

Cancer Research

Targeting Vacuolar H⁺-ATPases as a New Strategy against Cancer

Stefano Fais, Angelo De Milito, Haiyan You, et al.

Cancer Res 2007;67:10627-10630. Published online November 15, 2007.

Updated Version Access the most recent version of this article at:
doi:[10.1158/0008-5472.CAN-07-1805](https://doi.org/10.1158/0008-5472.CAN-07-1805)

Cited Articles This article cites 28 articles, 15 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/67/22/10627.full.html#ref-list-1>

Citing Articles This article has been cited by 6 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/67/22/10627.full.html#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Targeting Vacuolar H⁺-ATPases as a New Strategy against Cancer

Stefano Fais,¹ Angelo De Milito,¹ Haiyan You,² and Wenxin Qin²

¹Department of Drug Research and Evaluation, Section of Pharmacogenetic, Drug Resistance and Experimental Therapeutic, Istituto Superiore di Sanità, Rome, Italy and ²National Laboratory for Oncogenes and Related Genes, WHO Collaborating Center for Research on Cancer, Shanghai Cancer Institute, Shanghai Jiao Tong University, Shanghai, China

Abstract

Growing evidence suggests a key role of tumor acidic microenvironment 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⁺-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 *in vivo* 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 turn 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 1 α 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⁺ ions that otherwise may accumulate within cytosolic spaces, in turn fatally 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⁺-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

Note: W. Qin is the Visiting Scientist of the Institute for Systems Biology, Leroy Hood Laboratory, Seattle, WA 98103-8904.

Requests for reprints: Stefano Fais, Section of Pharmacogenetic, Drug Resistance and Experimental Therapeutic, Department of Drug Research and Evaluation, Istituto Superiore di Sanità, Rome, Italy. Phone: 39-06-49903195; Fax: 39-06-49903691; E-mail: stefano.fais@iss.it or Wenxin Qin, National Laboratory for Oncogenes and Related Genes, WHO Collaborating Center for Research on Cancer, Shanghai Cancer Institute, Shanghai Jiao Tong University, Shanghai, China. Phone: 86-21-64436581; Fax: 86-21-64432142; E-mail: qinwenxin@smail.cn.

©2007 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-07-1805

of protease activity (16). Cancer metastasis is an ultimate cause leading to the failure of clinical treatment for patients with malignant tumor (17). Throughout the entire process of cancer metastasis, degradation and remodeling of ECM almost exist in each step (18). Thus, blocking the ECM degradation has become a prospective approach in the development of treatment for cancer metastasis. However, previous trials whose end point was to target only one or several MMPs by MMP inhibitors did not provide positive results. A possible main reason for these failures is that MMP family consists of over 20 members and there is no MMP inhibitor (endogenous or exogenous) able to simultaneously inhibit all MMP members (19). Our data (16) and other reports (8, 11) have indicated that one way to inhibit protease activation in cancer metastasis is to increase the pH of extracellular microenvironment of metastatic cancer cells, in turn entirely suppressing the activation of proteases and blocking the process of degradation and remodeling of ECM. Some data suggest that different use of ion exchangers may help to distinguish tumor cells with different metastatic behavior (9). In fact, although breast cancer cells with low metastatic potential preferentially use Na^+/H^+ exchangers and HCO_3^- -based H^+ -transporting mechanisms, highly metastatic cells preferentially use plasma membrane V-ATPases (9).

Altogether, these findings have provided new *in vitro* and *in vivo* evidence that V-ATPases may represent a target of cancer therapy (Fig. 1A), by directly regulating the pH gradient within tumor microenvironment, indirectly avoiding activation of ECM proteases.

Proton Pump Inhibitors as Antineoplastic Drugs

The alkaline-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 chemotherapeutics (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 alkalinization 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.

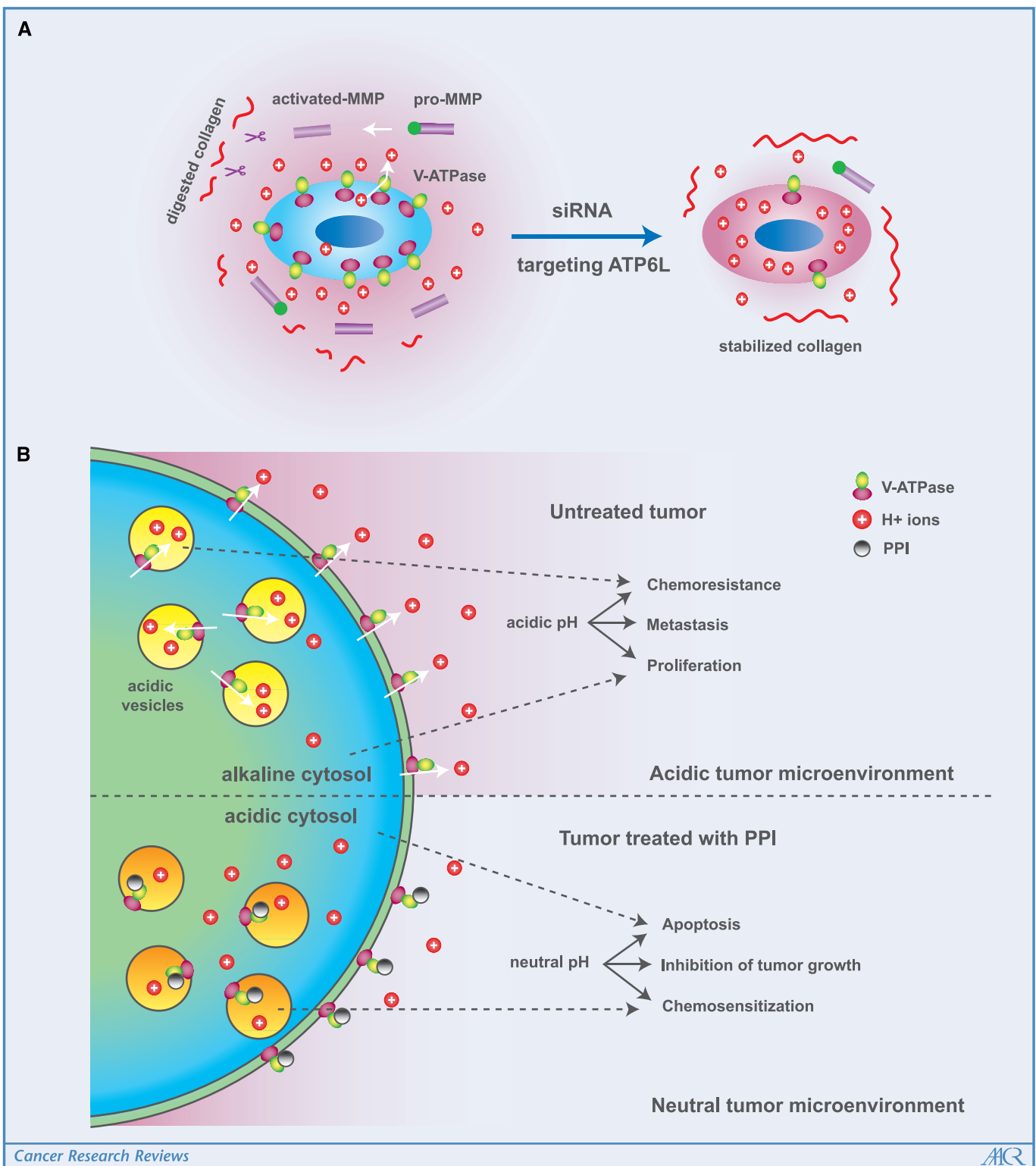


Figure 1. Effects of V-ATPase inhibition of tumor microenvironment. **A**, proton pump V-ATPase is overexpressed in cancer cells with metastatic potential. The 16 kDa subunit (ATP6L) of V-ATPase can provide the proton transmembrane path. To explore the role of V-ATPase in cancer metastasis, ATP6L expression 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 antiacidic 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 photodynamic 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-

ing from these new data. The mechanisms controlling the abnormal pH gradients in tumors should represent a selective and specific target in setting up new anticancer strategies. To our opinion, V-ATPases should be considered as the most important of these targets because of its crucial function in determining the acidification of tumor microenvironment and consequently the elimination of toxic molecules (such as H⁺ or ROS). Last, tumor acidity should be considered as a specific delivery system for new antitumor strategies, based on drugs that are specifically recruited within acidic environment (such as tumors) and activated *in situ*, thus hijacking an essential survival factor for tumors. Notably, tumor acidity is also related to another important tumor feature, in turn considered a survival option of malignant tumors, such as cannibalism (26). Tumor cannibalism is a function through which metastatic tumors feed off other cells, either dead or alive, including the T lymphocytes that should kill them. Experimental data have shown that cannibalism is increased in acidic culture conditions (26). It is therefore reasonable that PPI or V-ATPase inhibitors may well reduce cannibalism of tumors, thus inhibiting a way the tumors use to feed in low nutrient supply conditions, such as a growing tumor mass. In fact, it is very conceivable that tumors need proton pumps to allow their survival in such extremely adverse environmental conditions, the same conditions that do not allow survival of a normal cell.

Acknowledgments

Received 5/16/2007; revised 7/12/2007; accepted 8/8/2007.

Grant support: Italian NIH grant N33, Swedish Research Council grant (A. De Milito), and National Key Basic Research Program of China grant 2002CB513104 and Program of Shanghai Subject Chief Scientist grant 05XD14013 (W. Qin).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Xiaodong Lu for her help in drawing Fig. 1A.

References

- Raghunand N, Mahoney B, van Sluis R, Baggett B, Gillies RJ. Acute metabolic alkalosis enhances response of C3H mouse mammary tumors to the weak base mitoxantrone. *Neoplasia* 2001;3:227-35.
- Morita T, Nagaki T, Fukuda I, Okumura K. Clastogenicity of low pH to various cultured mammalian cells. *Mutat Res* 1992;268:297-305.
- Martinez-Zaguilan R, Seftor EA, Seftor RE, et al. Acidic pH enhances the invasive behavior of human melanoma cells. *Clin Exp Metastasis* 1996;14:176-86.
- Warburg O. On the origin of cancer cells. *Science* 1956;123:309-14.
- Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 2004;4:891-9.
- Newell K, Franchi A, Pouyssegur J, Tannock I. Studies with glycolysis-deficient cells suggest that production of lactic acid is not the only cause of tumor acidity. *Proc Natl Acad Sci U S A* 1993;90:1127-31.
- Yamagata M, Hasuda K, Stamato T, Tannock IF. The contribution of lactic acid to acidification of tumours: studies of variant cells lacking lactate dehydrogenase. *Br J Cancer* 1998;77:1726-31.
- Nishi T, Forgacs M. The vacuolar (H⁺)-ATPases—nature's most versatile proton pumps. *Nat Rev Mol Cell Biol* 2002;3:94-103.
- Sennoune SR, Bakunts K, Martínez GM, et al. Vacuolar H⁺-ATPase in human breast cancer cells with distinct metastatic potential: distribution and functional activity. *Am J Physiol Cell Physiol* 2004;286:1443-52.
- Sennoune SR, Luo D, Martinez-Zaguilan R. Plasma-lemmal vacuolar-type H⁺-ATPase in cancer biology. *Cell Biochem Biophys* 2004;40:185-206.
- Rofstad EK, Mathiesen B, Kindem K, Galappathi K. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer Res* 2006;66:6699-707.
- Montcourrier P, Mangeat PH, Valembois C, et al. Characterization of very acidic phagosomes in breast cancer cells and their association with invasion. *J Cell Sci* 1994;107:2381-91.
- Rozhin J, Sameni M, Ziegler G, Sloane BF. Pericellular pH affects distribution and secretion of cathepsin B in malignant cells. *Cancer Res* 1994;54:6517-25.
- Johnson LL, Pavlovsky AG, Johnson AR, et al. A rationalization of the acidic pH dependence for stromelysin-1 (matrix metalloproteinase-3) catalysis and inhibition. *J Biol Chem* 2000;275:11026-33.
- Kato Y, Lambert CA, Colige AC, et al. Acidic extracellular pH induces matrix metalloproteinase-9 expression in mouse metastatic melanoma cells through the phospholipase D-mitogen-activated protein kinase signaling. *J Biol Chem* 2005;280:10938-44.
- Lu X, Qin W, Li J, et al. The growth and metastasis of human hepatocellular carcinoma xenografts are inhibited by small interfering RNA targeting to the subunit ATP6L of proton pump. *Cancer Res* 2005;65:6843-9.
- Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med* 2006;12:895-904.
- Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell* 2006;127:679-95.
- Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002;295:2387-92.
- De Milito A, Fais S. Tumor acidity, chemoresistance and proton pump inhibitors. *Future Oncol* 2005;1:779-86.
- Murakami T, Shibuya I, Ise T, et al. Elevated expression of vacuolar proton pump genes and cellular pH in cisplatin resistance. *Int J Cancer* 2001;93:869-74.
- Torigoe T, Izumi H, Ishiguchi H, et al. Enhanced expression of the human vacuolar H⁺-ATPase c subunit gene (ATP6L) in response to anticancer agents. *J Biol Chem* 2002;277:36534-43.
- Raghunand N, He X, van Sluis R, et al. Enhancement of chemotherapy by manipulation of tumour pH. *Br J Cancer* 1999;80:1005-11.
- Luciani F, Spada M, De Milito A, et al. Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs. *J Natl Cancer Inst* 2004;96:1702-13.
- De Milito A, Iessi E, Logozzi MA, et al. Proton pump inhibitors induce apoptosis of human B cell tumors through a caspase-independent mechanism involving reactive oxygen species. *Cancer Res* 2007;67:5408-17.
- Lugini L, Matarrese P, Tinari A, et al. Cannibalism of live lymphocytes by human metastatic but not primary melanoma cells. *Cancer Res* 2006;66:3629-38.
- Kusuzaki K, Murata H, Matsubara T, et al. Acridine orange could be an innovative anticancer agent under photon energy. *In Vivo* 2007;21:205-14.
- Andreev OA, Dupuy AD, Segala M, et al. Mechanism and uses of a membrane peptide that targets tumors and other acidic tissues *in vivo*. *Proc Natl Acad Sci U S A* 2007;104:7893-8.