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## Review

Proton channels and exchangers in cancer<sup>☆,☆☆</sup>

Enrico Pierluigi Spugnini<sup>a</sup>, Pierre Sonveaux<sup>b</sup>, Christian Stock<sup>c</sup>, Mario Perez-Sayans<sup>d</sup>, Angelo De Milito<sup>e</sup>, Sofia Avnet<sup>f</sup>, Abel García García<sup>d</sup>, Salvador Harguindey<sup>g</sup>, Stefano Fais<sup>a,\*</sup>

<sup>a</sup> Anti-Cancer Drug Section, Department of Drug Research and Medicine Evaluation, Istituto Superiore di Sanità (National Institute of Health), Rome, Italy<sup>b</sup> Institut de Recherche Expérimentale et Clinique (IREC), Pole of Pharmacology, Université Catholique de Louvain (UCL), Brussels, Belgium<sup>c</sup> Department of Gastroenterology, Hannover Medical School, Hannover, Germany<sup>d</sup> Oral Medicine, Oral Surgery and Implantology Unit, Faculty of Medicine and Dentistry, Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela, Spain<sup>e</sup> Cancer Center Karolinska, Department of Oncology-Pathology, Karolinska Institute, Stockholm, Sweden<sup>f</sup> Laboratory for Orthopaedic Pathophysiology and Regenerative Medicine, Istituto Ortopedico Rizzoli, Bologna, Italy<sup>g</sup> Instituto de Biología Clínica y Metabolismo (IBCM), (Postas) Vitoria, Spain

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## ABSTRACT

Although cancer is characterized by an intratumoral genetic heterogeneity, a totally deranged pH control is a common feature of most cancer histotypes. Major determinants of aberrant pH gradient in cancer are proton exchangers and transporters, including V-ATPase, Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE), monocarboxylate transporters (MCTs) and carbonic anhydrases (CAs). Thanks to the activity of these proton transporters and exchangers, cancer becomes isolated and/or protected not only from the body reaction against the growing tumor, but also from the vast majority of drugs that when protonated into the acidic tumor microenvironment do not enter into cancer cells. Proton transporters and exchangers represent a key feature tumor cells use to survive in the very hostile microenvironmental conditions that they create and maintain. Detoxifying mechanisms may thus represent both a key survival option and a selection outcome for cells that behave as unicellular microorganisms rather than belonging to an organ, compartment or body. It is, in fact, typical of malignant tumors that, after a clinically measurable yet transient initial response to a therapy, resistant tumor clones emerge and proliferate, thus bursting a more malignant behavior and rapid tumor progression. This review critically presents the background of a novel and efficient approach that aims to fight cancer through blocking or inhibiting well characterized proton exchangers and transporters active in human cancer cells. This article is part of a Special Issue entitled: Membrane channels and transporters in cancers.

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\* Corresponding author at: Department of Therapeutic Research and Medicines Evaluation, National Institute of Health, Viale Regina Elena, 299, 00161 Rome, Italy. Tel.: +39 06 49903195; fax: +39 06 49902436.

E-mail address: [stefano.fais@iss.it](mailto:stefano.fais@iss.it) (S. Fais).<http://dx.doi.org/10.1016/j.bbamem.2014.10.015>

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Most often, tumor cells upregulate glycolysis and grow in a hypoxic microenvironment. Highly proliferative cancer cells produce a large amount of  $H^+$  generated by glycolysis and glucose utilization, amino acid metabolism, and ATP hydrolysis, all associated to proton efflux and extracellular acidification [1]. One interesting hypothesis is that the hostile microenvironment generated during tumor growth progressively selects cells suited to survive in these adverse conditions. Uncontrolled growth, lactic and carbonic acid production from tumor metabolism and low blood and nutrient supply all contribute to a tumor microenvironment with many molecules that are extremely toxic for either normal or more differentiated cells. It is therefore possible that the cells that survive in this unfavorable microenvironment possess the means for avoiding intracellular accumulation of toxic molecules, including the expression and activation of several proton extruders [2]. Among proton flux regulators are V-ATPase,  $Na^+/H^+$  exchanger (NHE), monocarboxylate transporters (MCTs) and carbonic anhydrase 9 (CAIX, Table 1). Their activity creates a disturbance of pH gradients typical of malignant cells, characterized by a reversed pH gradient between acidic extracellular and alkaline intracellular compartments. Acidity quickly kills normal or more differentiated cells, but not tumor cells that are well equipped with proton extruders thus promoting a transition towards more aggressive histotypes. Indeed, proton pumps and transporters efficiently export  $H^+$  out of the cell or sequester  $H^+$  inside internal vacuoles, thus avoiding potentially highly cytotoxic acidification of the tumor cell cytosol. In many tumors, a chronic exposure to acidic pH has been reported to promote invasiveness, metastatic behavior and resistance to cytotoxic agents [3–7]. Moreover, some evidence suggests that an abnormal pH may be involved in important tumor-associated cellular functions, such as acidic vesicular trafficking, drug resistance, lytic enzyme activation and aberrant phagocytic activity [1,2]. Detoxifying mechanisms may thus represent both a key survival option and a feature that progressively selects individual cells armed to survive to molecular insults. This makes malignant cells very similar to microorganisms [8]. In fact, proton pumps, as cellular mechanisms that counteract the accumulation of toxic agents, are very active in many microbial species [9]. Here, we

propose that an efficient anticancer approach could be to block or inhibit mechanisms involved in cell detoxification in order to deprive cancer cells of a key survival option, hence triggering tumor cell death.

## 1. Contribution of proton channels and exchangers to cancer progression and chemoresistance

### 1.1. V-ATPases

The peculiar anaerobic or aerobic metabolism of glucose by cancer cells leads to the accumulation of acid byproducts resulting in an acid milieu that strongly affects tumor cells and their host [10–12]. Low extracellular/intratumoral pH is a major cause of tumor unresponsiveness to the vast majority of cytotoxic drugs, mostly because the  $H^+$ -rich tumor microenvironment leads to protonation of the chemotherapeutic agent causing both its neutralization outside the cells and prevention of reaching its intracellular targets [10–12]. The prime cause of tumor microenvironment acidification is secondary to the byproducts of tumor metabolism, namely protons, coupled with reduced perfusion. However, this condition progressively selects cells adapted to survive in the acidic extracellular tumor microenvironment, which is due to overexpression and activation of membrane-bound pH-regulating systems that contribute to prevent intracellular acidification. Among them, vacuolar-type  $H^+$  ATPases seem to be involved in the acidification of tumor microenvironment [10–12]. Vacuolar  $H^+$  ATPase (V-ATPase) is a complex multisubunit protein devoted to the transport of protons from the cytoplasm towards intracellular compartments and from inside to outside of the cell through the cytoplasmic membrane [11–13]. V-ATPases are made of a transmembrane subunit, named  $V_0$  complex, devoted to proton transfer and a cytoplasmic portion, named  $V_1$  complex, that provides the necessary energy for proton translocation [13]. Because of its role in the regulation of cellular pH homeostasis, V-ATPase is involved in multiple cellular functions including endocytosis and activation of proteases [10,13], angiogenesis [14], autophagy [15] and amino acids sensing via interaction with mTOR [16]. Tumor cells located at the margin of neoplastic masses are often away from newly formed blood vessels, receiving and inadequate supply of oxygen and nutrients. Such cells survive and adapt to a highly selective environment characterized by hypoxic and acidic conditions caused by increased glycolysis and reduced tissue perfusion [17–19]. Augmented expression of V-ATPase is considered to be a well-designed compensatory mechanism that in fact confers survival and growth advantages to cancer cells [17–21]. Among its activities, V-ATPase contributes to lower extracellular pH ( $pH_e$ ) thus activating extracellular metalloproteinases that promote tumor cell survival, motility and invasion, resulting in enhanced malignancy ability. There is a bulk of evidence that points out the role of V-ATPase in tumor invasion and multidrug resistance in breast cancer [22–25], oral squamous cell carcinoma [26–28], esophageal carcinoma [29], hepatocellular and pancreatic carcinoma [30,31], lung carcinoma [32], sarcoma [33,34] and solid tumors in general [35].

Consequently, inhibition of V-ATPase has become a fascinating and promising strategy to counteract proton metabolism in cancer, which has been investigated *in vitro* and *in vivo*, in both preclinical and clinical settings. This section will summarize the results obtained so far in the different areas of investigation.

**Table 1**  
Proton exchangers and their role in cancer.

Type of pump	Cellular localization	Function
$H^+$ -ATPase	Plasma membrane and acidic organelles	Acidification of extracellular microenvironment and endolysosomal compartment
$Na^+/H^+$ -exchangers (NHE)	Plasma membrane	Alkalinization of cytosol and acidification of extracellular microenvironment
MCT1 ( $H^+$ /lactate symporter)	Plasma membrane	Elimination of lactate from glucose catabolism, and acidification of extracellular milieu
Carbonic anhydrase 9	Plasma membrane	Regulation of intracellular pH and pH gradients
$H^+/K^+$ -ATPase	Gastric epithelial cell line	Regulation of extracellular pH

## 1.2. Anti-V-ATPase compounds

The number of V-ATPase inhibitors is still rather small but they are currently being extensively studied to uncover their binding properties and their mode of inhibition [36,37]. Most of the known V-ATPase inhibitors are natural compounds of microbial origin such as bafilomycin, the first specific inhibitor isolated from *Streptomyces griseus* in the 1980s, or Concanamycin A isolated from *Streptomyces neyagawaensis* [36,38]. Archazolid, like bafilomycin A1 or concanamycin, binds to the V0 subunit c of V-ATPase and has also been recently investigated as an anticancer agent in vitro and in vivo [39]. Unfortunately, the use of these anti-V-ATPase compounds is limited to preclinical models since V-ATPase is a housekeeping complex widely expressed and active in all types of cells, and because their potential human utilization is bound to be extremely toxic [36]. In this context, omeprazole and other related drugs have sparked great interest among the different V-ATPase inhibitors. These gastric  $H^+/K^+$ -ATPase inhibitors can inhibit V-ATPase by binding to subunit A of the nucleotide binding domain [40]. An attractive feature of these compounds is that they require acidic conditions to be converted into the active form, therefore providing the possibility of tumor selectivity (Fig. 1).

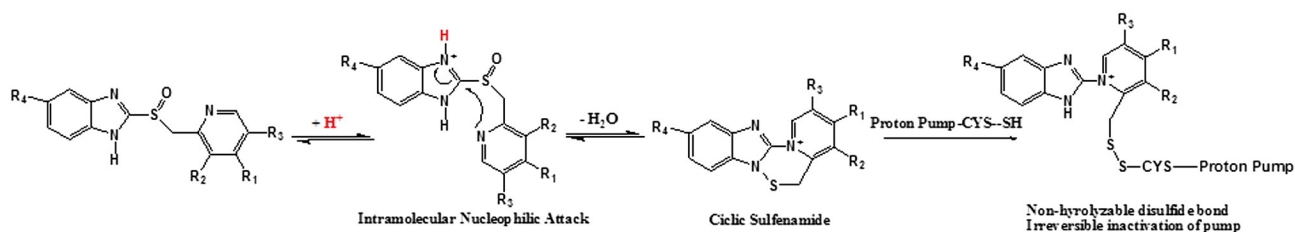
## 1.3. V-ATPase and acidic tumor pH in autophagy

Autophagy is a homeostatic self-digestive mechanism adopted by all cells under physiological conditions to maintain the integrity of organelles and to remove protein aggregates [41]. Cells under stress conditions use autophagy as a defense mechanism that recycles cytosolic material in order to counteract lack of nutrients or other metabolic and therapeutic insults. Lysosomes play a fundamental role in the autophagic process since the content of the autophagosome (autophagic cargo) is degraded after fusion with acidic lysosomes, thus providing optimal pH conditions for degradation activity [42]. Indeed, inhibition of V-ATPase by Bafilomycin A1 is a standard mechanism to block autophagy in the terminal stages [43]. Moreover, some human pathologies characterized by mutations in V-ATPase subunits are associated with defective autophagy [44,45]. As it has been shown that in many models aggressive metastatic cells are characterized by higher levels and/or increased activity of V-ATPase [46,47], it is likely that increased V-ATPase activity also contributes to the upregulated autophagy characteristic of metastatic cancers. Moreover, it was reported that treatment with esomeprazole in melanoma cells inhibits mTOR signaling, stimulates the formation of autophagosomes, and simultaneously slows down the autophagic flux, likely via inhibition of lysosome acidification [48]. An alternative mechanism by which proton pump inhibitors (PPI) may modulate autophagy is by affecting regulatory functions of V-ATPase without inhibiting its proton pump activity [49]. In fact, V-ATPase is part of the Regulator complex that regulates mTOR [16]. Since mTOR is a positive regulator of lysosomal biogenesis, it is possible that PPI affects the interaction of V-ATPase with mTOR. Autophagy has been reported as an essential mechanism used by melanoma and breast carcinoma cells to adapt to chronic acidosis [50,51]. This suggests that inhibition of autophagy may kill the cancer cells that are chronically

exposed to acidosis. The only available drug tested as autophagy inhibitor in clinical trials in cancer patients is the antimalarial compound chloroquine (CQ) [52]. Unfortunately, tumor acidosis has been recently reported to completely abrogate CQ activity both in vitro and in vivo due to lack of drug entry into acidic tumor regions [15,53]. Given the complex role of V-ATPase in both lysosome acidification and membrane trafficking, further investigation is required to understand how inhibition of V-ATPase may modulate autophagy and tumor growth [15,48].

## 1.4. Studies in murine models

Following the results of in vitro studies, the efficacy and mechanisms of action of V-ATPase inhibitors have been further substantiated by in vivo studies involving murine models. In 2004, Luciani et al. evidenced that pretreatment with PPI omeprazole greatly increased the in vitro sensitivity of tumor cell lines to chemotherapeutic agents [54]. Moreover, pretreatment with omeprazole of SCID mice carrying orthotopic melanoma xenografts resulted in an increased response to cisplatin with decreased tumor burden [54]. Another group of investigators evidenced that RNA interference with subunit ATP6L of V-ATPase resulted in growth inhibition and metastatic delay in murine xenografts of hepatocellular carcinoma [55]. The antitumor properties of omeprazole were further substantiated by another investigation that evaluated this feature both in vitro and in vivo [56]. In this study, SCID mice carrying B-cell tumor xenografts were treated with high dose oral omeprazole or placebo, and the treatment group evidenced significant tumor delay. This publication pointed out that PPI not only have chemosensitizing properties but also a direct anticancer activity, opening a whole new field for clinical applications [56]. The same year, Niikura showed that oral administration of a V-ATPase inhibitor to SCID mice carrying orthotopic breast cancer xenografts resulted in decreased tumor burden as well as in decreased bone metastasis [57]. Similarly, another group of investigators reported inhibition of local growth and distal metastases in a murine model of melanoma where mice were treated orally with a V-ATPase inhibitor [58]. In this study, in order to assess tumor growth inhibition, 7 weeks-old C57BL/6 mice were injected in the foot pad with tumorigenic melanoma cells and assigned to two groups: placebo or treatment with the inhibitor of the V-ATPase  $\alpha 3$  by oral gavage for 10 days. Similarly, to assess the effects on lung and bone metastases, two other groups were injected in the tail vein (lung metastasis model) or intracardially (bone metastasis model). In all three groups, treatment with the V-ATPase  $\alpha 3$  inhibitor resulted in decreased tumor burden. More recently, studies in rodents evidenced the involvement of V-ATPases in tumor-induced immunosuppression and in tumor resistance to biological therapy with targeted antibodies [23,59]. The study reported by Calcinotto and colleagues [59] is of particular interest since it unraveled a state of anergy of tumor-infiltrating lymphocytes induced by tumor acidosis, showing the possibility to reverse this situation through manipulation of the tumor acidic microenvironment. Thus, the possibility to modulate tumor acidity could potentially increase the tumor response to conventional and engineered drugs, as well as improve the recruitment of the immune system of the host for a better tumor control.



**Fig. 1.** Chemical representation of the proton pump inhibitor activation by protonation (due to the  $H^+$ -rich tumor microenvironment), its transformation into the active molecule, cyclic sulfenamide, and its binding to the proton pump.



### 1.5. Pilot studies

Comparative pathology by using spontaneous diseases in animals is a validated approach to find out novel therapies for human tumors [60], since their tumors have a behavior similar to those of humans and their shorter lifespan allows for a rapid generation of data [61,62]. Two studies have been conducted so far in companion animals with spontaneous neoplasms. The first one evaluated the capacity of high dose lansoprazole to reverse chemoresistance in dogs and cats with known refractory cancers or whose tumors no longer responded to conventional chemotherapy [63]. Lansoprazole is a PPI that is administered as a pro-drug and has a very high tropism towards acid environment where it is activated. It is used at low dose (1 mg/kg) for the treatment of gastric hyperacidity or at higher dose (2 mg/kg) for the therapy of gastrinoma [63]. In this study the drug has been used off-label with a three days loading dose followed by a maintenance to prevent a rebound of acidity due to drug withdrawal, following the schedule of 5 mg/kg Monday through Wednesday and then 1 mg/kg Thursday through Sunday. This strategy resulted in reversal of chemoresistance in 23 out of 34 treated animal patients (67% response rate). In this study, the most striking responses belonged to lymphoma patients that greatly benefited from the new therapy (responses ranging from 3 to 12 months) and to two osteosarcoma patients that experienced a partial response with dramatic decrease of the tumor-induced pain and improvement of their Karnofski performance status. The therapy was well tolerated with side effects limited to transient gastrointestinal toxicity (occasional vomiting, diarrhea, flatulence) [63]. A confirmatory study has been recently presented, showing the potentiation of metronomic chemotherapy by patients' alkalization through the administration of pulse lansoprazole and the addition of a water alkalinizer to the pets' drinking water in order to bring water pH to a value approaching 9 [64]. In this study, the cohort receiving alkalization showed improved tumor response (both in terms of number and duration of response) when compared to the group receiving metronomic chemotherapy alone. In particular, a dog with hepatic carcinoma and another one with inflammatory mammary carcinoma experienced complete remission lasting more than a year. Again, toxicity was limited and the pets experienced durable responses with improved quality of life. Patient alkalization will probably become a standard procedure in veterinary oncology due to its low cost, increased tumor control and improved quality of life.

In humans, treatment with PPIs as anticancer agents has only been evaluated in one pilot study on osteosarcoma patients [65]. In this cancer, 40% of patients treated with a neoadjuvant protocol develop chemoresistance and relapse with metastatic disease. The neoadjuvant protocol includes chemotherapy with methotrexate, cisplatin, doxorubicin and ifosfamide administered before and after surgical removal of the tumor. In this setting, the evaluation of necrotic rates in tumor sections allows for early assessment of response rate, which is highly correlated to prognosis [66]. Osteosarcoma patients with different histotypes were pretreated with PPI Esomeprazole before neoadjuvant treatment. Through mapping of the resected specimens, it appeared that pretreatment with Esomeprazole improved the response to chemotherapy only in the chondroblastic subtype that showed a histological response rate of 61% versus 25% in the non-pretreated group. On the contrary, no differences were seen in the osteoblastic or in the telangiectatic and fibroblastic groups. This preliminary result might indicate a direct correlation between the effectiveness of the anti-acidic therapy based on the use of high dosage PPI and the presence of a hypoxic environment in osteosarcoma. In fact, chondrocytic differentiation is associated with low oxygen tension [67] and with a metabolism largely, if not entirely, glycolytic, with little capacity for oxidative phosphorylation [68]. Under this condition, targeting V-ATPase with PPI inhibitors in chondrocyte-like cells would mean a strong and a quick reduction in intracellular pH, ultimately leading to cell death.

### 2. NHE1

The mechanisms by which the  $\text{Na}^+/\text{H}^+$  exchanger (NHE1) is thought to contribute to malignant transformation and cancerogenesis are manifold [69–72]. While in healthy cells NHE1 is usually quiescent and is activated primarily upon cytosolic acidification [70], it is hyperactive in cancer cells even at resting intracellular pH ( $\text{pH}_i$ ), this eventually leading to cytosolic alkalization. Increase in cytosolic pH is directly correlated with pathological processes that induce malignant transformation [71–73], uncontrolled proliferation [74], augmented DNA synthesis and the stability of spontaneously occurring mutations [75]. It also induces the expression of oncogenes, enhances the activity of growth factors [75], correlates with multiple drug resistance [76,77] and promotes metastasis [78,79]. Accordingly, hyperactivity of NHE1 contributes to uncontrolled proliferation, motility and invasion of cancer cells [69,72,80], as best evidenced for breast cancer [81–83], melanoma [78,84,85] and non-small lung cancer [86]. Since an elevated NHE1 activity can be correlated with both an increase in cell pH and a decrease in the extracellular pH of tumors, and such proton reversal is associated with the origin, local growth, activation and further progression of the metastatic process, NHE1 pharmaceutical inhibition by new and potent NHE1 inhibitors (e.g. cariporide, 2-Aminophenoxazine-3-one (Phx-3), compound 9 T) represents a potential and highly selective target in anticancer therapy [69].

NHE1 activity also plays a role in chronic and acute myeloid leukemia. The differentiation of K562 chronic myeloid leukemia cells is induced by hypoxia, itself enhanced by NHE1 inhibition possibly due to an upregulation of transcription factor C/EBP (CCAAT/enhancer-binding protein) via the p38 MAPK signaling pathway [87,88]. In acute myeloid leukemia (AML), tescalcin is upregulated and activates NHE1. Consequently, untreated AML cells show a higher  $\text{pH}_i$  compared to healthy hematopoietic cells [89]. In this context, inhibition of NHE1 by 5-(N,N-hexamethylene) amiloride has been shown to suppress growth and to induce selective apoptosis in various AML cell lines [74]. Usually, AML cells develop resistance fairly quickly and efficiently, which has been attributed to a high genetic variability of the *fms*-like tyrosine kinase 3 (*FLT3*) gene [90]. NHE1 inhibition, however, sensitizes AML cells to the tyrosine kinase inhibitor sorafenib [89,91]. Furthermore, treatment of chronic myeloid leukemia with NHE1-modulating therapeutic approaches (including NHE1 up- and downstream signaling) should be taken into consideration since a direct inhibition of NHE1 seems to be an option in the treatment of acute myeloid leukemia as well as in different solid tumors.

Hormone-dependent NHE1 activity may play a role in prostate cancer. In prostate cancer lines DU145 and LNCaP, testosterone-albumin conjugates activate membrane androgen receptors. This leads to a transient increase in NHE1 activity and to an elevated  $\text{pH}_i$  via serum and glucocorticoid-inducible and Rho-associated protein kinases [92]. The fact that hormones can potentially modulate NHE1 activity via their receptors may lead to new approaches taking advantage of hormone receptors that are mainly – if not even specifically – expressed in tumor cells and the tissue of origin. By therapeutically targeting the hormone of interest, NHE1 activity could be curtailed. Interestingly, an indirect  $\alpha_1$ -adrenergic receptor-mediated NHE1-dependent increase in matrix metalloproteinase (MMP) 9 activity and in cell invasion has been shown in CCL39 Chinese hamster lung fibroblasts [93].

In MDA-MB-231 breast cancer cells, the transmembrane glycoprotein CD44 regulates metastatic potential by regulating NHE1 expression [94]: downregulation of CD44 reduces the presence and activity of NHE1 and thus the cellular motility. Conversely, inhibition of NHE1 not only nearly abolishes CD44-stimulated breast cancer motility but also correlates with a reduced expression of p-ERK1/2 and MMPs. Based on their findings, Chang et al. [94] concluded that in MDA-MB-231 cells CD44 regulates the expression of MMPs via NHE1 through the ERK1/2 signaling pathway, and that simultaneously targeting CD44 and NHE1 may be a therapeutic strategy for treating breast cancer.

However, a recent study comparing cellular pH regulation in multicellular epithelial organoids isolated from human primary breast carcinomas of European women revealed that, in these cells, the  $\text{Na}^+/\text{HCO}_3^-$  cotransporter (NBCn1) rather than NHE1 manages pH homeostasis [95]. A clear and unambiguous assignment of either an increased NHE1 or an increased NBCn1 expression/activity to the malignancy of breast cancer becomes even more delicate since NBCn1 expression is downregulated in 64% percent of clinical tumor samples isolated from human primary breast carcinomas of Asian descendants [96].

Serious challenges become evident when mice are treated with the specific NHE1 inhibitor cariporide in order to control B16V melanoma metastasis [78]. Indeed,  $\text{pH}_\text{e}$  values as low as 6.8 neutralize the inhibitory effect of cariporide on B16V cell migration. Besides this, B16V cells show opposed migratory and invasive behaviors when seeded either on basement membrane-like (more adhesion, less invasion) or dermis-like (less adhesion, more invasion) matrices [78]. These observations indicate that locally present pH values or matrix components can counteract the effects of NHE1 inhibition and therefore would even attract metastasizing tumor cells. NHE1 is an attractive and potential drug target as it is, in fact, upregulated and/or overexpressed in cancer cells, and for these reasons on its inhibitors, cariporide shows some promises. Therefore it is necessary to find a way to use cariporide at low concentrations and to deliver it selectively to the tumor tissue. Cariporide, for instance, being one of the better studied specific and powerful NHE1 inhibitors, could become a new, slightly toxic and effective anticancer agent in different human malignancies. For this reason, it has been recently proposed as a new, powerful and selective NHE1 inhibitor with an important therapeutic potential in different solid human malignancies [69,97–101].

Even though NHE1 inhibition can reduce adhesion, invasion and metastasis, or even increase the sensitivity to efficient other chemotherapeutic agents, its status as a potential target in cancer therapy remains questionable. The efficiency of NHE1 inhibition will also depend on the prevailing local  $\text{pH}_\text{e}$  and on extracellular matrix composition, both of which strongly modulate cell adhesion and MMP activity [78]. Nevertheless, NHE1, as one out of a number of pH-regulating membrane proteins could be targeted by a broader drug cocktail of proton transport inhibitors (PTI) simultaneously directed against several pH-regulators, ion transporters and channels contributing to tumor malignancy, as has been previously proposed [69,101–103]. Finally, targeting signaling molecules that control NHE1 expression/activity may also become a combined therapeutic option assumed that healthy tissues would be less affected.

### 3. Carbonic anhydrases (CAs)

CAs are transmembrane Zn metalloenzymes that catalyze the reversible hydration of carbon dioxide to carbonic acid and are involved in respiration and acid–base equilibrium [104]. There are 14 known members of this family, which are subdivided according to their location: membrane-related, cytosolic, mitochondrial and secreted [105]. CA-IX and CA-XII are the two major tumor-related CA isoforms [106,107].

There is evidence that interfering with proteins related to proton translocation leads to changes in both  $\text{pH}_\text{i}$  and  $\text{pH}_\text{e}$  in tumors, which impair of tumor growth [106]. However, the main challenges of such approach are related to the fact that many of the proteins are also found in normal cells involved in physiological roles [108–111] and their inhibition may cause serious/lethal side effects. There are at least two exceptions for CAs: CA-IX and XII are predominantly found in hypoxic tumors with restricted expression in normal tissues in which their active site is closed and, therefore, the enzymes are kept inactive [112]. Furthermore, the inhibition of these two CA isoforms with small molecules and/or antibodies has an anticancer effect [113].

#### 3.1. CAs in human solid tumors

Tumor microenvironment plays a key role in the viability and evolution of solid tumors. Hypoxia and tumor-cell proliferation determine response to surgery, chemotherapy and radiotherapy [114]. Hypoxia delays tumor cell proliferation maintaining cell superpopulations in a proliferating situation under hypoxic conditions, and it is responsible for treatment failure [115]. In addition, hypoxia-related genes, formed by HIF-1 [116,117], related to the von Hippel–Lindau gene (vHL) during oncogenesis [118] also controls several target genes that involve energy metabolism (glucose and glycolytic enzyme transporters), angiogenesis (VEGF) and CAs, mainly CA IX [108,119–121].

CA IX has been determined by immunohistochemical and western blot studies and it has been thoroughly described in different malignancies, including lung [122], cervical carcinoma [123], esophagus [124], bladder [125], breast [126], and colorectal cancers [127]. For example, in head and neck cancer (HNSCC), CA IX seems to be overexpressed [128–132]. Furthermore, in advanced stage tumors these expression levels are higher than in initial stage tumors. For this reason, early diagnosis of these patients is essential to improve survival expectations. Additionally, survival in patients with moderate or negative expression improves significantly in contrast with those patients with intense CA-IX expression [133]. The same occurs in precancerous lesions, where the use of CA IX as an immunohistochemical marker may be useful as screening of dysplasia-free samples due to its high specificity as a diagnostic test [134].

#### 3.2. CA-IX and its relationship to resistance in cancer treatment

Resistance to chemotherapy and radiotherapy is the main reason for treatment failure in patients with solid tumors [27,135].  $\text{pH}_\text{e}$  is considerably more acidic in a solid tumor than in normal tissue (“Cancer Proton Reversal”). This increased acidity interferes with the absorption of chemotherapy drugs, reducing their effect on tumors [136,137]. CAs and other proton exchangers have been reported to be largely responsible for this acidic environment [101,138,139].

A clear association has been established between multidrug resistance and Pgp (P-glycoprotein) expression in some tumors, but the mechanism by which drug resistance occurs in many other solid tumors has not yet been fully elucidated [69,140,141]. The alteration of the pH gradient between the extracellular environment and the cell cytoplasm has been suggested as a possible mechanism of resistance to cytotoxic drugs [142]. The increase in tumor interstitial acidity interferes with the absorption of basic chemotherapy drugs, reducing their effect on tumors [136,137]. This is the reason why pretreatment with PPIs and other pH regulator molecules (proton transport inhibitors or PTIs) can sensitize tumor cell lines to the effect of different chemotherapy drugs, suggesting that tumor extracellular/intratranslational alkalization may be an extremely interesting additional target in future anticancer treatments [142–145].

CA-IX expression takes place mainly in tumors with low vascularization and necrotic areas and is related to a poor overall response [146,147]. The lack of microvessels in well-differentiated areas related to hypoxia and positive for CA-IX limits the use of chemotherapeutic drugs and induces resistance to therapy, confirming the hypothesis that hypoxia promotes the creation of resistant cell subpopulations as a CA IX-mediated drug resistance [148,149]. However, its role in radioresistance remains to be further elucidated [150,151].

#### 3.3. Interfering CAs in tumor cells

Through a catalytic reaction CAs contribute significantly to the extracellular acidification of solid tumors (in addition to lactic acid), for hence their inhibition is bound to revert this phenomenon [152]. It has been known for some time that many classes of aromatic/heterocyclic sulfonamides and sulfamates show good affinity for CA I–VII and

CAXII–XV [105,153], but generally they do not possess specificity for the inhibition of the tumor-associated isoform. During the last years several approaches that specifically target the tumor-associated isoforms CA IX and XII were discovered (which are extracellular proteins, with their active site outside the cell), namely:

- (i) positively or negatively-charged compounds: they cannot cross plasma membranes and inhibit selectively only extracellular CAs, like CA IX and XII [108]
- (ii) fluorescent sulfonamides: used for imaging purposes [112,152]
- (iii) sugar-containing sulfonamides/sulfamates/sulfamides: due to their highly hydrophilic character do not easily cross cell membranes and thus possess an enhanced affinity for extracellular sites [154]
- (iv) nanoparticles coated with CAs [155]
- (v) novel chemotypes: different than the sulfonamide compounds, such as coumarins, thiocoumarins, polyamines, phenols, etc. [156]
- (vi) monoclonal antibodies (mAbs): represent another avenue for the selective targeting of CA IX and CA XII [157,158]
- (vii) M75 is a highly specific anti-CAIX mAb targeting the PG domain of CA IX, discovered by Pastorekova's group [113].
- (viii) Interestingly, CA IX has also successfully been used as an antigen for the generation of monoclonal antibodies which have been subsequently radiolabeled for radioimmunotherapy applications [159].

All these data demonstrate that tumor-associated CAs are indeed almost ideal targets for designing novel and innovative anticancer drugs which interfere with tumor microenvironmental acidification.

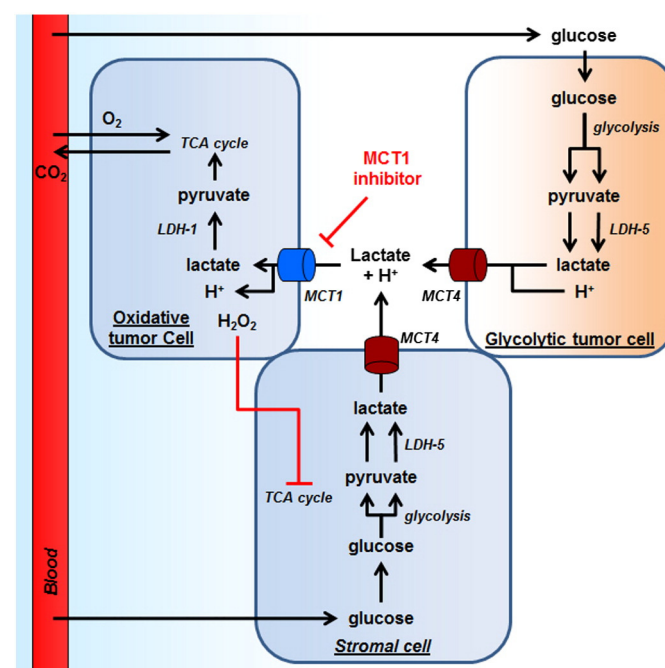
#### 4. Monocarboxylate transporters (MCTs)

Accelerated glycolysis is a main metabolic pathway for ATP generation and biosynthesis in hypoxic (anaerobic glycolysis) and proliferating (aerobic glycolysis) tumor cells characterized by low OXPHOS activities. It is associated to the conversion of lactate to pyruvate by lactate dehydrogenases (LDH, primarily LDH-5) followed by the export of lactate together with a proton, a process facilitated by monocarboxylate transporters (MCTs) expressed at the plasma membrane. The LDH-5 reaction (Fig. 2) has two main functions that contribute to maintain glycolysis at high rates. First, it ensures the oxidation of NADH to NAD<sup>+</sup>, which is thereby made available to fuel glyceraldehyde-3-phosphate dehydrogenase (GAPDH) upstream in the glycolytic cascade, ensuring energy production maintenance. Second, it buffers the cytosolic pH of glycolytic cells by incorporating the protons formed upstream in glycolysis into lactate anions, so that the production of 2 molecules of lactate from 1 molecule of glucose is not associated to a net production of protons by glycolysis [160]. A neutral to slightly alkaline intracellular pH supports glycolysis as the activity of phosphofructokinase-1 (PFK1) and LDH-5 itself increase with the pH [161,162]. Rather than directly released, acidity produced by glycolysis is incorporated into ATP and released during ATP hydrolysis, which occurs at a high rate in glycolytic tumor cells. Additional sources of acidity come from biosynthetic pathways (*ea.*, TCA cycle in the cataplerotic mode), 6-phosphogluconate decarboxylation in the oxidative arm of the pentose phosphate pathway,

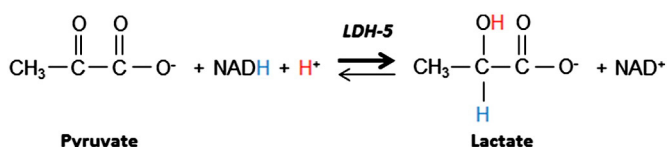
and amino acid metabolism producing NH<sub>4</sub><sup>+</sup> and decarboxylation reactions (*ea.*, malic enzyme reaction) [163,164].

According to mass action law, lactate removal would optimize the LDH-5 reaction. This activity depends on MCTs, a family of 14 members among which MCT1 to MCT4 are passive symporters that convey the transport of monocarboxylates, including lactate, together with a proton [165,166]. The transporters consist of 12 transmembrane domains with intracellular C- and N-terminals [167]. Their activity is primarily driven by the gradient of their substrate monocarboxylates across membranes, which differentiates them from other passive proton transporters (CAs and NHEs) that essentially depend on a steep gradient of protons [168]. With low affinity for lactate ( $K_m \approx 22$  mM) but a high turnover rate, the lactate transporter MCT4 is particularly well adapted to facilitate lactate export through the plasma membrane [169,170] (Fig. 3). As a target gene product of transcription factor hypoxia-inducible factor-1 (HIF-1), its expression increases with hypoxia [171], thereby linking glycolytic flux increase and transporter abundance/activity. Some tumor cells also use MCT1 ( $K_m \text{ lactate} \approx 2.5\text{--}5$  mM) for lactate efflux [165], whereas high affinity MCT2 ( $K_m \text{ lactate} \approx 1$  mM) has been found to be expressed in brain, prostate and colon cancers [172–174] where its role still remains elusive. To date, MCT3 has no ascribed a role in tumors. Collectively, MCT1 and MCT4 are responsible for extracellular lactic acid accumulation in cancer, raising average lactate levels from 1.8–2 mM [175] in normal tissues to 6–15 mM in clinical tumors, with peak levels as high as 40 mM [176]. The regulation of their expression and activities has been reviewed recently [165,166,177].

Once exported, lactic acid readily is dissociated into lactate and protons. The contribution of extracellular acidity to tumor progression has been well documented, in spite that the lactate anion has for a long



**Fig. 3.** MCTs convey lactate exchanges in tumors. The model represents lactate exchanges in a metabolic symbiosis between oxidative and glycolytic tumor cells and in the metabolic exploitation of stromal cells by oxidative tumor cells. Hypoxic/glycolytic tumor cells provide lactate to oxidative tumor cells, and therefore receive the glucose spared by the latter. Stromal cells are metabolically exploited by oxidative tumor cells that block their TCA cycle with H<sub>2</sub>O<sub>2</sub>, forcing them to supply lactate as a result of glycolytic compensation. At the core of lactate exchanges in cancer, oxidative tumor cells use lactate as an oxidative fuel preferred to glucose. Lactate is oxidized into pyruvate (lactate dehydrogenase [LDH]-1 reaction), and pyruvate fuels the TCA cycle. Monocarboxylate transporters MCT4 and MCT1 gate lactate export and uptake, respectively. MCT1 inhibitors, targeting oxygenated tumor cells close to blood vessels, can be used therapeutically to block lactate exchanges.



**Fig. 2.** The LDH-5 reaction. The reduction of pyruvate to lactate at the end of the glycolytic pathway serves to replenish the NAD<sup>+</sup> pool needed to maintain glycolysis at high rates, and buffers cytosolic pH by incorporating protons into the molecule of lactate.



time been considered as a biologically inactive byproduct of glycolysis. In 2008, however, Sonveaux et al. [111] reported that oxidative tumor cells can use lactate as an oxidative fuel in a two-step process involving MCT1-dependent lactate uptake and its oxidation to pyruvate by LDH-1 (Fig. 3). They further observed a metabolic preference of oxidative tumor cells for lactate compared to glucose, which can be explained by a competition between LDH-1 and GAPDH for  $\text{NAD}^+$  [178] and/or by an allosteric inhibition of glycolytic enzymes hexokinase and phosphofructokinase by lactate [179]. These findings supported the theory of a metabolic symbiosis in tumors [111,180] where the oxidative preference of oxygenated tumor cells for lactate would improve glucose delivery to hypoxic/glycolytic tumor cells (Fig. 3). In turn, glycolytic tumor cells would generate a lactate gradient fulfilling the respiration needs of oxidative ones. Sonveaux's hypothesis is supported by the observation of metabolic cooperativeness based on lactate exchanges in breast cancer [181,182], melanoma [183] and pancreatic cancer [184]. In an extension of the symbiosis theory, the group of Michael Lisanti [185,186] reported that oxidative tumor cells can secrete  $\text{H}_2\text{O}_2$  to convert oxidative stromal cells into glycolytic suppliers of lactate and ketone bodies (Fig. 4).

If MCT1 gates the oxidative use of lactate (and ketone bodies) in cancer, it also controls lactate signaling and tumor angiogenesis. Lu et al. [187,188] were the first to report that lactate can act as a hypoxia mimetic by activating HIF-1 independently of hypoxia. The pathway, which was reported to exist in oxidative tumor cells and in endothelial cells [189,190], involves lactate oxidation into pyruvate, a competition between pyruvate and 2-oxoglutarate to inactivate HIF-1 prolylhydroxylases (PHDs) and, consequently, the stabilization of HIF-1 subunit  $\alpha$  and HIF-1 activation. Consequently, lactate stimulates pro-angiogenic vascular endothelial growth factor (VEGF) signaling by increasing VEGF production by tumor cells and the expression of VEGF-receptor 2 in endothelial cells. It also triggers autocrine pro-angiogenic signaling in endothelial cells through basic fibroblast growth factor (bFGF, indirectly controlled by HIF-1) [189] and interleukin-8 (IL-8, a NF- $\kappa\text{B}$  target gene product) [191].

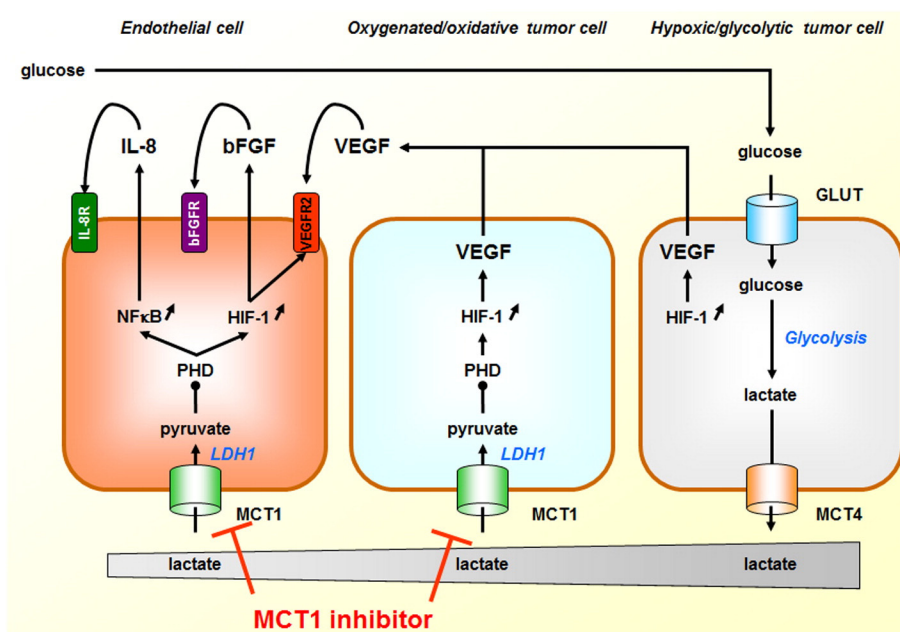
The key roles exerted by MCT1 in controlling lactate exchanges for the metabolic use of lactate and for its use as a signaling agent prompted

the development of MCT1 inhibitors. The historical inhibitor  $\alpha$ -cyano-4-hydroxycinnamate (CHC) [192] potentially inhibited metabolic symbiosis and lactate-induced angiogenesis [111,189–191] but lacks MCT1 specificity. AstraZeneca with Cancer Research UK launched a clinical trial where AZD-3965, a dual MCT1/MCT2 inhibitor having demonstrated sufficient clinical safety, is evaluated as an anticancer agent (NCT01791595). However, this agent does not specifically target MCT1-dependent lactate uptake, and dose-limiting toxicities could arise in tissues expressing MCT2 (brain, liver, kidney) or in cells using MCT1 for physiological functions (*ea.*, red blood cells and immune cell using MCT1 to export lactate produced glycolytically). MCT1 $^{-/-}$  mice die embryonically [193]. As an alternative to AZD-3965, Feron et al. [194,195] recently developed a first-in-class family of MCT inhibitors that blocks lactate influx but not efflux. Lead compound 7ACC2, a 7-aminocarboxycoumarin derivative devoid of any anticoagulant activity, is a dual MCT1/MCT4 inhibitor of lactate uptake ( $\text{IC}_{50} = 11 \text{ nM}$  on  $^{14}\text{C}$ -lactate flux inhibition) that does not inhibit lactate export. The future development of MCT1 inhibitors will require a better understanding of the roles of the different transporters in physiology and in cancer, and of its regulation by typical parameters of the tumor microenvironment and during tumor treatment. Finally, the recent discovery that MCT1 is involved in tumor cell migration [196] together with the fact that it is commonly upregulated in secondary versus primary tumors [197] could enlarge the therapeutic potential of this class of drugs to metastatic prevention and/or to the treatment of established metastases [198].

## 5. Conclusions

### 5.1. The present and future prospects of a new and integral paradigm in human cancer therapeutics

Cell acid–base balance, controlled by PTIs and PPIs is recognized to be the main parameter to define cellular homeostasis, the life of cells being possible only within a very narrow range of pH of less than one unit. In that context, the pH of normal and cancer cells has been repeatedly shown to deviate towards opposite ends of a metabolic spectrum.



**Fig. 4.** MCT1 gates pro-angiogenic lactate signaling in cancer. Lactate produced by hypoxic/glycolytic tumor cells diffuses along its concentration gradient towards blood vessels. Along the way, it enters into oxidative tumor cells and endothelial cells using MCT1-dependent transport. There, it competes with 2-oxoglutarate to inhibit PHDs, supporting normoxic HIF-1 activation in tumor and endothelial cells, and NF- $\kappa\text{B}$  in endothelial cells. Lactate thereby promotes paracrine VEGF and autocrine bFGF and IL-8 signaling, hence tumor angiogenesis. Because lactate signaling essentially depends on MCT1-facilitated uptake, tumor angiogenesis can be inhibited with MCT1 inhibitors.



This energetic abnormality represents the largest possible difference so far found between normal cellular physiology and cancer pathophysiology. At the same time, targeting the hydrogen-related dynamics of malignancy has become a new approach to tackle cancer that is helping to reach a better understanding of several, until now disparaged areas of cancer research both at basic and clinical levels. This unifying thermodynamic view of cancer metabolism has allowed to integrate under a unitarian perspective the hydrogen-related dynamics of malignancy (pH centric paradigm). This allows researchers belonging to different disciplines to embrace processes ranging from etiopathogenesis to cell transformation and metabolism, growth and local invasion, neovascularization, drug resistance and the activation and progression of the metastatic process [72,101].

The utilization of different PTIs and PPIs in cancer therapeutics was initially suggested as a novel approach for the pH-related treatment of malignant tumors because of its potential as a more selective and less toxic approach compared to conventional chemotherapy. From a therapeutic perspective, the primary aim of this originally pH-based approach was to manipulate the selective forces controlling the deregulated pH dynamics of all cancer cells and tissues in order to regress tumor growth, control local invasion and deactivate the metastatic potential of malignant tumors within this new and integral perspective and paradigm shift based upon the dynamics of the hydrogen ion  $[H^+]$  in cancer pathophysiology and treatment. This approach would have low toxicity and could be particularly efficient in combination with chemotherapy, because many chemotherapeutic drugs are weak bases that would benefit from a raise in extracellular pH. It has a real possibility to become a successful strategy for human cancers in general.

Any attempt to therapeutically induce a selective intracellular acidification using PTIs and at the same time alkalinizing interstitial tumor pH with PPIs in all cancer cells and tissues would secondarily inhibit the metastatic process and counteract drug resistance, thus representing a rational and firmly based approach for cancer treatment in all stages of development. Further, it has the potential of being selectively exploited in the treatment of many different human malignant solid tumors and leukemias.

As a final example, the new and potent NHE1 inhibitors of the amiloride series, like cariporide, as well as powerful and selective NHE1 inhibitors of the non-amiloride series, like Phx-3 and compound 9 t, have the potential of being highly promising, minimally toxic and truly effective anticancer agents in a wide array of malignant tumors and leukemias [65,101]. However, translation to the oncology clinic has yet to be realized because, unfortunately, the utilization of this drug in cancer treatment has not been explored [73,101]. The only non-amiloride based compounds with NHE1 inhibitory activity that have undergone clinical trials are cariporide and eniporide, and those trials were not in the field of cancer but in a cardiological setting and for ischaemic–reperfusion injury. Despite the cardioprotective value of cariporide in reducing myocardial infarcts in both the EXPEDITION and in the earlier GUARDIAN trials, use of the drug was associated in the EXPEDITION study with a significant increase in the rate of mortality (from 1.5% to 2.2% at day 5) due to an increase in cerebrovascular events [199–201].

The appearance of these adverse effects in the last trial can probably be ascribed to the higher cumulating dose of cariporide administered in the EXPEDITION trial with respect to the GUARDIAN trial [202]. Clearly, a clinically reasonable initial approach in an oncology setting would be to minimize the systemic dose of the drug in order to dissociate the adverse and probably off-target effects from beneficial effects. Interestingly, rats having a lifelong treatment with cariporide had a greatly extended lifespan and this was interpreted as being due to a reduced occurrence of cancer [202,203]. Besides, cariporide is orally bioavailable and by this route of administration has been used in thousands of patients in a cardiological setting but never to date in an oncological one and as anticancer drug [204–206]. Sanofi-Aventis, the patent holder (CARIPORIDE PATENT WO2004007480, SANOFI-AVENTIS, 2005), surprisingly writes in its patent on cariporide effects: “[...] there is also

surprisingly a prolongation of life to an extent which has to date been achievable by no other group of medicaments or by any natural products. This is a unique effect of NHE inhibitors like cariporide”. Furthermore, other patent holders for selective and potent NHE1 inhibitors like the 3-methyl-4-fluoro analog of 5-aryl-4-(4-(5-methyl-14-imidazol-4-yl) piperidin-1-yl)pyrimidine (Compound 9t) should also release and promote this drug for cancer research, more taking into account that Compound 9t is one of the most selective NHE-1 inhibitors ever known. It is 1400-fold more selective and 500-fold more powerful in inhibiting NHE1 than cariporide. Besides, it is orally available, has low side-effects in mice and may possess a significantly improved safety profile over other NHE1 inhibitors (BRISTOL-MYERS SQUIB PATENT WO 01 27107 A2, PCT/US00/27, 2001; US 6887870 B1; EP 1224183) [207]. Finally, Phx-3 (APO) (Japanese Research group patents US2010324285A1 and EP2098228A1) is another promising PTI and NHE inhibitor and anticancer drug. All these patent holders should review their efforts in this regard, all in order to make these drugs immediately available for oncology research to basic and clinical cancer researchers, so facilitating preclinical and clinical therapeutic attempts, both as anticancer drugs of their own and as adjuvants to overcome MDR (for a more detailed review of this subject, see Refs. [65,69,101,208]).

The new strategy against cancer based on targeting the main actors of tumor pH regulation is not a real molecular targeting approach but can rather be viewed as a targeting of an aberrant cancer phenotype that hampers the body reaction against cancer. Cancers live in this hostile condition thanks to proton exchangers that actually represent very efficient mechanisms of detoxification. Counteracting proton exchangers activity deprives cancer cells of their detoxification framework inducing a quick and fatal cancer cell death [209]. However, low extracellular pH is a key determinant for nanovesicles release by cancer cells that are involved in paracrine and systemic tumor spreading [210] and resistance to cytotoxic drugs [211]. It is therefore conceivable that inhibiting major tumor pH regulators may profoundly impair tumor natural history, at least contributing to control cancer growth and progression. It is mandatory that, to better understand the clinical application of the use of proton exchangers inhibitors, clinical trials should be promoted and supported by public funds, in order to contribute to refine the current paradigm where chemotherapy, surgery and radiotherapy occupy a central position.

Finally, we think that it is urgently needed that the new and potent proton transport inhibitors dealt with in this contribution, which represent a potential, highly selective and new avenue in anticancer therapy, should be immediately made available by patent holders in order to facilitate progress in this new area of modern cancer research instead of keep on hindering it any longer.

## Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be or become a potential conflict of interests.

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