Seminars in Cancer Biology 43 (2017) 35–41

Contents lists available at ScienceDirect

Seminars in Cancer Biology

journal homepage: www.elsevier.com/locate/semcancer

Review

Na^+/H^+ exchanger-mediated hydrogen ion extrusion as a carcinogenic signal in triple-negative breast cancer etiopathogenesis and prospects for its inhibition in therapeutics

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ARTICLE INFO

Article history:
Received 26 December 2016
Received in revised form 9 January 2017
Accepted 10 January 2017
Available online 16 January 2017

Keywords:
NHE1
Proton transport
Triple-negative breast cancer
Metastasis

ABSTRACT

Breast cancer is the leading cause of cancer-related death in women in Europe and North America, and metastasis is the primary cause of fatality in patients with breast cancer. While some breast cancers are quite treatable, the triple-negative breast cancers are more metastatic and resistant to chemotherapy. There is clearly an urgent need for better treatments for this form of the disease. Breast cancer is characterized by genetically complex intra-tumour heterogeneity, particularly within the triple-negative clinical subtype. This complicates treatment options, so the development of specifically targeted chemotherapy for less treatable forms is critical. Dysregulation of pH homeostasis is a common factor in breast tumour cells. This occurs in concert with a metabolic switch to aerobic glycolysis that occurs at the onset of oncogenic transformation. The Na^+/H^+ exchanger isoform 1 (NHE1) is the major pH regulatory protein involved in the increased proton extrusion of breast cancer cells. Its increased activity results in intracellular alkalinisation and extracellular acidification that drives cancer progression. The acidification of the extracellular tumour microenvironment also contributes to the development of chemotherapy resistance. In this review, we outline the role of H^+ as a carcinogenic signal and the role and regulation of NHE1 as a trigger for metastasis. We review recent evidence supporting the use of pharmacological inhibitors of NHE1 as a viable treatment option for triple-negative breast cancer.

Contents

1. Introduction

Breast cancer is the most commonly diagnosed cancer among women [1] and is the leading cause of cancer-related death in women in Europe and North America [2,3]. It will affect approximately 1 in 9 women in their lifetime. In breast cancer, it is the resulting metastasis that is the primary cause of fatality [4,5], with about 50% of all patients showing evidence of metastasis at first presentation [6]. Not all breast cancers are alike. Some are quite treatable and some are much less so. Triple-negative (TN) breast cancers (negative for estrogen, progesterone, and HER2 (ERBB2) receptors) are the most problematic, tending to be more metastatic and resistant to chemotherapy. While triple-negative breast cancer represents only 10–20% of breast cancers [7], this is still a large number of women. Only a minority of women with metastatic

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http://dx.doi.org/10.1016/j.semcancer.2017.01.004
1044-579X/

triple-negative breast cancer survive past 5 years, even with aggressive chemotherapy [8–10]. Despite advances in early detection and innovations in precision surgery aimed to conserve normal breast tissue, treatment strategies for patients with invasive breast cancer usually involve radiation and/or chemotherapy to curb the potential spread of malignant cells to sites beyond the breast. However, in both broad range and targeted chemotherapy regimens, the inherent heterogeneity of breast tumours presents a major challenge to treatment, so much so that breast cancer is categorized as multiple diseases based on subtype differentiation as opposed to one single disease [11]. The development of targeted chemotherapies where the complication of intra-tumour heterogeneity can be negated as an impediment to successful treatment is therefore an attractive and worthwhile prospect. Tumours are complex entities that include genetically and metabolically aberrant cancer cells, genetically stable stromal cells, and infiltrating immune cells. They are functionally dependent on the acidic, hypoxic, and poorly perfused serum-deprived microenvironment that surrounds them for cancer progression to occur [12,13]. Pharmacological targeting of this tumour microenvironment therefore represents an avenue for a putative widespread treatment strategy heretofore unexplored in great depth. pH homeostasis in cancer cells and, consequently, in the tumour microenvironment, is regulated by multiple pH regulatory proteins, chief of which is the Na^+/H^+ exchanger NHE1. NHE1 becomes activated by intracellular acidification in normal cells [14] but this process becomes dysregulated during oncogenic transformation [15]. In cancer cells, elevated NHE1 activity results in increased H^+ extrusion that leads to cellular alkalinisation and the establishment of the acidic extracellular tumour microenvironment [13]. Over the past decade, experts in the field of pH regulation and homeostasis in tumour cells have realized the potential of targeting the tumour microenvironment pH as an anti-cancer therapy [16–19]. In this review, we present evidence to highlight the role of H^+ ions as a key carcinogenic signal, regulating both intracellular pH of tumour cells and the extracellular pH of the tumour microenvironment. Several NHE1 inhibitors that curb H^+ extrusion and Na^+ uptake, and consequently re-establish acid-base balance in the tumour microenvironment, have been assessed for their efficacy in treatment in cancer models. We summarize recent progress and discuss options for tumour-targeted drug delivery.

2. Breast tumour heterogeneity: a hindrance to targeted therapy

Breast cancer is widely characterized as being of three major subtypes based on the expression of estrogen and/or progesterone receptors (ER+, PR+), an amplification of human epidermal growth factor receptor-2 (HER2++) expression, or the absence of all of these receptors (triple-negative breast cancer, TNBC) [20]. This taxonomy is based on histotyping; further classification by gene expression profiling reveals five intrinsic clinical subtypes: luminal A, luminal B, HER2-amplification, basal-like, and normal-like [21,22]. The luminal subtypes are hormone receptor-positive, while the basal-like subtype primarily exhibits a triple-negative profile [20]; the normal-like subtype is now known to have been identified from a mixed sample of breast carcinoma and normal breast cells [22,23]. Determination of treatment strategies depends on these characterizations: endocrine therapy targeted to hormone receptor-positive subtypes, and anti-HER2 targeted therapy (*e.g.* trastuzumab) for the HER2++ subtype; these targeted therapies generally translate into improved treatment outcomes. Treating triple-negative basal-like breast cancer is complicated by the inability to target hormone or HER2 receptors. Nonspecific cytotoxic chemotherapy therefore remains the cornerstone of treatment strategies for these patients and, while outcomes can initially be favourable, overall prognoses and patient survival rates are poor [23–25], and the development of chemotherapy resistance is problematic [11].

Amongst invasive breast cancers, the incidence of triple-negative breast cancer (TNBC) ranges from 15 to 20% [25]. TNBC is the most clinically aggressive of all the subtypes, with high recurrence rates in the early years post-treatment, and an increased tendency towards distant metastasis, higher grades, and large tumours with a greater infiltration of lymphocytes [23,26]. TNBC also occurs at higher frequencies in younger patients, those carrying the BRCA1 mutation, and those of African-American and Hispanic origin [23,27,28]. In recent years, gene expression profiling revealed further complications to therapy options by identifying six distinct subtypes of TNBC tumours: *1,* a highly proliferative basal-like subtype with an increased expression of cell cycle and DNA damage response genes (BL1); *2,* a second basal-like subtype with elevated expression of growth factor receptors and myoepithelial markers (BL2); *3,* an immunomodulatory (IM) subtype typified by an upregulation in genes involved in immune processes and associated signalling pathways; *4,* the mesenchymal (M) and *5,* mesenchymal stem-like (MSL) subtypes characterized by an enrichment of Rho-mediated cell motility pathways, and enhanced cell differentiation pathways and interactions with extracellular matrix receptors; and *6,* a luminal androgen receptor (LAR)-expressing subtype that, while being ER-negative, has gene ontologies highly enriched in hormonal regulation and androgen receptor signal pathways [8]. While this more detailed TNBC taxonomy may lend itself to increased avenues in the search for targeted therapies, the incredible level of potential genetic heterogeneity within a single TNBC breast tumour (intra-tumour heterogeneity) can significantly limit the efficacy of current cytotoxic chemotherapies. In some cases, this could be because certain drugs are ineffective against a particular tumour cell subtype, enabling cell survival and the potential for metastasis. Once metastasis does occur, surgery and radiotherapy are no longer feasible options. Thus, a chemotherapeutic strategy that is less cytotoxic than routinely used chemotherapy agents but is still effective enough to target tumour cells irrespective of their gene profiles would be a major step forward in the battle against triple-negative breast cancer.

3. H^+ ions as a carcinogenic signal

In their seminal review, Hanahan and Weinberg [29] identified the unifying hallmarks of all cancers as their ability for: sustained proliferation, clonogenic replication, upregulation of tumour suppressor genes, resisting apoptosis, inducing angiogenesis, and initiating invasion and metastasis. Since then, the development of novel chemotherapies has aligned with targeting the molecular mechanisms underpinning many of the above traits. A decade later, the same authors updated the list adding that some, if not all, cancers were also defined by their ability to evade immune destruction and their capacity for reprogramming cellular metabolism to promote neoplastic proliferation [30]. This reprogramming involves a switch in glucose metabolism from the oxidative phosphorylation that occurs in normal cells to aerobic glycolysis in cancer cells, as was first observed by Otto Warburg [31,32]. Initially, it was thought that the hypoxic conditions within growing tumours limited the more energy efficient oxidative phosphorylation in favour of glycolysis, but this energy inefficient metabolic pathway still persists in cancer cells even when oxygen is present [33]. Indeed, the switch to aerobic glycolysis in leukemic and lung tumour cells occurs despite exposure to oxygen in the bloodstream and lungs respectively [34–37]. Commonly, though, hypoxic conditions can activate hypoxia-inducible factors 1 and 2 alpha (HIF-1α/2α) transcription factors that can independently upregulate glycolysis

[30,38]. Numerous other allosteric factors can also influence glycolysis: hormones, oncogenes, oxygen, temperature, by-products of metabolism, and ions [reviewed in [39]]. Of these, H^+ ion concentration (and, as a consequence, pH) is arguably the most significant, whereby elevated H^+ extrusion results in an alkaline intracellular pH that drives aerobic glycolysis, while increased H^+ uptake gives rise to a more acidic intracellular pH that drives oxidative phosphorylation [39,40]. However, despite evidence supporting the fact that NHE1-mediated intracellular alkalinisation is an early event in oncogenic transformation [15,41,42], the idea that the metabolic switch to aerobic glycolysis in cancer cells is primarily due to perturbed H^+ extrusion resulting in intracellular alkalinisation, is still controversial.

4. NHE1: the major cellular regulator of pH homeostasis

There are ten known isoforms of the *SLC9A* solute carrier family of ion transporters encoding the mammalian Na^+/H^+ exchangers (NHEs), including the ubiquitously expressed NHE1 (*SLC9A1*) [43]. NHE1 is a plasma membrane-bound glycoprotein comprised of 815 amino acids [44]. The precise crystal structure of the full-length NHE1 protein has not yet been resolved but topology models and cysteine accessibility experiments [45–47] reveal a hydrophobic transmembrane NH_2-terminal domain of 12 transmembrane segments (amino acids 1–500) through which ion exchange occurs. This is followed by a hydrophilic COOH-terminal cytosolic domain (amino acids 501–815). The cytosolic domain regulates ion flux and its actions are modified by phosphorylation and the binding of intracellular proteins and lipids [44,48]. ATP-binding to NHE1 is necessary for its activation, but NHE1 does not require the energetic input from ATP hydrolysis for transport. Proton (H^+) extrusion uses the energy from the inwardly directed sodium (Na^+) gradient [49]. Intracellular H^+ ions are extruded in a 1:1 electroneutral exchange for extracellular Na^+. Ion exchange is dependent on the transmembrane concentration gradients for both Na^+ and H^+ and the directionality of ion exchange is reversible. When a more alkaline intracellular pH is reached, a "proton-sensor" site results in cessation of the NHE1 activity. NHE1 activity therefore serves the important function of preventing intracellular acidosis due to an accumulation of excess intracellular H^+, a process that has immense relevance in cancer cells. NHE1 is also the major route for Na^+ uptake into cells and its activity, when coupled with the uptake of Cl^- and water, therefore also controls cell volume and shape [50].

In addition to intracellular protons, NHE1 can be activated by external factors like hormones and growth factors. The activation of several classes of cell surface receptors (*e.g.* receptor tyrosine kinases, G protein-coupled receptors, and integrins) can modify the activity the exchanger. They shift the response to intracellular protons so the protein is more active at more alkaline intracellular pH. Receptor-mediated intracellular signal kinases can either directly or indirectly activate NHE1 *via* phosphorylation of specific amino acids in its C-terminal domain. NHE1 can also be activated by the binding of intracellular signal proteins and lipids to the C-terminal regulatory domain [43,48,51]. While the transmembrane domain of NHEs are highly conserved across the different isoforms, the cytosolic domain exhibits considerable variability. Deleting the C-terminal tail of the protein does not block ion exchange but does affect proton-sensing and activation of the protein [52]. Many of NHE1′s interactions with its lipid and protein binding partners tend to be on the C-terminal domain proximal to the membrane, while the distal end of the tail, spanned by amino acids 636–815, is where the majority of phosphorylation sites are located [48].

Several different serine/threonine kinases can activate NHE1 by phosphorylation, in addition to the exchanger's activation by its intracellular binding partners (extensively reviewed in [48,51,53]. Examples of kinase-mediated activators of NHE1 include: extracellular signal regulated kinases ERK1/2 which are important in the activation of NHE1 in response to intracellular acidosis, and for which NHE1 serves as a signal scaffold [54,55]; AKT (protein kinase B) which activates NHE1 in response to insulin and platelet-derived growth factor in fibroblasts [56], but inhibits NHE1 activity in cardiomyocytes [57]; β-Raf which phosphorylates NHE1 at amino acid threonine 653 [58]; and p90 ribosomal S6 kinase ($p90^{RSK}$) which phosphorylates NHE1 at serine 703, activating the exchanger in response to serum [59]. Adaptor protein 14-3-3 binds NHE1 at the phosphorylated serine 703 residue and prevents its dephosphorylation [60].

NHE1 activity can also be regulated by its interactions with calcium-binding proteins like calmodulin and calcineurin homologous protein CHP 1 and 2, and tescalcin (CHP3). A key regulator of NHE1 activity is autoinhibition via a high affinity calmodulin-binding domain in the region between amino acids aspartic acid 626 to threonine 653 of the NHE1 cytosolic tail. The Ca^{2+}-binding protein calmodulin binds to NHE1 at a high (or a secondary low) affinity binding site. Ca^{2+}-calmodulin binding is thought to release an autoinhibitory interaction with the proton modifier site of the transmembrane domain [61]. The CHP isoforms increase NHE1 localization at the plasma membrane and bind to a juxtamembrane region of NHE1′s cytosolic tail. This increases activity by stabilizing more protein at the cell surface [62].

NHE1 has also been shown to bind to carbonic anhydrase II, which is involved in acid-base regulation [33]. In addition, NHE1 is tethered to the actin cytoskeleton by binding to ezrin-radixin-moesin (ERM) proteins, an association that aids in the maintenance of cell shape [63].

Though this review primarily focuses on NHE1, we acknowledge that there are other key regulators of pH homeostasis in tumour cells (some of which are discussed in greater detail elsewhere in this edition), including: vacuolar-type proton pump ATPases (V-ATPases), monocarboxylate cotransporters (MCTs), carbonic anhydrases (CAs), and sodium-bicarbonate cotransporters (NBCs) [33]. The first three classes of transporters are involved in H^+ extrusion [64], but all have been implicated in cancer progression [65]. These H^+ transporters interact with NHE1 in cancer cells and contribute to H^+ signalling; however, we present evidence that, in triple-negative breast cancer, particularly of the basal-like clinical subtype, it is the manipulation of NHE1 activity that shows the most promise in terms of therapeutic gain. Additionally, earlier evidence has shown that NHE1 plays a more critical role in pH regulation in breast cancer cells compared with other cell types. In most breast cancer types, NHE1 may be more critical under conditions associated with tumours, such as with larger acid loads [66,67].

5. Beyond "housekeeping": NHE1′s definitive role in triple-negative breast cancer

A role for NHE1 in cancer was first reported almost thirty years ago by Pouysségur's group who observed that NHE1-deficient hamster lung fibroblasts formed fewer xenograft tumours in athymic nude mice in comparison to cells expressing the wild-type exchanger [68]. The first firm evidence that dysregulation of pH homeostasis may be a unique feature of tumours came from the observation that the extracellular pH around malignant breast tumour cells from pleural effusions acidified their culture media up to 200-fold more than normal mammary cells [69]. In general, cancer cells have been shown to have a reversed pH gradient with an intracellular pH (pH_i) ranging from 7.2 to 7.7 and an extracellular pH (pH_e) ranging from 6.2 to 6.8. This compares with normal cells that have a pH_i ranging from 6.9 to 7.1 and a pH_e between 7.2 and 7.4 [13,70]. In NIH3T3 murine fibroblasts and

human keratinocytes, this kind of early cytoplasmic alkalinisation, as a result of E7 oncogene-induced transformation, was found to be a direct result of an increase in NHE1 activity. The intracellular alkalinisation was a precursor to the metabolic reprogramming of glucose metabolism to aerobic glycolysis (the so-called "Warburg Effect") that is often observed in cancer cells [15]. The concept of metabolic reprogramming as a reflection of upregulated glycolysis and hypoxia-induced transcription has been widely acknowledged for years, but the idea that dysregulated pH homeostasis and perturbed H^+ dynamics may also play key roles in cancer progression has been largely overlooked. Only of late is this pH-centric paradigm of cancer progression gaining both weight and momentum. Consider how NHE1 becomes activated by external factors in normal cells *versus* in cancer cells: in normal cells, growth factors and hormones in serum activate NHE1, whereas it was shown that, in hormone receptor-positive MCF-7 breast cancer cells, it is serum deprivation (and thus, hormone and growth factor deprivation) that activates NHE1 [71,72]. This is not due to changes in Na^+ kinetics or NHE1 expression, but is due to an alkaline shift in the pH_i activation curve [72]. In highly invasive triple-negative basal-like MDA-MB-231 breast cancer cells, there is a similar pattern of NHE1 activation by serum deprivation [53]. NHE1 activation in low serum in MCF-7 and MDA-MB-231 cells is initiated through a sequential RhoA/p160ROCK/p38MAPK signal cascade gated by protein kinase A (PKA)-mediated phosphorylation of RhoA [73]. Serum deprivation in both these cell types resulted in a redistribution of NHE1, RhoA, and phospho-RhoA to the leading edges of cells, priming the cells for directed migration [73]. NHE1 was also found in the invadopodia of invasive MDA-MB-231 cells where it co-localizes with phosphorylated cortactin, an invadopodial marker [74]. NHE1 activity acidifies the peri-invadopodial space, establishing the optimal pH for the proteolytic activity of serine proteases and matrix metalloproteinases that aid in the digestion of extracellular matrix at the leading edge of invading cells [75,76]. NHE1-mediated H^+ extrusion therefore facilitates every step of metastasis from the primary tumour. This includes: directed migration to exit the tumour; the subsequent dissociation of cells from the extracellular matrix; cell-matrix interactions with integrins, selectins, and cadherins, that are important in focal adhesion at the leading edge; and focal digestion of the extracellular matrix. This culminates in intravasation of invading tumour cells into the bloodstream [77].

Various studies have examined the importance of NHE1 and other pH regulatory proteins in breast cancer cells. In one study, 3-D spheroids of MCF-7 and MDA-MB-231 breast cancer cells were cultured in extracellular matrix to mimic conditions in the tumour microenvironment and to assess how spheroid growth affects the spatial distribution of acid-extruding transporters involved in pH regulation [78]. They examined NHE1, the Na^+/HCO_3^- cotransporter NBCn1, and the lactate/H^+ co-transporters of the monocarboxylate cotransporter family, MCT1 and MCT4. In luminal A MCF-7 spheroids, NHE1 and MCT4 were evenly distributed while MCT1 expression increased at the spheroid core. NBCn1 was highly expressed in the periphery. Triple-negative MDA-MB-231 spheroids showed a similar distribution except for MCT1, which these cells do not express. Stable but partial knockdown of MCT1 (↓75%) and NBCn1 (↓80%) decreased MCF-7 spheroid growth, but only a complete knockout of NHE1 had a similar effect. In contrast, in triple-negative MDA-MB-231 cells, both transient and stable knockdown of NHE1 (↓85%), and complete knockout of NHE1 by CRISPR/Cas9 significantly reduced spheroid growth [78]. The work demonstrated that there are distinct expression and localization patterns of acid-extruding transporters that are cell specific. Our own studies have shown that, when NHE1 is knocked out from highly invasive MDA-MB-231 cells, there are marked decreases in migration and invasion through the extracellular matrix in comparison with cells containing wild-type NHE1. Additionally, when NHE1 knockout cells were injected into athymic nude mice, xenograft tumour growth was almost completely abolished [71].

To determine how NHE1 regulation affects its role in migration and invasion in MDA-MB-231 cells, we generated a series of mutations to the NHE1 protein *via* mutation of the regulatory cytosolic domain. Phosphorylatable serine 703 was replaced with a non-phosphorylatable alanine (S703A). This prevents its activation *via* phosphorylation by $p90^{RSK}$ and, subsequently, prevents 14-3-3 binding to the phosphorylated serine residue. Another mutation was in the ERK1/2 phosphorylation sites, where serine residues 766, 770, and 771 were replaced by alanines (SSSA). A third mutation modified the autoinhibitory (calmodulin-binding) region of the exchanger to prevent autoinhibition, thus resulting in a constitutively active exchanger (1K3R4E). Here, positively charged lysine 641 (1K) and arginines 643, 645 and 647 (3R) were replaced by negatively charged glutamic acid (4E) [79]. Cells expressing SSSA-NHE1 were not very different from the 231-wtNHE1 cells, except for having lower rates of migration and colony formation. In contrast, the constitutively active 1K3R4E-NHE1 cells exhibited a much greater degree of metastatic potential (faster migration, invasion, and increased spheroid growth in extracellular matrix) than cells with wild-type NHE1. Clearly, these data indicate a reliance on NHE1 and its various intracellular regulators for its functional effects in TNBC cells. Of all the mutations to NHE1, though, S703A was the most remarkable. Cells expressing S703A-NHE1 exhibited a drastic change in morphology, seemingly reverting from their characteristic mesenchymal morphology to a more epithelial-like phenotype. This change was accompanied by a dramatic decrease in metastatic potential, as demonstrated by many parameters including: low rates of migration, low invasion through extracellular matrix, poor anchorage-dependent clonogenic growth, poor anchorage-independent colony growth in soft-agar, and reduced 3D-spheroid growth in extracellular matrix. In fact, S703A-NHE1 cells behaved much like 231-NHE1ko cells, though the same morphological changes were not observed in the NHE1-knockout cells [79]. We noted that the observed changes in morphology in the S703A-NHE1 cells correlated with a marked downregulation in both the mRNA and protein expression of vimentin intermediate filaments. Vimentin is usually upregulated in the epithelial-to-mesenchymal transition that occurs in tumour cells [80]. In S703A-NHE1 cells, we hypothesized that the mutation may have triggered a reverse process of mesenchymal-to-epithelial transition, resulting in cells that had reduced potential for metastasis. Presently, it is unclear why the change in morphology was so drastic. It could be that replacement of serine 703 with alanine also prevents the binding of 14-3-3 to NHE1. Interestingly, 14-3-3 is also known to bind phospho-vimentin, and it is important in the dephosphorylation and disaggregation of vimentin [81]. We have not yet been able to demonstrate a direct association between NHE1 and vimentin, but we have shown that several isoforms of 14-3-3 bind to and co-immunoprecipitate with NHE1 [82]. Whether and how 14-3-3 links NHE1 to the vimentin intermediate filament cytoskeleton remains unclear.

In another study, we used bioinformatics to assess gene profiles of primary patient breast tumours using the Cancer Genome Atlas [82]. We found that *NHE1* mRNA expression in primary breast tumours (N = 1062) was significantly higher compared to normal associated breast tissue (N = 113). However, when this database was reanalysed for tumour subtype, luminal A (N = 421), luminal B (N = 192), and HER2-amplified (N = 67) tumours did not show a difference in *NHE1* mRNA expression compared to normal associated breast tissue (N = 23). Intriguingly, NHE1 mRNA expression in basal breast tumours (N = 141) was significantly lower than in normal breast tissue [82]. This trend is comparable to the NHE1 protein expression we observed in luminal A MCF-7 cells, which expressed higher levels of NHE1 protein, but had lower metastatic

capacity, compared to basal-like, triple-negative MDA-MB-231 and MDA-MB-468 cells [71]. It should be noted that despite the lower protein expression of NHE1 in both triple-negative cell types, both were more migratory and invasive than MCF-7 cells. We believe that the upregulation of NHE1 activity in these cells, particularly in the low-serum culture conditions that mimic the tumour microenvironment, is sufficient for the increased rates of migration and invasion. We suggest it is the activity of the protein, and not its amount per se, that is critical. In this regard, we found that active (phosphorylated) $p90^{RSK}$, which regulates NHE1 by phosphorylation of serine 703, was positively correlated with NHE1 in some triple-negative tumour types [82]. Further studies in the area could examine the correlation between NHE1 regulation and metastatic behaviour.

6. NHE1 inhibition as a chemotherapy strategy: the challenge of targeted drug delivery

Elevated H^+ produced as a result of upregulated aerobic glycolysis in cancer cells needs to be extruded in order for cells to survive potential intracellular acidosis. The resultant H^+ extrusion acidifies the microenvironment around tumours, setting up a perfect milieu for the selection and survival of an aggressively metastatic cancer cell phenotype. This is particularly true in the case of triple-negative breast cancer, for which targeted therapies do not exist and, where intra-tumour heterogeneity further complicates treatment with cytotoxic chemotherapy. Unfortunately, the highly acidic pH of the tumour microenvironment is also a major contributing factor in the development of resistance to chemotherapy drugs, most of which are mildly basic and therefore neutralized in acid [19]. Chemotherapy agents that are weakly basic can also become protonated in an acidic environment, preventing their entry into cells to reach their target sites [70,83]. The ability to modulate microenvironmental pH has the potential to curb the development of a metastatic cell phenotype as well as provide a means to avoid the development of multi-drug resistance, at least in the case of weakly basic chemotherapy agents.

Upregulated glycolysis is the most significant common denominator determining the tumourigenic phenotype of most cancer cells, but targeting the aberrant H^+ dynamics underpinning this metabolic shift has rarely been considered a therapeutic option [84]. pH homeostasis in normal and tumour cells is regulated by a variety of plasma membrane-bound transporters and pumps including the: Na^+/H^+ exchangers, sodium bicarbonate co-transporters, anion exchangers, monocarboxylate transporters, carbonic anhydrases, and vacuolar proton pump ATPases (V-ATPases) [Reviewed in Refs. [33,64]]. While combinatorial approaches targeting more than one transporter may be an option, to the best of our knowledge, there is currently no evidence to suggest pharmacological inhibition of multiple transporters would have an additive anti-cancer effect and this could be an area for further study. NHE1 is the best studied of the above transporters in cancer. We focus on NHE1 because its role in cancer – and triple-negative breast cancer in particular – has been shown to be significant. This significance seems to occur at virtually every step in the metastatic pathway. This includes oncogenic transformation and dysregulation of pH homeostasis that leads to neoplastic proliferation, directed migration, invasion through extracellular matrix, colony and spheroid growth *in vitro*, and xenograft tumour growth *in vivo*. Inhibition of NHE1 activity could potentially moderate intracellular alkalinsation and neutralize the acidic extracellular pH of the tumour microenvironmental as a means to slow or stop metastasis and cancer progression. This strategy would also impede the development of resistance to routinely used chemotherapies since protonation of weak bases would not readily occur in a more neutral pH.

The use of pharmacological inhibitors of NHE1 as anti-cancer therapies was recently reviewed [39,85]; in summary, they comprise of two main chemical classes: amiloride and its derivatives, and benzoyl (acyl) guanidines. Amiloride, a potassium-sparing diuretic, was the earliest developed NHE1 inhibitor, and the only drug clinically used in humans. It inhibits NHE1 activity by binding to its transmembrane domain [18]. Sustained use as a diuretic is tolerated well. There are occasional reports of it achieving remission when used in patients with cancer [39], but because of its of low potency and specificity towards NHE1 it seems unlikely to be a first line of treatment. Amiloride's more potent derivatives include 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) and 5-(*N*,*N*-hexamethylene)-amiloride (HMA). These have strong anti-proliferative and pro-apoptotic effects in cancer cells *in vitro*, but they have not been used or tested clinically. Cariporide and eniporide, both representative of the benzoyl guanidine class of NHE1 inhibitors, are non-amiloride-based drugs to be used in human clinical trials; however, these were tested for treatment of ischemia-reperfusion injury in cardiac disease [39]. Unfortunately, cariporide had cerebrovascular side effects [85] that seem to be related to the high dose used and the method of intravenous administration used [86]. This however should not preclude preclinical and clinical studies in cancer patients and bedside oncology especially with different dosing and methods of administration. Other compounds also have promising potential. Some are well absorbed by the gastrointestinal tract and can be given orally such as compound 9 t [85,87].

We used HMA and EMD87580 (2-methyl-4,5-di-(methylsulfonyl)-benzoyl-guanidine) in combination with paclitaxel to determine whether pharmacological inhibition of NHE1 could amplify the anti-cancer effects of paclitaxel. We found that, in triple-negative MDA-MB-231 and MDA-MB-468 cells examined in culture, low dose paclitaxel (1 nM) was effective in reducing the rates of migration and invasion in the presence of low doses of EMD87580 (10 μM) and HMA (10 nM). The combination of paclitaxel plus NHE1 inhibitor, was significantly more effective than either paclitaxel or NHE1 inhibitors alone [71]. This combinatorial approach may present a viable avenue for the use of NHE1 inhibitors as co-adjuvants to chemotherapy; however, further characterization of this effect is necessary, including in *in vivo* experiments. Though slow, progress on the use of NHE1 inhibitors as either anti-cancer therapeutics or co-adjuvants to chemotherapy has been steady. We have recently tested a new, more selective and potent NHE1 inhibitor, KR-33028, [88] in MDA-MB-231, MDA-MB-468, and Hs578T cells, which are representative of multiple TNBC subtypes. It was very effective in reducing rates of migration, invasion and clonogenic colony growth, all of which are characteristics that are predictive of metastasis in patients, A related approach to decrease NHE1 activity is to affect NHE1 regulation. To this end, we tested the effect of $p90^{RSK}$ inhibitor BI-D1870 on TNBC cells. BI-D1870 caused significant decreases in rates of migration and invasion through extracellular matrix [79].

7. Perspectives

In 2015, there were 2.4 million breast cancer cases worldwide, with 0.5 million deaths related to the disease [89]. Patient fatality is usually due to metastasis or development of resistance to chemotherapy. In the case of triple-negative breast cancer, treatment is further confounded by intra-tumour heterogeneity and the aggressively metastatic nature of the cancer itself. The issue of intra-tumour heterogeneity may be approachable, at least in some cases, by using a pH-centric paradigm of cancer progression.

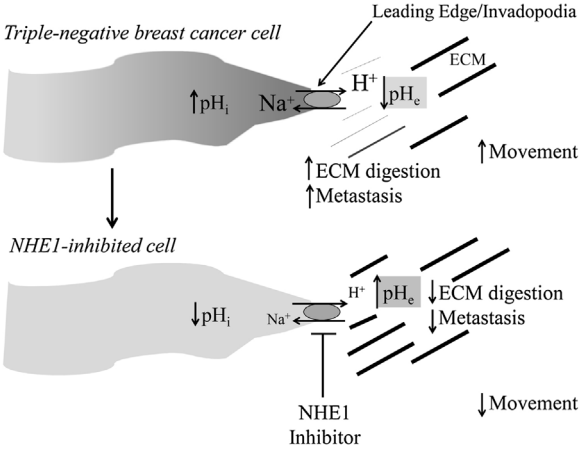

Fig. 1. Schematic diagram of hypothetical process whereby NHE1 inhibition reduces metastasis in triple-negative breast cancer cells. In an uninhibited cell, the basal elevated NHE1 activity increases intracellular pH and decreases extracellular pH. NHE1 tends to become redistributed to the leading edge of migrating cells, and to invadopodia of invading cells, creating a localized acidic microenvironment at these sites. This facilitates extracellular matrix (ECM) digestion that promotes cell movement and metastasis. Inhibition of NHE1 reverses the intracellular to extracellular gradient and reduces these effects, resulting in decreased cell metastasis. Darker shading indicates more alkaline pH.

Here, it is the acidic microenvironment pH that, not only drives the selection of an aggressive, more metastatic cancer cell phenotype, but also contributes to the development of chemotherapy resistance. Targeting the pH_e of the tumour microenvironment and the elevated pH_i of solid tumours with pharmacological inhibition of NHE1 has incredible potential in theory but lags when it comes to practice. Fig. 1 illustrates a summary of an idealized scenario whereby NHE1 inhibition of breast cancer cells causes reduced acid extrusion. This results in a less acidic extracellular pH, a more acidic intracellular pH and, thus, reduced cell movement, migration, and metastasis of triple-negative breast cancer cells. So far, amiloride, which is arguably the least selective and potent of NHE1 inhibitors, is the only one used – with very limited success – to treat cancer patients. Perhaps an impediment to the successful use of amiloride as an anti-cancer agent is current limitations in drug delivery; amiloride is administered orally. Ideally, and particularly in the case of triple-negative breast tumours, tumour-targeted drug delivery would be the most effective treatment route. While this may not yet be possible in practice, there are multiple new drug delivery systems to consider, including nanoparticles, micelles, and extracellular vesicles. In TNBC, the focus is on the development of targeted chemotherapies. We instead suggest that a pH-centric treatment approach with tumour-targeted drug delivery may be efficacious at least in types of breast cancer such as triple-negative breast cancer, where NHE1 appears to play a more critical role.

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