Review

Value of pH regulators in the diagnosis, prognosis and treatment of cancer

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ABSTRACT

Altered metabolism, associated with acidification of the extracellular milieu, is one of the major features of cancer. As pH regulation is crucial for the maintenance of all biological functions, cancer cells rely on the activity of lactate exporters and proton transporters to regulate their intracellular pH. The major players in cancer pH regulation are proton pump ATPases, sodium-proton exchangers (NHEs), monocarboxylate transporters (MCTs), carbonic anhydrases (CAs) and anion exchangers (AEs), which have been shown to be upregulated in several human malignancies. Thanks to the activity of the proton pumps and transporters, tumours acidify their microenvironment, becoming more aggressive and resistant to therapy. Thus, targeting tumour pH may contribute to more effective anticancer strategies for controlling tumour progression and therapeutic resistance. In the present study, we review the role of the main pH regulators expressed in human cancer cells, including their diagnostic and prognostic value, as well as their usefulness as therapeutic targets.

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1. Introduction

Uncontrolled proliferation is an intrinsic characteristic of cancer cells and requires adaptations in energy metabolism to fuel cell division and tumour growth [1]. Thus, a common feature of invasive cancer cells is a preference for glucose metabolism, in which cells exhibit high rates of glycolysis, with increased glucose uptake, culminating in the production of high amounts of lactic acid, which lead to acidification of the microenvironment [2]. While oxygen inhibits lactate production in most normal mammalian cells, promoting oxidation of pyruvate to CO₂ and H₂O in mitochondria (Pasteur effect), cancer cells present increased glycolysis independently of the oxygen levels. This phenomenon was first described by Otto Warburg and is currently known as “aerobic glycolysis” or “Warburg effect” [2,3].

As a consequence of this metabolic phenotype, high amounts of protons are generated, mainly deriving from glucose metabolism [4]. To cope with this, cancer cells rely on proton exchangers and transporters, which export protons to the microenvironment, allowing malignant cells to survive in the hostile environment that they have created. These pH regulators mainly include ATPases, Na⁺/H⁺ exchangers (NHEs), monocarboxylate transporters (MCTs), carbonic anhydrases (CAs) and anion exchangers (AEs).

ATPases are a group of enzymes that couple ATP synthesis or hydrolysis to the transport of ions across membranes. The members H⁺/K⁺-ATPases and V-ATPases are the most important in the cancer setting, and will be the ones further described below, although some reports also point at ATP synthase as being involved in pH regulation, when ectopically expressed at the plasma membrane [5–7]. NHEs are sodium–hydrogen antiporters that comprise eleven human isoforms, which are involved in normal and pathological cellular events. NHE-1 is the isoform involved in pH regulation in cancer [8]. MCTs play a dual role in the cancer cell glycolytic metabolism, since they mediate lactate transport coupled with a proton. Thus, they allow the maintenance of the glycolytic metabolism by removing lactate from the cell, and, on the other hand, they also help in the regulation of pH [9]. The most important isoforms in cancer are MCT1 and MCT4, which are overexpressed in many cancer types. The CA family is responsible for the reversible conversion of carbon dioxide into carbonic acid, and comprises 15 members [10]. CAIX and CAXII are the isoforms designated as cancer-related, due to their abnormal expression in tumours. Carbonic acid is rapidly dissociated into H⁺ and HCO₃[−], and the released protons contribute to the microenvironment acidosis. The resulting bicarbonate can be uptaken to the cytosol by bicarbonate transporters, like AEs, in exchange for Cl[−], being responsible by intracellular buffering. CAIX can interact directly with AEs, maximizing this mechanism [11]. The AE family comprises 3 members AE1, AE2 and AE3, which are mainly involved in the exchange of Cl[−] and HCO₃[−] across the plasma membrane. Even though AEs are still poorly explored in cancer, there is evidence for altered expression of AE1 and AE2 in malignant tumors.

Acidification of the tumour microenvironment has been associated with certain key features of cancer aggressiveness, including

invasion, evasion from the immune system, increased angiogenesis and resistance to therapy, making this hallmark of cancer an attractive target for therapy [12]. However, clinical exploitation of pH regulators in the cancer setting is still at its infancy, and there is still much to be done in this field.

In the present review, we will focus on the pH regulators described above which play a role in the cancer setting, summarizing their expression in human malignant tumours, their role as biomarkers of diagnosis and prognosis, as well as exploitation as therapeutic targets, including results of existing clinical studies.

2. Monocarboxylate transporters

Monocarboxylate transporters (MCTs) are proteins belonging to the family of plasma membrane transporters SLC16A, with 14 members identified so far. MCTs are transmembrane proteins, with 12 predicted transmembrane domains (TMDs), with both amino and carboxyl terminal located intracellularly, and a large loop between TMDs 6 and 7 [13]. The first four isoforms, MCT1–4 catalyze the proton-coupled transport of monocarboxylates, and play an important role in cell metabolism. The transport mechanism starts by H⁺ binding, which is then followed by binding of the monocarboxylate to the protonated transporter [13,14]. Despite transporting lactate, MCTs transport other metabolically important monocarboxylates, including pyruvate, branched-chain oxoacids, and ketone bodies [13].

The expression pattern of each MCT isoform varies according to the function and the metabolic requirements of each tissue (reviewed in [9]). MCT1 has an intermediate affinity for the substrates, being present in most human tissues, with high levels in heart and muscle. MCT2 is a high affinity transporter, and is more adapted to the cellular uptake of monocarboxylates. MCT2 is mainly expressed in tissues that use lactate as energy source, such as brain, cardiac and red skeletal muscle and is also expressed in tissues that use lactate as a gluconeogenic substrate, such as kidney and liver. MCT3 was described in the retinal pigment and choroid plexus epithelia as being associated with the efflux of lactate in the retina. MCT4 is a low affinity transporter that has been described in tissues with high glycolytic activity, specifically white skeletal muscle fibers, astrocytes and white blood cells [13,15]. Due to the limited expression of MCT3 in tissues and in the cancer context, in this review we will focus on MCT1, MCT2 and MCT4.

2.1. Expression and prognostic value of MCTs

During the last decades, MCT1 and MCT4 have been described as crucial players on the maintenance of pH homeostasis of cancer cells, being associated with cancer aggressiveness. For that reason, the role of MCTs in several cancer types, including expression analysis in human cancer tissues have been widely explored in the past years (for an extensive review see [16]). MCT1 and MCT4 are upregulated in tumour cells when compared to adjacent normal tissue in a variety of human malignancies including lung [17], breast [18], head and neck [19], renal [20], brain [21], adrenal [22],

melanomas [23], pancreatic [24], colorectal [25], ovarian [26], bladder [27] and cervix [28]. Interestingly, in liver and prostate cancer, MCT1 appears downregulated, while MCT4 is upregulated [29,30]. In what concerns MCT2, fewer tumour types express this high affinity isoform, however it has been reported to be overexpressed in lung [31], brain [32], pancreas [24], colorectal [33] and prostate cancer [34].

Importantly, analysis of associations between MCT expression and clinic-pathological data identified MCTs as potential markers of poor survival in some tumour types. In fact, MCT1 and MCT4 showed associations with some poor prognostic variables including advanced tumour staging, high grade tumours, as well as shorter overall and disease free survival in a variety of human cancers such as adrenocortical malignant tumours, breast, renal cancers, prostate, head and neck, hepatocellular and pancreas tumours (reviewed in [16]). In opposition, MCT2 expression showed association with favourable prognostic parameters. For instance, in adrenocortical malignant tumours the presence of MCT2 was associated with lower mitotic index, small tumour size, absence of metastasis and good prognosis [22].

2.2. Exploitation of MCTs as therapeutic targets

Tumour cells are characterized by an excessive anaerobic glycolysis that is a hallmark of cancer cells [1] leading to an accumulation of glycolytic intermediates, upregulation of glycolytic enzymes and consequently a higher consumption of glucose and production of lactate. MCTs are key players in this aggressive cancer phenotype since they have a double role in the adaptation of cancer cells to hypoxia. They are responsible for lactate export, essential for the maintenance of the hyper-glycolytic phenotype, and for pH regulation, contributing to the acid-resistant phenotype. Thus, taking into account the role of MCTs in cancer metabolic adaptations, inhibition of MCTs will have important implications in cancer homeostasis by interfering with intracellular pH homeostasis and also with the acidic tumour microenvironment. Therefore, these lactate transporters represent attractive targets in cancer therapy.

There is a number of MCT inhibitors described to inhibit MCT activity [14], with different affinity and specificity for each MCT isoform. Among these, are aromatic compounds including α -cyano-4-hydroxycinnamate (CHC) and phenylpyruvate, stilbene disulfonates, such as 4,40-di-isothiocyanostilbene-2,20-disulfonate (DIDS) and 4,40-dibenzamidostilbene-2,20-disulfonate (DBDS), and the bioflavonoids phloretin and quercetin. At lower concentrations, these compounds display higher affinity for MCT1 and 2, although they can also inhibit other isoforms at higher concentrations. However, these compounds have also other targets besides MCTs. For example, CHC was described as a strong inhibitor of the mitochondrial pyruvate carrier [35], and both DIDS and DBDS also inhibit the activity of the chloride/bicarbonate exchanger AE1 [13].

CHC is the most well studied classical inhibitor of MCTs. Investigations were undertaken to study the effect of CHC in *in vitro* glioma [21], colorectal [36], cervix [37] and breast tumour cells [38]. CHC was effective in reducing lactate transport, cell proliferation, invasion and migration and increased cell death. This effect was also corroborated using RNAi experiments [21,38]. Promising results using *in vivo* models have already been reported. Treatment with CHC retards tumour growth [21,39], renders tumour cells sensitive to radiation [37], induces tumour necrosis and decreases tumour invasion [39].

AstraZeneca developed recently a group of specific and high-affinity inhibitors for MCT1, aiming to prevent transplant rejection. However, due to the growing interest of MCTs in the cancer field, this inhibitor has achieved great results. One of the compound, AR-C155858, is active against MCT1 and MCT2 but not MCT4 [40].

The use of chimeric transporters combining different domains of MCT1 and MCT4 revealed that the binding site is located in the C-terminal of MCT1, also involving the TMDs 7–10 [40]. This compound has been shown to influence lactate transport [41]. Recent improvements resulted on the design of AZD3965, a selective MCT1 inhibitor, whose effect was already tested in small cell lung carcinoma cell lines and also in a tumour xenograft model. MCT inhibition led to an accumulation of intracellular lactate in cells and in the xenografts and consequently reduced *in vivo* tumour growth [42].

Nevertheless, inhibition of one MCT isoform can be compensated by another MCT isoform, leading to resistance to treatment [41]. Importantly, these transporters require a protein chaperone for membrane localization and activation. Previous results show that CD147 and MCTs are co-expressed in a diversity of human cancer tissues [21,26,30,41,43–46]. Also, the major pro-tumoural action of CD147 is mediated by its fundamental function of chaperoning MCTs [41]. Therefore, targeting CD147 to inhibit MCTs appears to be a rational approach. In this context, CD147 silencing has been described to inhibit MCT1/MCT4 function, decreasing lactate efflux [47] and consequently reducing intracellular pH [24,41,48] and the *in vivo* malignant potential of cancer cells [24,41]. Moreover, CD147 expression is also associated with tumour progression, prognosis and chemoresistance [49]. However, there are no clinical studies with respect to inhibition of CD147 in cancer patients, probably because effective and specific inhibitors are scarce. Exciting clinical progress has recently been made with the development of CD147-directed monoclonal antibodies [50].

2.3. MCT inhibitors in clinical trials

The specific inhibitor of MCT1, AZD3965, is now being tested in a phase I/II clinical trial in patients with advanced solid tumours (prostate and gastric) or lymphomas (NCT01791595). This trial is supported by Cancer Research UK and started patient recruitment in 2013. This trial aims to find the highest dose that can be safely administered to patients with cancer and to understand the effects in certain types of tumours. This trial will be recruited until 2017 and there are no results available about the efficacy of this treatment [51].

3. ATPases

ATPases are a group of enzymes that couple ATP synthesis or hydrolysis to the transport of ions across membranes. A gradient of protons is the driving force for ATP synthesis, whereas the ATP hydrolysis releases the energy necessary for ion pumping. ATPases are expressed as membrane-bound transporters in diverse cell types and have been functionally classified as F-ATPases (primarily used in ATP synthesis), V-ATPases (vacuolar-ATPases), A-ATPases, P-ATPases (phosphorylated-ATPases) and E-ATPases [52]. All the above families exist in mammalian cells, with exception of A-ATPases, which are only found in Archaea.

V-ATPases and P-ATPases are involved in H^+ pumping, using the energy gathered from ATP hydrolysis in the homeostasis of cellular pH [6,53]. Regarding ATP synthase (F-ATPase), although previously assumed to be located only at the mitochondrial inner membrane, different reports evidenced its presence also on the surface of many cell types. ATP synthase plays its major role in the mitochondrial inner membrane, where is responsible for the synthesis of most cellular ATP, driven by the proton motive force generated by the electron transport in the respiratory chain. However, more recent reports have shown that ATP synthase is also expressed at the plasma membrane, participating in other cellular processes, includ-

ing pH regulation of cancer cells [54,55]. The ectopic expression of ATP synthase is still unclear, but it was reported that tumour-like environments present higher activity of the enzyme at cell surface, being associated to tumour cell proliferation [56].

V-ATPases and P-ATPases will be further discussed below due to their role in cancer, where they play a major role in pH regulation.

3.1. P-type ATPases

P-type ATPases are characterized by a cytoplasmic domain, which includes a nucleotide binding, a phosphorylation and an anchor domain, and by a transmembrane domain containing a central core of six α -helices [6]. The main mechanism that distinguishes P-ATPases from the other ATPase families is that P-ATPases display a phosphorylated intermediate state after the binding of the ion [57]. The phosphorylation of a conserved aspartate residue in the catalytic subunit is driven by ATP hydrolysis and promotes conformational changes that triggers ion translocation [6].

P-type ATPases are divided in five subfamilies, P1 to P5-type ATPases, with further sub-classifications A, B, C, and D [6]. They are involved in different cell physiologic processes and control numerous secondary transports, imposing a strict regulation on different signalling pathways, such as nerve impulse propagation, relaxation of muscle fibers, acid-base balance regulation in the kidney, acidification of the stomach and nutrient absorption in the intestine [58]. Because of their key role in some tissues, these pumps have been associated to human disorders, including heart failure, neurodegenerative disease, and cancer [6,59].

The best characterized members of the P-type ATPase family are the Na^+/K^+ -ATPase, H^+/K^+ -ATPase, Ca^{2+} -ATPase from sarco(endo)plasmic reticulum (SERCA) and H^+ -ATPase [59]. Since H^+/K^+ -ATPases are proton pumps involved in carcinogenesis, they will be detailed below [6].

3.1.1. H^+/K^+ -ATPases

H^+/K^+ -ATPases, like Ca^{2+} and Na^+/K^+ ATPases, are members of the P2-type ATPase family and are responsible for the exchange of intracellular protons by extracellular potassium ions [60]. They are α,β -heterodimeric enzymes, and transport ions against a gradient concentration by consuming ATP. The α subunit contains the catalytic site and is composed by 10 TMDs (TMD1 to TMD10), as well as by the phosphorylation and activation domains [61]. A lysine residue located in the fifth TMD is essential for the outward transport of the proton. The β subunit contains a short cytoplasmic domain and a single TMD. The extracellular domain of the β subunit contains three disulfide bridges and six or seven putative N-glycosylation sites, being N-glycosylation important for enzyme assembly, maturation, and trafficking [61,62].

Two isoforms of H^+/K^+ -ATPases have been identified, the gastric H^+/K^+ -ATPase (HKalpha1) and the non-gastric or colonic H^+/K^+ -ATPase (HKalpha2), the last found in prostate, kidney, uterus, placenta, skin, brain, pancreas and colon [63]. The gastric H^+/K^+ -ATPase is located at the plasma membrane of the parietal cells of the gastric mucosa, being involved in gastric acid secretion [61,64], by exchanging cytoplasmic protons by potassium.

3.1.1.1. Expression and prognosis value of H^+/K^+ -ATPases. Despite their role in pH regulation, studies regarding the association of H^+/K^+ -ATPases with human malignancies are still scarce. Regarding the non-gastric (HKalpha2) isoform, encoded by the gene ATP12A, Streif and coworkers [65] showed that HKalpha2 expression is mainly restricted to the plasma membrane of basal cells in normal prostate tissue, while in benign prostate hyperplasia and tumour tissues the expression is increased and the pattern is markedly altered, being found in the cytoplasm of epithelial cells. Another study described that this isoform was overexpressed (mRNA levels)

in human colorectal adenocarcinomas when compared to normal mucosa [66]. In what concerns the gastric H^+/K^+ -ATPase, to the best of our knowledge, there are no studies in the literature showing its association with clinicopathological data in human cancers.

3.1.2. Vacuolar-type H^+ -ATPases

V-ATPases are multimeric protein complexes with two functional domains: the peripheral V1 domain composed by eight subunits (A-H), involved in ATP hydrolysis and the integral V0 domain consisting in five subunits (a, c, c', d, e), involved in the translocation of protons across the membrane [67,68]. Although firstly described in a vacuolar system, V-ATPases are also present in the plasma membrane of specialized cells, being involved in physiological pH regulation, including regulation of systemic acid-base balance, bone resorption, among others [69]. Further, V-ATPases play a critical role in biosynthetic and endocytic pathways, due to acidification of intracellular organelles, including endosomes, lysosomes and Golgi-derived vesicles [70–72]. Some reports refer to V-ATPases as being involved in the multidrug resistance phenotype in cancer cells, through ion trapping in these cellular acidic compartments. V-ATPases remove protons from the cytosol to intracellular vesicles or to the extracellular environment resulting in lower pH, which increases tumour aggressiveness, being thus attractive targets for cancer therapy [73].

3.1.2.1. Expression and prognostic value of V-ATPases. V-ATPases are functionally expressed at the plasma membranes of human cancer cells, where they may contribute to cell growth, regulating signal transduction, differentiation, angiogenesis, and metastasis [74].

In the last years, several studies revealed that V-ATPases are overexpressed in human malignancies including pancreatic ductal adenocarcinoma [75], esophageal squamous cell carcinoma [76], oral squamous cell carcinoma [77], hepatocellular carcinomas [78], non-small cell lung carcinomas (NSCLC) [79], glioblastomas [80], ovarian [81], gastric [82], cervical [83] and breast cancer [84]. Moreover, the presence of V-ATPases in some types of cancer was associated with different prognostic variables. For instance, in esophageal squamous cell carcinoma, V-ATPases are associated with lower patient disease-free survival, tumour invasion and lymph node metastasis [76], in glioblastomas V-ATPases are associated with shorter overall survival and high grade tumours [80] and in NSCLC their expression was correlated to the pathological type (lung adenocarcinoma) and higher grade [79].

3.2. Exploitation of ATPases as therapeutic targets

Maintenance of the intracellular pH is crucial to normal cell function, and therefore upregulation of proton extrusion systems, such as ATPases, are essential for tumour cell survival in a hypoxic and acidic microenvironment.

V-ATPase is the main responsible for the efflux of protons to the extracellular milieu [85] and its expression is associated with migration and metastasis processes in ovarian cancer [81] and with the invasiveness of various types of cancer, including breast [84,86], oral [87], hepatocellular [88], pancreatic [75], and prostate cancer [89]. Furthermore, V-ATPase is involved in vesicular trafficking, and its involvement in regulation of other signalling pathways such as Wnt, Notch and mTOR, important for tumourigenesis, was also described [76,80].

V-ATPase overexpression in cancer is also correlated to multidrug resistance, what can be due to the ion-trapping phenomenon or to the altered pH of the microenvironment, that is critical for the anticancer agent action [71]. Besides being expressed at the plasma membrane, V-ATPases are also present in intracellular compartments like lysosomes or endosomes, leading to their acidification and to altered intracellular drug distribution [90]. In acidic con-

ditions, weak base drugs (majority of antitumour drugs) become charged (ionized form), which compromises their transport across the plasma membrane and their further cytoplasmic accumulation, leading to a lower cytotoxicity [91].

For example, in NSCLC tissues samples, resistance to chemotherapeutic drugs (cyclophosphamide, gemcitabine, doxorubicin, paclitaxel and cisplatin), is associated with V-ATPase expression, being this expression increased at higher tumour grades [79]. The reduced intracellular accumulation of anticancer drugs may be also due to a described putative role of V-ATPase as cooperating factor of drug efflux pumps like P-glycoprotein (Pgp) [53,92] and it has been shown that V-ATPase is overexpressed in both vincristine-resistant (Pgp-overexpressing) and adriamycin-resistant (MRP (multidrug resistance protein)-overexpressing) cancer cells [91]. However, the literature is controversial, concerning this association. Some authors refer that pH, either intra or extracellular, has no effect in drug extrusion [93], while others present opposite results, showing an increased Pgp activity at lower extracellular pH values [94].

Thus, these proton pumps are good candidates for the development of anticancer therapies. The most frequently used V-ATPase inhibitors are the plecomacrolide antibiotics, bafilomycins and concanamycins (lipophilic compounds) that have been reported to induce apoptosis in human cancer cells. Isolated from *Streptomyces* species, these antibiotics were originally described as inhibitors of P-ATPases, nevertheless, they are more potent against V-ATPases with concentrations in the nanomolar range [5,85,95]. However, many reports evidenced high cytotoxicity of these ATPase inhibitors for normal cells, probably due to the ubiquitous expression and the key role of these proton pumps in many cells and organs [96].

Another promising class of ATPase inhibitors in cancer are the proton pump inhibitors (PPIs), that include omeprazole, esomeprazole, lansoprazole, pantoprazole and rabeprazole, already in clinical use [96]. These compounds are weak bases which when protonated accumulate selectively in acidic spaces, where they are activated, and are non-toxic to normal cells [97,98]. PPIs were developed to suppress the production of acid in excess in the stomach, by irreversibly inhibiting H^+/K^+ -ATPases in the epithelial parietal cells. The active form binds irreversibly to cysteine residues of H^+/K^+ -ATPase, leading to its inactivation, by forming disulphide bonds [99]. They are commonly indicated for a wide range of gastro-intestinal disorders, including gastroesophageal reflux disease, peptic ulcers or functional dyspepsia [100].

Esomeprazole was the first drug to be marketed for various gastropathies and its efficacy and tolerability has been proved [101]. Some reports also refer that PPIs could be useful in blocking ATPase activity in tumour cells. The similarity between V-ATPase and the target H^+/K^+ -ATPase, prompted the investigation of PPIs for V-ATPase inhibition also [102]. The use of PPIs constitutes a targeted strategy in cancer, since they require an acidic environment to be activated, such as that found in the tumour microenvironment, which provides the possibility for tumour specific selection [53]. The activated PPIs increase caspase activity and accumulation of reactive oxygen species, inducing cell apoptosis [103]. Beside the direct toxic effects, PPIs are also able to inhibit mTOR signalling, a major regulator of cell proliferation [96].

Evidence shows that the use of omeprazole or pantoprazole led to a decrease in cell migration and cell death in gastric cancer [104] and melanoma cells [105], and increase tumour sensitivity to chemotherapy agents in breast cancer [106]. Regarding *in vivo* experiments, esomeprazole treatment lead to the elevation of extracellular pH and decrease of intracellular pH in melanoma mice xenografts [105].

The efflux of protons has been implicated in drug resistance, being probably possible to reverse drug resistance using PPIs. Treatment with PPIs has been found to sensitize cancer cells through

changes in extracellular/intracellular and cytosol/lysosomal compartment pH gradients, with retention of the drugs in the cytoplasm, and also in the nucleus [107,108]. Ferrari et al. showed that the esomeprazole sensitized human osteosarcoma cell lines to cisplatin [109]. In another study, pre-treatment with this PPI impaired the growth of triple-negative breast cancer cells and conducted to higher treatment efficacy with the weak-base doxorubicin, with no significant effect on non-neoplastic breast epithelial cells [106]. In esophageal cancer cell lines, cell viability was significantly reduced after pre-treatment with esomeprazole, as well as cell adhesion and cell migration. Furthermore, the cytotoxic effects of cisplatin and 5-fluorouracil were potentiated with this pre-treatment [110]. Recently, another research group showed that ovarian cancer cells treated with chemotherapeutic agents, namely paclitaxel with or without V-ATPase siRNA or omeprazole pre-treatment, induced a drug synergistic effect. These results were confirmed in *in vivo* experiments, on tumour growth, in orthotopic and patient-derived xenograft (PDX) mouse models [111].

Therefore, this class of drugs presents advantages as they can selectively target cancer cells in their acidic environment, where they become active. They have been successfully used to suppress tumour growth *in vitro* and *in vivo* and can have an important role overcoming drug resistance. In addition, they are generally well tolerated and safe, even in long-term therapy, with severe side effects rarely described.

Besides H^+/K^+ -ATPase and V-ATPase, ATP synthase can be also considered as an anticancer target, when expressed at the plasma membrane of cancer cells, where it contributes to proton efflux [56]. The association between ATPases and cancer was already described in 1990, when the heterologous expression of the yeast proton-pumping ATPase in fibroblasts increased intracellular pH and induced cell tumourigenic transformation [112]. However, only one study reports overexpression of ATP synthase in human samples of breast cancer [113].

Different reports showed that angiostatin, aurovertin or resveratrol can act as cell surface ATP synthase inhibitors, promoting tumour cell death. Cancer cell treatment with resveratrol (a type of natural phenol) inhibited tumour proliferation in breast [114], colon [115], pancreas [116], stomach [117] and prostate [118] cancers as well as in leukemia [119]. Another ATP synthase inhibitor, angiostatin, induced a cytotoxic effect in lung cancer cells in a pH-dependent manner [120], whereas aurovertin showed strong inhibition of cell proliferation in breast cancer cells [113].

3.3. ATPase inhibitors in clinical trials

There are three main clinical trials that investigated the effect of combined application of PPIs and chemotherapy treatment in cancer.

One of the studies is a phase II clinical trial aiming to evaluate the effect of the combination of high-dose PPI (esomeprazole 200mg/day) with chemotherapy (docetaxel and cisplatin) treatment in metastatic breast cancer (NCT01069081). This study was designed to explore whether a PPI improves efficacy of docetaxel and cisplatin treatment and does not influence drug tolerability in metastatic breast cancer patients [121]. This study was completed in February 2012 and the results showed that high dose PPI proved beneficial and improved chemotherapy efficacy.

Another phase II clinical trial assessed the effectiveness and safety of the combined use of pantoprazole and docetaxel (with prednisone) in metastatic castration-resistant prostate cancer patients who have not received prior chemotherapy (NCT01748500). The main objectives of this trial are: to assess the activity and safety of the PPI, evaluate archival prostate cancer tissue of men included in the clinical trial for evidence of autophagy, and evaluate pharmacokinetic interactions of pantoprazole with

docetaxel. This study is ongoing, but not recruiting participants, and the estimated study completion date is December 2016.

More recently, a phase II randomized trial evaluating the use of omeprazole in combination with chemotherapy, in patients with recurrent unresectable or metastatic cancers of the head and neck started in December 2013. However, this study has been withdrawn prior to enrolment due to lack of funding. The final data collection for primary outcome measure is December 2016 (NCT02013453) [122].

Despite the promising results described above, a recent study aiming to assess the role of gastric acid suppressants such as PPIs in capecitabine efficacy, revealed that PPIs decreased the efficacy of capecitabine on overall survival. This study was a secondary analysis of a previous phase III trial which compared capecitabine and oxaliplatin (with or without lapatinib) in HER2-positive metastatic gastroesophageal cancer, in which PPIs were part of the medication records [123]. A possible explanation for this fact is that raising of the gastric pH by PPIs might have compromised the dissolution and absorption of capecitabine. Due to the extensive use of capecitabine in breast and colon cancer, additional studies are ongoing.

4. Na^+/H^+ -exchangers (NHEs)

Sodium-proton exchangers (NHEs) belong to the SLC9A solute carrier family of ion transporters that comprise eleven human isoforms (NHE1–11) involved in various cellular processes like cell-cycle regulation, apoptosis or cell movement, as well as in pathological conditions like heart disease and cancer [124,125]. Among the NHEs, NHE-1 is one of the most important systems involved in pH regulation in cancer development [8]. NHE1 presents a hydrophobic N-terminal TMD that performs the ion flux, and a hydrophilic C-terminal cytosolic domain that regulates exchanger activity [126]. Regulation of NHE1 activity involves amino acid phosphorylation in the C-terminal domain and also interactions of the C-terminal with intracellular proteins and lipids. NHE1 extrudes intracellular protons, a by-product of metabolism or electrochemical transport, in an electroneutral manner by exchange with extracellular Na^+ in a ratio 1:1. The directionality of Na^+/H^+ exchange is reversible and dependent on the transmembrane concentration gradients for both ions, as well as of intracellular pH [127].

4.1. Expression and prognostic value of NHEs

NHE1 is a transporter ubiquitously expressed in various cancer cell types, involved in cell volume regulation and pH homeostasis, contributing to cell transformation, proliferation, motility, migration, resistance to chemotherapy *in vitro*, and tumour growth, metastasis, and probably also to tumour spontaneous regression [128–130]. However, there are only a few studies regarding the expression of NHE1 in human cancer samples and the clinic-pathologic significance of NHE1 is also poorly explored. For instance, NHE1 has been shown to be overexpressed in cervical cancer [131], hepatocellular carcinoma [132], esophageal adenocarcinomas [133], breast cancer [134] and glioblastomas [135] when compared with normal tissues. In cervical cancer, the expression of NHE1 was associated with tumour invasion and pelvic lymph node metastasis, whereas in hepatocellular carcinomas NHE1 expression was associated with increased tumour size, venous invasion and advanced tumour stage [131,132]. Moreover, NHE1 mRNA increased levels were associated with shorter overall survival in patients with glioblastoma and hepatocellular carcinomas [135,136].

4.2. Exploitation of NHEs as therapeutic targets

The role of NHE1 in the cancer aggressiveness features such as invasion, migration and metastasis is explained by its localization in the invading portion of cell, in which there is increased intracellular pH and acidification of extracellular milieu [128]. Therefore, this pH regulator controls different steps of tumour progression, and for that reason it is seen as a promising therapeutic target [137].

Many studies showed the involvement of NHE1 in distinct patterns of neoplastic progression in different types of cancers [127,138,139]. Amith *et al.*, showed that the knockout of NHE1 sensitized MDA-MB-231 cells to paclitaxel and decreased the ability of triple-negative breast cancer cells to form subcutaneous xenograft tumours in athymic nude mice [126]. Recently, Cardone *et al.* showed that the expression of NHE1 was correlated with aggressiveness of different pancreatic adenocarcinoma cell lines. A strong interaction of the epidermal growth factor receptor (EGFR) with NHE1 in pancreatic adenocarcinoma cell lines was observed, suggesting an important role of the NHE1 in transducing the EGFR signal, playing an important role in both basal and EGFR-stimulated 3D colony growth, invasion and invadopodia-dependent extracellular matrix digestive ability, therefore related with induction of metastasis [140].

The two major families of NHE inhibitors are: the pyrazine derivatives [e.g. 5-(N,N-hexamethylene) amiloride (HMA), 5-(N,N-dimethyl) amiloride (DMA), 5-(N-ethyl-N-isopropyl-amiloride) (EIPA)], and the benzoylguanidines (e.g. cariporide, eniporide, HOE-694) [141,142].

Amiloride, a diuretic drug, was the first NHE1 inhibitor developed. Since the initial report, in 1983 [143], where hepatoma growth was inhibited by this drug, different studies have demonstrated that amiloride can suppress the proliferation of metastases in different tumours. In the human breast cancer cell lines MDA-MB-231 and MDA-MB-436 and also in xenografts using the same cells, it has been reported that amiloride decreased vascular endothelial growth factor (VEGF) expression, as well as the activity of urokinase-type plasminogen activator (μPA), metalloproteinases and other proteases, involved in invasion and metastasis [144]. It was also shown that treatment of human K562 myeloid leukemia cells with amiloride significantly reduced VEGF mRNA levels [145]. Furthermore, inhibition of NHE1 by amiloride has been demonstrated to inhibit proliferation of gliomas [146], hepatocellular carcinoma [147] and breast cancer cells [148].

Recently, powerful amiloride analogues, like EIPA (200-times more potent), and HMA and DMA have been studied for their anticancer potential in different cancer settings [141]. It has been demonstrated that the administration of HMA and DMA decreases intracellular pH and induce apoptosis in leukemic cells, with low toxicity to normal cells [149]. HMA compromises the growth and viability of human and rat hepatocarcinoma cells, possibly through a decrease in glutathione levels and loss of lysosomal integrity [150]. Co-administration of paclitaxel with DMA demonstrated synergistic effects in breast cancer cells compared to paclitaxel or DMA administered alone [151]. Concerning NHE1 inhibition by EIPA, a recent study showed that this drug impaired DNA replication, inhibited proliferation and migration and promoted apoptosis, reducing hepatocellular carcinoma invasion and motility [147]. Co-administration of EIPA with clinical anti-cancer agents, increased intracellular accumulation of co-administered doxorubicin, in resistant colon cancer cells [145].

Non-amiloride based compounds, such as cariporide, were also developed to target NHE1. Cariporide is a specific and powerful well studied NHE1 inhibitor, being well tolerated by humans in a cardiac disease context. It has been demonstrated that the invasive capacity of various kinds of cancer cells are suppressed by the use of selective and potent inhibitors of NHE1, including cari-

poride [152,153]. Some authors have also shown that the selective inhibition of NHE1 by cariporide reduced proliferation and induced apoptosis in cholangiocarcinoma cells, due to the subsequent acidification of the intracellular pH [141]. It was also observed that cariporide reduced hypoxia-mediated tumour invasion of human tongue squamous cell carcinoma [154].

Other non-amiloride and non-guanidine derived compounds have been developed as promising anticancer drugs, more potent and with good efficacy, such as SL-591227, Phx-3, and compound 9t. Phx-3 displays high selectivity for NHE1 and was shown to promote apoptosis in different cancer cells [155,156]. Compound 9t was reported to be 500-fold more potent against NHE1 than cariporide and to have much greater selectivity [157]. The non-amiloride and non-guanidine-derived compound 9t is a potent and highly selective inhibitor of NHE1, leading to higher specificity and thus to lower toxic side effects. Furthermore, is well absorbed in the gastrointestinal tract, allowing oral use. Despite its promising potential use as anticancer agent, there are no further studies either *in vitro* or *in vivo* and the effort to put it in clinical trials/translational studies has been unsuccessful so far [141].

Importantly, the potency of NHE1 inhibitors is related to the ionization state of the guanidine residues. Thus, the lower pH of the tumour microenvironment can constitute an advantage to the activity of these inhibitors, which would lead to a better response [158,159].

4.3. NHE inhibitors in clinical trials

The only non-amiloride NHE1 inhibitors that have undergone clinical trials were cariporide and eniporide. However, those trials were not in the cancer field but in the cardiology setting and in the context of ischaemic-reperfusion injury [141,160].

Two trials have been done with cariporide (Expedition) [161] and there was also a trial utilizing eniporide (Escami) [162]. The clinical trial Expedition with cariporide in cardiovascular disease progressed to Phase III, but was abandoned, due to adverse side effects, which could be explained by the ubiquitous expression of NHE1 [163]. The results are disappointing in this research field, however, as the clinical trials have only been carried out in patients with cardiac disease and the side effects are also related to cardiac function, studies of NHE1 inhibition in cancer patients in good cardiac conditions, remain to be undertaken [102].

5. Carbonic anhydrases

Carbonic anhydrases (CAs) form a family of enzymes responsible for the reversible hydration of carbon dioxide to carbonic acid, being key molecules in two vital tumour processes – cell metabolism and pH regulation [164]. This family of enzymes comprises 15 members, that are classified according with their cell location: membrane-associated, cytosolic, mitochondrial or secreted (for review see [10,165]).

The zinc ion, in the active site of CAs, is essential for catalysis. Thus, a hydroxide bound to the zinc ion attacking the CO₂ molecule to the hydrophobic pocket in its neighbourhood, leading to formation of bicarbonate. The bicarbonate ion is then replaced by a molecule of water and released. [10].

The membrane-associated CAIX and CAXII isoforms, in contrast to the other isoforms, are markedly increased under tumour hypoxic conditions [166], and are the ones designated as cancer-related, due to their abnormal expression in tumours. Therefore, these will be the ones reported in this review.

5.1. Expression and prognostic value of CAs

Overexpression of CAIX and CAXII was reported for the first time in renal cell carcinoma cells, where the tumour suppressor gene the von Hippel–Lindau (VHL) is inactivated and hypoxia-inducible factor 1- α (HIF-1 α) is constitutively active [166].

Diverse immunohistochemical studies have reported CAIX to be expressed in several cancers, including malignancies of the breast [167,168], colon/rectum, head and neck [19,169], lung [170], brain [171], bladder [172], ovarian [173], and kidney [166] (for review see [174]). Importantly, its expression is scarce in normal human tissues, being only detected in the gastrointestinal tract [175]. This atypical differential expression pattern elects CAIX as a potent tumour hypoxic biomarker that could be useful in the clinic.

CAIX expression has been widely correlated with poor prognostic variables such as high tumour grade, necrosis, bad treatment outcome, advanced stage and poor prognosis in a variety of human malignancies, including breast [168], adrenocortical tumours [22], lung cancer [170], among others (for an extensive review see [164,174]). Lately, predictive value of CAIX for further clinical decision has been explored. For instance, in metastatic clear cell renal cell carcinoma (CCRCC), CAIX was shown to be predictive of outcome of anti-VEGF and sunitinib (multi-targeted receptor tyrosine kinase (RTK) inhibitor) therapy [176], and useful in selecting patients for systemic therapy [177]. In rectal adenocarcinoma, CAIX expression also indicates preoperative chemotherapy response [178]. Also, in breast cancer patients with early stage tumours, CAIX expression can predict doxorubicin resistance and chemosensitivity to some neoadjuvant therapies [179,180]. Though, this evidence still needs further investigation to introduce CAIX in the clinical practice. Interestingly, expression of CAIX in the tumour microenvironment, namely in cancer-associated fibroblasts was also reported and it has been associated with decreased patient survival in head and neck cancers [181].

CAXII, also regulated by hypoxia, has received less attention than CAIX but is usually expressed in the same tissues as CAIX. Accordingly, CAXII expression is reported in human malignancies such as breast [167], ovarian [173], colorectal [182], cervical [183], renal cell carcinomas [184], lung carcinoma [185] among others [182,186]. Despite being a hypoxia induced marker and present in different tumour types, it has been strongly associated with several good prognostic parameters in invasive breast cancer [187] and NSCLC [185]. However, this characteristic is still controversial and apparently is tissue-dependent, since an association of CAXII with poor prognosis it was described in oral cell carcinomas [188] and diffuse astrocytomas [189].

Importantly, the serum levels of CAIX and CAXII may also be of value as diagnostic biomarkers for lung cancer [190] and as molecular markers for the detection of breast cancer lymph node metastasis [191]. This could be clinically exploited for screening or monitoring patients with cancer.

Overall, these CA isoforms regulate intracellular pH and regulate tumour growth, thus being labelled as important molecular players in human malignancies.

5.2. Exploitation of CAs as therapeutic targets

Accumulating experimental evidence recognizes that CAs strongly contribute to the acidification of the tumour extracellular milieu, and consequently to the maintenance of the alkaline intracellular pH of tumour cells, being key players in tumour aggressiveness [102]. Thus, CAIX and CAXII represent promising targets for efficient anticancer therapies.

Numerous pre-clinical studies have validated CAIX as an imaging and treatment target [192]. Due to its rare distribution in normal tissues, CAIX has become an attractive biomarker of hypoxia in

solid tumours. The development of different radionuclide labelled antibodies [193] or fluorescent labelled compounds [194] targeting CAIX have been designed and tested to validate this molecule as a hypoxic marker for clinical imaging and tumour following [175]. These pre-clinical studies using *in vitro* and *in vivo* experiments demonstrated that CAIX, more than CAXII, is a suitable target to trace hypoxic regions and could be used to select candidate patients for anti-CAIX therapies [175].

CAIX and CAXII promote tumour cell survival, by counteracting acidosis through regulation of the intracellular pH, thus targeting and inhibiting their catalytic activity demonstrated to successfully decrease tumour growth [195,196]. A collection of studies has reported that disrupting CAIX by gene knockdown or inhibiting its catalytic domain alters both extracellular and intracellular pH, inhibits or even stops the growth of tumour cells [197] and decreases cell migration/invasion [181,198], *in vitro* and *in vivo* models [199]. CAIX also contributes to therapy resistance, since inhibition of its catalytic activity significantly improves chemo- or radiosensitivity [200,201].

As a consequence, there has been an effort to develop pharmacological inhibitors in the last years. Antitumour activity of the different CAIX inhibitors has been widely tested (for review see [192,202]). Sulfonamides are the most studied CAIX inhibitors. Unfortunately, most of them besides having strong activity are not selective for CAIX but also inhibit CAXII. The mode of action of these small molecules consists on binding the catalytic site of CAs and their potent inhibitory growth effect was observed in several cancers including leukemia, NSCLC, ovarian cancer, melanoma, colon cancer, central nervous system, renal, prostate and breast cancer cell lines [203]. Different sulfonamide derivatives have been created and tested in the last years and all of them have shown to inhibit the catalytic activity of CAs with more or less specificity to CAIX and CAXII. Pre-clinical studies have shown that some compounds led the inhibition of tumour growth while others have great *in vivo* anti-metastatic effects [192]. Supuran and coworkers' investigations contributed highly to the development of a sulfonamide-derived compound (compound 6) that strongly and specifically inhibits CAIX [204]. This drug inhibits tumour growth, cell invasion and decreased cancer stem cell population in breast cancer models, and due to its promising effects, was submitted to a clinical trial phase I study [192].

The second class of inhibitors against CAIX is coumarins. 7-Glycosyl coumarin has demonstrated to be selective for CAIX and CAXII at low concentrations, being ineffective against the broadly expressed isoforms CAI and CAII. Coumarins efficiently inhibit both tumour growth and metastasis formation of the highly aggressive 4T1 syngeneic mouse mammary tumour cells. Despite their potency and selectively, these inhibitors are still under investigation in pre-clinical studies [192,205].

Another methodology in the design of CAIX inhibitors is the "dual drug" approach that consists in combining a pharmacophoric moiety inhibiting CAIX activity, with a moiety with a different pharmacological action, in the same molecule. Compound 13 showed *in vivo* tumour growth reduction and radiosensitization and is currently being prepared in preclinical Phase (for pharmacology studies and oral formulation) by DualTPharma [192,206].

Despite CAXII tumour activity is not so well explored, evidence shows that CAXII silencing or pharmacological inhibition also lead to alteration in tumour cell intracellular pH and consequent chemosensitization [207], suppressing tumour growth, cell migration and invasion [208]. The first anti-CAXII monoclonal antibody (6A10) was created by Battke and coworkers [209] and binds to the catalytic domain of CAXII, inhibiting its activity at nanomolar concentrations. This inhibitor has shown to

be successfully in inhibiting the growth of multicellular tumour spheroids *in vitro* and efficiently decreased tumour growth *in vivo* [196].

A point of consideration is that the expression of CAIX and CAXII is interdependent in some type of tumours and therefore the expression pattern and functional role of both enzymes should be better explored in order to understand if a broad-spectrum CA inhibitor could be a better strategy for treatment than inhibiting a single isoform. Finally, some drugs already used for the treatment of some tumours, such as the tyrosine kinase inhibitors imatinib and nilotinib and statins, also inhibit the catalytic activity of all CAs isoforms, being CAI and CAII the most efficiently inhibited isoforms [210]. This fact could be of important value for tumours that benefit of tyrosine kinase inhibitors and with overexpression of CAs.

5.3. CA inhibitors in clinical trials

CAIX has been validated as an imaging and treatment antitumour/anti-metastatic target [192] and some therapeutic strategies against CAIX have already entered in clinical studies. The University Hospital of Saint Etienne developed a pilot human trial to test the serum levels of CAIX as potential biological marker for treatment response in patients with metastatic renal cell cancer (mRCC) (NCT00942058). This study is already finished. The authors collected blood samples from 91 patients with mRCC and 32 healthy individuals [211]. They observed a significant association between serum CAIX levels and stage, tumour grade, tumour size, recurrence and metastasis, suggesting that CAIX may be a valuable diagnostic and prognostic tool in RCC.

CAIX-based therapy using small molecule CAIX inhibitors has advanced in 2014, by SignalChem Lifescience in a Phase 1a clinical trial for the treatment of advanced, metastatic solid tumours with compound 6 (SLC-0111 – NCT02215850). This study had the propose to investigate the safety, tolerability and pharmacokinetics of SLC-0111. This study was completed in March 2016 however, no results have been posted yet.

Other inhibitors such as dual drug 13 (DTP-348 – NCT02216669), is currently in a Phase I clinical trial sponsored by Maastricht Radiation Oncology. This dual drug has two mechanisms of action: it has a CAIX inhibitor, the sulfamide component, and a radiosensitizer for hypoxic cells through its 5-nitroimidazole moiety. The main objective of this study is to find the recommended dose for combination with radiotherapy and will be tested in patients with solid tumours, mainly head and neck neoplasms. Nevertheless, this study is not yet open for participant recruitment.

Regarding antibodies, monoclonal antibody G250 known under the commercial names RENCAREX[®] or GIRENTUXIMAB[®], was the first monoclonal antibody anti-CAIX introduced in a clinical phase I/II trial in combination with interferon-alpha-2a in mRCC patients. In this study, 31 patients with mRCC were treated with both drugs and the results showed that the treatment was safe, well tolerated and led to clinical disease stabilization [212]. A recent completed phase III clinical trial investigated RENCAREX[®] use as an adjuvant monotherapy in patients with advanced renal cancer (NCT00087022). Treatment was safe and well tolerated, however, the patients treated with G250 showed no clinical benefit. Importantly, data analysis showed that patients with tumours with high CAIX expression have a prolonged disease-free survival of about 22 months [213]. Radiolabelled cG250 was also tested as a valuable imaging agent for the diagnosis of patients with RCC. A phase II/III clinical trial is currently recruiting participants to show the impact of the Zr-89-girentuximab in clinical management (NCT02883153).

Table 1

Expression of different pH regulators in human cancer tissues.

Human cancer expression					
Tumour type	MCTs	ATPases	NHEs	CAs	AEs
Brain	↑MCT1 [21] ↑MCT2 [32] ↑MCT4 [21]	↑V-ATPase [80]	↑NHE1 [135]	↑CAIX [171]	**
Head and Neck	↑MCT1 [19] ↑MCT4 [19]	↑V-ATPase [77]	**	↑CAIX [19]	**
Breast	↑MCT1 [18] ↑MCT4 [18]	↑V-ATPase [84] ↑ATP Synthase [113]	↑NHE1 [134]	↑CAIX [167] +CAXII [167]	**
Lung	↓MCT1 [17] ↓MCT2 [31] ↑MCT4 [17]	↑V-ATPase [79]	**	↑CAIX [170] ↑CAXII [185]	**
Liver	↑MCT1 [29] ↑MCT2 [29] ↑MCT4 [29]	↑V-ATPase [78]	↑NHE1 [132]	↑CAIX [247]	↑AE2 [225]
Pancreas	↑MCT1 [24] ↑MCT2 [24] ↑MCT4 [24]	↑V-ATPase [75]	+NHE1 [140]	↑CAIX [186] +CAXII [186]	**
Stomach	+MCT1 [45] ↑MCT4 [45]	↑V-ATPase [82]	**	↑CAIX [248] +CAXII [248]	↑AE1 [221] ↓AE2 [226]
Esophageal	**	↑V-ATPase [76]	↑NHE1 [133]	**	**
Colorectal	↑MCT1 [25] ↑MCT2 [25] ↑MCT4 [25]	↑H ⁺ /K ⁺ -ATPase [66] +ATP Synthase [249]	**	↑CAIX [174] +CAXII [182]	**
Bladder	↑MCT1 [43] ↑MCT4 [43]	**	**	↑CAIX [172]	**
Prostate	↓MCT1 [30] ↑MCT2 [34] ↑MCT4 [30]	↑H ⁺ /K ⁺ -ATPase [65]	**	↑CAIX [174]	**
Kidney	↑MCT1 [20] ↑MCT4 [20]	**	**	↑CAIX [166] ↑CAXII	**
Ovarian	↑MCT1 [26] ↑MCT4 [26]	↑V-ATPase [81]	**	↑CAIX [173] +CAXII [173]	**
Cervix	↑MCT1 [28] ↑MCT4 [28]	+V-ATPase [83]	↑NHE1 [131]	↑CAIX [174] +CAXII [183]	**
Skin	↑MCT1 [23] ↑MCT4 [23]	**	**	**	**
Sarcoma	+MCT1 [250] +MCT2 [250] +MCT4 [250]	+V-ATPase [251]	**	**	**
Adrenal	+MCT1 [22] +MCT2 [22] ↑MCT4 [22]	**	**	+CAIX [22]	**
Hematological	↑MCT1 [252]	**	**	**	**

** No studies reported; ↑/↓ upregulation/downregulation (compared to non-tumoural tissues); + expressed.

Hereupon, CAIX represents an ideal target for anti-tumour therapy and imaging of hypoxic solid tumours.

6. Anion exchangers

SLC4 family of genes, also known as the bicarbonate-transporter family, comprises 10 members, including Na⁺-coupled bicarbonate transporters (NCBT) and Na⁺-independent anion exchangers (AEs). NCBT can be either electrogenic or electroneutral, and include the electroneutral Na⁺-driven Cl⁻-bicarbonate exchanger (Cl⁻/HCO₃⁻). Slc4 proteins move HCO₃⁻ either into or out of cells and, playing an important role the regulation of intracellular pH, being also involved in other physiological functions, including carriage of CO₂ in erythrocytes and the trans-epithelial movement of electrolytes [214].

The AE family comprises 3 members of structurally and functionally related genes, AE1, AE2 and AE3, which mediate the electroneutral exchange of two monovalent anions through the plasma membrane, of which Cl⁻ and HCO₃⁻ are the most common substrates. AE1 was the first member of the SLC4 family to be identified and cloned, which was initially called band 3. This designation corresponds to the third major band identified by SDS-PAGE from the membrane proteins of erythrocytes [215]. The N-terminal domain of human AE1 functions as a binding site for different pro-

teins, including proteins from the cytoskeleton, glycolytic enzymes and hemoglobin. The anion-exchange function of the protein is located at the transmembrane domain of AE1, whereas the cytoplasmic C-terminal portion contains an anchorage site for different proteins, including GAPDH, cytoplasmic CAlI and the tumour suppressor p16 [216,217]. Further, the extracellular loop 4 of AE1 binds CAIV [218].

6.1. Expression and prognostic value of AEs

In humans, AE1 is commonly expressed at the erythrocyte cell membrane, where it was first identified, as well as in kidney. Human AE2 is expressed in the gastric parietal cells and throughout the gastro-intestinal tract, with the highest expression in the colon [219], while AE3 is predominantly expressed in brain neurons and heart. However, expression of AE members and function in cancer, as well as the effect of the inhibition of their activity, is still poorly characterized.

AE1 protein was found expressed in gastric and colon cancer models, likely taking part in the carcinogenic process, by two different mechanisms. One of them involves the interaction of AE1 with the tumour suppressor p16, which promotes cytoplasmic accumulation of p16, deregulating cell cycle; and the other mechanism is related to the role of AE1 in the alkalization of gastric can-

Table 2
Studies targeting the different pH regulators in human cancer cells.

pH regulator	Inhibitors	<i>In vitro</i> studies	Results	<i>In vivo</i> studies	Results
MCTs	Aromatic compound (eg. CHC, DIDS)	Glioma [21] Colorectal cancer [253] Cervical cancer [37] Breast cancer [38]	x lactate transport x cell proliferation x cell migration x cell invasion ✓ cell death ✓ chemosensitization	Glioma [21] Cervical cancer [37] Colorectal cancer [37]	x tumour growth ✓ radiosensitization ✓ tumour necrosis x tumour invasion
	ARC155858	Lung cancer [17] Lymphomas [254]	x lactate transport x cell proliferation	**	**
	AZD3965	Lung Cancer [42]	x lactate transport	Lung Cancer [42]	x lactate transport x tumour growth
CAs	Sulfonamides (eg. compound 6) reviewed in [203]	Leukemia Melanoma Lung cancer Ovarian cancer Colorectal cancer Renal carcinoma Prostate cancer Breast cancer Brain tumours Breast cancer [204]	x cell proliferation x cell invasion x cell migration ✓ chemosensitivity	Breast cancer Colorectal cancer	x tumour growth x metastasis x cancer stem cell population x cell invasion
	Coumarins (eg. 7-Glycosyl)		x cancer stem cell expansion x cell viability x extracellular acidification	Breast cancer [205]	x tumour growth x metastasis x cancer stem cell population
	Dual drug (eg. Compound 13) Monoclonal antibody (6A10)	Colorectal cancer [255] Cervical cancer [255] Lung cancer [196] Gastric cancer [196] Breast cancer [196] Colon cancer [196]	x extracellular acidification x cell proliferation x tumour spheroids growth	Breast cancer [206] Colorectal cancer [256] Lung Cancer [196]	x tumour growth ✓ radiosensitization x tumour growth
	Concanamycin A/Bafilomycin	Pancreatic cancer [75] Hepatocellular adenocarcinoma [78] Ovarian cancer [81] Glioblastoma [80] Breast cancer [84]	x cell proliferation x cell migration x cell invasion ✓ cell death	Hepatocellular adenocarcinoma [78]	x tumour growth
V-ATPase/ H ⁺ /K ⁺ - ATPase	Omeprazole	Ovarian [111] Leukemia [108]	✓ chemosensitivity x cell proliferation ✓ cell death	Ovarian [111] Leukemia [108]	x tumour growth ✓ chemosensitization
	Esomeprazole	Esophageal cancer [110] Gastric cancer [104] Melanoma [105] Breast cancer [106] Sarcoma [251]	✓ chemosensitivity x cell proliferation ✓ cell death x cell migration x cell invasion ✓ cell death x proliferation ✓ chemosensitivity	Melanoma [105]	x tumour growth x pH gradient
	Lansoprazole	Melanoma [257]	✓ cell death x proliferation ✓ chemosensitivity	Melanoma [257]	x tumour growth ✓ chemosensitization
NHEs	Amiloride	Gliomas [146] Breast cancer [144] Esophageal cancer [133] Hepatocellular adenocarcinoma [132] Pancreas [258] Leukemia [149] Hepatocellular adenocarcinoma [150] Breast cancer [151]	x cell migration x cell invasion x cell proliferation ✓ chemosensitivity ✓ cell death	Esophageal cancer [133]	x tumour growth
	HMA		✓ cell death x pH gradient	**	**
	DMA		✓ cell death ✓ chemosensitivity	**	**
	EIPA	Hepatocellular adenocarcinoma [147] Colorectal cancer [145] Gastric cancer [259]	x cell proliferation ✓ cell death x cell motility x cell invasion	**	**
	Cariporide	Tongue squamous cancer [154] Cholangiosarcoma [153] Melanoma [139] Pancreatic cancer [140] Gastric cancer [155]	x cell proliferation x cell migration x cell invasion	**	**
	Phx	Colorectal cancer [260] Leukemia; Breast cancer; Glioma; Pancreatic cancer; Lung adenocarcinoma [156]	x cell proliferation ✓ cell death x cell motility	**	**

Table 2 (Continued)

pH regulator	Inhibitors	<i>In vitro</i> studies	Results	<i>In vivo</i> studies	Results
AEs	DIDS	Hepatocellular adenocarcinoma [234]	✓ cell death	**	**
ATP synthase	Resveratrol	Colon cancer [115] Pancreatic cancer [116] Gastric cancer [117] Prostate cancer [118] Leukemia [119]	x cell proliferation x cell proliferation ✓ cell death	**	**
	Aurovertin B	Breast cancer [113]	x cell proliferation	**	**
	Angiostatin	Lung adenocarcinoma [55]	x cell proliferation	**	**

** No studies reported; ✓ induction; x inhibition.

Table 3

Details of clinical studies targeting pH regulators in different types of cancer.

Target	Compound	Type of cancer	Trial phase	Results	Status	Observations	Clinical trial identifier
MCT1	AZD3965	Prostate, gastric cancer and lymphomas	Phase I/II	–	Recruiting		NCT01791595
ATPases	Esomeprazole	Breast cancer	Phase II	Treatment safe, well tolerated; increase efficacy of chemotherapy	Completed	Combination with chemotherapeutic drugs	NCT01069081
	Pantoprazole	Prostate cancer	Phase II	–	Not recruiting. Estimated study completion: December 2016	Combination with chemotherapeutic drugs	NCT01748500
	Omeprazole	Head and neck cancer	Phase II	–	Primary outcome measure: December 2016	Combination with chemotherapeutic drugs	NCT02013453
CAIX	SLC-0111	Advanced and metastatic solid tumours	Phase Ia	Not posted yet	Completed		NCT02215850
	DTP-348	Solid tumours, mainly head and neck neoplasms	Phase I		Planning the recruitment		NCT02216669
	G250	Metastatic renal cell carcinomas	Phase I/II	Treatment safe, well tolerated; disease stabilization	Completed	Combination with combination with interferon-alpha-2a	NCT00087022
	G250	Advanced kidney cancer	Phase III	Treatment safe, well tolerated; no clinical benefit	Completed	Adjuvant monotherapy	NCT02883153

cer cells [217,220]. In human samples, AE1 was described to be overexpressed in gastric carcinomas, being negative in normal gastric tissue. Additionally, AE1 positivity was associated with bigger tumour size, deeper invasion, shorter survival and non-lymph node metastasis [221]. Further, AE1 expression was described in transformed lymphocytes, while normal lymphocytes express AE2 and not AE1 [222]. AE1 expression was also associated with human erythroleukemia cells and transformed erythropoietic cells, not occurring in normal erythropoiesis [223].

AE2 expression was described in colon cancer cells, where it was associated with higher expression of Ki67, a marker of cell proliferation [224]. In the same study, the authors showed that AE2 expression in colon cancer patient samples was associated with poor prognosis. AE2 was also found overexpressed in human hepatocellular carcinoma, compared to adjacent non-tumour tissues [225]. On the other hand, AE2 was found down-regulated in gastric body samples, with low intensity and a diffuse profile in the gastric antrum [226]. A more recent study confirms AE2 downregulation in gastric cancer tissue, and also described that AE2 decreased levels were associated with poor cell differentiation and prognosis [227]. The same study revealed that p16 binds both AE1 and AE2, and AE1 or p16 silencing led to increased AE2 plasma membrane expression, where it regulates intracellular pH and plays a role in gastric cancer suppression.

6.2. Exploitation of AEs as therapeutic targets

The aromatic monocarboxylate CHC and the stilbene disulfonates DIDS and DBDS are known to inhibit the activity of AE1 [228]. However, these compounds are not specific for AE1, since they also inhibit the function of other proteins such as monocarboxylate transporters (MCTs) [13,229]. The flavonoids quercetin and phloretin, as well as some carbonic anhydrase inhibitors were also reported to inhibit AE1 activity [230,231]. Further, oxonol dyes and polyaminosterol analogs of squalamine (shark antibiotic) were reported to inhibit differentially AE1 and AE2 activity, showing important differences in the respective IC₅₀s [232].

Downregulation of AE1 in gastric mucosa by specific siRNAs inhibited the growth of gastric cancer in experimental mice [233]. Additionally, treatment with DIDS inhibited proliferation of highly malignant hepatocellular carcinoma cells, inducing apoptosis [234]. In another study, transfection of miR-24 into gastric cancer cells reduced AE1 expression, resulting in AE1-sequestered p16 to the nucleus, with consequent inhibition of cell proliferation [235].

Targeting of AE2 expression by the antisense oligonucleotide-AE2 decreased cell viability, induced cell cycle arrest, and induced apoptosis in poorly differentiated hepatocellular carcinoma cells [236].

Fig. 1. Role of pH regulators and targeting in cancer. Broadly, the activity of pH regulators leads to acidification of the tumour microenvironment promoting cell migration, invasion and resistance to therapy (left); on the other hand, inhibition of pH regulator activity prevents extracellular acidification, decreases cell migration, invasion and improves response to therapy (right). The inhibitors used in preclinical (red) and clinical (green) studies to target pH regulators in cancer are shown on the right. (Lac-lactate; Gluc-glucose; Pyr-pyruvate; PPIs-proton pump inhibitors).

6.3. AE inhibitors in clinical trials

To the best of our knowledge, there are no clinical studies exploring inhibition of AEs in cancer treatment. The lack of specificity of AE inhibitors might have constituted a limiting factor in these studies.

7. Discussion and conclusions

Due to the Warburg effect, tumours present a constitutive and persistent upregulation of glycolysis, which results in chronic acidification. As a way to adapt and survive in adverse conditions, which include local hypoxia and poor vasculature perfusion, cancer cells upregulate membrane pH regulators, as a self-defensive strategy. The increased expression of these proteins at the plasma membrane keeps an intracellular pH ranging from neutral to slightly alkaline, due to an increased secretion of protons to the tumour environment, leading to its acidification [237].

The acidic tumour microenvironment contributes to cancer cell proliferation, also increasing several malignant features like migration, invasion and metastasis [102,238]. The multiple families of plasma membrane pH regulators present in cancer cells are co-expressed and redundant, being the role of such functional redundancy still unclear, although making probably part of a self-protective mechanism to avoid an apoptotic cascade triggered by intracellular acidification [239,240]. These families include CAs, namely CAIX and CAXII, the proton exporter NHE1, the ATPases V-ATPase, H⁺/K⁺-ATPase and ATP synthase, the AE isoforms AE1 and A2, and MCTs, in particular MCT1 and MCT4, which are addressed in this review. The role of these membrane proteins in pH regulation, the prognosis value of their expression and their potential use as targets in cancer therapy, including clinical studies, were here reported and discussed.

Owing to the role of tumour microenvironment acidosis in the aggressive characteristics of cancer cells, the expression of pH regulators in clinical samples of several cancer types has been largely investigated during the past years. On the other hand, the capacity of cancer cells to maintain a self-protective high or at least normal pH, which is crucial to avoid apoptosis, being also involved in proliferation, invasion capacity, metastasis and drug resistance [241].

The variety of studies on the expression of these pH regulators, demonstrated that the expression profile is quite different in normal and tumour samples, where, with few exceptions, a higher expression of these proteins has been found (see Table 1). From the addressed pH regulators, this difference is even more relevant for CAIX, as it is highly expressed in cancer but scarce in normal tissues, being present only in the gastrointestinal tract [175]. Further, with the exception of MCT2, where its upregulation was associated to a more favourable prognosis, in general, the increased expression of pH regulators favours poor prognostic factors such as advanced stage and high grade tumours, increased drug resistance, invasion, metastasis, shorter overall and disease free survival rates, in different cancer types.

Such knowledge can be important in clinical research, through the putative use of these pH regulators as cancer biomarkers. Their identification as predictive biomarkers can be helpful in early diagnosis and also to identify patients that potentially take advantage of being treated with inhibitors that target such proteins. There is already evidence supporting the use of the different pH regulators as prognosis and/or predictive biomarkers in several cancer types, what can lead to a more personalized and effective medicine. Namely, CAIX is considered one of the best biomarkers associated to renal cell carcinoma, where its use as prognostic biomarker is gaining attention [242,243]. CAIX is regulated by HIF-1 α and its expression in tumours is associated with hypoxic regions (a common hallmark of solid tumours), being thus a promising biomarker for cancer cells adapted to a hypoxic environment, which normally present a more aggressive phenotype and are associated to a poor outcome [244,245]. However, some studies present controversial results, being the association between CAIX expression and the hypoxic status of the cell, dependent of the cancer type [175,246]. Nevertheless, determination of CAIX expression can be useful, by identifying candidates that can benefit from a CAIX directed treatment. Regarding the other pH regulators described in this review, they are not also in clinical use and no clinical trials for their use as prognosis/predictive biomarkers have yet been done, but the results obtained so far in *in vitro* and in *in vivo* models, as well as with clinical samples, are encouraging in this direction.

The expression levels of these proteins can have prognostic value, but can also be used for the development of innovative therapies targeting these pH regulators, which can contribute to improve cancer patient survival. Small molecules targeting and inhibiting each of these pH regulators have been developed and assayed in *in vitro* and in *in vivo* studies and/or in different phases of clinical assays (see Table 2 and 3). Most of them were effective in inhibiting proton transport and tumour environmental acidification, reducing cell proliferation, invasion and migration and metastasis in some cases, and increasing apoptosis and cancer cell death, besides rendering tumour cells more sensitive to conventional anti-tumour drugs. Additionally, some of them (PPIs, NHE1 inhibitors like cariporide) only become active in acidic conditions, what turns to be an advantage as they can selectively target cancer cells in their distinctive acidic environment. Nevertheless, due to the redundancy of the expression of the pH regulators, inhibition of one isoform of these proteins, can be compensated by an increased expression/activity of other(s), leading to resistance to therapy. As so, higher concentrations have to be used, likely leading to toxic side-effects. Considering this, and also the effect that pH regulators have in chemosensitization, it is more likely that their future use will involve combination with other conventional anti-tumour drugs, rather than being used as single agents. Also, a combined use of inhibitors in therapeutic non-toxic doses, targeting different pH regulators, can be necessary to overcome the redundancy of pH regulators. Several pH regulator inhibitors have already been evaluated in different phases of clinical trials. Some of these clinical trials have already finished, whereas others are still ongoing, but

no final results on the safety and effectiveness of the drugs are yet available. The ones that have already finished, point to the benefits of their use in a combined therapy approach.

In summary, a better appreciative analysis of the influence of the acidic microenvironment in cancer is needed for the discovery of the desired specific magic “poison” for cancer cells. There is increasing amount of data and a high number of both preclinical and clinical studies on this issue are ongoing. The goal of cancer treatment is to specifically target cancer cells, and exploiting the altered cell metabolism and microenvironment, recognized as hallmarks of cancer, can be the answer. Disrupting the activity of pH regulatory proteins can be an effective mean to achieve a metabolic catastrophe, causing a decrease in cancer aggressiveness (Fig. 1). However, these regulators are redundantly expressed, and inhibition of one of them can be compensated by other(s). Thus, considering the potential killing power that altered proton dynamics has in cancer, but also the limitations of its use as therapeutic target, we believe that a combined inhibition of proton-exporting systems would lead to more effective anticancer therapies.

Conflicts of interest

The authors declared no conflicts of interest.

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