

# A Periodic Regulation of the Na<sup>b</sup>/H<sup>b</sup> Exchanger (NHE1) and PH values in Breast Cancer Metastasis

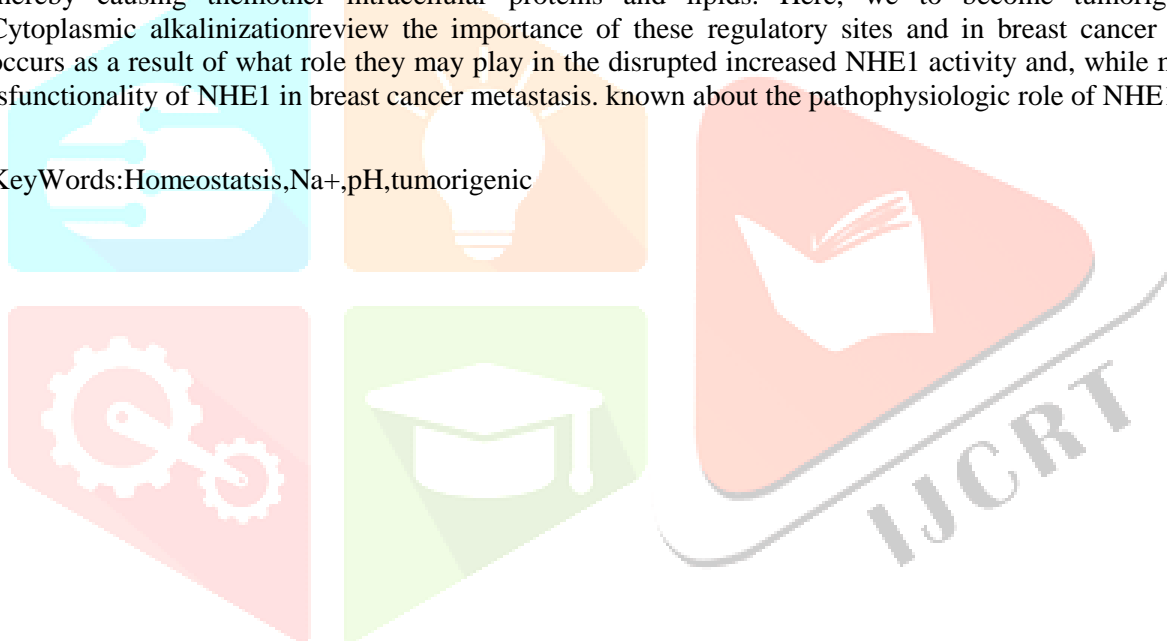
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**Abstract:** The pH gradient in normal cells is tightly in tumor progression with regard to ion flux, the controlled by the activity of various pH-regulatory regulation of its activity on a molecular level is membrane proteins including the isoform protein only recently becoming evident. The membrane of the Na<sup>b</sup>/H<sup>b</sup> exchanger (NHE1). NHE1 is domain of NHE1 is sufficient for ion exchange.

constitutively active in a neoplastic. However, its activity is regulated through the microenvironment, dysregulating pH homeostasis phosphorylation of key amino acids in the and altering the survival, differentiation, and cytosolic domain as well as by its interaction with proliferation of cancer cells, thereby causing them other intracellular proteins and lipids. Here, we to become tumorigenic. Cytoplasmic alkalinization review the importance of these regulatory sites and in breast cancer cells occurs as a result of what role they may play in the disrupted increased NHE1 activity and, while much is functionality of NHE1 in breast cancer metastasis. known about the pathophysiologic role of NHE1

**Key Words:** Homeostasis, Na<sup>+</sup>, pH, tumorigenic



## Introduction

Dysregulation of the pH gradient in and around cancer cells is a crucial step in tumor progression leading to metastasis. Abnormal increased proliferation of cells, loss of cell-cell contact, and detachment from the extracellular matrix results in a tumor metabolic microenvironment that is hypoxic, acidic, and deprived of serum (1). Extracellular acidification around tumor cells can be attributed to disrupted pH homeostasis as these cells adapt to the harsh extremes of the neoplastic milieu. Normal cells may undergo apoptosis under these conditions; however, cancer cells survive by manipulating and exploiting a host of ion exchangers including NHE1, the most common isoform of the Na<sup>+</sup>/H<sup>+</sup> exchanger family that is ubiquitous to all mammalian cells (2). The pathophysiologic function of NHE1 in various cell types is well-documented (3), but of particular interest is that proton extrusion plays a pivotal role in cell migration, which, in cancer, is necessary for tumors to invade and metastasize at sites distant from the primary tumor

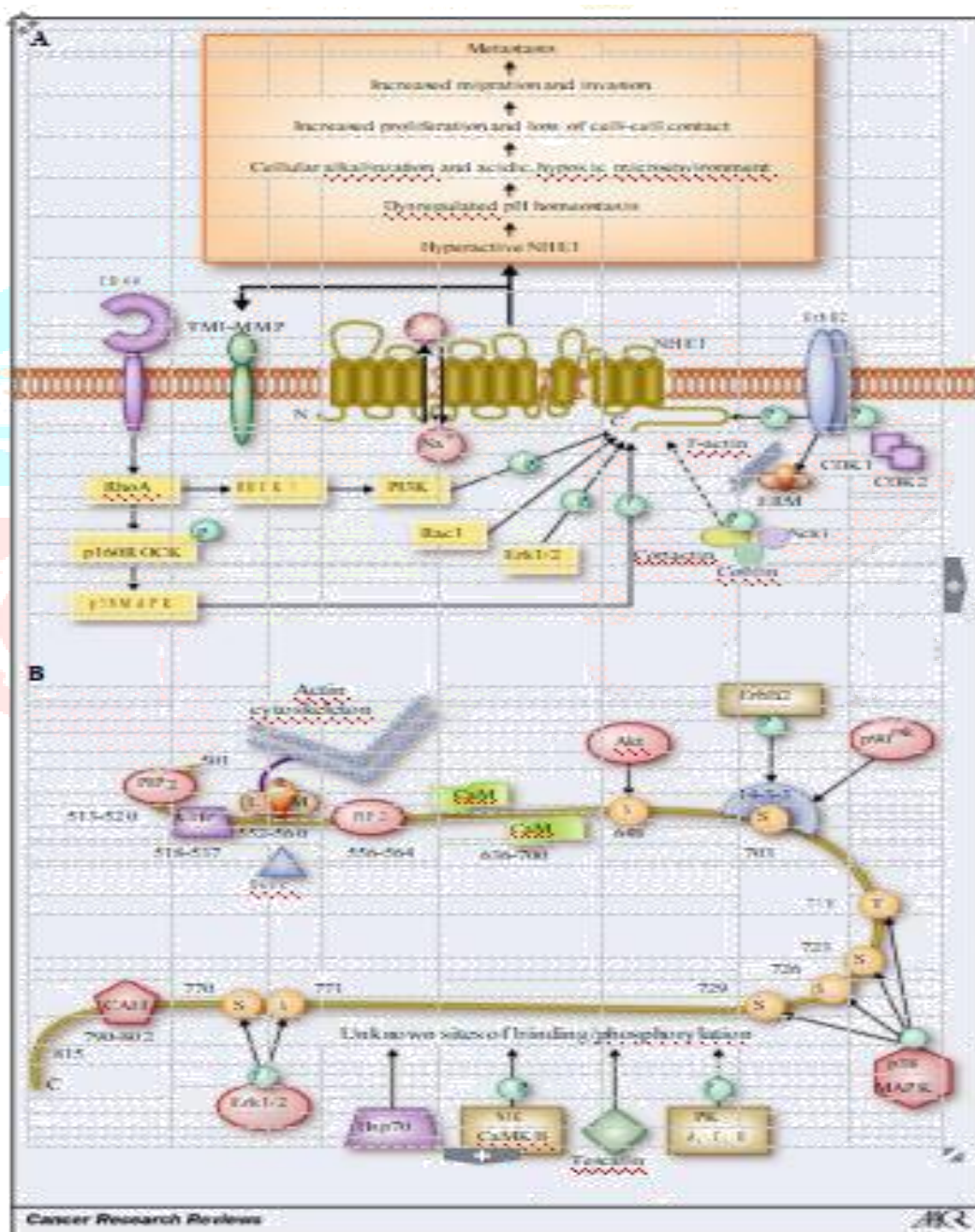
(4). In breast cancer, it is suggested that dysregulation of NHE1 activity is the predominant factor leading to tumor metastasis (1); however, this claim remains to be fully substantiated. In this review, we discuss how the multiplex regulation of NHE1 activity impinges on breast cancer disease progression and current treatment regimes.

Human NHE1 is a membrane protein that is 815 amino acids in length, with residues 1 to 500 comprising the membrane domain and residues 501 to 815 comprising the cytoplasmic tail (Fig. 1A). The membrane domain of NHE1 extrudes 1 intracellular proton in exchange for 1 extracellular sodium ion, protecting the cell from acidification and regulating intracellular pH (pH<sub>i</sub>). Ion flux is driven by the transmembrane Na<sup>+</sup> gradient and requires no direct metabolic input in normal cells. In addition, the activity of the exchanger is allosterically increased with decreasing pH<sub>i</sub>, resulting in a rapid activation of NHE1 and a subsequent elevation of pH<sub>i</sub> as a consequence of increased proton extrusion (5). NHE1 activity is regulated by the phosphorylation of key amino

tissues. In many cases, it is unclear if these regulatory pathways are retained in breast cancer cells, although a review of the known regulators of NHE1 can illuminate potential pathways in tumor cells. These regulators of NHE1 are activated or controlled by extracellular growth factors, hormones, and other agonists (serum, thrombin, epi-dermal growth factor, insulin, angiotensin II, and others; ref. 6). Ultimately, NHE1 regulators alter transport activity by altering the affinity for intracellular H<sup>+</sup> such that it is more active at a more alkaline pH<sub>i</sub> (6, 7). Much of the hormonal regulation of NHE1 is not just due to phosphorylation, but is mediated by several regulatory proteins and cofactors including calcium-binding proteins that associate with the cytosolic regulatory tail. The approximate binding sites of regulator proteins and cofactors is illustrated in Figure 1B. This includes calmodulin (CaM), which binds to a high- and low-affinity site located between amino acids 636 and 700, and blocks an autoinhibitory site, thereby activating NHE1 (8). It also includes the calci-neurin homologous protein (CHP) group of regulatory calcium-binding proteins. CHP1 and CHP2 bind to NHE1 in the region of amino acids 518 to 537 (Fig. 1B), where CHP1 is

acids in its C-terminal cytosolic domain, as well as by interactions of the C-terminal tail with intracellular proteins and lipids (Fig. 1A and B; ref. 3). Regulation of NHE1 at the molecular level has been elucidated in a variety of cell types and

figure 1. Schematic diagram summarizing NHE1 regulation by proteins, cofactors, and protein kinases. A, NHE1 protein in the plasma membrane with regulatory pathways in the tumor microenvironment indicated. B, the cytosolic regulatory tail of NHE1 is enlarged and indicates the location of regulatory binding sites and sites of phosphorylation. CaM, calmodulin; CHP, calcineurin homologous protein; ERM, ezrin, radixin, moesin; PIP2, phosphatidylinositol 4,5-bisphosphate.



thought to be important for NHE1 activity and its stabilization and localization to the plasma membrane (9). CHP2 is highly expressed in tumor cells; it is protective against serum deprivation-induced cell death by increasing pH<sub>i</sub> (10) and may play a role in the enhanced proliferation of tumor cells (11). CHP3, or tescalin (binding site uncertain), is thought to promote maturation and cell surface stability of NHE1 (12, 13). Another regulatory factor of NHE1 is phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), which binds at 2 sites between amino acids 513 and 520 and 556 and 564 (Fig. 1B). Depletion of PIP<sub>2</sub> results in ATP-dependent inhibition of NHE1 (14). Carbonic anhydrase II (CAII) is an enzyme that catalyzes the production of HCO<sub>3</sub><sup>-</sup> and H<sup>+</sup> from the hydration of CO<sub>2</sub>, and it associates with NHE1 at amino acid residues 790 to 802 and increases its activity (15). In addition, the actin-binding ERM (i.e., ezrin, radixin, and moesin) proteins bind to NHE1 between amino acids 552 and 560, and direct the proper localization of NHE1 to the plasma membrane as well as maintaining cell shape (16). Daxx is a death domain-associated protein that competes with ezrin in binding to the ERM-interacting domain of NHE1 (17), while the 14-3-3 adaptor protein only binds to NHE1 when it is phosphorylated at S<sup>703</sup> (Fig. 1B), thus activating NHE1 by protecting S<sup>703</sup> from dephosphorylation (18). Finally, heat shock protein 70 (Hsp70) binds to NHE1 and may play a role in protein folding (19).

Several protein kinases are also involved in the regulation of NHE1 and their actions have been described in a number of studies in varying cell types. They are known to phosphorylate specific amino acids of the regulatory cytosolic domain. Figure 1B illustrates the relative position of several phosphorylation sites in this domain. Briefly, p160ROCK is a serine/threonine protein kinase and downstream target of RhoA. It facilitates the assembly of focal adhesion and actin stress fibers and was shown to mediate the RhoA activation of NHE1 in fibroblasts (20). In vascular smooth muscle cells, angiotensin II stimulates NHE1 by ERK-dependent p38MAPK phosphorylation and, in pro-B cells, NHE1-mediated apoptosis is regulated by phosphorylation at amino acids T<sup>718</sup>, S<sup>723</sup>, S<sup>726</sup>, and S<sup>729</sup> (21).

Interestingly, the mutation of S<sup>726/729</sup> to nonphosphorylatable alanine is protective against

serum deprivation-induced cell death (22). In myocardial cells, Erk 1/2-dependent pathways are critical in the regulation of NHE1; Erk 1/2 phosphorylates NHE1 at S<sup>770</sup> and S<sup>771</sup>, while p90<sup>rk</sup>, a kinase downstream of Erk

1/2, phosphorylates NHE1 at S<sup>703</sup> and, by doing so, forms a 14-3-3 ligand binding site (18). Protein kinase B (Akt) was shown to phosphorylate S<sup>648</sup> and inhibit myocardial NHE1 activity, possibly by interfering with CaM binding (23); however, in other cell types, Akt phosphorylation stimulates NHE1 activity and is thought to be important for cell growth and survival and, possibly, in metastasis (24). Other kinases that are involved in the direct phosphorylation of NHE1, but are less defined in their site of action, include Nck-interacting kinase (NIK) and Ca<sup>2+</sup>/calmodulin-dependent kinase (CaMKII; refs. 25, 26). In addition, protein kinase A, C, and D are known to regulate NHE1 activity indirectly (27). Lysophosphatidic acid (LPA), a bioactive phospholipid that mediates its effects through protein kinases and by association with G-protein coupled receptors (GPCR), stimulates G<sub>α13</sub>-mediated activation of both the RhoA and Cdc42 pathways. Activation of the RhoA pathway results in p160ROCK activation, which potentiates NHE1 activation possibly by direct phosphorylation. In contrast, activation of the Cdc42 pathway mediates Erk 1/2 activation leading to NHE1 stimulation (28).

Although NHE1 plays a demonstrable role in breast cancer metastasis (outlined in Fig. 1A), the exact mechanism by which the activity of NHE1 is elevated is not well known. In breast cancer cells, early work showed that mimicking the tumor microenvironment by serum deprivation stimulates NHE1 activity in metastatic breast cancer epithelial cell lines but not in nontumorigenic cells. This was a direct result of alteration of the H<sup>+</sup> affinity of the exchanger. Phosphoinositide-3-kinase (PI3K) inhibition impaired NHE1 activity in serum-supplemented conditions but potentiated the serum-deprived activation of NHE1. This upregulation of NHE1 under serum deprivation is thought to occur via a PI3K-dependent mechanism, but direct phosphorylation of NHE1 was not shown in that study (29). More recently, it was observed that in serum-deprived human breast cancer cells, the activation of NHE1 is regulated by a



sequential RhoA/p160ROCK/p38MAPK signaling pathway that is gated by direct protein kinase A phosphorylation and inhibition of RhoA, suggesting that serum deprivation leads to a dynamic remodeling of the cytoskeleton forming long leading-edge pseudopodia that are uniquely poised for invasion and metastasis (30). Invadopodia, which are actin-rich invasive protrusions of these pseudopodia, are capable of proteolytic degradation of the extracellular matrix (ECM), thereby allowing invading cells to detach from the ECM and metastasize elsewhere (1). Several actin regulatory proteins including cortactin and cofilin are upregulated in these invasive cells and are specifically associated with invadopodia formation (31, 32). In MDA-MB-231 breast cancer cells, cortactin phosphorylation was found to be important in invadopodia maturation, the regulation of Nck1 binding, and cofilin activity, as well as promoting the recruitment of NHE1 to regulate pH in the invadopodia (33). CD44, a cell surface glycoprotein that serves as a receptor for hyaluronic acid (Fig. 1A), has also been shown to localize to the invadopodia of invasive breast cancer cells, stimulating NHE1 activity and invasion through the RhoA effector ROCK1. This activation of NHE1 was shown to create an acidic extracellular microenvironment that facilitates protease-mediated degradation of the extracellular matrix and promotes breast cancer cell invasion (34). The same authors also show that a RAC1 and RhoA–ROCK–PI3K pathway are involved in CD44-induced cell invasion (35), which, taken together, suggest that these signaling molecules drive NHE1 activation, cellular alkalization, and extracellular acidification, all of which facilitate invasion. Similarly, hypoxia triggers the activation of the p90 ribosomal S6 kinase (p90<sup>rsk</sup>) in HT-1080 fibrosarcoma cells resulting in invadopodia formation and increased invasive capability via site-specific phosphorylation and activation of NHE1 (36), but whether or not the same is true in breast cancer cells is not known.

In a comparison between noninvasive MCF-7 cells and invasive MDA-MB-231 cells, the role of MAPK signaling pathways in NHE1-mediated breast cancer metastasis was

examined. It was found that the constitutively phosphorylated levels of Erk 1/2 and p38MAPK were

higher in MDA-MB-231 cells, but both cell types expressed a similar level of phosphorylated c-Jun N-terminal kinase JNK. Treating MDA-MB-231 cells with either an NHE inhibitor (cariporide) or MAPK inhibitors suppressed the invasive capability of these cells, while treatment with a JNK-specific inhibitor had no effect (37). It is of interest to note, however, that while many studies have shown that NHE1 is regulated by MAPK in various cell types, there is also mounting evidence to suggest a role for NHE1 in the regulation of MAPK pathways (38); this could be of particular importance in the pathophysiology of breast cancer progression and could shed light on the complex interaction between the exchanger and its associated intracellular signaling molecules.

Together with the multitude of key regulatory sites on NHE1 acting as potential drug targets, several therapeutic applications may exist for the use of NHE1 inhibitors as adjuvants, to reverse dysregulated pH in the tumor environment, thereby ameliorating the efficacy of anticancer drugs on tumor growth and survival. Although there is some evidence to show that NHE1 inhibition or downregulation has an effect on the invasiveness of breast cancer cells in vitro, it should be noted that many studies are based on cells cultured in a well-buffered 2-dimensional (2D) milieu, generally in the absence of bicarbonate, while the tumor microenvironment in vivo can be different from these experimental conditions. For example, Boedtker and colleagues (39) showed that, while both  $\text{Na}^b/\text{H}^b$  exchange and  $\text{Na}^b/\text{HCO}_3^-$  cotransport contribute to pH regulation, net acid extrusion by the  $\text{Na}^b, \text{HCO}_3^-$  cotransporter NBCn1 is the major determinant of intracellular pH regulation in primary human breast cancer carcinomas at  $\text{pH}_i$  levels less than 6.6, with NHE activity prominent under more acidic  $\text{pH}_i$  conditions (39). In spite of this, however, there is significant evidence suggesting that inhibition or deletion of NHE1 has significant effects on tumor growth in several in vivo models (40–43). Although these studies were not done in breast cancer cells, they are suggestive that NHE1 could play a similar role in breast cancer. Therefore, approaches to modulate NHE1 activity are of interest. Aside from targeting kinases that modulate NHE1 activity, its function could be impaired with inhibitors of various cell surface receptors, intracellular regulatory proteins, or signaling molecules, and potentially with inhibitors of extracellular agonists of the exchanger.

surface receptors, intracellular regulatory proteins, or signaling molecules, and potentially with inhibitors of extracellular agonists of the exchanger. All of these could be diverse treatment options for patients with breast cancer. One example is the receptor tyrosine kinase ErbB2 (Fig. 1A). This kinase is upregulated in approximately 30% of patients with metastatic breast cancer and is associated with resistance to paclitaxel, a chemotherapeutic agent used in the treatment of breast cancer. In a phase 2 clinical trial, ErbB2-positive mammary carcinomas became susceptible to paclitaxel treatment in vivo when paclitaxel was administered in combination with trastuzumab, a humanized monoclonal anti-ErbB2 antibody (44). N-terminal truncation of ErbB2 renders it constitutively active as is common in breast cancer cells where its expression is associated with increased metastatic potential. Interestingly, in MCF-7 cells expressing the truncated ErbB2 kinase, inhibition or knockdown of NHE1 sensitizes these cells to cell death induced by the chemotherapeutic drug cisplatin. This indicates that the resultant cathepsin release may be amplified by NHE1 inhibition (45). ErbB2 upregulation is also generally associated with the inhibition of cyclin-dependent kinase CDK1 (46). In breast cancer xenograft tumors, those with significantly higher CDK1 specific activity were sensitive to paclitaxel in vivo, while tumors without increased CDK activity were resistant to the drug (47); however, there is no evidence to suggest a more direct link between CDK1 and NHE1 activity. Reshkin and colleagues, in 2003, also identified NHE1 as an essential component of paclitaxel-induced apoptosis in breast cancer cells and showed that simultaneous inhibition of NHE1 results in a concurrent enhancement of low-dose paclitaxel cell death (48). Taken together, these data indicate that the efficacy of paclitaxel and cisplatin chemotherapy may potentially be enhanced in the presence of NHE1 inhibitors.

NHE1 is also shown to mediate invasion in MDA-MB-231 cells through the regulation of membrane-type 1 matrix metalloproteinase (MT1-MMP; see Fig. 1A; ref. 37), and several MMP inhibitors exist that could potentially have a synergistic effect on decreasing invasiveness if used

in combination with NHE1 inhibitors. The same may be true when using agonists or antagonists of GPCRs or inhibitors of other receptors associated with NHE1. For example, the expression of the peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is greater in breast cancer cells compared with normal breast epithelium. Kumar and colleagues (49) showed that, in breast cancer cells over-expressing PPAR $\gamma$ , ligand-induced activation of the receptor by its natural and synthetic agonists inhibits the proliferation of tumor cells by downregulating NHE1 transcription as well as protein expression in vitro. Furthermore, histopathologic analysis of breast cancer biopsies from patients treated for type 2 diabetes with the PPAR $\gamma$  agonist rosiglitazone showed a marked decrease in NHE1 protein expression in the tumor tissues (49). Troglitazone, another member of the PPAR $\gamma$ -ligand family, is known to induce severe acidosis mediated by the inhibition of NHE1 activity in MCF-7 and MDA-MD-231 cells, leading to a discernible reduction in cell proliferation, although this effect is highly dependent on the delivery and concentration of the drug, where it is stimulatory at low concentrations and inhibitory at higher concentrations (50). Studies like this and others highlight the need for chemotherapeutic strategies that exploit the additive anticancer effects of using drugs that inhibit NHE1 activity as well as those that target sites of NHE1 regulation.

Despite the progress made in both diagnostic and therapeutic approaches in the treatment of breast cancer, the leading cause of fatality in patients with the disease is still metastasis: the invasion and spread of the primary tumor to other sites in the body. The development of novel strategies to either inhibit tumor progression or prevent tumors from metastasizing is, therefore, essential. Because pH regulation plays such an integral role in the switch from the normal to the neoplastic microenvironment, it is imperative that the Na<sup>b</sup>/H<sup>p</sup> exchanger be considered as an important target in the fight against breast cancer. With in vitro data suggesting an increased efficacy of chemotherapeutic drugs when used in synergy with NHE1 inhibitors, a multifaceted approach taking

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