

Cancer: fundamentals behind pH targeting and the double-edged approach

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Abstract: The highly regulated pH of cells and the less-regulated pH of the surrounding extracellular matrix (ECM) is the result of a delicate balance between metabolic processes and proton production, proton transportation, chemical buffering, and vascular removal of waste products. Malignant cells show a pronounced increase in metabolic processes where the 10- to 15-fold rise in glucose consumption is only the tip of the iceberg. Aerobic glycolysis (Warburg effect) is one of the hallmarks of cancer metabolism that implies excessive production of protons, which if stayed inside the cells would result in fatal intracellular acidosis (maintaining a strict acid–base balance is essential for the survival of eukaryotic cells). Malignant cells solve this problem by increasing mechanisms of proton transportation which expel the excess acidity. This allows cancer cells to keep a normal intracellular pH, or even overshooting this mechanism permits a slightly alkaline intracellular tendency. The proton excess expelled from malignant cells accumulates in the ECM, where chronic hypoxia and relative lack of enough blood vessels impede adequate proton clearance, thus creating an acidic microenvironment. This microenvironment is quite heterogeneous due to the tumor’s metabolic heterogeneity and variable degrees of hypoxia inside the tumor mass. The acidic environment (plus other necessary cellular modifications) stimulates migration and invasion and finally intravasation of malignant cells which eventually may result in metastasis. Targeting tumor pH may go in two directions: 1) increasing extracellular pH which should result in less migration, invasion, and metastasis; and 2) decreasing intracellular pH which may result in acidic stress and apoptosis. Both objectives seem achievable at the present state of the art with repurposed drugs. This hypothesis analyzes the altered pH of tumors and its implications for progression and metastasis and also possible repurposed drug combinations targeting this vulnerable side of cancer development. It also analyzes the double-edged approach, which consists in pharmacologically increasing intracellular proton production and simultaneously decreasing proton extrusion creating intracellular acidity, acid stress, and eventual apoptosis.

Keywords: metabolic processes, malignant cells, acid, apoptosis, Warburg effect

Introduction

Enhanced growth and proliferation in cancer cells require a nutrient- and oxygen-rich environment in order to fuel anabolic processes that increase metabolism more than 10-fold. But such a rich environment does not exist. As soon as increased proliferation and growth start, high demand depletes oxygen and nutrients from the tumor milieu.

The direct consequence of this hypoxic state is the overexpression of HIF, which may be already increased due to oncogenic mutations.

HIF induces the expression of multiple genes, most of them related to a metabolic switch toward a low (or nil) oxygen consumption/metabolism and stimulation of growth of new vessels (neangiogenesis). The process of new vessels production achieved

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through the stimulation of HIF/VEGF in cancer cells and in peritumoral cells like fibroblasts, macrophages, myofibroblasts, and endothelial cells is an imperfect process because the new vessels usually do not reach a fully functional entity. This means that the hypoxic environment becomes a permanent feature in cancer, in spite of these new vessels.

The metabolic shift, which is a consequence of oncogenic mutations and HIF overexpression, is toward a glycolytic phenotype (Warburg effect). Abandoning partially oxidative phosphorylation (OXPHOS) and adopting the glycolytic pathway as the main source of energy has two important advantages for the cancer cells, in spite of this pathway's low energetic efficiency:

- 1) Decreases reactive oxygen species production that is much higher under OXPHOS metabolism.
- 2) Generates biological building blocks for other molecules needed in the anabolic process of biomass building.

But at the same time, it has a disadvantage: an excess of protons is produced through different mechanisms; three outstanding are:

- 1) Excess lactic acid production; lactic acid dissociates into lactate plus a proton.¹
- 2) ATP hydrolysis also generates excess of protons.
- 3) Hydration of CO₂ forming CO₃H⁻ through the activity of carbonic anhydrases (CAs), particularly CA IX.

Cancer cells adapt to this excessive intracellular acidity by increasing the number and function of proton-exporting mechanisms. This adaptation keeps intracellular pH at normal or slightly alkaline levels, while the ECM receives the burden of the exported acidity.² In this way, a pH gradient is quickly established with a normal or slightly alkaline interior and an acidic exterior.³

This picture favors cancer growth and progression through diverse mechanisms:

- 1) Intracellular alkaline pH stimulates proliferation.
- 2) Extracellular acidity is a necessary feature for the activation of matrix-degrading enzymes like cathepsin and metalloproteases. This matrix degradation is necessary for migration, invasion, and eventual metastasis.
- 3) ECM acidity blocks immunologic attacks against malignant cells and decreases tumor access of certain chemotherapeutics.

Hypoxia plus extracellular acidity contribute to tumor progression and the Darwinian selection of resistant cells that may survive in this harsh environment.

The highly regulated pH of cells and the less-regulated pH of the surrounding ECM is the result of a delicate balance between metabolic processes, proton production, proton

transportation, chemical buffering, and vascular removal of waste products, supported by sophisticated mechanisms.

Microenvironmental and intracellular pH are major issues that influence processes like proliferation, differentiation, metastasis, and angiogenesis.

Malignant cells show a pronounced increase in metabolic processes where a 10- to 15-fold increase in glucose consumption is only the tip of the iceberg. Aerobic glycolysis (Warburg effect) is one of the hallmarks of cancer metabolism which implies excessive production of protons, which if stayed inside the cell would result in fatal intracellular acidosis (maintaining a strict acid–base balance is essential for the survival of eukaryotic cells). Also, an anaerobic glycolysis is found in hypoxic areas of the tumor. So, the main cause of environmental acidification is the accumulation of protons in the ECM, as byproducts of intracellular metabolism. Cancer cells require a normal or slightly alkaline pH inside the cell which favors protein synthesis and mitosis. Zetterberg and Engstrom⁴ in 1981 described that a pH between 7.3 and 8.2 produced a linear increase in DNA synthesis in quiescent serum-starved cells. According to Tannock and Rotin,⁵ the cytoplasmic alkalization may precede proliferation in certain cells. Malignant cells are in a permanent fight against excessive acid load, and solve this chronic problem on a short- and a long-term basis. In the short term, malignant cells use chemical buffering and transfer of acids into organelles.⁵

In the long term, the solution comes through increasing mechanisms of proton transportation which expel the excess acidity. This allows cancer cells to keep a normal intracellular pH, or even overshooting this mechanism permits a slightly alkaline intracellular tendency.

Not all cancer cells express or overexpress the same combination of proton transporters, and sometimes, there may be important differences among different tumors.⁶

The proton excess expelled from malignant cells accumulates in the ECM, where chronic hypoxia and relative lack of functional blood vessels hinder adequate proton clearance, thus creating an acidic microenvironment which shows a pH 0.5–1 unit lower than nonmalignant tissues. Cancer actually shows an inverted pH gradient.⁷ This microenvironment is quite heterogeneous due to the metabolic heterogeneity of the tumor and varying degrees of hypoxia inside the tumor mass, which is partially a consequence of a highly disorganized neovascular structure. Darwinian selection is responsible for the survival and evolution of the best adapted cells to the acidic microenvironment, so almost all malignant tumors show heterogeneous extracellular acidity with well-adapted malignant cells.

The acidic environment (plus other necessary cellular modifications) stimulates migration and invasion and finally intravasation of malignant cells which eventually may result in metastasis. This acidic environment also plays an important role in drug uptake, facilitating the entry of weak acids (cyclophosphamide and cisplatin) into the cells and working the opposite way with weakly alkaline drugs (doxorubicin).

Targeting tumors pH may go in three directions:

- 1) increasing extracellular pH which should result in less migration, invasion, and metastasis (“acid-mediated invasion hypothesis”);
- 2) decreasing intracellular pH which may result in acidic stress and apoptosis; and
- 3) increasing extracellular pH and at the same time decreasing intracellular pH.

These objectives seem achievable at the present state of the art with repurposed drugs.

This review analyzes the altered pH of tumors and its implications for progression and metastasis and also possible repurposed drug combinations to target this vulnerable side of cancer development. Targeting acidic microenvironment should not represent an aggression against normal tissues because they lack this feature, and it may be part of conventional chemoradiotherapy treatments because it does not interfere with them. On the contrary, in certain cases, targeting the acidic microenvironment may be an advantageous companion of conventional treatments.

Many recent studies have emphasized the role of extracellular pH in cancer, while to a certain extent neglecting the importance of intracellular pH in cancer growth and progression. We think that both should be addressed simultaneously in order to achieve better therapeutic results. This is what we have called double-edged pH targeting.

The double-edged pH targeting

Having said this, an interesting question arises: what would happen if proton extrusion is handicapped?

Protons would remain in the cell, decreasing the pH of the intracellular milieu, and the ECM would not receive an acid burden so that acidity of this nano-environment would be reduced. An increase in intracellular acidity would eventually drive the cells toward apoptosis due to acidic stress and migration, and invasion would be decreased.

What would be the result if in addition to handicapping proton extrusion we increase intracellular proton production?

More proton production and less proton extrusion would increase the acidic stress and its consequence: cell death.

Can this be achieved with repurposed nontoxic drugs?

The answer is absolutely “yes”.

Drugs like metformin and doxycycline are inhibitors of mitochondrial complex I decreasing OXPHOS and increasing aerobic glycolysis. Atovaquone is a selective inhibitor of mitochondrial complex III, and it also decreases OXPHOS. The consequence of this process is an increase in lactic acid production. We shall call these drugs “mitochondrial poisons”.

Amiloride, quercetin, proton pump inhibitors (PPIs), and voltage-gated sodium channel (VGSC) blockers like phenytoine decrease the activity of different proton exporters. We shall dub these drugs as “proton extrusion inhibitors”.

The combination of mitochondrial poisons with proton extrusion inhibitors may achieve this double-edged effect we are looking for: increased acidic intracellular environment with decreased extracellular acidity.

But how will this affect noncancer cells?

Cancer cells have a 10- to 17-fold higher consumption of glucose than nonmalignant cells, and glycolysis is the predominant metabolic pathway under normoxia (aerobic glycolysis or Warburg effect) and under hypoxia (anaerobic glycolysis) in cancer cells. Nonmalignant cells on the other hand use the tricarboxylic acid OXPHOS pathway as the predominant metabolic pathway.⁸ This means that the lactic acid burden is significantly higher in malignant cells and consequently intracellular pH will decrease markedly in relation to nonmalignant cells when treated with mitochondrial poisons.

In addition to the extracellular and the intracellular compartments that are classically considered for pH targeting, there is a third compartment which is frequently forgotten: the intra-organelle compartment with the intra-lysosomal compartment. This compartment is usually acidic in tumor cells and “helps” the cell “put away” part of the excess of acidic load.

The importance of tumor pH targeting is clearly visible in the work of Robey et al,⁹ where using oral sodium bicarbonate to increase tumor pH, in an in vivo trial of a mouse model of metastatic breast cancer, the number of metastases was decreased.

The review by Leanza et al¹⁰ gives an excellent view of ion channel targeting in cancer; nevertheless, it does not discuss the pH modifications that this targeting produces. pH targeting is essentially (though not solely) a targeting of ion channels and ion transporters.

This concept of targeting cancer cells from two different sides was described for the first time in 2011 by McCarty and Whitaker,¹¹ but it had no follow-up in practice.

The double-edged effect of increasing intracellular proton production and inhibiting proton extrusion simultaneously has a proof of concept in the research by Lee et al.¹² To achieve this, they used hydrogen sulfide which increases glucose uptake and lactate production through an increased glycolytic rate, and simultaneously reduces the activity of proton extrusion mechanisms by sodium–proton exchanger (NHE) activity reduction. The final result was increased intracellular acidity in cancer cells which selectively killed malignant cells but not the nonmalignant counterparts. The results found in this research showed that at the cellular level at least, it was possible to:

- 1) increase intracellular acidity selectively in cancer cells by increasing glycolytic pathways;
- 2) use increased intracellular acidity as a lethal weapon against cancer cells; and
- 3) preserve normal cells from any major damage due to this procedure.

There are partial aspects of this double-edged approach with sufficient and adequate evidence to support the double-edged hypothesis as described in this review.

Cellular pH sensors

Normal cells keep intracellular pH within a narrow range, which runs between 7.1 and 7.2 by regulating membrane proton pumps and proton transporters that are under the control of intracytoplasmic pH sensors. These sensors recognize pHi and induce adequate cellular responses to keep it in the above-mentioned range. In many cases, this implies acidifying the extracellular pH due to excessive proton extrusion.

Expression of proton-sensing G protein-coupled receptors (GPCRs) regulates certain aspects of tumorigenesis, migration, and invasion. There is no evidence of a relation between intracellular and extracellular pH sensors.

Decreasing proton export produces apoptosis in cancer cells

Six important pH regulators have been identified at the cellular level (there are probably more, but these six account for most of the activity; Figure 1):

- 1) Vacuolar ATPase proton pump.
- 2) NHE family.
- 3) Bicarbonate transporter family.
- 4) Monocarboxylate transporter (MCT) family.
- 5) VGSCs.
- 6) CA isoforms family.

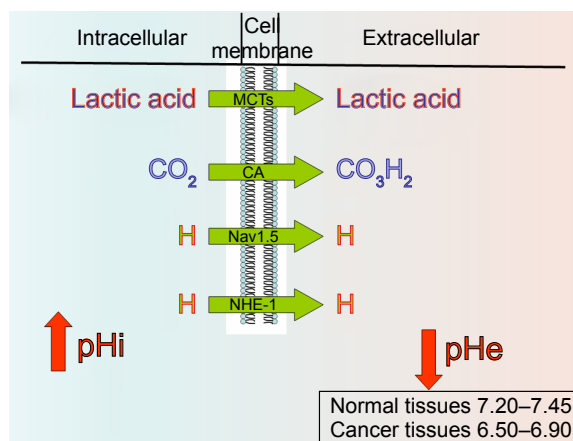


Figure 1 Main pH regulators' mechanism of action. Nav1.5 represents the voltage-gated sodium channel.

Abbreviations: MCTs, monocarboxylate transporters; CA, carbonic anhydrase; NHE-1, sodium–proton exchanger-1.

Another pH regulator that is showing increased importance in cancer is $\text{Na}^+/\text{CO}_3\text{H}^-$ cotransporter (NBCn1).

Vacuolar ATPase

Vacuolar H^+ ATPase is a highly conserved enzyme and a vital component of all eukaryotes, located in the membranes of many organelles and is responsible for low intravacuolar pH, mainly in lysosomes and endosomes. It can also be found in the cell surface.¹³ V-ATPase pumps H^+ from the cytoplasm into vacuoles with energy expenditure (ATP hydrolysis) regulating cytoplasmic pH.

Overexpression of a yeast plasma membrane V-ATPase in normal mouse fibroblasts increased intracellular pH and induced a tumorigenic phenotype.^{14,15}

Inhibition of tumor vacuolar ATPase produces intracellular acidification and induces apoptosis^{16,17} and increases the cytotoxic activity of chemotherapeutic drugs like paclitaxel.¹⁸ Unfortunately, there are no highly effective and specific V-ATPase inhibitors that could reach medical practice, due to their usually high toxicity (like Bafilomycin). Disulfiram is a V-ATPase inhibitor which has been used in the treatment of alcoholism and shows interesting antitumor activity. Probably, most of its activity is not related to V-ATPase inhibition. PPIs usually used for the treatment of gastroduodenal ulcers, gastroesophageal reflux disease, and Zollinger–Ellison syndrome have shown antitumor activity. PPI's effect is particularly elicited in acidic environment¹⁹ like the one usually found in tumors.

In 1993, Martinez-Zaguilan et al²⁰ identified the presence of functional V-ATPases in cell membranes of various human tumors. Bafilomycin A1, an experimental

V-ATPase-specific inhibitor, significantly lowered the intracellular pH of those tumors with high V-ATPase expression. A few years later, Sennoune et al²¹ found that breast cancer cells with high metastatic potential expressed high levels of V-ATPase. The opposite was found in cell lines with low metastatic risk. V-ATPase also regulates Notch signaling in triple-negative breast cancer²² and modulates metalloprotease isoforms in pancreatic cancer cells,²³ immunomodulates neutrophils associated with tumors²⁴ and is an essential proton pump for cancer invasion.²⁵ Inhibition of V-ATPase also decreased the population of macrophages associated with the tumor microenvironment which develop a pro-tumor activity,²⁶ decreased tumor growth, and overcame cisplatin resistance in ovarian cancer cells.¹⁶ V-ATPases also play an important role as a mediator of cancer-related inflammation.²⁷

These publications represent a proof of concept that V-ATPase is not only present in membranes of intracellular organelles but also located in plasmatic membranes of malignant cells, and its presence has a functional significance: homeostasis of cytoplasmic pH plus other functions related to malignant phenotype. But V-ATPases not only acidify the extracellular compartment through proton extrusion and consequently increase cytoplasmic pH but also acidify intracellular compartments like lysosomes and endoplasmic vesicles.²⁸

It was the work of Yeo et al²⁹ which called attention to the apoptotic effect on gastric cancer of repurposed PPIs already being used to treat gastritis and acid gastroduodenal syndromes.

Selecting the PPI

According to Lugini et al,³⁰ lansoprazole is the best choice for targeting tumor pH because it showed better results when compared to other PPIs in the experimental setting (cell culture). Furthermore, lansoprazole is an inhibitor of fatty acid synthase, which is a necessary enzyme in lipogenic phenotype malignancies. Omeprazole too has been recognized as a fatty acid synthase inhibitor.

Clinical trial NCT02595372 (<https://ClinicalTrials.gov>) is based on a preliminary retrospective study that showed that PPIs intake in breast cancer patients during chemotherapy significantly improved overall survival. The trial tries to determine the exact role of PPIs in cancer (omeprazole is used). The study is still ongoing at the Indiana University School of Medicine.

Clinical trial NCT01748500 ongoing at the University Health Network in Toronto is an interventional study to

determine the benefits of pantoprazole in the treatment of castrate-resistant prostate cancer treated with docetaxel.

Clinical trial NCT01069081 explores whether adding a PPI (esomeprazole at high doses) to docetaxel and cisplatin chemotherapy improves efficacy and does not affect tolerability in metastatic breast cancer.

Clinical trial NCT01163903 is a dose-finding, phase I study to determine the recommended phase II dose for the combination of doxorubicin and pantoprazole in patients with advanced tumors and no standard treatment options.

Additional evidence of PPIs' effects in cancer is summarized in Table 1.

As can be seen in Table 1, many antitumor effects of PPIs are independent of V-ATPase inhibition.

Table 2 shows other anticancer effects of PPIs independent of V-ATPase inhibition.

Recently, Canitano et al³¹ found that lansoprazole, and to a lesser extent omeprazole, showed significant antitumor activity in multiple myeloma cells. Lansoprazole's cytotoxicity was caspase independent.

NHE-1 and VGSC

The expression of VGSCs appears increased in cancer cells in many cases where it is not expressed in their normal counterparts, and plays a significant role in disease progression. The overexpressed VGSCs are usually Nav1.5, Nav1.6, and Nav1.7 and their splicing variants. The embryonic or neonatal isoform of Nav1.5 was identified in breast cancer. The mechanism of action of VGSCs is depicted in Figure 2.

This overexpression has been identified in breast, cervical, non-small-cell lung, small-cell lung, prostate, ovarian, colon, pancreas, and mesothelial cancer.³² This increased expression induces invasiveness by increasing the proteolytic activity in the ECM without regulating cellular multiplication or migration.³³

Activities of NHE-1 and VGSC are summarized in Figure 3.

In 1981, Moolenaar et al³⁴ observed in neuroblastoma cells that there was a Na-proton interchange that increased intracellular pH upon proton extrusion or Na entering the cell. They also described that this mechanism was blocked by amiloride. Three years later, Comoli et al³⁵ described that in a hepatoma model of ascitic cells, pH was higher in proliferating cells than in nonproliferating ones, and this difference was eliminated by amiloride. When glucose was added to the culture, pH decreased in the extracellular medium of proliferating and nonproliferating cells due to lactate production, but intracellular pH only decreased in proliferating cells.

Table 1 PPIs in cancer

Study	PPI effect
Mattsson et al ¹²⁰	In 1991, omeprazole was found to exhibit specific inhibitory activity on the H ⁺ /K ⁺ -ATPase
Mizunashi et al ¹²¹	In 1993, this research group established that omeprazole decreased bone reabsorption through inhibition of V-ATPase at lysosomal level in osteoclasts in a human clinical setting
Luciani et al ¹²²	PPI pretreatment (omeprazole or esomeprazole or pantoprazole) sensitized tumor cell lines (melanoma, adenocarcinoma, and lymphoma) to cisplatin, 5FU, and vinblastine. PPI pretreatment inhibited V-ATPase activity and increased pHe and the pH of lysosomes. Oral pretreatment with omeprazole induced sensitivity of human solid tumors to cisplatin
De Milito et al ¹²³	PPIs affected viability of human B cells and increased sensibility to vinblastine. They also induced lysosomal permeabilization which was probably related to apoptosis, which induced cytosolic acidification. PPIs resulted in cytotoxicity for leukemic cells in ALL
Capodicasa et al ¹²⁴	Omeprazole induced apoptosis in polymorphonuclear cells
Ferrari et al ¹²⁵	PPIs chemosensitized human osteosarcoma cells to chemotherapy with cisplatin in cell cultures and xenografts
Patel et al ¹²⁶	Pantoprazole increased the cytotoxicity of doxorubicin in solid tumors (cell culture). Pantoprazole increased endosomal pH
Avnet et al ¹²⁷	The targeting of V-ATPase with siRNA and omeprazole in Ewing sarcoma produced a reduction in cell viability
Chen et al ¹²⁸	Pantoprazole decreased multidrug resistance in gastric adenocarcinoma and decreased cell viability. Pantoprazole decreased pHi and reversed pHi–pHe gradient. Experiments were carried out on cell cultures and xenografts. Also, downregulation of the V-ATPases-mTOR-HIF-1 signaling was found
Shen et al ¹²⁹	Pantoprazole inhibited tumor growth and decreased HIF-1 expression in human gastric adenocarcinoma
Patlolla et al ¹³⁰	Rats fed with omeprazole showed decreases in aberrant crypt formation in a murine model of azoxymethane-induced crypt formation. Omeprazole also increased p21 expression in colon cancer cell lines and decreased antiapoptotic proteins expression
Perut et al ¹³¹	Sarcomas show increased numbers of acidic lysosomes. Esomeprazole induced dose-dependent cytotoxicity by interfering with proton compartmentalization
Azzarito et al ¹³²	Lansoprazole increased sensitization of human melanoma cells to low doses of paclitaxel. This was confirmed in a xenograft model
Huang et al ¹³³	Pantoprazole induces apoptosis in gastric cancer cells probably through inhibition of STAT3
Goh et al ¹³⁴	Esomeprazole increased the antitumor effect of doxorubicin on triple-negative breast cancer cell MDA-MB-468 and showed growth-suppressive activity when used alone
Zhang et al ¹³⁵	Human breast cancer cells treated with lansoprazole showed apoptosis in a dose-dependent way. In xenografts, lansoprazole produced alkalization of lysosomes and increase in ROS
Jin et al ¹³⁶	Omeprazole showed ligand capacity to aryl hydrocarbon receptor, decreasing cell invasion and metastasis in ER-negative breast cancer
Salerno et al ¹³⁷	Rhabdomyosarcoma stem cells showed a very high level of V-ATPase and lysosomal acidity with high invasiveness and reduced cytotoxicity with doxorubicin. Omeprazole increased doxorubicin cytotoxicity, and decreased growth and invasion even at low concentrations of omeprazole
Yeo et al ¹²⁹	Pantoprazole in vivo and in vitro induced apoptosis in gastric cancer cells
Udelnow et al ¹³⁸	Omeprazole inhibited proliferation of pancreatic cancer cells and modulated autophagy
Marino et al ¹³⁹	Esomeprazole induced apoptosis in melanoma cells but also induced autophagic defenses. The administration of an autophagy inhibitor increased malignant cell death due to esomeprazole
Vishvakarma and Singh ¹⁴⁰	Pantoprazole in a murine model of T cell lymphoma produced an increase in tumoricidal activity of TAMs
Yeo et al ¹⁴¹	PPIs induced apoptosis in gastric cancer cells

Abbreviations: PPIs, proton pump inhibitors; 5FU, 5-fluorouracil; ALL, acute lymphoid leukemia; siRNA, small interfering RNA; ROS, reactive oxygen species; TAMs, tumor associated macrophages.

Table 2 Other anticancer effects of PPIs

Study	Other effects of PPIs related to cancer therapy
Shen et al ¹⁴²	Pantoprazole downregulates pyruvate kinase M2
Zhang et al ¹⁴³	Pantoprazole decreases Wnt/beta catenin signaling and epithelial–mesenchymal transition in gastric cancer cells, decreasing invasiveness
Tan et al ¹⁴⁴	Pantoprazole decreases autophagy and increases docetaxel effects increasing growth delay in human breast carcinoma MCF-7 cells, human vulvar epidermoid cells and prostate cancer PC-3 cells
Hahm ¹⁴⁵	Pantoprazole has anti-inflammatory activity
Vishvakarma and Singh ¹⁴⁶	Pantoprazole decreases tumor-induced myelosuppression in T cell lymphoma
Mishima et al ¹⁴⁷	Lansoprazole increases osteoblast genesis through enhancement of nuclear accumulation of Runx2 and stimulating osteoblast differentiation
Matsui et al ¹⁴⁸	Omeprazole inhibits melanogenesis in rat melanoma cells and in normal human melanocytes by blocking ATP4A (a membrane P-type H ⁺ /K ⁺ ATPase) and also increases tyrosinase degradation
Shiizaki et al ¹⁴⁹	Omeprazole activates aryl hydrocarbon receptor in human hepatoma cells and human hepatocytes

Abbreviation: PPIs, proton pump inhibitors.

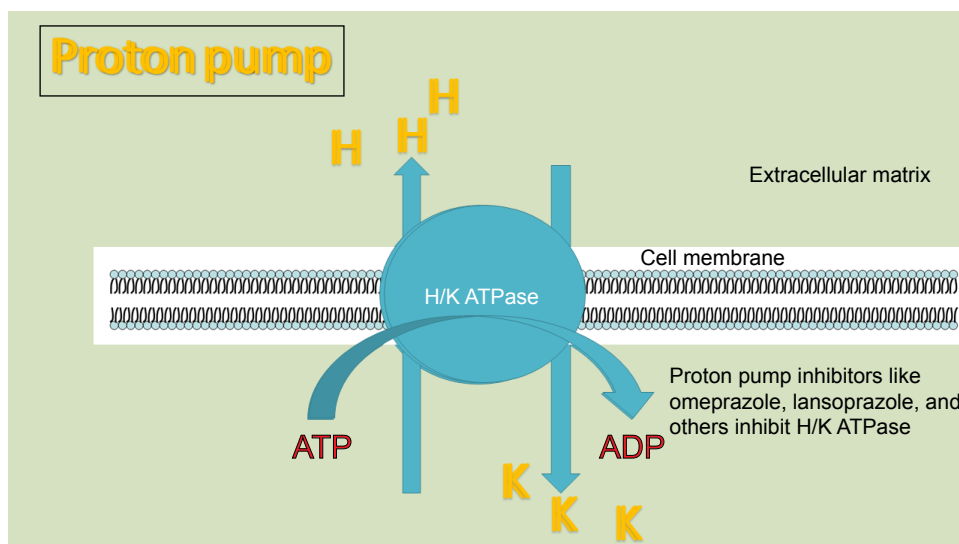


Figure 2 V-ATPase using energy and extruding H⁺.

Sparks et al³⁶ showed that amiloride had the ability to inhibit H6 hepatoma growth and mammary adenocarcinoma growth in a dose-dependent manner in mice. Not only do the injections of amiloride inhibited tumor growth and proliferation, but there was also a correlation with an important decrease in tumor cell sodium content.

Greco et al³⁷ demonstrated that the acidification of the ECM by proton extrusion through NHE-1 was a fundamental condition for the activation of proteases at the invadopodial extracellular level (Figure 2), and in 1990, Delvaux et al³⁸ found that amiloride and analogs had the capacity to inhibit NHE-1 and malignant cell proliferation.

NHE-1 has three basic functions, all of which are related to cancer evolution:

- 1) Extrusion of protons.
- 2) Structural anchor for actin filaments.
- 3) Assembler of signaling complexes in specialized plasma membrane domains.³⁹

NHE-1 is activated by growth factors, GPCRs, integrins, different tyrosine kinase receptors and p42/p44 MAPK cascade,^{40,41} HIF-1,⁴² p38 MAPK,⁴³ cytoplasmic acidification,⁴⁴ Akt,⁴⁵ CD44,⁴⁶ and insulin.⁴⁷

There is now enough evidence about amiloride's activities and its analogs to consider this diuretic as an NHE-1 blocker and a pH modifier, including antiproliferative activity. The evidence is summarized in Figure 4.

But there are other remarkable effects of amiloride which were described by Davis and Czech:⁴⁸ it blocks EGFR autophosphorylation, and also the PDGFR autophosphorylation. The authors attribute the antiproliferative activity of amiloride to this anti-tyrosine-kinase activity.

Rich et al⁴⁹ studying leukemia cells found that pHi was increased not only in leukemia cells but also in peripheral blood cells in relation to normal hematopoietic tissues. Treating leukemia cells with an amiloride analog (5-(N,N-hexamethylene) amiloride) decreased pHi and induced apoptosis.

In addition to its actions on proton extrusion, NHE-1 has other effects that are of capital importance in cell migration: cytoskeletal anchoring. This is particularly important at the invadopodia level where NHE-1 not only creates polarity through proton extrusion that increases pHi but also acts as an anchor of actin filaments to plasma membrane.⁵⁰ Inhibiting NHE-1 decreases or even eliminates cell migration.

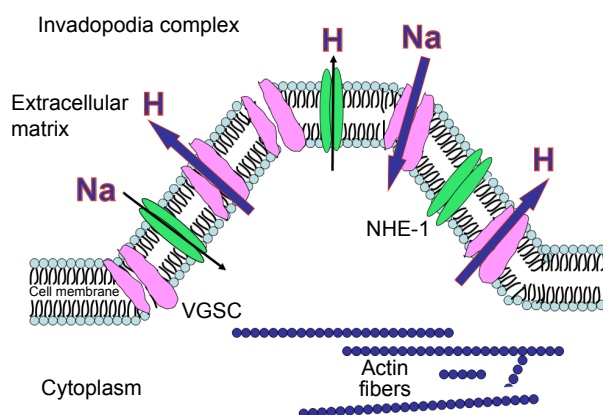


Figure 3 NHE-1 and VGSC working as H⁺ extruders.¹⁵⁰ (Invadopodia complexes are actin-rich protrusions of the cell membrane with active degradation of the extracellular matrix and invasion.)¹⁵¹ The presence of high levels of NHE-1 is fundamental for invadopodia formation.¹⁵² There is a relationship between NHE-1 enhanced activity and VGSC, but it has not been fully elucidated yet. Reproduced from Koltai T. Voltage-gated sodium channel as a target for metastatic risk reduction with re-purposed drugs. *FI000Res*. 2015;4:297.³²

Abbreviations: NHE-1, sodium-proton exchanger-1; VGSC, voltage-gated sodium channel.

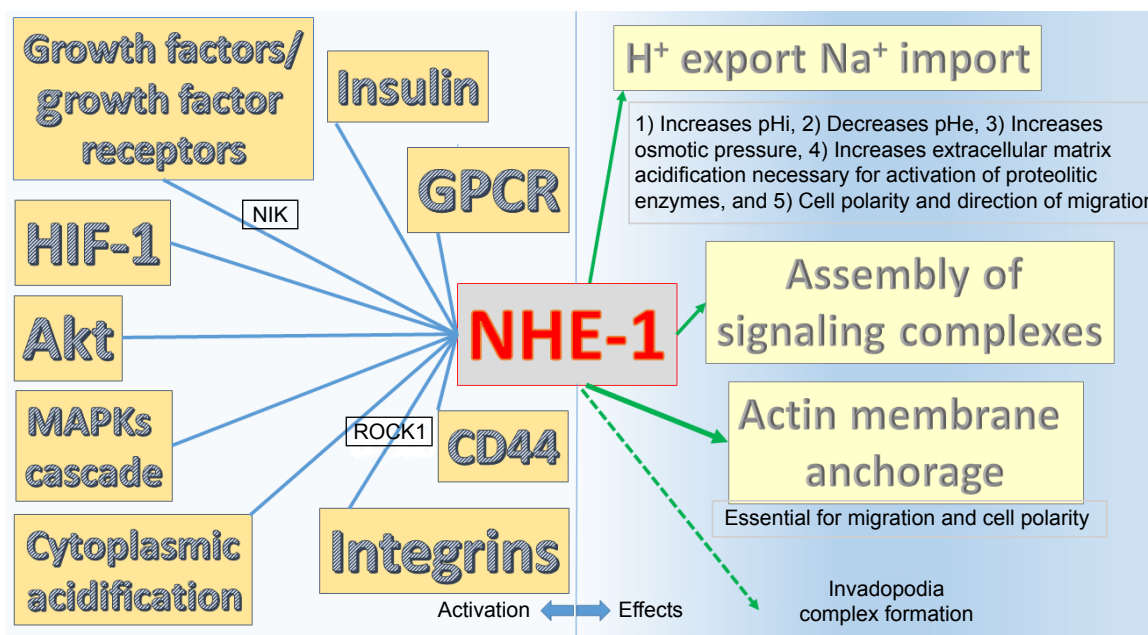


Figure 4 Summary of NHE-1 activators on the left side and effects on the right side. **Abbreviation:** NHE-1, sodium-proton exchanger-1.

Table 3 provides further evidence of amiloride's anticancer activity.

Cariporide is a sodium-hydrogen ion exchange inhibitor developed by Aventis Pharma (Mumbai, India) with the purpose of decreasing myocardial ischemic damage during the reperfusion process, but it has shown interesting anticancer activities. Research in the cardiovascular area has slowed down, due to adverse effects when used at high dose. In the field of oncology, cariporide has demonstrated apoptotic effects on cancer cells over-expressing NHE-1.

Cariporide mesylate may be administered by the oral or parenteral route.

Cariporide and amiloride have a guanidine function that is probably responsible for NHE-1 inhibition.⁵¹

In cancer, cariporide had been shown to reduce hypoxia-induced invasion in human squamous cell carcinoma of the tongue,⁵² and cholangiocarcinoma,⁵³ and overcome multidrug resistance.³³

Cariporide has not been clinically tested in oncology.

Another mechanism that has been found to partially decrease the activity of NHE-1 is ATP depletion, in spite of the fact that NHE-1 activity is not energy consuming, and therefore, it should not theoretically depend on the ATP level.⁵⁴ Although it has not been tested experimentally, but based on this finding, we may assume that ATP depletion produced by mitochondrial poisons, as we propose here, may have an inhibitory effect on NHE-1.

Another possible way to decrease NHE-1 activity is through PPAR γ agonists in those tumors that overexpress

Table 3 Evidences of amiloride's anticancer activity

Study	Activity
Rowson-Hodel et al ¹⁵³	The amiloride derivative 5-(N,N-hexamethylene) amiloride has cytotoxic properties against breast cancer cells
Sanhueza et al ¹⁵⁴	The authors proposed amiloride as a possible treatment of ovarian cancer
Acevedo-Olvera et al ¹⁵⁵	The authors demonstrated a suppressive effect of 5-(N-ethyl-N-isopropyl) amiloride on the proliferation of leukemia cell line stimulated with SCF, by decreasing the mitochondrial membrane potential and decreasing intracellular alkalization
Pieri et al ¹⁵⁶	Amiloride blocks the growth of murine leukemia cells
Sparks et al ¹³⁶	Amiloride decreases intranuclear sodium content and inhibits cell proliferation in hepatoma and breast adenocarcinoma cells
Kellen et al ¹⁵⁷	Amiloride inhibits the urokinase-type activity of plasminogen activator
Zheng et al ¹⁵⁸	Amiloride increases erlotinib anticancer activity on human pancreatic cancer cells through Akt inhibition
Hrgovic et al ¹⁹⁶	Ion pump inhibitors reduce tumor growth. Amiloride decreases tumor growth, and synergizes with other ion pump inhibitors

Abbreviation: SCF, stem cell factor.

PPAR γ , like certain breast cancers. Exposure of these cell lines to natural or synthetic PPAR γ agonists like rosiglitazone decreases NHE-1 gene expression.⁵⁵

Voltage-gated sodium channels

In 1995, Grimes et al⁵⁶ studied differential electrophysiological characteristics in two different rodent prostate cancer cell lines: Mat-Ly-Lu cell line (highly metastatic) and AT-2 cell line (lower metastatic potential). These two cell lines exhibited different electrophysiological features that maintained direct relationship with *in vitro* invasiveness. Inward sodium currents were detected only in the Mat-Ly-Lu cell line, and inhibition of VGSC protein with tetrodotoxin (TDX) reduced significantly the capacity for invasion. TDX showed no effect on invasion of AT-2 cell lines. The TDX-induced reduction in invasion kept a direct correlation with the amount of cells expressing VGSC in the culture.

They concluded that ion channels may be involved in malignant cell behavior and VGSCs could play a role in the metastatic process.

Laniado et al⁵⁷ found similar differences in two human prostate cell lines: one was highly metastatic and the other was not. As in the work by Grimes et al, they found that PC-3, the more malignant cell line, expressed VGSC protein and the inhibition of this channel protein with TDX reduced invasion in a significant way. LNCap cells did not express VGSC.

One conclusion reached by the authors is that cells expressing functional VGSC develop a selective advantage regarding migration and distant metastasis.

The correlation between VGSC protein expression and invasiveness in human and rat prostate cancer cells was confirmed by Smith et al⁵⁸ by comparing seven lines of rat prostate carcinoma cells with different metastatic ability, and nine human prostate carcinoma cell lines. In general, invading capacity of the basement membrane and metastatic ability kept a positive correlation with the percentage of cells expressing VGSC. But this positive correlation occurred only in a certain range of cells being invasive (27% in rats and 12% in humans). The authors suggest that the discrepancies may be due to the need of other factors besides the presence of VGSC so that this protein may represent a prerequisite for the invasive phenotype but other requirements must also be met for a full-blown invasive phenotype. Fraser et al⁵⁹ determined the key role played by VGSCs in prostate cancer cells regarding invasion and motility and showed that TDX and phenytoin that are known VGSC blockers decreased motility and invasiveness, while channel openers increased motility.

The increased invasion capacity in VGSC-expressing cancer cells is not limited to prostate. It can be found in breast cancer cell lines.⁶⁰

Batcioglu et al⁶¹ showed the importance of VGSCs inhibition in a rat model of induced breast cancer in order to inhibit antioxidant response. They observed a survival improvement in rats treated with a VGSC blocker.

An important location of VGSCs in cancer cells is in a cellular region directly involved in migration and invasion: the invadopodia.

Invadopodias are actin-rich protrusions of the plasma membrane, which are strongly related to degradation of the ECM.

The Na(+)/HCO₃(-) cotransporter SLC4A4

The Na/HCO₃ cotransporter SLC4A4 plays an important role in intracellular pH regulation because it intervenes in bicarbonate recapture and helps maintain a slightly alkaline intracellular environment. Parks and Pouyssegur⁶² found that hypoxia induces overexpression of SLC4A4 cotransporter in a colon adenocarcinoma cell line in a HIF-dependable manner. Inhibition of SLC4A4 reduced proliferation and increased apoptosis during external acidosis. In a breast cancer line overexpressing SLC4A4, the knockdown of this transporter had an important impact on proliferation, migration, and invasion.

Carbonic anhydrase

CAs are a family of hypoxia-inducible enzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton.



There are 15 CA isoforms expressed in humans; two of them, CA IX and CA XII, were found to be associated with tumors. Both are transmembrane isoforms where the catalytic domain is extracellular. Both may be highly expressed in tumors and almost insignificantly expressed in non-tumor cells.⁶³

CA IX plays a key role in extracellular pH regulation in the tumor environment.

Oncogenic metabolism is characterized by high production of lactic acid and carbon dioxide which are exported to the environment generating an acid extracellular milieu. Bicarbonate generated by CA IX is incorporated into the cell-buffering pHi.

Inhibition of CA IX has shown important antitumor effects and is actually considered a valid target in cancer treatment. Many small molecules with selective ability to inhibit CA IX are in the experimental phase. Also, monoclonal antibodies have been developed and are now under clinical trial.

Acetazolamide is a pan-CA inhibitor with no selectivity for CA IX but has been tested with good results in many tumors like bronchial carcinoid,⁶⁴ renal carcinoma cells,⁶⁵ breast cancer cells,^{66,67} colon cancer cells,⁶⁸ bladder cancer,⁶⁹ glioblastoma,⁷⁰ and gastric carcinoma.⁷¹

Acetazolamide improves the efficacy of mTOR inhibitors by increasing its activity in hypoxic areas of the tumor⁷² and potentiates bevacizumab in cholangiocarcinoma.⁷³

It has been suggested that saccharin may be a potential CA IX and CA XII inhibitor.⁷⁴

Monocarboxylate transporters

MCT isoforms 1–4 are enzymes that catalyze the proton-linked transport of monocarboxylates such as L-lactate, pyruvate, and ketone bodies across the plasma membrane. MCT4 expression is increased in response to hypoxia by mediation of HIF-1 α . It is frequently overexpressed in malignant cells.

MCT1 is present in almost all tissues, and its main role is to catalyze lactic acid influx or efflux from the cell.

When aerobic or anaerobic glycolysis is increased, as in cancer cells, MCT1 reduces intracellular acidity by exporting lactate with a proton. This prevents the toxic accumulation of lactate and acidification in the intracellular milieu. Very aggressive tumors may overexpress MCT4 with similar functions.⁷⁵

MCTs play a fundamental role in shuttling lactic acid between different cells (Figure 5). This is particularly noticeable in cancer cells. Izumi et al⁷⁶ found that MCT1 and MCT4 expression in human lung cancer cell lines was significantly correlated with invasiveness.

There are no MCT inhibitors currently used in clinical practice. Astra Zeneca developed an oral molecule (AZD3965) that inhibits MCT1 but not MCT4 which is being tested in clinical trials. It seems to be useful in small-cell lung cancer with overexpression of MCT1 and no overexpression of MCT4.⁷⁷ The problem with MCT inhibitors is that these drugs are ineffective in hypoxic regions of the tumor because HIF-1 α induces MCT4 production.⁷⁸

The inhibition of MCTs becomes a serious impediment for cancer cell growth if both MCT1 and MCT2 are inhibited.

MCT1, besides its transporter activity, also seems to intervene in cell motility, migration and metastasis.

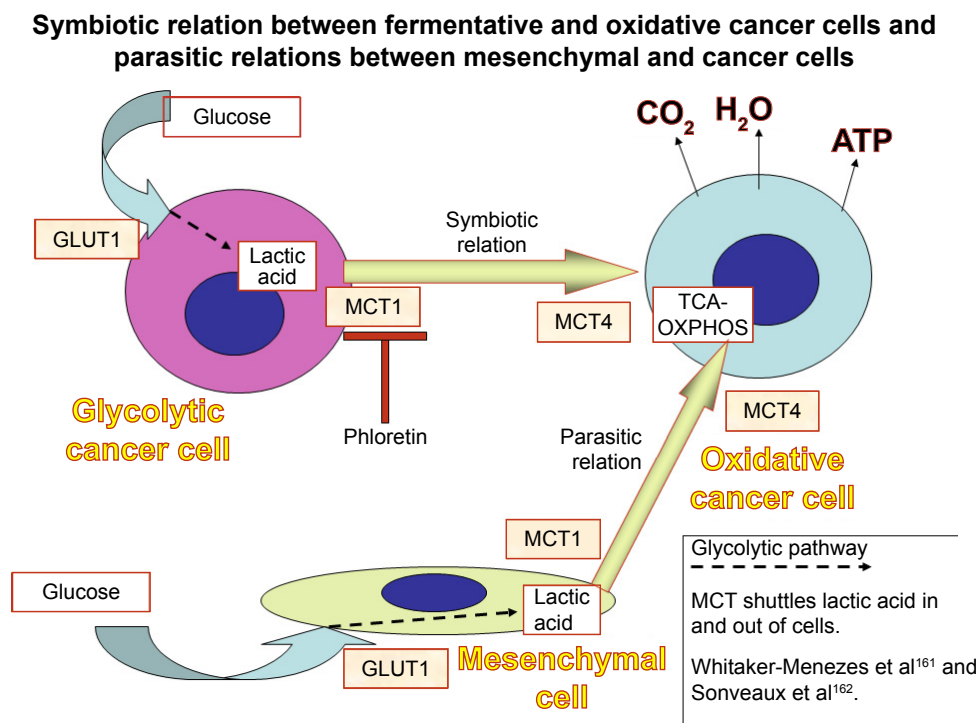


Figure 5 Glycolytic cancer cells and “enslaved” mesenchymal cells expel lactic acid through the activity of MCT1. Oxidative cancer cells uptake lactic acid and complete its catabolism through OXPPOS. MCT4 intervenes in this step.

Abbreviations: MCT, monocarboxylate transporter; OXPPOS, oxidative phosphorylation; GLUT, glucose transporter; TCA, tricarboxylic acid.

The knockdown of MCT1 decreased HGF-induced and EGF-induced cell motility.⁷⁹

Flavonoid quercetin seems to inhibit MCTs.⁸⁰ Simvastatin decreases the activity of MCT4,⁸¹ but almost all statins have an inhibitory effect (atorvastatin, fluvastatin, cerivastatin, simvastatin, lovastatin).⁸²

Mitochondrial poisons

Atovaquone is an antimalarial drug used for the treatment of pneumocystis pneumonia and toxoplasmosis and is approved by the US Food and Drug Administration. At the molecular level, it is a potent and selective inhibitor of OXPHOS by targeting mitochondrial complex III and inducing aerobic glycolysis and oxidative stress in cancer stem cells.^{83,84} Atovaquone decreases the pyrimidine synthetic pathway in *Plasmodium falciparum* dependent on mitochondria.⁸⁵

Metformin is the most widely prescribed drug for the treatment of diabetes. Its main mechanism of action is inhibition of mitochondrial complex I, increasing the glycolytic pathway through reduction of OXPHOS. Due to lower production of mitochondrial ATP, the AMP/ATP index increases and activates AMPK which further inhibits mTOR.⁸⁶ Used at high doses, it may produce lactic acidosis due to increased lactic acid production.

Phenformin is metformin's predecessor with similar effects on lactic acid production but is a more powerful inhibitor of the mitochondrial respiratory chain which entails an increased risk of lactic acidosis. This adverse effect led

to the withdrawal of this drug from the market.⁸⁷⁻⁸⁹ As the effect we are looking for is precisely a strong inhibition of lactate oxidation, phenformin may be more appropriate for this purpose than metformin, although it is more toxic. Regarding cancer cytotoxicity, phenformin also seems to be more powerful than metformin.⁹⁰

Doxycycline is an antibiotic that exerts inhibition of mitochondrial protein synthesis and reduces mitochondrial complex I activity.⁹¹⁻⁹⁴ (mechanism described in Figure 6).

All of the three pharmaceuticals described as mitochondrial poisons have in common their inhibitory activity on OXPHOS and increase in lactic acid production through increased aerobic glycolysis.

Metformin, phenformin, and doxycycline are weak inhibitors of mitochondrial complex I with no effect on the rest of the mitochondrial complexes,⁹⁵ so for a more potent inhibition of the OXPHOS process, it would be convenient to associate atovaquone as an inhibitor of complex III and possible synergistic activity with biguanides. This needs experimental testing.

By reducing OXPHOS activity, mitochondrial poisons decrease ATP production, and NHE activity is reduced at a low intracellular concentration of ATP.⁵² This means that in theory, at least, mitochondrial poisons may increase inhibition of proton extrusion mechanisms. In spite of this finding, NHE-1 is not energy dependent, and its inhibition is due to modulation of intracellular proton-dependent mechanisms.⁵²

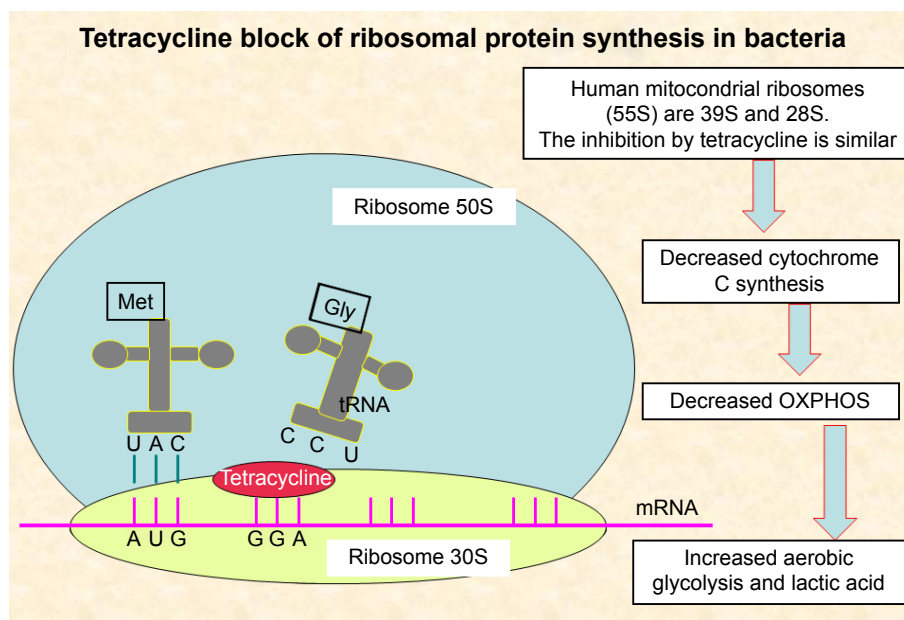


Figure 6 Mechanism of action of tetracyclines.

Abbreviation: OXPHOS, oxidative phosphorylation.

Hypothesis

It has been demonstrated that the different ion pump inhibitors decrease tumor cell proliferation. Using inhibitors that act on different mechanisms show synergy in antiproliferative activity.⁹⁶ In this review, we propose the use of multiple ion pump inhibitors plus an increase in intracellular acidity by increasing the lactic acid production through mitochondrial poisons like metformin, atovaquone and doxycycline. The excess of intracellular acidity that cannot be extruded due to proton pump inhibition should generate an acidic stress that induces apoptosis.

Precisely, we propose using the association of eight pharmaceuticals (and a possible ninth) to achieve this goal:

- 1) Lansoprazole or pantoprazole
- 2) Amiloride or an analog of amiloride and cariporide could be another option
- 3) Phenytoin
- 4) Quercetin
- 5) Lipophilic statins like simvastatin, atorvastatin, cerivastatin and lovastatin
- 6) Metformin or phenformin
- 7) Doxycycline
- 8) Atovaquone
- 9) If the tumor overexpresses CA, acetazolamide should be added to the combination.

Each of these drugs has low toxicity at therapeutic doses. Except for cariporide, there is adequate experience with all of them, and they are FDA-approved. The drugs are inexpensive and require no phase I clinical trials. They can all be associated with conventional chemotherapy and radiotherapy.

Discussion

The importance of acid–base homeostasis in keeping normal cellular responses has long been known, and the importance of targeting cancer pH has been recognized by the scientific community, to the extent that in 2010, the International Society for Proton Dynamics in Cancer was created⁹⁷ with the intention of bringing together basic and clinical investigators to stimulate translational research and interdisciplinary work for the development of therapeutic strategies based on proton dynamics in cancer.

The peculiarities of proton dynamics in cancer are a direct consequence of deep metabolic changes in cancer cells. Interfering adequately with the extracellular and intracellular acidity of cancer cells may represent a resource that shows low or no toxicity for normal cells and at the same time decrease proliferation, migration, invasion, and metastasis.

Inhibiting the compensatory mechanisms that tumor cells use to adapt themselves to a high load of toxic

metabolites may deprive these cells of a capital resource for detoxification. And what is more important: this inhibition can be achieved with already existing drugs that have no toxic effect on normal cells.

Despite the ease and low cost of interference in proton dynamics as an anticancer strategy, it has been neglected in the clinical setting.

The role of pH in cancer development, evolution, and metastasis has been underlined by many fundamental investigations.^{98–103} The role of the acidic environment in cancer is a serious drawback for natural and induced immunotherapy,^{104,105} and neutralizing this acidity improved immunological defenses in an experimental *in vivo* setting. As proof of concept, we should mention that the combination of bicarbonate with anti-PD-1 drugs or anti-CTLA-4 drugs improved antitumor responses.¹⁰¹

At the same time, an increase in intracellular pH is responsible for increased DNA and protein synthesis, increased metabolic rate, and cell proliferation.¹⁰⁶

The simultaneous attack (double-edged) on intracellular pH leading it to acidosis through increased lactic acid production, and extracellular pH pushed toward a higher pH by reduction of the proton export mechanism will have two desired effects:

- 1) acidic intracellular stress that may increase apoptosis; and
- 2) lower extracellular acidity that decreases migration and invasion by reduced cathepsin and matrix metalloproteinases activity.

The eight drugs (eventually nine) chosen to achieve the double-edged approach were selected on the assumption that they will probably act in synergy to reach the goals outlined. No MCT inhibitors were included in this multidrug compound because no effective drug has been developed yet. But there are evidences that the combination of two flavonoids, phloretin, and luteolin, may show inhibitory activity on MCT1⁷⁸ or phloretin alone.^{107,108} The interesting issue with phloretin is that it also inhibits MCT4.¹⁰⁹ Phloretin has also other anticancer properties and deserves further research.^{110–112}

More than one proton extrusion mechanisms have to be inhibited because tumors show heterogeneous expression of these transporters and there is redundancy in the mechanisms for acid extrusion. This explains why it is necessary to use at least four different compounds to eliminate the main transporters activity.

Targeting extracellular acidity in cancer with a simple and nontoxic resource as PPIs may overcome immune escape that is unleashed by low pHe.^{113,114} Another simple salt like sodium bicarbonate administered orally

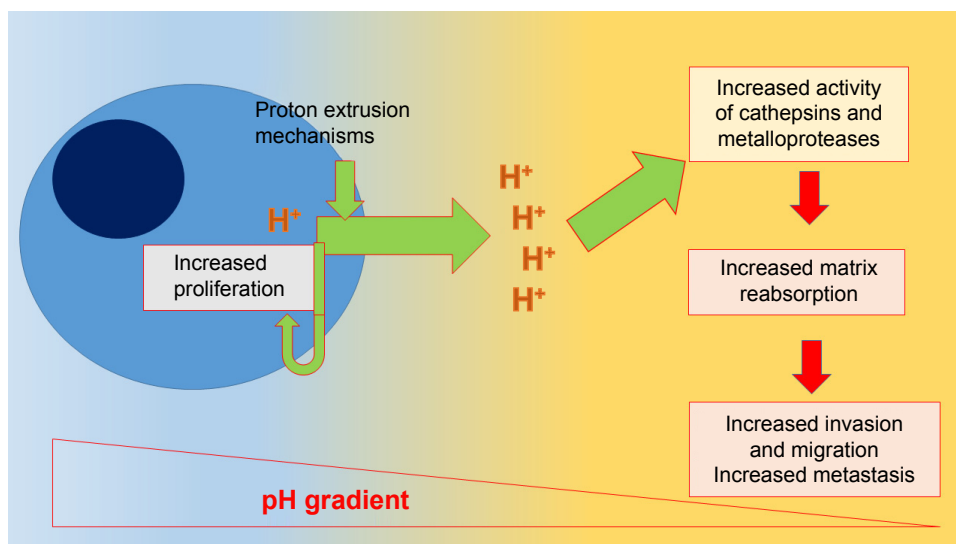


Figure 7 Intracellular and extracellular pH in tumor cells. Proton extrusion mechanisms create an extracellular acidic milieu and a slightly alkaline intracellular environment. Low extracellular pH contributes to the activation of enzymes that digest extracellular matrix, while the slightly alkaline cytoplasm is appropriate for increased proliferation. Organelles like lysosomes are highly acidic in cancer cells. Migrating cells exhibit an intracellular pH gradient along the migration axis which is related to NHE-1 activity.¹⁵⁹
Abbreviation: NHE-1, sodium-proton exchanger-1.

elevated peritumoral pHe and inhibited local growth and invasion.¹¹⁵

Tumor pH has a strong influence on therapeutic response:³

- 1) Acidity suppresses radiation-induced apoptosis.
- 2) Weakly acid drug uptake is enhanced.
- 3) Retards the uptake of weakly basic drugs.

The simplicity and low toxicity of pH targeting is so important that there are no sound reasons for neglecting it in cancer treatment. (The fundamentals of the double-edged approach are illustrated through Figures 7–9.)

Final comment

pHi changes are proportional to the difference between acid extrusion and acid loading.¹¹⁶ The double-edged approach

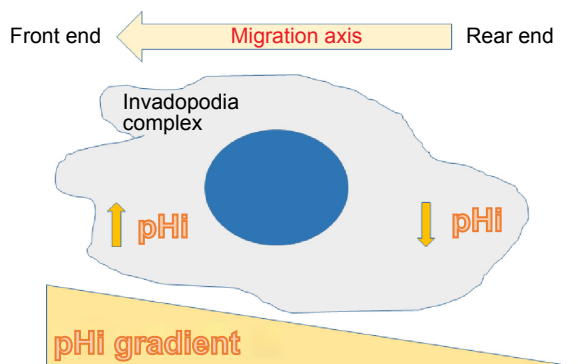


Figure 8 Intracellular pH gradient in migrating cells.¹¹² Malignant cells show a tendency to a higher gradient between the front and rear ends. Inhibition of NHE-1 makes the gradient flatten or disappear. There is also an NHE-1 distribution gradient similar to the pHi gradient¹¹² which cannot be modified by pHe¹⁶⁰ when NHE-1 is inhibited.
Abbreviation: NHE-1, sodium-proton exchanger-1.

increases acid loading through mitochondrial poisons and reduces acid extrusion by inhibiting acid extruders. The net result is a decrease in pHi which carries cellular acid stress and cytotoxicity. The acid load is strongly enhanced in malignant cells as compared to nonmalignant cells because the production of lactic acid in these tumor cells is much higher than in their normal counterparts. So, we can expect low toxicity in normal cells and a high toxicity in malignant cells.

Inhibition of ion transport alone should have minimal or no cytotoxic effects on malignant cells. Mild mitochondrial poisons alone should have no cytotoxic effects at usual doses, either. But the association of both types of drugs would create a significant acid stress inside the cell that produce cytotoxicity and a decrease of extracellular acidity which may result in decreased migration, invasion, and eventual metastasis. By reducing OXPHOS, mitochondrial poisons produce inhibition of stem cell proliferation, which is an additional feature favoring the use of these kinds of drugs.

Although the acid extrusion mechanisms are highly redundant, a partial inhibition of many of these mechanisms is enough to reduce cancer proliferation and invasion because a complete inhibition of proton extrusion would result in unacceptable toxicity for normal cells.¹¹⁷

The synergistic effect of the association of lansoprazole with an inhibitor of CA IX against human melanoma cells has been recently demonstrated.¹¹⁸

Many of the drugs proposed in the double-edged approach are in clinical use and approved by the FDA and other

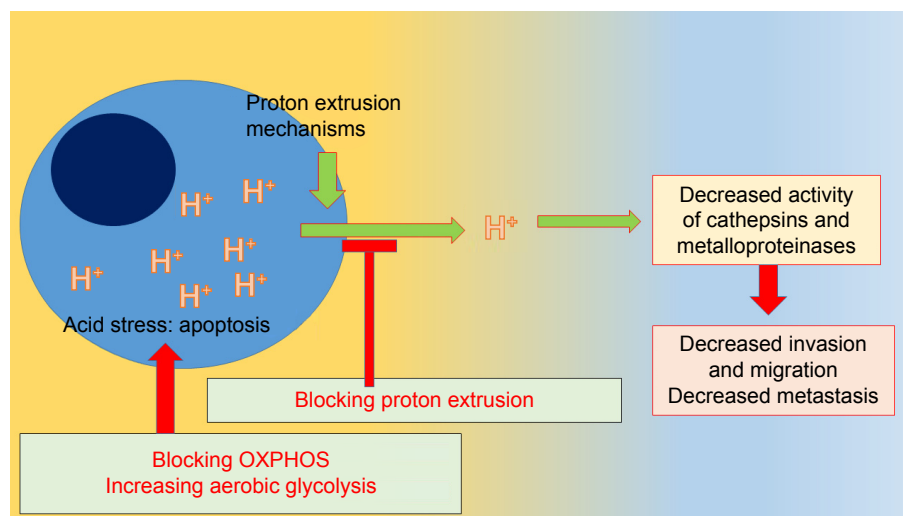


Figure 9 The double-edged approach increases intracellular acid burden and decreases extracellular acidity by limiting exportation of protons.
Abbreviation: OXPHOS, oxidative phosphorylation.

regulatory authorities. The combination of pharmaceuticals proposed in this review can be associated with most of the chemotherapy protocols currently in use.

This scheme deserves adequate and well-planned clinical trials as an adjunct cancer treatment.

Future perspectives

Based on a mathematical theoretical study, Webb et al¹¹⁹ determined that the transfer of acids from the cytosol into acidic organelles like endoplasmic reticulum, endosomes, Golgi apparatus, and lysosomes had a capital importance in keeping an alkaline pHi. Up to now, no specific drugs have been developed to reduce or abort this sequestration of protons. Probably, the future will present us with advances in this area.

Anticancer vaccines, activated T lymphocytes utilized against tumors, anti-PD-1 and anti-CTLA-4 monoclonal preparations, and anticancer immunotherapy in general will be benefited by modulating tumor acidity that causes a reversible state of anergy.

As new molecules for selective CA IX inhibition, new monocarboxylate inhibitors and new amiloride derivatives are developed and brought into medical practice, the spectrum of pH-targeted therapies will increase and probably become part of the oncological armamentarium. In the meantime, there are excellent drugs that may do the job when adequately combined.

Disclosure

The author reports no conflict of interest in this work.

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