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Targeting Vacuolar H\textsuperscript{+}-ATPases as a New Strategy against Cancer

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Abstract
Growing evidence suggests a key role of tumor acidic micro-environment in cancer development, progression, and metastasis. As a consequence, the need for compounds that specifically target the mechanism(s) responsible for the low pH of tumors is increasing. Among the key regulators of the tumor acidic microenvironment, vacuolar H\textsuperscript{+}-ATPases (V-ATPases) play an important role. These proteins cover a number of functions in a variety of normal as well as tumor cells, in which they pump ions across the membranes. We discuss here some recent results showing that a molecular inhibition of V-ATPases by small interfering RNA as well as a pharmacologic inhibition through proton pump inhibitors led to tumor cytotoxicity and marked inhibition of human tumor growth in xenograft models. These results propose V-ATPases as a key target for new strategies in cancer treatment. [Cancer Res 2007;67(22):10627–30]

Introduction
Evidence is accumulating that hypoxia and acidity are involved in cancer progression and in the tumor sensitivity to chemotherapy. The hypoxic and acidic tumor microenvironment may in part induce the selection of tumor cells able to survive in this unfavorable condition and contribute to the progression from benign to malignant growth. Acidity, in particular, has been shown to have a role in resistance to chemotherapy (1), proliferation (2), and metastatic behavior (3). Up to five decades ago, it was postulated that tumors were acidic due to their marked rate of lactic acid production (4). In fact, an important determinant of tumor acidity is the anaerobic metabolism that allows the selection of cells able to survive in a hypoxic-anoxic environment through the up-regulation of hypoxia-inducible factor \(\alpha\) and the adaptation of a glycolytic phenotype with generation of lactate (5). However, tumors are able to create the acidic environment also in conditions of reduced production of lactate via glycolysis, suggesting that the aerobic metabolism is not the major mechanism responsible for the development of an acidic environment within solid tumors (6, 7). Particularly, the causes for the acidic pH in tumors may include (a) deficiencies in tumor perfusion, due to the abnormal vascularization of the tumor mass; (b) hypoxia and metabolic abnormalities associated with transformation and uncontrolled cell growth; and (c) increased capacity for transmembrane pH regulation. These conditions may create a sort of vicious circle that, on one hand holds up the same conditions and on the other hand may favor the selection of highly malignant tumor cells able to survive in this hostile environment. A variety of ion exchangers expressed by tumor cells seem to have a key role in the establishment of the malignant tumor microenvironment. These transmembrane proteins pump ions from the inside to the outside of the cells, and in some cases they exchange ions between the internal and the external spaces. Of course, this mechanism substantially helps the cells in managing to free themselves from the very dangerous H\textsuperscript{+} ions that otherwise may accumulate within cytosolic spaces, in turn fataly activating a cascade of lytic enzymes. Among these proton pumps, one of the most studied, as responsible for both the establishment and the maintenance of the acidic pH of tumors, are the vacuolar H\textsuperscript{+}-ATPases (V-ATPase).

Role of V-ATPases in Cancer Metastasis
V-ATPases, as a specific proton pump of the cell, have an important role in the control of intracellular pH and extracellular pH. V-ATPases are involved in maintaining a relatively neutral intracellular pH, an acidic luminal pH, and an acidic extracellular pH (8), through pumping protons into extracellular environment or lumen of some membrane-bound organelles. V-ATPases are overexpressed in many types of metastatic cancers and positively correlated to their invasion and metastasis (9, 10). In cancer tissues, the extrusion of protons via V-ATPase causes extracellular acidification and contributes to the maintenance of an aberrant pH gradient between the alkaline cytosol and the acidic extracellular environment. The low pH of tumor extracellular microenvironment may induce the increased secretion and activation of proteases. Moreover, low extracellular pH may promote the degradation and remodeling of extracellular matrix (ECM) through proteolytic enzyme activation, thus contributing to cancer invasion and metastasis (3, 11). In fact, the promoting effect of V-ATPases on cancer invasion and metastasis mainly relies on their ability to maintain an acidic pH of extracellular microenvironment and very acidic luminal pH. This pathway is in turn related to the activation, secretion, and cellular distribution of many proteases involved in the digestion of ECM. The proteases needing a low extracellular pH to optimize their activation (12–15) include matrix metalloproteinases (MMP), bone morphogenetic protein-1-type metalloproteinases, tissue serine proteases, and adamalysin-related membrane proteases. Among them, MMPs are proteases essentially involved in degradation and remodeling of ECM, due to their ability to collectively degrade all the structural components of the ECM. We have recently shown that the inhibition of V-ATPase function via knockdown of ATP6L (c subunit gene) expression using RNA interference technology could effectively suppress cancer metastasis by the decrease of proton extrusion and the down-regulation...
HCO₃-based H⁺-transporting mechanisms, highly metastatic cells

The specific targets of PPI pump, dramatically inhibiting proton translocation and acidification of acidic spaces of the stomach, PPI irreversibly bind the proton acidic outside pH gradient featuring cancer cells is generated by the up-regulated activity of proton transporters like the Na⁺/H⁺ exchangers and the V-ATPases that maintain an alkaline intracellular pH and an acidic extracellular pH. In cancer cells, it also altered the pH gradient between the cytoplasm (alkaline) and the lumen of intracellular vesicles (very acidic), whose regulation is fully controlled by V-ATPases. The homeostatic regulation of such abnormal pH gradients by V-ATPases is a crucial factor involved in proliferation, tumorigenesis, drug resistance, and tumor progression, and may represent a suitable and specific target for novel anticancer strategies (8, 10, 20). The expression of V-ATPases is increased in chemoresistant cancer cells and can be induced by chemotherapeutic agents (21, 22). Molecular silencing and pharmacologic inhibitors of the V-ATPases can delay cancer growth, but such approaches may result in severe toxicity and be unfeasible and problematic. Proton pump inhibitors (PPI) have been largely and successfully used for the treatment of peptic diseases, due to their antiacidic properties. After protonation in the acidic spaces of the stomach, PPI irreversibly bind the proton pump, dramatically inhibiting proton translocation and acidification of the extracellular environment. The specific targets of PPI are H⁺-ATPases normally contained within the lumen of gastric parietal cells. However, PPI also inhibit the activity of V-ATPases, thus blocking proton transport across membranes. Because of the importance of the reversed pH gradients in malignant progression of tumor cells, we tested the specific effects of PPI on (a) drug resistance in a variety of human tumor cells and (b) growth of B-cell tumors, both in vitro and in vivo. Being weak bases, most chemotherapeutics are neutralized either in the acidic tumor microenvironment or in the lumen of acidic intracellular vesicles, representing these two factors as the bases for cellular resistance to drugs (20, 23). We observed that physiologic concentrations of PPI significantly increase the pH of acidic intracellular vesicles and the extracellular pH, inducing accumulation of acidic vesicles in the cell. Indeed, low-dose pretreatment in vitro and in vivo with PPI (a) reverted chemoresistance of different tumor cells to cisplatin, 5-fluorouracil, and doxorubicin; and (b) increased the sensitivity of drug-sensitive cells to anticancer agents. These effects were mediated by the intracellular retention of chemotherapeutic agents, associated with a “normalization” of the pH gradients of the tumor cells (Fig. 1B; ref. 24).

It seems highly conceivable that tumor acidity may induce a selective accumulation of PPI in the tumor tissues, as it occurs normally in the stomach. By analogy with the gastric compartment, PPI may be protonated and transformed in the active form in the acidic tumor microenvironment, thus blocking the H⁺-ATPase and altering the tumor pH gradients. In fact, we found that effect of PPI on tumor cells were directly related to the level of acidity of the culture medium (25). PPI induced selective cytotoxicity in B-cell tumors that passed through an early massive reactive oxygen species (ROS) activation and lysosomal membrane perturbation, leading to a caspase-independent cell death (25). In line with the expected inhibition of pH regulation, PPI caused alkalization of acidic vesicles and acidification of the cytosol. The antineoplastic activity of PPI was observed also in pre-B acute lymphoblastic leukemia cells obtained from patients with acute lymphoblastic leukemia, as well as in severe combined immunodeficient mice engrafted with B-cell lymphomas, whose growth was significantly reduced after PPI p.o. administration. Of course, the effect of PPI may depend on the level of acidity within the target tumor tissues. Indeed, it is conceivable that solid tumors, such as carcinoma or melanomas, may create a more acidic environment, in turn allowing a more potent PPI activation. We know that melanoma cells may survive at very low pH (lower than 5; ref. 26), whereas B-cell tumor-derived cells mostly die at these pH.3 This suggests that at least melanomas are more acidic than B-cell lymphomas, and that melanoma cells are armed to bear extreme acidic conditions. Altogether, these results suggest that PPI-mediated antitumor activity may pass through a strong inhibition of a crucial mechanism that allows tumor cells to efficiently eliminate toxic molecules, including protons and ROS. ROS accumulation is an early event in the PPI-mediated antineoplastic effect and permeabilization of acidic vesicles is crucial in this apoptotic cascade. The following acidification of the cytosol may create the optimal conditions for massive activation of protease and other very dangerous lytic enzymes, thus leading to cell death through a sort of autodigestion. However, these results provided the proof of concept that PPI may be considered not only as chemosensitizer agents, but also as a new class of antineoplastic drugs. We reckon that the mechanisms of action of PPI are based on the strong reduction of the reversed cellular pH gradient characterizing tumor cells, eventually leading to tumor cell death (Fig. 1B). In fact, tumor microenvironment is characterized by reversed pH gradient, with an acidic extracellular pH and an alkaline cytosol. This condition is involved in both tumor malignancy and tumor resistance to chemotherapeutics (Fig. 1B, top). PPI treatment clocks up the pH gradient by stopping the V-ATPase-mediated H⁺ efflux, in turn allowing anticancer drugs to enter and exert their action within tumor cells (24) and triggering apoptotic pathways in tumor cells that lead to tumor growth inhibition (ref. 25; Fig. 1B, bottom).

3 Unpublished data.
A, proton pump V-ATPase is overexpressed in cancer cells with metastatic potential. The 16 kDa subunit (ATP6L) of V-ATPase can provide the proton transmembrane path. To explore the role of V-ATPase in cancer metastasis, ATP6L expression in highly metastatic HCCLM3 cells was knocked down by ~60% using small interfering RNA. The invasion, the expression of MMP-2, and the protease activity of small interfering RNA–treated cells were decreased in vitro. In vivo, the protease activity and the metastasis were also drastically reduced. These results implicate the potential of V-ATPase as a candidate target for antimetastasis treatment (16).

B, tumor cells are characterized by an alkaline cytosolic pH and an acidic extracellular pH whose gradient is involved in tumor progression and malignancy, resulting in chemoresistance, metastasis, and increased proliferation rate. Such pH gradients are maintained by the up-regulated activity of V-ATPases that extrude protons outside the cell and acidify intracellular vesicles. In the presence of PPI, which are protonated and activated in the tumor environment, the activity of V-ATPase is inhibited and the abnormal pH homeostasis of tumor cells is dramatically altered, thus depriving them of a crucial mechanism contributing to tumor malignant behavior (24, 25). The action of PPI include chemosensitization, inhibition of proliferation, and induction of apoptosis.
Tumor Acidity as a New Delivery for Anticancer Drugs

Pharmacologic inhibitors of V-ATPases activity have been used in the past with high level of efficacy in vitro, but their potential application in clinical settings is hampered by predicted toxicity on normal cells (20). The great potential of PPI as V-ATPase inhibitors is that they need protonation to be transformed in the active drug (20). This, in turn, means at least two important things: (a) they are recruited by the acidic compartments and (b) they are activated to work as anticadi drugs, only in these compartments. The evidence is that PPI need an acidic or at least unbuffered medium to work as active drug in culture condition. It has been also shown that human tumor cells may be cultivated in really extreme environmental conditions, such as very acidic medium, whereas in the same conditions normal cells die (26). In fact, PPI are not toxic to normal cells, whereas they exert their action against tumor cells, and this is proven to occur in vivo as well (25). Additional strategies using tumor acidity as a delivery system for anticancer drugs imply the use of acridine orange and that of pH-low insertion peptides nanotechnology. In fact, acridine orange selectively accumulates within tumor tissues due to reversed pH gradients, and after photo- and radiodynamic therapy acridine orange is activated within cancers (such as musculoskeletal tumors) and exerts a very selective cytotoxic effect (27). Recently, interesting nanotechnology data identified peptides able to selectively insert into the membrane of cancer cells only at acidic pH, thus providing a powerful tool for selective delivery of therapeutic agents to the acidic site of the tumors (28).

Concluding Remarks

Our results and recent published observations indicate a new path to anticancer treatment, and important suggestions are emerg-