

Na⁺-H⁺ Exchanger, pH Regulation and Cancer

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Abstract: Cancer cells and tissues, regardless of their origin and genetic background, have an aberrant regulation of hydrogen ion dynamics leading to a reversal of the intracellular to extracellular pH gradient ($\Delta p\text{Hi}$ to $\Delta p\text{He}$) in cancer cells and tissue as compared to normal tissue. This perturbation in pH dynamics rises very early in carcinogenesis and is one of the most common patho-physiological hallmarks of tumors. Recently, there has been a very large increase in our knowledge of the importance and roles of pHi and pHe in developing and driving a series of tumor hallmarks. This reversed proton gradient is driven by a series of proton export mechanisms that underlie the initiation and progression of the neoplastic process. In this context, one of the primary and best studied regulators of both pHi and pHe in tumors is the Na⁺/H⁺ exchanger isoform 1 (NHE1). The NHE1 is an integral membrane transport protein involved in regulating pH and in tumor cells is a major contributor to the production and maintenance of their reversed proton gradient. It is activated during oncogene-dependent transformation resulting in cytosolic alkalinization which then drives subsequent hallmark behaviors including growth factor- and substrate-independent growth, and glycolytic metabolism. It is further activated by various growth factors, hormone, the metabolic microenvironment (low serum, acidic pHe and hypoxia) or by ECM receptor activation. This review will present the recent progress in understanding the role the NHE1 in determining tumor progression and invadopodia-guided invasion/metastasis and recent patents for NHE1 inhibitors and novel therapeutic protocols for anti-NHE1 pharmacological approaches. These may represent a real possibility to open up new avenues for wide-spread and efficient treatments against cancer.

Keywords: Amiloride, angiogenesis, cariporide, growth factors, HOE642, invasion, tumor microenvironment, MDR, Na⁺/H⁺ exchanger, NHE1, pH and cancer, proton transport in cancer.

INTRODUCTION

A major paradigm shift is occurring from the gene-centric view which has predominated cancer biology for the last 20 years towards the search for the fundamental underlying principles that could form a unified theory of transformation, progression and metastasis. The gene-centric approach has produced a perception of cancer as a complex collection of diseases unrelated amongst themselves and has led to the idea of a tailored therapy for each patient based on the tumors' pattern of gene expression. The inherent difficulties in this approach are self-evident, whereas the reductionist 'recasting' of cancer as a single disease could correspondingly permit the development of more general therapeutic strategies that exploit common underlying forces. This approach to cancer at the level of its metabolic character and constraints has led to the unifying paradigms that tumors depend on angiogenesis (endothelial-centric paradigm) and on aerobic glycolytic metabolism (metabolic-centric paradigm). Importantly, these two processes interact between themselves and both interact with and help to develop the tumor metabolic microenvironment (defined later on).

Both ion transport and cytoplasmic pH play crucial roles in multiple cell functions including control of cell membrane

potential, mitochondrial activity, cell volume, enzyme activity, DNA synthesis, cell growth and proliferation, growth factor activity, differentiation, oncogenesis, oncogene action and malignant transformation [1, 2]. A great deal of accumulating data over the last years has amply demonstrated that practically all tumors have in common a pivotal characteristic: the aberrant regulation of hydrogen ion dynamics [1-3]. Cancer cells have an acid-base balance that is completely different than that observed in normal tissues and that increases with increasing neoplastic state: an extracellular acid microenvironment (pHe) linked to a 'malignant' alkaline intracellular pH (pHi). Indeed, tumor cells have the alkaline pHi values of 7.12-7.7 vs 6.99-7.05 in normal cells while producing acidic pHe values of 6.2-6.9 vs 7.3-7.4 in normal cells. This creates a reversed pH gradient ($\Delta p\text{Hi}$ to $\Delta p\text{He}$) across the cell membrane that increases as the tumor progresses. This specific and pathological reversal of the pH gradient in cancer cells and tissues compared to normal tissue is now considered to be one of the main characteristics defining tumor cells and completely alters their thermodynamic molecular energetics, regardless of their pathology and genetic origins [3-5]. Indeed, the induction and/or maintenance of intracellular alkalinization and its subsequent extracellular acidosis [2-5] have been repeatedly implicated as playing a pivotal role both in cell transformation as well as in the maintenance and active progression of the neoplastic process [1-3]. Further, the increased diffusion of the proton ions along concentration gradients from tumors into adjacent normal tissues creates a

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peritumoral acidic microenvironment involved in driving invasion and metastasis [6-8].

The development and maintenance of this reversed pH gradient is directly due to the ability of the tumor cells to secrete protons (H^+) and this ability increases with increasing tumor aggressiveness [2]. This proton secretion depends on the buffering capacity of the cell and is driven by a series of transporters and enzymes including carbonic anhydrases (CAs), vacuolar H^+ -ATPases, the H^+/Cl^- symporter, the monocarboxylate transporter (MCT, mainly MCT1) (also known as the lactate-proton symporter), the Na^+ -dependent Cl^-/HCO_3^- exchangers, ATP synthase (for reviews see [1-3, 9].

Although this reversed tumor pH gradient is driven and maintained by these numerous cellular mechanisms, an activated sodium/proton exchanger isoform 1 (NHE1) is considered to be the major factor in promoting tumor acidity from even the earliest pre-cancer stage of oncogene-driven neoplastic transformation [10] and to play fundamental roles in regulating motility, invasion and the tumor cells response to a variety of anti-neoplastic agents as will be discussed below. The NHE1 is a member of a family of integral membrane secondary active acid extruders that mediate the electroneutral 1:1 exchange of extracellular sodium for intracellular protons across the cell membrane (the K_m for extracellular sodium ranges from 10-50mM). Through its action the inwardly directed sodium gradient can drive the uphill extrusion of protons that alkalinizes pH_i and acidifies pHe . The first physiological evidence for the existence of an NHE activity in mammalian cells was provided in 1967 in mitochondria [11] and in the plasma membrane in 1976 [12],

while the NHE1 isoform was cloned in 1989 by the Pouyssegur group [13]. To date, nine mammalian isoforms have been identified [14]. NHE1 is the most extensively characterized member of this family and is present in most cell types. A model showing the factors regulating NHE1 activity and the tumor hallmark activities regulated by NHE1 are shown in Fig. (1) and will be discussed in the sections below.

REGULATION OF NHE1 ACTIVITY

For a detailed review of the structure and biophysical characteristics of NHE1 please refer to the very recent review [15]. In brief, the NHE1 is composed of 12 transmembrane segments and a long c-terminal cytoplasmic tail that plays a role in both its regulation and function through three processes. Firstly, there is an exquisite sensitivity to pHi through an internal allosteric proton binding regulatory site. When pHi drops below a threshold level it is activated and, in this way, intracellular protons are an important allosteric regulator of NHE1 activity independently of their function as a substrate for the exchange with external sodium [16]. Secondly, the cytoplasmic tail contains numerous ser/thr residues, some of which are constitutively phosphorylated in quiescent cells [13] and are further phosphorylated in response to extracellular stimuli [17]. Lastly, since the cytoplasmic tail also contains numerous binding sites for multiple protein partners, the NHE1 is also able to act as a scaffolding protein [18, 19]. These partner proteins include the 14-3-3 adaptor protein, calcineurin homologous protein (CHP), carbonic anhydrase II, calmodulin, ERM proteins (ezrin, radixin, moesin), heat shock protein 70 (HSP70) and

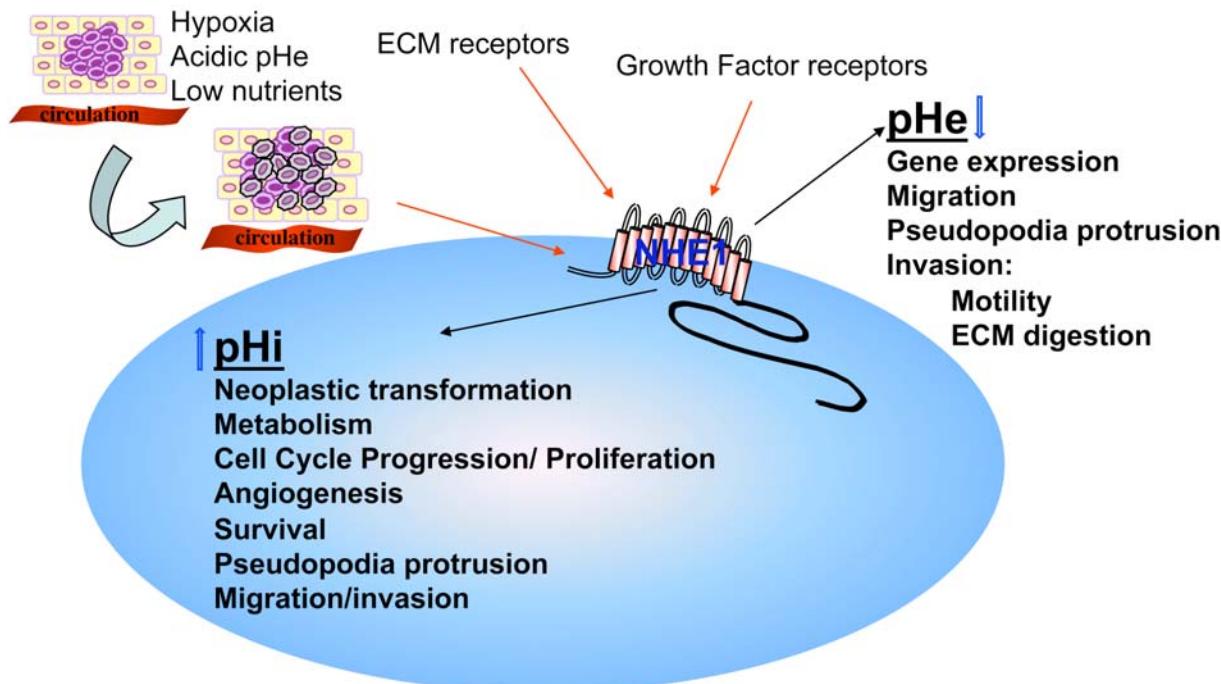


Fig. (1). The regulation of NHE1 and its roles in driving tumor hallmark behaviors. A general scheme showing the major systems regulating the activity of NHE1 with the resultant alkalinization of intracellular pH (pHi) and acidification of extracellular pH (pHe). These altered intra- and extra-cellular environments, in turn, drive a series of tumor cell behaviors resulting in progression to more aggressive characteristics. See main text for further details.

PI(4,5)P2 [15, 20]. Recently, a direct binding with B-Raf that activates NHE1 was described [21]. Additionally, through its binding to the actin binding protein ezrin, NHE1 can directly regulate cytoskeleton dynamics independently of its ion transporting capabilities [22]. Together with transport, these three activities make the NHE1 a very important membrane bound integrator for many signaling networks and cellular processes and this aspect of the role of NHE1 in the regulation of tumor processes is just beginning to be studied in tumor cells.

In normal and tumor cells NHE1 activity regulation is mediated by multiple extracellular stimuli comprised of three major categories: receptor activation from (i) soluble growth factors, hormones or cytokines acting through receptor tyrosine kinases and G-protein coupled receptors; (ii) extracellular matrix (ECM) ligand receptors (integrin [23] and CD44 [24]); and (iii) physical stimuli such as osmotic cell shrinkage and shear stress (for reviews, see [25-27]). As stated above some of these receptors are known to stimulate NHE1 phosphorylation at S648 by Akt [17], at S703 by p90RSK [28] and by stimuli that regulated apoptosis through NHE1 that also phosphorylated NHE1 at S726 and S729 [29]. However, how these extracellular cues and their signalling systems are altered in regulating tumor cell NHE1 and its down-stream action is still poorly understood.

There is now ample evidence that in addition to these above stimuli tumor cell NHE1 is further activated by the components of the tumor metabolic microenvironment (TMM) previously described [30]: low serum [31, 32], acidic pH_e [33] and hypoxia [28, 32, 34, 35] which links these components into a dynamic, reciprocal system that drives further microenvironmental acidification and malignant progression. Further, it has been shown that interaction with the stromal microenvironmental compartment in breast cancer cells, via activation of the CD44 receptor, acidifies the extracellular medium via activation of the NHE1 [24]. Altogether, these data lead to the recognition of a synergistic, positive feedback interaction between the tumor cell and both the metabolic and stromal microenvironments in tumors and suggests that NHE1 may have an important role in integrating these interactions.

Another level of regulation of NHE1 activity and its downstream tumor-promoting functions has been described in breast cancer cells where the sodium transporting activity of the sodium channel, Na_v1.5, is necessary for full NHE1 activity and subsequent invasion. The stimulated NHE1 acidifies the extracellular environment with subsequently activation of extracellular cathepsin B which digests the extracellular matrix making invasion possible [36, 37]. Presumably Na_v1.5 permits the maintenance of the necessary sodium gradient for maximum sustained NHE1 activity.

ROLES OF NHE1 IN CANCER

The Role of NHE1 in Tumor Cell pH Homeostasis

As stated above, one of NHE1s' fundamental characteristics is the exquisite sensitivity to pH_i through an internal allosteric proton binding regulatory site such that when pH_i drops below a threshold level it is activated. This pH_i sensitivity determines its activity set-point, i.e. the pH_i at which it

first starts to be activated and, in normal cells, the set-point is at their physiological, resting pH_i such that the NHE1 is quiescent. It becomes activated only when the cell is acidified and functions to return the cell to neutral pH_i and this activation results in a sigmoid regulatory dependence of NHE1 activity on the intracellular proton concentration. This same process is utilized to increase NHE1 activity in tumor cells. Oncogene-driven neoplastic transformation constitutively activates NHE1 and raises pH_i by increasing the affinity of this allosteric proton regulatory site which mimicks the lowering of cytosolic pH [10]. Further, in a study to determine the mechanism of tumor cell activation by serum removal demonstrated that this treatment stimulated NHE1 activity specifically in tumor cells though a PI3K-dependent increase of the affinity of this allosteric site [31]. However, two studies have suggested that the NHE1 may function as a dimer and that the above described sigmoidal dependence on intracellular proton concentration may instead reflect the two substrate binding sites in the dimer rather than an allosteric proton binding site on the NHE1 monomer [38, 39]. That the activated NHE1 in tumor cells could be the result of increased dimerization is a potentially important aspect that needs to be further analyzed.

Carbonic anhydrase (CA) activity has been found to be important in maintaining uniformly alkaline pH_i in small tumor spheroids [40] and CA IX was recently found to be broadly localized in the interior of rat brain C6 tumor [41]. Interestingly, the activity of NHE1 has also been shown to be enhanced via its direct binding to CA II [42, 43], although the relevance of this interaction in tumor cells has yet to be determined. Furthermore, NHE1 is often co-expressed with and regulates pH_i in cooperation with bicarbonate transporting systems (i.e., Na⁺-HCO₃⁻ cotransporters (NBC), Na⁺-dependent HCO₃⁻/Cl⁻ exchangers (NCBE) and Cl⁻/HCO₃⁻ exchangers (AE). A recent series of papers shows that oncogene overexpression (activated erbB2 receptor) in the MCF-7 breast cancer cell line increases pH_i through the activation of both NHE1 and NBCn1 but the underlying mechanism is still unknown [44, 45]. Thus, the NHE1 in tumor cells is always active and these cells can have pH_i values as high as 7.8. Interestingly, although both transporters contributed to regulate pH_i in MCF-7 cells inducibly expressing the activated erbB2 receptor, only the NHE1 played a role in regulating either motility [44] or response to cisplatin chemotherapy [45]. This relative importance of NHE1 in motility compared to Na⁺-HCO₃⁻ cotransporter (NBC1) was also observed in NHE1-deficient Madin-Darby canine kidney (MDCK-F) cells [46] and, altogether, these studies suggest that NHE1 contributes to these processes through one of its other two functions outlined above and further demonstrate its importance as a potential anti-neoplastic target.

ROLE OF NHE1 IN ONCOGENE-DRIVEN NEOPLASTIC TRANSFORMATION AND THE FIRST APPEARANCE OF THE PROTON GRADIENT

This cancer cell-specific increased proton secretion with the resultant initiation of the reversed proton gradient appears during the very first steps of neoplastic transformation. Indeed, oncogene-dependent transformation results in a rapid cytoplasmic alkalinization An elevated pH_i was very early on implicated as a crucial factor in neoplastic transformation

driven by the ras and v-mos oncogenes [47, 48]. They observed that these oncogene-dependent transformations resulted in an elevated pH_i, increased NHE1 activity and increased glycolysis, although it was not clear from those experiments if the driving factor was the stimulated NHE1 or the increased glycolysis. This question was resolved in a study utilizing the inducible expression of an oncogene (HPV16 E7) to dissect time-dependence of the appearance of the hallmarks demonstrated that the first step in oncogene-dependent transformation of normal cells is the activation of the NHE1 with the subsequent cytosolic alkalinization [10]. A kinetic analysis of the activation of the NHE1 demonstrated that the oncogene-driven neoplastic transformation constitutively activates NHE1 by increasing the affinity of this allosteric proton regulatory site increasing the sensitivity of the NHE1 to the intracellular protons and increasing its activity with a resultant intracellular alkalinization and extracellular acidification. This alkalinization was the driver of a series of transformation hallmarks such as increased growth rate, substrate-independent growth, growth factor independence, glycolysis in aerobic conditions and tumor growth in nude mice [10]. Altogether, these data demonstrate that oncogenes utilize NHE1-induced alkalinization to produce very early the unique cancer specific altered pH regulation with the resulting pH-profile and the hallmark phenotypes characteristic of cancer cells [49].

THE ROLE OF pH IN DEVELOPING AND MAINTAINING WARBURG METABOLISM

Another unique hallmark of cancer cells that is receiving ever increasing attention is their shift to glycolytic metabolism relative to oxidative phosphorylation (OxyPhos), even under aerobic conditions. This was first described by Otto Warburg [50] and is known as the Warburg effect. It is thought to be downstream of oncogene activation and was shown to be an early effect/consequence of oncogene-driven transformation of normal cells [47, 48].

There is ever more evidence that both pH_i and pH_e are important in driving this ever increasing dependence on glycolysis and decreasing dependence on OxyPhos as the tumor cell progresses (reviewed in [4, 5]. Briefly, as both the processes of OxyPhos and glycolysis are exquisitely but oppositely pH sensitive, a rapid shift of cell metabolic patterns follows alkalinization. On the one hand, alkaline pH_i even slightly above steady-state levels stimulates the activity of glycolytic enzymes such as phosphofructokinase-1 (PFK-1) and inhibits gluconeogenesis [51-54] while, on the other hand, the proper functioning of numerous mitochondrial proton transporters and proton driven transporters that are involved in regulating OxyPhos metabolism have a strong dependence on a relatively high cytosolic proton concentration [4]. In all, at least, 9 transporters regulating mitochondrial activity depend on a constant, regulated cytosol-mitochondrial proton gradient. This reciprocal metabolic shift may well be the most sensitive pH_i sensor of all.

Altogether, this evidence supports the hypothesis that it is the alkaline pH_i that is the driver of this metabolic shift and this pH_i-dependent shift is one of the 'corner-stones' in the altered metabolism that the pH perturbation creates. Indeed, a recent paper added further weight to this conclusion ob-

serving, with a new NHE1 inhibitor, that the Warburg effect may be explained simply through the elevation of pH_i in cancer cells [55]. An added depth and complexity to this field comes from the demonstration that lower pH_e (in both the presence and absence of extracellular lactate) has profound effects on tumor cell gene expression, including genes involved in glycolysis [56] and that inhibition of the NHE1 results in changes in expression patterns of a number of genes including many that regulate metabolism [57].

THE FIRST STEPS IN THE DEVELOPMENT OF THE TUMOR MICROENVIRONMENT

As stated above, this increase in pH_i of the transformed cell drives obligate tumor DNA synthesis, cell cycle progression, and both substrate-independent and serum-independent growth, resulting in a pathological and disorganized increase in cell number and density [3, 30]. A consequence of increased tumor cell density is a corresponding decrease in access to circulation which creates a hypoxic condition reducing the cells ability to run their mitochondrial oxidative respiratory chain and increasing the need to satisfy their energy demand through glycolytic metabolism and increased glucose consumption.

Glycolysis is much less efficient than oxidative metabolism in producing ATP (2 molecules of ATP per molecule of glucose, compared to up to 38 ATP per glucose in a full cycle of glycolysis-Krebs cycle-oxidative phosphorylation). More importantly, each round of glycolysis produces 2 protons, which challenges the tumor cell with an ever increasing acid load [58] and pH_i would rapidly decline which could be lethal if not compensated for by increased proton extrusion which results in additional pH_e acidification [30].

Therefore, an adaptative feature of cancer cells, and especially of highly aggressive cancer cells, is the overexpression and the increased activity of multiple pH-regulating transporters and enzymes such as V-ATPase [3, 59], carbonic anhydrases [60, 61], the proton linked monocarboxylate transporter MCTs [62, 63], and Cl⁻/HCO₃⁻ exchangers. As an example NHE1 is overexpressed in cervical cancer [64] and hepatocellular carcinoma [65] and is correlated with clinical outcome, while its activity is upregulated in glioma [66] and breast cancer cells [10, 36].

These complex dynamics of the pH-metabolism interaction engages a vicious cycle from very early on: the oncogene-driven alkalinization increases glycolysis and proliferation, generating a need for a high energy consumption which maintains a high proton production that, through stimulated proton efflux transport systems, further alkalinizes the cell that even further reduces OxyPhos and increases glycolysis.

The increasing hypoxia of the tumor also necessitates a new blood supply that is achieved through neoangiogenesis, whereby new blood vessels are formed from preexisting ones [30]. However, neoplastic vascularization occurs uncoordinatedly, resulting in a chaotic, functionally poor vasculature incapable of meeting tumoral demands of oxygen and serum and causing an efficient washout of metabolic products (i.e. carbonic acid) which even further exacerbates the low pH_e. The physiological environment, tumor metabolism, angio-

genesis and vascularization are, therefore, inextricably linked.

Altogether, these processes give rise to the tumor specific metabolic microenvironment defined as extracellular areas within tumors characterized by dynamic, interacting areas of (i) hypoxia, (ii) low serum nutrients and (iii) acidic pHe (Fig. (2)). Multiple studies have strongly supported a pathogenic role of both the low nutrients and the acidic interstitial pHe of tumors by giving a selective advantage for tumor progression and metastasis. Low pHe together with low nutrients [67] or low pHe alone has been shown to drive large changes in gene expression independently of hypoxia [56, 68, 69] and has also been associated with tumor progression by impacting multiple processes including increased invasion [56, 69-71] and metastasis [7, 67, 72]. In this context, low nutrient concentrations [31, 32] or low pHe [33] have been shown to preferentially stimulate NHE1 activity in tumor cells but not in normal cells. Accordingly, emphasis is shifting toward elucidating the unique responses of cancer cells to their own microenvironment and determining how this contributes to metastasis.

This tumor specific increase in glucose consumption induces a higher glucose transporter expression of the GLUT1 isoform [73], and the resulting increased glucose uptake is used in 18-fluorodeoxyglucose (FdG) positron-emission tomography to very efficiently visualize even small tumors

[74-76], demonstrating that this tumor metabolism is a widespread and perhaps ubiquitous trait of tumor cells.

NHE1 AND THE METASTATIC PROCESS

Tumor invasion and metastasis associated with neoplastic progression are the major causes of cancer deaths and understanding the mechanisms determining metastatic spread of malignant cells via invasion to distant tissues is perhaps the central question in oncology [77, 78]. Even though metastasis represents the most relevant aspect of cancer in terms of therapy and survival it remains the least studied and known aspect. Of particular importance is the identification of the fundamental driving forces involved in metastatic progression. Some of the most relevant physiological processes required for metastasis to occur are to evade apoptosis, to promote angiogenesis and to invade (together with intra- and extra-vasation) both from the primary tumor and at the secondary site. Invasion may well be the deadliest aspect of the metastatic cascade as it results in the progressive disruption of both the primary tissue and especially the secondary colonized tissue. Invasion occurs through a complex series of interactions with the host tissue in which the infiltration and penetration of the normal tissue by the cancer cell takes place by three biochemical and physiological steps: tumor cell attachment to basement membranes or extracellular matrices, local degradation of these structures directly by acid extrusion and secretion of

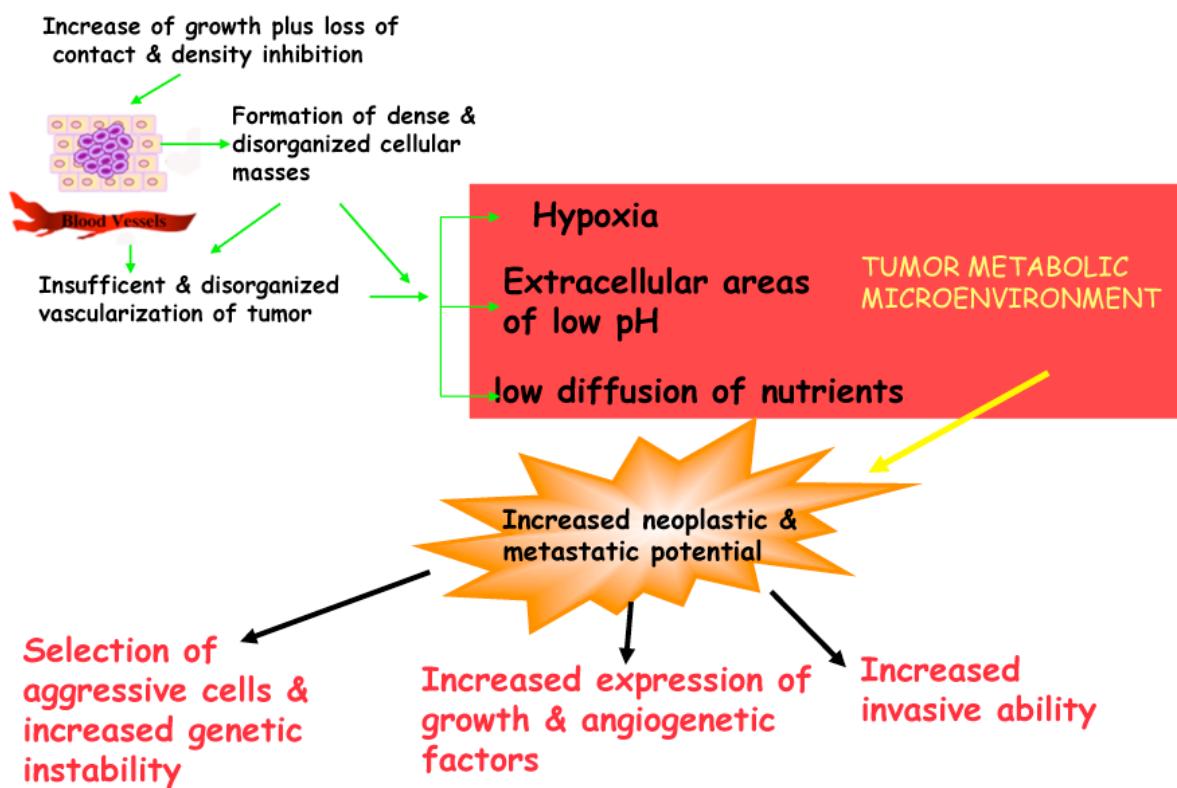


Fig. (2). Development of tumor metabolic microenvironment. General scheme showing how the dense, disorganized tumor interacts with a reduced circulatory availability to produce the tumor metabolic microenvironment, which is composed of low serum availability, hypoxia and acidic extracellular pH. Exposure to this microenvironment further drives metastatic progression. See main text for further explanation.

acid-dependent proteases and increased tumor cell locomotion into the modified region. Both the second and third processes are regulated by extra- and intracellular pH, respectively. The tumor microenvironment and particularly the acid component of the tumor microenvironment has been shown to be critical in controlling invasive capacity and subsequent malignant progression by increasing the activity one or more of the above steps and can be considered to be a strategic principle utilized by the tumor rather than only a side effect of tumor metabolism [7]. This can occur directly or through the alteration of the extracellular matrix (ECM) compartment through up-regulation of protease secretion/activation and in an altered tumor-stromal interaction via an inverse stimulation of pro-angiogenic factors paired with impaired immune functions [1, 7].

ROLE OF TUMORAL pHe IN INVASION AND EXTRACELLULAR PROTEASE ACTION

Proteolytic ECM remodeling is a prerequisite for the invasive process. Indeed, the proteolytic breakdown of proteins of the ECM is one of the first steps in invasion in primary cancer lesions. The theoretical basis for the role of low extracellular pH in driving invasion has been put forth in a series of modeling papers showing that tumor driven extracellular acidification of the tumor pericellular space can directly drive the destruction of the surrounding normal tissue [79, 80]. A recent up-dated model has included the pHe stimulation of the activity of proteases secreted by the tumor cells themselves and by other cell types in the tumor stromal microenvironment [7]. There is now a growing body of experimental data in support of this aspect of their model. During invasion, cancer cells use secreted, surface-localized and intracellular cathepsins, serine proteases, and matrix metalloproteinases (MMP) to proteolytically cleave, remove and remodel different types of ECM substrates at the cell surface, including collagens, laminins, vitronectin, and fibronectin [81].

Indeed, acidic pHe can also indirectly drive ECM proteolysis and invasion by increasing protease production and secretion of the active forms of the cathepsin family of proteases cathepsin D [72, 82], cathepsin B [24, 83], [36, 71], cathepsin L [72] and the secreted metalloproteases MMP-9 [35, 65, 70-72, 84-86], MMP-2 [71, 72] and the membrane-bound metalloprotease MT1-MMP [87, 88]. A recent paper has shown that also the Urokinase plasminogen activator receptor induction of invasion and metastasis requires extracellular acidification [89, 90]. In an ample sub-set of these studies, the NHE1 was identified to be the transporter involved in the pHe acidification-dependent activation of cathepsin B [24, 36], MMP-2 [35, 65], MMP-9 [65, 86], the MT1-MMP [87, 88] and the Urokinase plasminogen activator receptor [89]. Further, the low pHe-driven activation of MMP-9 and MMP-2 was dependent on the up-stream activation of cathepsin B [71]. Lastly, the pHe-dependent anterograde lysosome trafficking and Cathepsin B secretion were driven by the NHE1 [90].

LOCALIZATION OF NHE1 TO INVASIVE STRUCTURES

One fundamental question that had until recently remained unresolved concerned the cellular localization of the

NHE1 and acidic pHe in driving invasion through the ECM. As stated above, invasion requires increased directed cell motility combined with a remodeling of the extracellular matrix and the organized driving of these processes requires that the assembly of multimolecular complexes be restricted to unique intracellular locations at the cellular site of action. It is now well established that tumor cells have acquired two morphological characteristics to facilitate their increased chemotactic and invasive ability: the migratory leading edge of the cell [91] and the $\beta 1$ -integrin and protease and actin rich plasma membrane structures, called invadopodia, that are involved in directed proteolysis of the ECM [92, 93]. The creation of these specific cellular domains of focal proteolytic action is one of the most intriguing properties of tumor cells and we still know fairly little concerning the interplay of biochemistry and cell structure that underlies their development and function [94].

Since activation of $\beta 1$ integrin recruits proteases to invadopodia and induces membrane protrusive and ECM degrading activity [92, 93], integrin-mediated cell substrate adhesion at point contacts probably constitutes the primary spatial cue leading to the recruitment of ECM-degrading enzymes and formation of a polarized plasma membrane extraflection domain which can penetrate the underlying matrix permitting the focal proteolysis of the ECM and favoring the invasion of the tumor cell. Recent work has demonstrated that NHE1 is localized to invadopodia and its activity has a double function in driving invadopodia formation and proteolytic activity through (i) the acidification of the extracellular per-invadopodia nanospace which is necessary for ECM proteolysis [33] and (ii) the alkalinization of the invadopodia cytosol which causes the release of cofilin from cortactin to stimulate the dynamic process of invadopodia protrusion [95]. This cortactin-directed localization of NHE1 is much like that reported for cortactin in the trafficking and localization of MMPs to invadopodia [96], suggesting a generalized mechanism for the regulated trafficking of the invasive machinery to invadopodia. All together these data suggest that there exists a concordance between NHE1 localization and extracellular acidification, gelatinase/proteinase activity on the cell surface at invadopodia and the formation of the cytoskeleton necessary of human malignant breast carcinoma cells. Interestingly, tumor hypoxia associated with the microenvironment enhances invadopodia formation and cancer cell invasiveness by promoting NHE1 activity through the phosphorylation of serine 703 by p90RSK [28]. Interestingly, in this regard a recent study demonstrated that glycolytic enzymes are enriched into invadopodia [97], leading to localized proton production that can favor local NHE1 activity.

This importance of NHE1 localization in invasion was recently corroborated at the tissue level in rat brain C6 gliomas where NHE1 had a sharp peak expression at the invasive front of the tumor while other pH regulatory proteins (carbonic anhydrase IX, MCT1 and MCT4) were found to be more broadly localized in the interior of the tumor [41].

It has been shown that more rigid ECM stimulates invadopodia formation and proteolysis while a less rigid ECM is conducive to motility [98, 99]. On this basis it has been hypothesized that a tumor cell progresses through a cycle of

NHE1/invadopodia-directed ECM proteolysis followed by NHE1-directed leading-edge pseudopodial motility into the digested, semi-liquid areas and then followed by a new round of invadopodia formation when the cell again encounters a more solid ECM [3, 100]. A fundamental question concerns the mechanisms underlying the cycling of NHE1 function and localization to regulate cytoskeletal dynamics and resulting cell shape during this invasion-motility cycle. There must be a system(s) by which the cell communicates between the different compartments to turn on or off the 'localized' NHE1 or its functional interactions so that the cell can coordinate this complex cycle. It has been hypothesized that members of the ERM family of proteins are the probable physical linkers of the NHE1 to the actin cytoskeleton since

one of the members, ezrin, has been shown to bind to both NHE1 and to actin [101, 102]. A model showing these known and hypothesized relationships is shown in Fig. (3).

NHE1 PHARMACOLOGY

Due to the importance of NHE1 in numerous physiological and pathophysiological processes, a number of inhibitors have been developed. The most part belong to two groups of modifications of the structure of the K^+ -sparing diuretic, amiloride (3,5-diamino-6-chloro-*N*-(diaminomethylene) pyrazinecarboxamide), the first compound found to have inhibitory activity. Amiloride, however, also inhibits the epithelial Na^+ channel ENaC, the Na^+ / Ca^{2+} exchanger (NCX) and the acid sensing cation channel-1 (ASIC-1) which is part

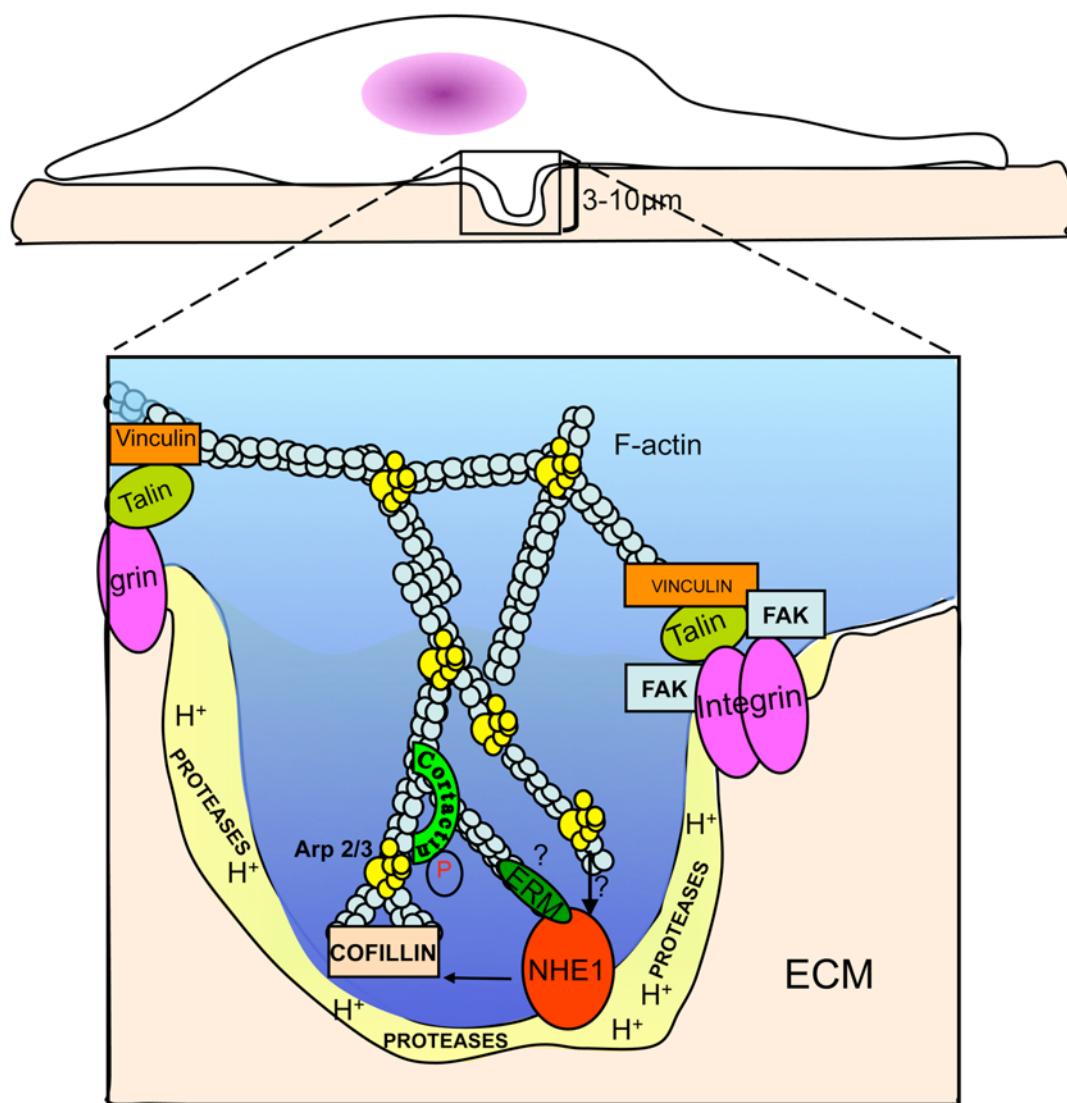


Fig. (3). Role of NHE1 in invadopodia formation and function The insert is a magnification of the cellular extrusion into the ECM call invadopodia. Invadopodia are F-actin-enriched cellular protrusions responsible for ECM degradation whose formation is activated by integrin binding to the ECM and where the proteases cathepsin B, D and L, Urokinase Plasminogen Activator and the matrix metalloproteinases MMP-2 and MMP-9 are released extracellularly while MT1-MMP is associated to the membrane and participates together with cathepsin B in the processing of inactive pro-MMP-2 into active MMP-2. Glycolytic enzymes are enriched in invadopodia, leading to the localized production of protons which are secreted via an active NHE1, resulting in a peri-invadopodial acidification favorable to the activity of the various proteases localized in this sub-cellular region. Furthermore, the NHE1-dependent alkalinization of the invadopodia cytosol results in a phosphorylation of cortactin with the subsequent release of cofilin and the growth of the cytoskeleton within the invadopodia.

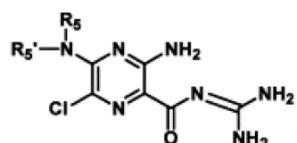
of the ENaC family. Furthermore, while NHE1 is the isoform most sensitive to amiloride, NHE2 is also inhibited and to a lesser extent NHE5 [103].

The first series of other NHE1 inhibitory drugs based on the chemical scaffold of amiloride were designed using double substitutions of the nitrogen of the 5-amino pyrazine derivatives at the R5 and R5' groups (for structures see Table 1, part A) and had a slightly higher inhibitory activity and specificity for NHE1 and very low activity towards NCX and ENaC [104]. Some of the best known and most studied of these pyrazines are DMA (dimethylamiloride; R5: -CH₃ and R5': -CH₃), EIPA (N-ethylisopropylamiloride; R5: -C₂H₅ and R5': -CH(CH₃)₂) and HMA (-(CH₂)₆-).

Somewhat later two sets of alterations gave rise to a new series of inhibitors where the pyrazine moiety of amiloride was substituted with a phenyl ring or a heterocycle pyridine to produce benzoylguanidines (for structures see Table 1, part B). For example, the replacement of the pyrazine ring of amiloride by a pyridine or a phenyl ring improved the NHE inhibitory potency (36- and 54-times more active than amiloride on human platelet NHE1, respectively) [105]. The simultaneous substitution of the 6-chloro by a sulfomethyl with the deletion of the 2-amino or its replacement by a methyl group gave rise to the benzoylguanidine group of inhibitors such as HOE-694 [106], cariporide (HOE-642; R2: -H and R5: -CH(CH₃)₂) [107], eniporide (EMD85131; R2: -CH₃ and R5: -N ring; [108]) and BIIB-513 [109]. These compounds no longer inhibit the ENaC and the Na⁺/Ca⁺ exchanger and became much more selective towards NHE1.

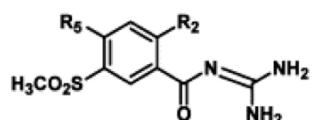
Table 1. Structure of Major NHE1 Inhibitors.

A. Pyrazine derivatives:

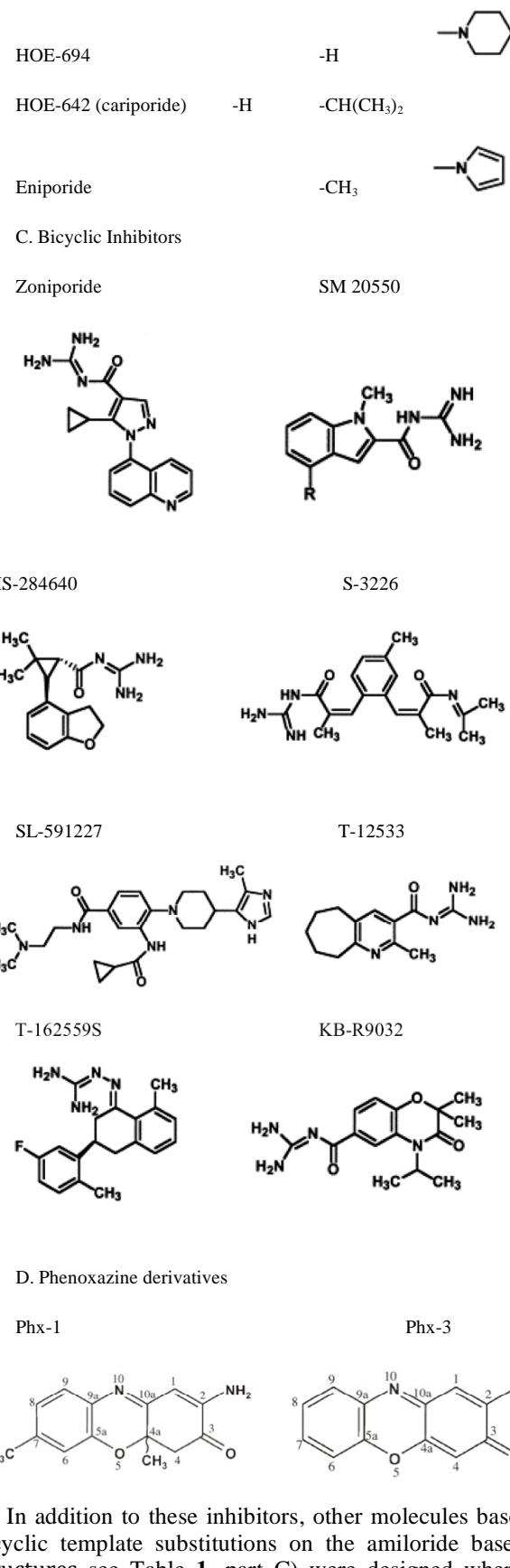


Drug	R5	R5'
Amiloride	-H	-H
DMA	-CH ₃	-CH ₃
EIPA	-C ₂ H ₅	-CH(CH ₃) ₂
HMA		-(CH ₂) ₆ -

B. Benzoylguanidines



Drug	R2	R5



In addition to these inhibitors, other molecules based on bicyclic template substitutions on the amiloride base (for structures see Table 1, part C) were designed where the bicyclic ring was either a quinoleine (zoniporide; [110], an indole (SM-20220; [111], and SM-20550; [112]), a dihydro-

benzofurane (BMS- 284640; [113]) a tetrahydrocycloheptapyridine (TY-12533; [114]) or a tetrahydronaphthalene (T-162559; [115]). All these compounds, except for T-162559, have an unsubstituted acylguanidine group. Recent patented advances have been pentafluorosulfanylbenzoylguanidine substitutions in R1 and R4 described [116] and guanidine derivatives having a condensed tricyclic ring have been described [117]. Further, US7875625 [118] describes a compound obtained by substituting the hydroxyl group on the methyl group at the 9-position of a 9-hydroxymethylcyclohepta[b]pyridine-3-carbonylguanidine amiloride derivative, which had an excellent *in vitro* and *in vivo* NHE1 inhibitory effect while having little degradation of the product in the blood and a very reduced toxic effect on the central nervous system due to low transferability to the brain. An interesting concept has been the conjugation of amino acids and peptides to amiloride such that endogenous proteases cleave and activate them *in situ* has been described [119]. This is the only NHE1 inhibitor patent to date that has included cancer as a therapeutic target for the described product. The authors demonstrated cytotoxic and/or antiproliferative effects on glioma cells and intracerebral glioma xenografts.

Non Amiloride Derived Compounds

There is an additional series of NHE1 inhibitor compounds whose structure is independent of amiloride. (A) One of this is SL-591227 which was the first potent and NHE1 selective non-guanidine inhibitor [104, 120]. (B) the group of Tomoda developed a phenoxazine derivative (2-amino-4,4,4-dihydro-4,7-dimethyl-3H-phenoxazine-3-one (Phx-1), and Phx-3; for structures see Table 1, part D) that is highly selective for NHE1 which stimulated apoptosis in a variety of cancer cell lines [121] and that in animal studies effectively reversed subcutaneous injected adult T-cell leukaemia cell tumor growth without noticeable toxicity (personal communication). (C) Finally, researchers at Bristol-Meyers synthesized a 5-aryl-4-(4-(5-methyl-1*H*-imidazol-4-yl)pyridin-1-yl)pyrimidine analog (compound 9t) that was reported to have a very high inhibitory activity ($IC_{50} = 0.0065\mu M$; i.e. as much as 500-times more potent than cariporide) and much greater selectivity for NHE1 over NHE2 (1400-fold) with a 52% oral bioavailability and a plasma half-life of 1.5 hr in rats [122]. Unfortunately, there have been no further publications utilizing compound 9t either *in vitro* or *in vivo*.

Implications for Therapy

Possible Clinical Exploitation of NHE1 Inhibition

The idea of an acid-base approach to the treatment of cancer dates back from the early 30s [123]. Inhibitors of the amiloride series have been shown effective in retarding tumor development in mice [10] or in rendering chemotherapy more effective [45, 124]. While not being a specific inhibitor of NHE1, amiloride has been used as a cancer therapy in animal models and clinically [125]. A very recent and complete historical review on the use of amiloride in cancer therapy discussed tens of older but still valid animal studies where its use had clear anti-neoplastic effects with few side-effects [125].

Besides amiloride, the only compounds with NHE1 inhibitory activity that have undergone clinical trials are cariporide and eniporide, however these trials were not in the field of cancer but for ischaemic-reperfusion injury. An early study on the effect of cariporide in 100 patients waiting to receive perfusion therapy via primary coronary angioplasty within 6 hours of the onset of symptoms suggested that reperfusion injury could be a target for NHE inhibitors and these results led to further clinical trials to confirm the therapeutic potential of NHE inhibitors [126]. Two were with cariporide: The “Guard During Ischemia Against Necrosis” (Guardian) [127, 128] and “The Na^+/H^+ Exchanger Inhibition to Prevent Coronary Events in Acute Cardiac Conditions” (EXPEDITION) [129]. The “Guardian” trial included a total of 11590 patients with unstable angina or a myocardial infarction who received placebo or different doses (30, 80 and 120mg) of cariporide. There were an early clinical benefit and elevated six month survival rate in only a patient group requiring urgent coronary bypass graft surgery and at a cariporide level of 120mg [127, 128]. There was also a trial utilizing eniporide: “The Evaluation of the Safety and Cardioprotective Effects of Eniporide in Myocardial Infarction” (ESCAMI) [130].

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 [129]. 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 [131]. Clearly, a clinically reasonable approach would be minimize the systemic dose of the drug in order to dissociate the adverse effects, and probably off-targets effects, from the beneficial effects. This could probably already be the case due to the increase in efficacy at low pHe for cariporide described in the next paragraph and is also precisely the idea considered in utilizing the combined therapeutic strategies described below. Interestingly, in this context, 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 [107].

Importantly, the potency of cariporide and some other NHE inhibitors is related to the ionization state of the guanidine residues (Table 2). In this respect, the acidic extracellular pH of tumors (which can be as low as 6.2) will render zoniporide ($pK_a = 7.2$), TY-12533 ($pK_a = 6.93$) and, especially, cariporide ($pK_a = 6.28$) positively charged [104, 110, 114, 115]. Therefore, the acidic tumor microenvironment could turn out to be an advantage in terms of dose-dependent side effects as these compounds would be more efficient at inhibiting NHE1. Indeed, particularly cariporide will be even more active at very low pHe (i.e. $IC_{50} = 22nM$ vs. $120nM$ at pHe 6.2 and 6.7, respectively, [132]).

Therapeutic Implications Using NHE1 Inhibitors

While promising advances in pharmacogenetics have allowed the development of effective agents which will enable personalized cancer chemotherapy to become routine

Table 2. Characteristics of major NHE1 inhibitors.

Drug Inhibitory Potency	IC50 [mM]	pKa
Amiloride	5.3	pKa = 8.78
EIPA	25.1	--
HOE694	--	--
Cariporide	0.03 - 3.4	pKa = 6.28
Eniporide	0.005 – 0.38	--
Zoniporide	0.059	pKa = 7.2
SM 20550	0.010	--
BMS-284640	0.009	--
T-162559 (S)	0.001	--
T-162559 (R)	35	pKa = 8.4
TY-12533	0.017	--
SL-591227	0.003	--
S-3226	3.6	--

Concentrations are given as half maximal inhibitory concentration (IC50) and the pKa as the pH. The table was modified after Masereel *et al.* (93)

for the clinical practice, a major problem facing oncologists is the outstandingly varied efficacy of treatment.

In this respect, there have been new directions in 'pH-based therapies' either singly or in combination. In this context, combination can mean both (i) cocktails of inhibitors directed against the various proteins regulating or orchestrating the reversed pH gradient of tumors and (ii) the strategy of targeting NHE1 in combination with a 'traditional' pharmacological agent against one or more of its up-stream activators.

These strategies have been presented in a review [1] and in a perspective [133] and finally present the promise of a real paradigm shift in cancer treatment towards manipulating the selective forces controlling the dysregulated pH dynamics to reduce both the growth and the metastatic potential of tumors.

Here, we present some of what we believe could be some of the more promising directions in combined strategies.

Growth Factor Receptors

Additional papers showing the potential of this strategy are for the important and common clinically used chemotherapeutic agent, paclitaxel [134], and for the biological-based compound acting against Bcr-Abl, imatinib, where they observed an increased sensitization and, more importantly, a resensitization of leukemic cells to imatinib by co-treatment with amiloride to block the NHE1 [135].

These examples provide other possible combinations of proton transport inhibitors and the new 'biological' targeting of certain receptors. An example is the well known role of EGFR and/or integrins in driving tumor progression and it is well known that both of these classes of receptors stimulate NHE1 activity. As several anti-EGFR compounds (e.g. er-

lotinib) have been approved to inhibit metastasis [136] and an anti-integrin drug (cilengitide) is in Phase II trials [137, 138] while cariporide, eniporide and/or amiloride have passed all clinical phases, a highly potential future direction could be a combinatorial therapy of NHE1 inhibitors with inhibitors of one or both of these receptors.

Anti-angiogenic Therapies

Suppression of tumor angiogenesis is emerging as a new therapeutic approach in several advanced and metastatic cancers [139]. However, in patients with some advanced and metastatic disease, such as metastatic colon cancer or recurrent glioma, treatment with bevacizumab, a monoclonal antibody to vascular endothelial growth factor (VEGF), failed to show an improvement in overall survival duration after an initial improved response in progression-free survival. This relapse probable was due to the development of acquired resistance mechanisms [140, 141]. Resistance has been confirmed in experimental models, in which antiangiogenic therapies while restraining tumor burden initially, might select for more aggressive variants and accelerate progression later by promoting a phenotypic shift to a predominantly infiltrative pattern of tumor progression [142, 143]. Moreover, sustained inhibition of angiogenesis worsens tumor hypoxia as it forces cells to switch to an anaerobic metabolism and increases cell survival, invasion and metastasis [144, 145].

Since hypoxia is part of the tumor metabolic microenvironment and has been shown to hyperactivate NHE1 and consequent invasion [28, 146], and since NHE1 inhibitors are already available (e.g. Cariporide) one might consider designing innovative combination trials with antiangiogenics. Indeed, in addition to being stimulated by hypoxia, VEGF release and therefore, angiogenesis has also been

linked to acidic pH [147] and to the NHE1-dependent changes in pH in that blocking NHE1 reduces the release from the tumor cell [35, 148]. Systemic amiloride treatment also reduced experimentally induced neovascularisation in an animal model; probably through inhibition of NHE1 [149]. For more detailed information please refer to the following review [150].

Hyperthermic Therapy and pH/NHE1

Recently, there has been a renewed interest in treating tumors with hyperthermia (<http://www.cancer.gov/cancer-topics/factsheet/Therapy/hyperthermia> and more than 60 papers in 2011) and there is a group of studies showing that the lowering of pH (almost all by targeting the NHE1) can strongly enhance the thermosensitivity of the cancer cell [151-155]. Therefore, there are very real and important future possibilities for the combined use of proton transporter inhibitors together with hyperthermia.

CURRENT & FUTURE DEVELOPMENTS

While much research shows the link between NHE1 and cancer, further research on the mechanisms by which NHE1 activity is up-regulated in tumor cells is still needed and development of useful therapeutic agents for its selective inhibition in anti-cancer strategy remains a final goal. Based on the significant role of NHE1 in enhancing tumor malignancy and on the extremely high therapeutic potential of NHE1 inhibitors in blocking tumor progression a number of papers showed that blocking NHE1 activity together with more than one type of chemotherapeutic agent greatly sensitizes the cells to their growth inhibition and/or apoptosis [124, 134, 135]. However, even if this targeted therapy approach might increase chemotherapy's efficacy and selectivity (thus reducing toxicity), often a targeted therapy's effects are not durable when the therapy is designed to target a single biological molecule. This is because cellular pathways operate like webs with multiple redundancies or alternate routes that may be activated in response to the inhibition of a pathway.

For this reason, combination therapies are often needed to effectively treat many tumors screened for pertinent pathway dependence. In line with this, the relatively high concentration of growth factors (such as IGF, EGF, PDGF) in tumors and their positive role in NHE1 activation [22, 156-158] represents a perfect platform for a locally NHE1 inhibition, through the combination of the NHE1 inhibitors and the new biological targeting of some of these growth factor-receptors. In this regard, a highly potential future direction would be a multi-combined therapy of NHE1 inhibitors, such as cariporide, with inhibitors of one or more of these receptors, such as EGFR or integrins, having a role in both activating NHE1 and promoting tumor progression. As anti-EGFR agents are currently in clinical use for some cancers and cariporide has passed all clinical phases, the development of a two-drug combination therapy for tumors with abnormal activation of NHE1-and EGFR signaling pathways has real possibilities.

However, we believe that to definitively prove the fundamental of concerted utilization of NHE1 inhibitors, alone or in combination with other forms of chemotherapy and/or biological therapy, in primary, adjuvant and/or neoadjuvant

treatment of different solid tumors in humans, will be required multidisciplinary collaborations from experimental researchers, clinical investigators and industry. Also in this line for age related disorders, is a combination of subthreshold cariporide concentrations combined with inhibitors of angiotension converting enzyme (ACE) described [159]. Another interesting development and the only patent to date that considers the product as a possible therapeutic compound for cancer is the use of the conjugation of amino acids and/or peptides to amiloride to produce a pro-drug such that endogenous peptidases cleave and activate them *in situ* where they can then function [119]. This strategy would render the product functional only in environments rich in proteases, such as tumors and would reduce toxic side-effects. The authors report that the amiloride conjugates exhibit high specificity and potency, low toxicity, and should have a particular activity against hypoxic-ischemic tumor cells (i.e., tumor cells with little or no blood supply) that are not normally killed by conventional therapy.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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