with a lower risk of metastasis in primary cervical cancer (Kim et al. 2006). High expression of CAXII in non-small cell lung cancer (NSCLC) was associated with better prognosis lower grade and with the squamous cell carcinoma type (Ilie et al. 2011). The shed extracellular domain of CAXII has been identified as a blood serum marker for diagnosis of NSCLC (Kobayashi et al. 2012).

7 Bicarbonate Transporters

Bicarbonate transporter proteins can be broadly subdivided into two main categories. These are the sodium-coupled bicarbonate transporters and the chloride–bicarbonate exchangers (Alper 2006; Romero et al. 2004; Cordat and Casey 2009). There are 14 genes encoding bicarbonate transporters, and these are split between 2 gene families that have distinct evolution and little amino acid homology (Alper 2006; Romero et al. 2004; Cordat and Casey 2009). These are the SLC4 and SLC26 families (Alper
Within these families, there are two main subtypes of bicarbonate transporter. These are the chloride–bicarbonate anion exchangers and the sodium-dependent bicarbonate cotransporters. One of the bicarbonate transporters, NBCDE, fits into both categories (Alper 2006; Romero et al. 2004; Cordat and Casey 2009). In normal cells, bicarbonate transport is important mostly in the regulation of HCO$_3^-$ removal as a by-product of the Krebs cycle (Alper 2006; Romero et al. 2004; Cordat and Casey 2009).

There are eight chloride–bicarbonate anion exchangers. These transporters exchange Cl$^-$ for HCO$_3^-$ and consist of (gene name(protein name)): SLC4A1 (AE1), SLC4A2 (AE2), SLC4A3 (AE3), SLC26A3 (chloride anion exchanger (CLD)), SLC26A4 (pendrin), SLC26A6 (PAT-1), SLC26A7 (SLC26A7) and SLC26A9 (SLC26A9) (Alper 2006; Romero et al. 2004; Cordat and Casey 2009; Kopito and Lodish 1985; Alper et al. 1988; Kudrycki et al. 1990; Walker et al. 2009; Scott et al. 1999; Waldegger et al. 2001; Lohi et al. 2002; Chernova et al. 2005). Within this grouping, there is also some difference in the electrogenicity. AE1-3, CLD, pendrin and PAT-1 are electroneutral and therefore result in no exchange of charge, and one Cl$^-$ is exchanged for one HCO$_3^-$ (Kopito and Lodish 1985; Alper et al. 1988; Kudrycki et al. 1990; Walker et al. 2009; Scott et al. 1999; Waldegger et al. 2001; Lohi et al. 2002; Alper 2006; Romero et al. 2004; Cordat and Casey 2009; Chernova et al. 2005). SLC26A7 and SLC26A9 are electrogenic, although there is some disagreement within the literature (Cordat and Casey 2009; Lohi et al. 2002). Diseases associated with defects in these transporters include haemolytic anaemia (AE1) (Southgate et al. 1996; Bouhassira et al. 1992; Jarolim et al. 1992), idiopathic epilepsy (AE3) (Sander et al. 2002) and Pendred syndrome (pendrin) (Everett et al. 1997).

Six sodium-dependent bicarbonate cotransporters consist of (gene name (protein name)): SLC4A4 (NBCe1), SLC4A5 (NBCe2), SLC4A7 (NBCn1), SLC4A8 (NDCBE), SLC4A9 (AE4) and SLC4A10 (NBCn2). These transporters cotransport Na$^+$ and HCO$_3^-$ across the membrane (Romero et al. 1997; Virkki et al. 2002; Choi et al. 2001; Griechtenko et al. 2001; Parker et al. 2008a, b). NBCe1 and NBCe2 are electrogenic, whilst NBCn1, NDCBE, AE4 and NBCn2 result in no exchange of charge across the membrane (Romero et al. 1997; Virkki et al. 2002; Choi et al. 2001; Grichthchenko et al. 2001; Parker et al. 2008a, b). In addition, NBCe1 can also cotransport CO$_3^{2-}$ (Grichthchenko et al. 2001) although the physiological significance is unclear due to the greater than 100-fold higher levels of HCO$_3^-$ in the cell than CO$_3^{2-}$ (Cordat and Casey 2009). NDCBE is a Na$^+$-dependent Cl$^-/HCO_3^-$ exchanger (Parks et al. 2011).

AE1–AE3 bicarbonate transporters are most likely restricted to bicarbonate efflux as theses anion exchangers are driven by Cl$^-$ and HCO$_3^-$ gradients except in the case of red blood cells (Cordat and Casey 2009), although if the Cl$^-$ gradients were reversed, the anion exchangers can equally take up bicarbonate (Sterling and Casey 1999). The sodium-dependent bicarbonate transporters similarly are dependent on Na$^+$ and HCO$_3^-$ gradients, the levels of which are higher extracellular than intracellular (Cordat and Casey 2009).
Bicarbonate transporters have roles in many normal physiological processes including gastric acid neutralization as it enters the intestine (Kuijpers and De Pont 1987) and multiple roles in the nervous system (Majumdar and Bevensee 2010). Bicarbonate transporters have three main functions: efflux of CO$_2$ and HCO$_3^-$ produced by respiration, pH regulation and regulation of cell volume (Cordat and Casey 2009).

HCO$_3^-$ is membrane impermeant and, therefore, unlike CO$_2$, needs to be transported across the membrane (Cordat and Casey 2009). However, bicarbonate transport via the bicarbonate transporters does not require ATP. This means that in situations of reduced ATP production such as stress induced by hypoxia, the lack of energy requirement should increase the importance of bicarbonate transport over other methods of pH regulation, and this has been shown in (Hulikova et al. 2011, 2012a). Investigation into the role of bicarbonate transport in normoxia versus hypoxia revealed a greater importance for bicarbonate transport in hypoxia (Hulikova et al. 2012a). Modelling the three-dimensional microenvironment of tumours in vitro using spheroids highlighted the dual importance of both bicarbonate transport and NHE activity. This work used the NHE inhibitor 5-(N,N-dimethly) amidoride (DMA), and also the SLC4 family bicarbonate transport inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS). The dependence on the bicarbonate transport and NHE activity for pH regulation was cell line dependent and therefore possibly also cancer-type dependent (Hulikova et al. 2011). This work also highlighted the importance of carbonic anhydrase function in cellular use of bicarbonate as a mobile buffer. Finally, the dominance of bicarbonate transport and NHE activity in pH regulation depends on regional variation in spheroids. In general, the further from the spheroid periphery, the greater the reliance on bicarbonate transport (Hulikova et al. 2011). Figure 4 shows data from Hulikova et al. 2011 identifying the importance of bicarbonate transport on intracellular pH regulation in the hypoxic core of spheroids, in two colorectal cancer cell lines.

Increased levels of bicarbonate transporters have been reported in tumours. For example, SLC4A1 levels are raised in gastric cancer and its knockdown reduced tumour progression (Suo et al. 2012). SLC4A2 expression is increased in hepatocellular carcinoma and colorectal cancer (Gorbatenko et al. 2014). SLC4A4 expression is increased in chronic myeloid leukaemia (Gorbatenko et al. 2014), and knockdown in a colorectal tumour cell line increased sensitivity to methotrexate (Gorbatenko et al. 2014). SLC4A7 expression is regulated by ErbB1, ErbB2 and ErbB3 in breast cancer cell lines via AKT, ERK, Src and KLF4 (Gorbatenko et al. 2014). Furthermore, recent analysis of bicarbonate transporter gene expression from TCGA data sets for lung colorectal adenocarcinoma and breast cancer revealed the heterogeneous patterns of expression across and within the tumour types (Gorbatenko et al. 2014). High levels of SLC26A9 were associated with high levels of CA9 in breast cancer, but the opposing relationship was seen in lung cancer
In breast cancer, expression of \textit{SLC4A5} tended to be raised, whilst in colon cancer, increased expression of \textit{SLC4A2} and \textit{SLC4A5} was identified. In lung cancer, there was an increased in expression of \textit{SLC4A3}, \textit{SLC4A7} and \textit{SLC26A6} (Gorbatenko et al. 2014). In colon cancer, \textit{SLC4A4} expression was increased in adenocarcinoma compared to squamous cell carcinoma which had increased expression of \textit{SLC4A3} and also tended to have higher expression of \textit{CA9} and \textit{CA12} (Gorbatenko et al. 2014). Furthermore, hypoxia increases \textit{SLC4A4} expression in the Ls174T colorectal cancer cell line (Parks and Pouyssegur 2015).

\textbf{Fig. 4} Bicarbonate transport is key in pH regulation in the hypoxic area of spheroids. A 20 mM ammonium prepulse was performed to impose an acid load on HT29 and HCT116 spheroids. Spheroids were allowed to recover for 12 min, and the data shown here are of the end point analysis of pH\textsubscript{i} gradients for spheroids untreated or treated with 150 uM of the bicarbonate transport inhibitor DIDS (4,4’-diisothiocyanate-stilbene-2,2′-disulfonic acid). The superfusate used in these experiments was buffered by 5 % CO\textsubscript{2}/22 mM HCO\textsubscript{3}\textsuperscript{-}, pH 7.4. \textbf{a} pH\textsubscript{i} map of HT29 spheroids with and without DIDS treatment. pH\textsubscript{i} gradients for untreated HT29 spheroids = 0.071 ± 0.023. pH\textsubscript{i} gradients for DIDS treated HT29 spheroids = 0.180 ± 0.037 (mean spheroid radius = 182.6 ± 15.3um)(bar = 100um). \textbf{b} Histograms of the pH\textsubscript{i} gradients between the spheroid core and periphery (periph) for HT29 and HCT116 spheroids untreated or treated with DIDS. DIDS treatment increased the pH\textsubscript{i} gradient within spheroids due to reducing pH\textsubscript{i} in the core of spheroids (*p < 0.05, Student’s t test). This figure is modified from Figs. 3a and b and 6a of Hulikova et al., 2011. Modified with permission © 2011 The Journal of Biological chemistry

(Gorbatenko et al. 2014).
8 Metabolons

Interactions between members of the families of proteins involved in pH regulation have been identified, and the formation of pH regulation metabolons proposed, examples of these interactions are described below. Carbonic anhydrase II (CAII) increases the transport activity of MCT1 and MCT4 in a catalytic and non-catalytic way (Becker et al. 2011). CAII function is facilitated by a H⁺ shuttle, which was key for this function. The authors hypothesize that the mechanism by which CAII positively regulates MCT1 and MCT4 transport is though removal of high local H⁺ around MCT1 and MCT4 (Becker et al. 2011). Similarly, the transport activity of MCT2 was enhanced by the extracellular carbonic anhydrase CAIV (Klier et al. 2011). This effect was only slightly reduced in response to mutation of the intermolecular H⁺ shuttle of CAIV (Klier et al. 2011). It could be hypothesized that CAIX may also potentiate extracellular transport by MCTs dependent on localized H⁺ concentrations.

Interactions have also been identified between members of the bicarbonate transporter and carbonic anhydrase families. The bicarbonate transporter SLC26a6 binds directly to CAII through a CAII binding (CAB) site, mutation of which greatly reduced SLC26A6 activity. This highlights the role of metabolon formation in bicarbonate transport and could be a possible therapeutic target (Alvarez et al. 2005). Binding of SLC26A6 to CAII was negatively regulated by protein kinase C (PKC) (Alvarez et al. 2005). CAIX co-immunoprecipitated with bicarbonate transporters such as AE1, AE2 and AE3 but not with SLC26A7 (Morgan et al. 2007). Co-expression of CAIX with AE1, AE2 or AE3 increased the transport rate of these bicarbonate transporters by 32, 28 and 37 %, respectively (Morgan et al. 2007). Further to this, interactions between CAIX and two bicarbonate transporters, AE2 and NBCe1, were identified by a proximity ligation assay (Svastova et al. 2012).

8.1 pH and Cell Phenotype

8.1.1 Proliferation and Growth

An intracellular pH of approximately higher than 7.2 has been identified as a threshold for growth factor-induced DNA synthesis to reinitiate G-/S-phase transition (Pouyssegur et al. 1985). This work compared wild-type and Na⁺/H⁺ mutant fibroblasts (Pouyssegur et al. 1985). Similarly, an acidic microenvironment (pH 6.6) suppressed G₂-/M-phase transition, after G₂ arrest induced by radiation, by modulating the kinase activity of the cyclin B1–Cdc2 complex (Park et al. 2000). Regulation of intracellular pH by NHE1 activity affected the entry of G₂/M. This work, carried out using NHE1 mutant and wild-type fibroblasts, identified an increase in intracellular pH just before G₂/M which if attenuated in the NHE1 mutant, delays S phase and inhibits G₂/M entry (Putney and Barber 2003). Further
work also showed a reduction in the kinase activity of the cyclin B1–Cdc2 complex in response to the more acidic intracellular pH and showed an increase in the expression of Wee1 kinase (Putney and Barber 2003). Knockdown of NHE1 in a small cell lung cancer cell line reduced proliferation and resulted in increased number of cells in G1 in cell cycle analysis (Li et al. 2009). Ethyl-isopropyl amiloride (EIPA) is an inhibitor of NHE (Hosogi et al. 2012) and EIPA treatment of a gastric cancer cell line reduced proliferation and induced a G0/G1 arrest; however, it did not affect intracellular pH (Hosogi et al. 2012). In addition, NHE activity regulates cell volume (Grinstein et al. 1992).

Knockdown of CD147, which acts as a chaperone protein and is required for the correct membrane localization of MCT1 and MCT4, reduced in vitro and in vivo growth of pancreatic cancer cells (Schneiderhan et al. 2009). A further study in a colon cancer cell line found that inhibition or knockdown of MCT1 or MCT4 by shRNA or by targeting CD147 reduced xenograft growth rate (Le Floch et al. 2011). Conversely, constitutive MCT4 expression in RAS-transformed fibroblasts increased growth in xenografts (Chiche et al. 2012). Knockdown and inhibition of MCT4 and MCT1, respectively, increased cellular dependence on oxidative phosphorylation (Marchiq et al. 2015). MCT1 and MCT4 deficiency sensitized cells to a mitochondrial complex 1 inhibitor, phenformin, the combination of which reduced cellular ATP levels and inhibited xenograft growth (Marchiq et al. 2015).

CAIX expression increased 3D spheroid culture growth rate in 2 colon cancer cell lines (McIntyre et al. 2012). CAIX knockdown, investigated in 3 separate studies, reduced the growth rate of xenografts in two human colon, one human breast, one human glioblastoma and one mouse breast cancer cell line model in vivo (McIntyre et al. 2012; Chiche et al. 2009; Lou et al. 2011). Further to this, the reduced xenograft growth rate effect with CAIX knockdown was increased in combination with Bevacizumab, an anti-VEGF inhibitor, which increased the hypoxic fraction of cells within the xenograft (McIntyre et al. 2012). The effects of CAIX knockdown or inhibition with and without Bevacizumab treatment in colon carcinoma and glioblastoma xenografts from McIntyre et al. 2012 are shown in Fig. 5. A role for CAIX in the cancer stem cell niche has been identified. The stem cell niche increases in hypoxia (Lock et al. 2013). mTORC1 signalling that regulates cell metabolism, proliferation and invasion is inhibited by acidic intracellular pH (Balgi et al. 2011). CAIX regulates the expression of markers of cancer stem cells such as Notch and Jagged1 via maintenance of mTORC1 signalling in the hypoxic tumour microenvironment (Lock et al. 2013). Knockdown of the bicarbonate transporter SLC4A4 in the colorectal cancer cell line Ls174T reduced 2D growth and increased cell mortality in acidic conditions in vitro (Parks and Pouyssegur 2015).
The Effect of pH on Migration and Metastasis

Culturing of two tumour cell lines long term in more acidic media (pH 6.8) increased migration and invasion in both (Martinez-Zaguilan et al. 1996). Acidic extracellular pH altered lysosome location to the periphery of the cell and increased

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Fig. 5 CAIX knockdown reduces growth rate and enhances Bevacizumab treatment in colon cancer and glioblastoma xenografts. a Xenograft growth curves of HCT116 clones ± Bevacizumab treatment. shCA9 (CA9 knockdown by shRNA) xenografts grow slower than EV (empty vector) (*p < 0.05, n = 5). Bevacizumab treatment reduced EV growth rate (***p < 0.01, n = 5). shCA9 xenografts treated with Bevacizumab grow slower than EV treated with Bevacizumab (**p < 0.01, n = 5). B. Xenograft growth curves of HT29 wt ± acetazolamide (ATZ) ± Bevacizumab treatment. Acetazolamide and Bevacizumab combination treatment reduced xenografts growth rate compared to the untreated xenografts (*p < 0.05, n = 5) and Bevacizumab treatment alone (**p < 0.05, n = 5). c Xenograft growth curves of U87 doxycycline (Dox) inducible shCTL (control shRNA) ± Dox and shCA9—Dox xenografts. These xenografts grow equally. d Xenograft growth curves of U87 doxycycline (Dox) inducible shCA9 ± Dox ± Bevacizumab. Dox-induced shCA9 (**p < 0.05, n = 5) and Bevacizumab treatment (*p, 0.05, n = 5) alone significantly reduced growth rate. Dox-induced shCA9 and Bevacizumab in combination significantly reduced xenografts growth rate compared to the untreated xenografts (***p < 0.001, n = 5) and Dox (*p < 0.05, n = 5) or Bevacizumab treatment (*p < 0.05, n = 5) alone. Red arrows denote the start of Bevacizumab treatment (10 mg/kg/3 times a week) and/or acetazolamide treatment (20 mg/kg/day) at 150 mm³ xenograft volume. This figure is reproduced from Fig. 6 of McIntyre et al. (2012). Reproduced with permission © 2012 The AACR
lysosome size in more metastatic cell lines (Glunde et al. 2003). Acidic pH increased the expression of MMP2, MMP9 and the Cathepsin B and Cathepsin L in melanoma cell lines (Rofstad et al. 2006). Increased secretion and activity of these proteinases were also identified in response to more acidic pH (Rofstad et al. 2006). Further to this, using a tail vein injection model of metastasis in immune-compromised mice identified increased metastasis with cells cultured under more acidic conditions (Rofstad et al. 2006). Culturing cells in acidic extracellular pH increased the expression of a number of genes including MMP9, and this was mediated by MAPK activation and NF-Kappa B activity both of which were up-regulated in response to low extracellular pH (Kato et al. 2005). Further work identified a role for transient Ca\(^{2+}\) increase triggering phospholipase D and acidic sphingomyelinase pathways, in increasing expression of MMP9 (Kato et al. 2007). Human stromelysin-1, a member of the MMP family, has a pH-dependent activity showing highest activity between pH 5.75 and 6.25 (Holman et al. 1999). This pH-dependent activity is regulated by protonation of a histidine residue in the flexible loop of the protein that contributes to the binding pocket, mutation of this reduced activity at more acidic pH (Holman et al. 1999). Oral bicarbonate treatment of mice growing xenografts reduced metastasis of a spontaneously metastatic breast cancer model (Robey et al. 2009). Bicarbonate treatment increased the extracellular pH of the xenografts whilst not affecting the intracellular pH (Robey et al. 2009).

10 The Expression of PH Regulators and Metastasis

V-ATPases were more highly expressed at the membrane of a highly metastatic breast cancer cell line compared with a less metastatic line. This was also associated with greater V-ATPase activity in isolated membranes in the highly metastatic line an effect that was decreased by V-ATPase inhibitors (Sennoune et al. 2004b). Migrating cells have an extracellular pH gradient which is more acid pH at the leading edge and less acid environment at the rear of the cell (Stock et al. 2007). This gradient was related to NHE activity which was increased at the leading edge compared to the rear of the cell (Stock et al. 2007). In a separate study in Madin–Darby canine kidney (MDCK) cells, NHE1 and AE2 localized to the leading edge of lamellipodia (Klein et al. 2000). NHE1 localized to the leading edge of human melanoma cells (Stock et al. 2007). A more recent study identified CAIX localization to the leading edge of lamellipodia upon hepatocyte growth factor (HGF) stimulation (Svastova et al. 2012). At the leading edge of lamellipodia, CAIX colocalizes with 2 bicarbonate transporters, anion exchanger 2 (AE2) and NBCe1 (Svastova et al. 2012). A further study identified that CAIX localizes in focal adhesion structures of lamellipodia and plays a role in focal contact regulation enabling enhanced migration (Csaderova et al. 2013). MCT4 is also localized in the leading edge of migrating cells (Gallagher et al. 2009). Immunohistochemistry analysis of tumour material has identified a correlation with levels of MCT1 and MCT4 and tumour invasiveness (Izumi et al. 2011). In addition, co-expression of
MCT1 and CD147 was associated with metastasis in cervical adenocarcinoma (Pinheiro et al. 2009).

11 Functional Studies of pH Regulators and Their Effects on Migration and Metastasis

MCT4 knockdown reduced migration in ARPE-19 and MDCK cells (Gallagher et al. 2009). MCT4 knockdown in the breast cancer cell line MDA-MB-231 also reduced migration (Gallagher et al. 2007). In addition, another study showed reduced invasion but not migration in response to MCT1 or MCT4 knockdown by siRNA in vitro (Izumi et al. 2011).

Inhibition of NHE reduced migration and acidified Madin–Darby canine Kidney (MCDK-F) cells (Klein et al. 2000). Application of the NHE inhibitors just to the lamellipodium reduced migration, whereas when the inhibitors were directed to the cell body, no effect was seen (Klein et al. 2000). Serum starvation increased cell migration and invasion via increased NHE1 activity (Paradiso et al. 2004). A further study showed that NHE inhibition reduced serum starvation increased cell motility and invasiveness in breast cancer cell lines (Reshkin et al. 2000). In this study, inhibition of PI3-Kinase signalling NHE activity and reduced invasion suggests that PI3-Kinase regulates NHE in serum starvation conditions (Reshkin et al. 2000). Inhibition of NHE1 (using EIPA) suppressed hypoxia-induced migration of HepG2 cells (Yang et al. 2010). Overexpression of NHE1 or NHE7 in a breast cancer cell line increased invasion (Onishi et al. 2012). Lysosomal exocytosis hypothesized to be required for protease secretion and was inhibited by NHE inhibition with EIPA (Steffan et al. 2009). Further to this, NHE inhibition with EIPA or combination of NHE inhibition with cariporide and NHE3 inhibition with s3266 reduced Cathepsin B secretion and HGF-induced invasion in prostate cancer cells in vitro (Steffan and Cardelli 2010). Additionally, NHE1 inhibition also reduced MMP2 and MMP9 expressions via an ERK1/2-dependent mechanism (Yang et al. 2010).

Invasion was reduced in the breast cancer cell line MDA-MB-231 by isoform-specific knockdown of V-ATPase subunit a3 (Hinton et al. 2009). Knockdown of the a4 subunit also reduced invasion but did not affect intracellular pH (Hinton et al. 2009). MMP-9 activity was reduced in vitro in response to V-ATPase inhibition or knockdown with siRNA in pancreatic cell lines. In addition, V-ATPase colocalized with cortactin a factor directing MMP release (Chung et al. 2011). Further to this, FR167356 a specific V-ATPase a3 subunit inhibitor reduced bone metastasis of B16 melanoma cells in vivo (Nishisho et al. 2011). TM9SF4 is a recently identified V-ATPase-associated protein that interacts with the VATPase subunit ATP6V1H, resulting in aberrant constitutive activation of VATPase (Lozupone et al. 2015). Knockdown of TM9SF4 acidified cytosolic pH, alkalinised the extracellular pH and reduced the invasive capacity of colon cancer cell lines (Lozupone et al. 2015).
CAIX overexpression in fibroblasts increased wound healing (migration) at a pH of 6.5 where no difference was seen at a pH of 7.4 (Chiche et al. 2010). Inhibition of CAIX also reduced migration in HeLa cells (Svastova et al. 2012). Knockdown or inhibition of CAIX with small molecular inhibitors reduced metastasis in a mouse breast cancer cell line model in vivo (Lou et al. 2011). CAIX is required for the NF-κB-regulated expression of granulocyte colony-stimulating factor (G-CSF) which drives the mobilization of granulocyte myeloid-derived suppressor cells to the lung metastatic niche of breast cancer (Chafe et al. 2015). This colonization of bone-marrow-derived cells to the premetastatic niche enables metastasis (Chafe et al. 2015). Reactive oxygen species-driven expression of CAIX in prostate cancer-associated fibroblasts (CAF) was identified in normoxia and was found to increase extracellular acidity and expression of matrix metalloproteinase expression by CAF which enabled increased invasion in vitro and metastasis in vivo (Fiaschi et al. 2013). Similarly, inhibition of bicarbonate transport using the inhibitor 4,4′-diisothiocyanate-stilbene-2,2′-disulfonic acid (DIDS) inhibited cell migration in cell culture (Klein et al. 2000). Furthermore, knockdown of the bicarbonate transporter SLC4A4 in the breast cancer cell line MDA-MB-231 reduced migration and invasion in vitro (Parks and Pouyssegur 2015).

12 Therapeutic Considerations

12.1 The Effects of pH on Chemotherapy and Radiotherapy

Protonation of weakly basic drugs in the acidic extracellular environment is proposed to inhibit uptake of these drugs (Gerweck and Seetharaman 1996). Doxorubicin, a weakly basic drug, increased in concentration and toxicity with a corresponding increase in extracellular pH (Gerweck et al. 1999). Similarly, the toxicity of doxorubicin treatment on a breast cancer cell line in vitro was reduced by reducing the pH from 7.4 to 6.8 (Raghunand et al. 1999). Bicarbonate treatment of mice growing xenografts reduced extracellular pH and increased toxicity of doxorubicin to significantly further reduce xenograft growth rate (Raghunand et al. 1999). A further study of three weakly basic chemotherapeutic compounds (mitoxantrone, doxorubicin and danorubicin) and three weekly acidic chemotherapeutic compounds (cyclophosphamide, 5-flourouracil and chlorambucil) identified a significant difference in IC₅₀ response to these compounds in a breast cancer cell line in vitro in media of pH 7.4 versus pH 6.8 (Mahoney et al. 2003). More acidic pH reduced the toxicity of weakly basic drugs whilst increasing toxicity of weakly acidic drugs (Mahoney et al. 2003). This study also identified reduced uptake of weakly basic drugs in more acidic medium, identifying ion trapping as a physiological and microenvironmental explanation for drug resistance (Mahoney et al. 2003). An in vivo study altering the extracellular pH of xenografts by administration of glucose (resulting in a 0.2 pH increase in pH gradient) changed the ratio
of the growth delay effect of a weakly acidic drug (Chlorambucil) versus a weakly basic drug (doxorubicin) (Gerweck et al. 2006).

Increased doxorubicin resistance was associated with increased expression and activity of NHE. Inhibition of NHE with EIPA increased doxorubicin uptake and sensitized the resistant line to doxorubicin treatment (Miraglia et al. 2005). The authors hypothesized that NHE resulted in a higher intracellular pH, which reduced doxorubicin accumulation in the resistant cells (Miraglia et al. 2005). NHE1 knockdown in a drug-resistant SCLC cell line significantly increased sensitivity to a number of chemotherapeutic drugs including cisplatin, etoposide and vincristine (Li et al. 2009).

Expression of V-ATPase was increased in 3 cisplatin-resistant cell lines, which had significantly more alkali intracellular pH than the parental cells (Murakami et al. 2001). Knockdown of the V-ATPase subunit ATP6L, identified in this previous study to be overexpressed in cisplatin-resistant cell lines, sensitized a drug-resistant breast cancer cell line to basic chemotherapeutics including doxorubicin and vincristine (You et al. 2009).

In addition to effecting chemotherapeutic response, pH modulation has also been displayed to effect response to radiation therapy. CAIX knockdown and inhibition enhanced the effect of tumour irradiation in a colon cancer xenograft model (Dubois et al. 2011). Similar experiments in a second colon cancer xenograft model with knockdown of CAIX showed a similar effect where CAIX knockdown enhanced radiotherapeutic treatment (Doyen et al. 2012). Furthermore, MCT1 inhibition or siRNA knockdown resulted in increased tumour sensitivity to irradiation (Sonveaux et al. 2008).

13 pH Modulators as Therapeutic Targets

The effects of pH regulation on tumour growth, proliferation and survival in addition to their role in chemotherapy and radiotherapy resistance described above make the pH regulators appealing targets for cancer therapy. Therefore, a number of inhibitors for these compounds have been designed. AR-C155858, an inhibitor of MCT1 and MCT2, has been identified (Ovens et al. 2010). However, it remains to be investigated whether this has any effect on pH regulation in tumours. However, MCT1 may not be a good target for cancer therapy given its role in the rapid proliferation of T cells upon activation that plays an important in immune response (Murray et al. 2005). There is a phase I clinical trial for the MCT1 inhibitor AZD3965 (AstraZeneca) (Table 2).

A number of NHE inhibitors have been developed (reviewed in (Masereel et al. 2003)). Cariporide, a NHE inhibitor, was used in preclinical studies (Masereel et al. 2003; Karmazyn 2000) and in a phase III clinical study investigating the inhibition of NHE on death or myocardial infarction in patients undergoing coronary artery bypass graft surgery (Mentzer et al. 2008). The results showed a decrease in myocardial infarction whilst increasing mortality due to an increase in cerebrovascular events possibly due to cariporide (Mentzer et al. 2008).
<table>
<thead>
<tr>
<th>Target</th>
<th>Compound</th>
<th>Generic name</th>
<th>Study title</th>
<th>Indications</th>
<th>Company/sponsor</th>
<th>Phase</th>
<th>Clinical trials identifier</th>
<th>Status</th>
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<tr>
<td>MCT1</td>
<td>AZD3965</td>
<td></td>
<td>A Phase I Trial of AZD3965 in Patients With Advanced Cancer</td>
<td>Solid tumours</td>
<td>AstraZeneca</td>
<td>Phase I</td>
<td>NCT01791595</td>
<td>Active recruiting</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Omeprazole</td>
<td></td>
<td>Docetaxel and Cisplatin Chemotherapy With or Without High Dose Proton Pump Inhibitor in Metastatic Breast Cancer</td>
<td>Metastatic breast cancer</td>
<td>Fudan University</td>
<td>Phase II</td>
<td>NCT01069081</td>
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</tr>
<tr>
<td>V-ATPase</td>
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<td>A Study to Examine the Effects of Esomeprazole on The Pharmacokinetics of Orally Administered Lapatinib in Subjects With Metastatic ErbB2 Positive Breast Cancer</td>
<td>Metastatic ErbB2 positive breast cancer</td>
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<td>V-ATPase</td>
<td>Pantoprazole</td>
<td></td>
<td>Pantoprazole and Docetaxel for Men With Metastatic Castration-Resistant Prostate Cancer</td>
<td>Metastatic castration-resistant prostate cancer</td>
<td>University Health Network, Toronto</td>
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<td>NCT01748500</td>
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<tr>
<td>V-ATPase</td>
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<td></td>
<td>Study Evaluating Pantoprazole With Doxorubicin for Advanced Cancer Patients With Extension Cohort of Patients With Solid Tumours</td>
<td>Solid tumours</td>
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<td>Phase I</td>
<td>NCT01163903</td>
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<tr>
<td>CAIX</td>
<td>cG250</td>
<td>Girentuximab</td>
<td>Monoclonal Antibody Therapy (Rencarex®) in Treating Patients Who Have Undergone Surgery for Non-metastatic Kidney Cancer</td>
<td>Kidney cancer</td>
<td>Wilex</td>
<td>Phase III</td>
<td>NCT00087022</td>
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</table>

(continued)
A table detailing some of the clinical trials targeting proteins which regulate pH. Information obtained from clinicaltrials.gov (a service of the US National Institutes of Health), which is a database of worldwide publicly and privately supported human clinical studies. Clinical trials targeting MCT1, V-ATPase and CAIX have been started and in some cases completed. The clinical trials utilising a CAIX-specific chimeric monoclonal antibody (cG250) to target CAIX are not inhibiting CAIX CO₂ hydration capacity. The CAIX-targeting trials use cG250 to induce antibody-dependent cell mediated cytotoxicity, image CAIX-expressing tumours using a radiolabelled version of this antibody and specifically target CAIX-positive tumour cells using a cytotoxic drug–antibody conjugate.

<table>
<thead>
<tr>
<th>Target</th>
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<tr>
<td>CAIX</td>
<td>¹²⁴I-cG250</td>
<td>Girentuximab</td>
<td>Pre-surgical Detection of Clear Cell Renal Cell Carcinoma Using Radiolabeled G250-Antibody</td>
<td>Cancer imaging</td>
<td>Wilex</td>
<td>Phase III</td>
<td>NCT00606632</td>
<td>Completed</td>
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<tr>
<td>CAIX</td>
<td>3ee9-MMAE</td>
<td>BAY-79-4620</td>
<td>cG250 coupled to the cytotoxic drug auristatin</td>
<td>Solid tumours</td>
<td>Bayer</td>
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<td>CAIX</td>
<td>SLC-0111</td>
<td>Safety Study of SLC-0111 in Subjects With Advanced Solid Tumours</td>
<td>Solid tumours</td>
<td>SignalChem Lifesciences Corporation</td>
<td>Phase I</td>
<td>NCT02215850</td>
<td>Active Recruiting</td>
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<tr>
<td>CAIX</td>
<td>DTP348</td>
<td>Trial to Determine Optimal Phase II Dose of the Oral Dual CAIX Inhibitor/Radiosensitizer</td>
<td>Solid tumours</td>
<td>Maastricht Radiation Oncology</td>
<td>Phase I</td>
<td>NCT0221669</td>
<td>Not yet open to participant recruitment</td>
<td></td>
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</table>
Inhibition of V-ATPase can be achieved with proton pump inhibitors (Spugnini et al. 2010). Proton pump inhibitors including esomeprazole have been used for many years clinically for the treatment of gastric acid disorders with few clinical side effects (Der 2003). Treatment of melanoma xenografts with 2.5 mg/kg of esomeprazole reduced tumour tissue pH gradients and tumour growth (De Milito et al. 2010). In addition, NiK-12192, a V-ATPase inhibitor, induced cell death in colon carcinoma cell lines (Supino et al. 2009). This work and other have provided a strong rationale for more testing of V-ATPase inhibitors in the treatment of tumours (Spugnini et al. 2010; De Milito et al. 2012). There are multiple clinical trials using the proton pump inhibitors, omeprazole or pantoprazole, in combination therapy (examples are shown in Table 2).

Many carbonic anhydrase inhibitors have been developed (see (Supuran 2008) for a review). Much work has been done in recent years to identify CAIX-/CAXII-specific inhibitors (Neri and Supuran 2011). CAIX/CAXII inhibitors are derivatives of sulphonamides, sulphamates, sulphamides, coumarins and thio-coumarins (Supuran 2010; Vullo et al. 2005; Pastorekova et al. 2004; Cecchi et al. 2005). Acetazolamide (Diamox) a general carbonic anhydrase inhibitor (Supuran 2008) is used clinically in a number of non-cancer treatment schedules including glaucoma (Kaur et al. 2002). Acetazolamide has also been used in CAIX inhibition in in vivo tumour models (McIntyre et al. 2012; Dubois et al. 2011). However, many drug studies have used much higher concentrations than needed to inhibit CA activity, suggesting off target effects. In addition to chemical inhibition, antibodies targeting CAIX and also CAXII function have been developed (Murri-Plesko et al. 2011; Battke et al. 2011). The CAXII inhibitory antibody (6A10) inhibits CAXII very effectively at low concentrations (Battke et al. 2011). However, the CAIX inhibitory antibody can inhibit a maximum of 76% of CAIX CO2 hydration capacity (Murri-Plesko et al. 2011). The CAXII inhibitory antibody (6A10) reduced spheroid growth in vitro and significantly reduced the growth rate of xenografts (Gondi et al. 2013). An antibody against CAIX, which does not inhibit CAIX activity, has also been developed (cG250) and is in clinical trials for clear cell renal cell carcinoma, where it induces natural killer cell interactions via antibody-dependent cellular toxicity (Surfus et al. 1996; Siebels et al. 2011) (Table 2). cG250 is also in 2 additional clinical trials. A phase III study for presurgical detection of clear cell renal cell carcinoma uses a radiolabelled 124I-cG250. Additionally in a phase I study, G250 is coupled to the cytotoxic drug auristatin for the treatment of solid tumours (Table 2). A dual targeting bioreductive nitroimidazole-based anti-CAIX sulfamide drug (DH348) has been developed (Dubois et al. 2013). DH348 reduced xenograft growth and sensitized CAIX-positive tumours to radiotherapy (Dubois et al. 2013). A phase I clinical trial to optimize dosing has been initiated (Table 2).

Bicarbonate transport by the SLC4A and SLC26A families is largely inhibited by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (Hulikova et al. 2011). There are also two more specific bicarbonate transport inhibitors. S0859, a specific Na+-dependent bicarbonate transport inhibitor, has been identified (Ch’en et al. 2008; Larsen et al. 2012). In addition, NDCBE is specifically inhibited by S3705.
(Wong et al. 2002). S3705 treatment reduced the intracellular pH of cholangiocarcinoma cells, reduced proliferation, increased apoptosis and inhibited activation of the ERK and AKT pathways upon serum stimulation (Di Sario et al. 2007).

14 Conclusion

The identified phenotypic roles of proteins involved in pH regulation, in proliferation, invasion and metastasis, make these good therapeutic targets. This highlights the importance of further developing drugs against these for clinical assessment. With regard to the development of therapeutic targeting agents, it is worth also considering the numerous normal physiological roles of the monocarboxylate transporters, NHE, the V-ATPases and the bicarbonate transporters. These normal roles make these a less cancer-specific target than CAIX. CAIX is more tightly regulated by hypoxia and tends to be tumour associated, and a CAIX knockout mouse shows little effect on pathology (Gut et al. 2002; Leppilampi et al. 2005). Development of compounds, which inhibit the protein interactions, required for formation of metabolons, or which inhibit membrane localization of the pH regulation proteins, would also be worth pursuing. Future research directions should include the effect of deregulating tumour pH controls on metabolism (Parks et al. 2013) given that this is the source of the acidity and also on signalling cascades following up the effects of pH on signalling described earlier. It is clear that the increased acidity of the tumour microenvironment has many effects and adaptation to this, and other microenvironmental stresses are key in cancer development and progression. Furthermore, understanding this heterogeneous tumour microenvironment will be crucial in developing effective therapeutic strategies.

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References


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1, 2, and 4 in colorectal carcinomas. Virchows Arch 452(2):139–146. doi:10.1007/s00428-007-0558-5


