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Proton pump inhibitor-induced tumour cell death by inhibition of a detoxification mechanism

S. Fais

From the Anti-Tumour Drugs Section, Department of Therapeutic Research and Medicines Evaluation, Istituo Superiore di Sanità (National Institute of Health), Rome, Italy

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This review presents a possible new approach against cancer, as represented by inhibition of proton pumps, a mechanism used by tumour cells to avoid intracellular accumulation of toxic substances. Proton pump inhibitors (PPIs) belong to a family of pro-drugs that are currently used in the treatment of peptic diseases needing acidity to be activated. PPIs target the acidic tumour mass, where they are metabolized, thus blocking proton traffic. Proton pump inhibition triggers a rapid cell death as a result of intracellular acidification, caspase activation and early accumulation of reactive oxygen species into tumour cells. As a whole, the devastating effect of PPIs on tumour cells suggest the triggering of a fatal cell toxification. Many human tumours, including melanoma, osteosarcoma, lymphomas and various adenocarcinomas are responsive to PPIs. This appears highly conceivable, in as much as almost all human tumours are acidic and express high levels of proton pumps. Paradoxically, metastatic tumours appear to be more responsive to PPIs being more acidic than the majority of primary tumours. However, two clinical trials test the effectiveness of PPIs in chemosensitizing melanoma and osteosarcoma patients. Indeed, tumour acidity represents a very potent mechanism of chemoresistance. A majority of cytotoxic agents, being weak bases, are quickly protonated outside and do not enter the cells, thus preventing drugs to reach specific cellular targets. Clinical data will provide the proof of concept on the use of PPIs as a new class of antitumour agent with a very low level of systemic toxicity as compared with standard chemotherapeutic agents.

Keywords: cancer, cancer chemotherapy, detoxification enzymes, drug resistance, reactive oxygen species, therapeutics.

The hostile cancer microenvironment

Cancer is an area with significant unmet medical need. Worldwide, more than 20 million people are diagnosed with cancer annually and, in spite of currently available therapies, more than a million patients die from this disease each year. There is a continuing need for safe and effective new treatments resulting in durable disease remission and increased overall survival. In recent decades, the war against cancer was based on the principle of Paul Ehrlich's 'magic bullets', introduced more than 100 years ago, which led to the success of antibiotics 50 years later. The successful use of antibiotics against infectious agents supported the use of the same approach against malignant tumours: to set up new drugs that selectively target and kill tumour cells [1]. After so many years we are still waiting for the magic bullet against malignant tumours. New approaches are now being proposed, such as developing therapeutic strategies aimed at controlling cancer rather than trying to cure it [2]. However, it is also possible to approach new anticancer therapies by trying to understand the mechanism/s through which cancer cells avoid growth control. It is possible that cancers also use the same mechanisms to overcome the cytotoxic effect of chemotherapeutic agents, which very often induce more adverse side effects than inhibition of cancer growth, progression and metastatic spread. Further understanding of the molecular mechanisms involved in the growth and progression of malignant tumours is required. In general, tumour cells upregulate glycolysis and grow in a hypoxic microenvironment. Highly proliferative cancer cells produce a large amount of H^+ generated by glycolysis, glucose utilization, lactic acid production and proton efflux [3, 4]. One interesting hypothesis is that the hostile microenvironment generated during

tumour growth progressively selects cells suited to survive in these adverse conditions. The uncontrolled growth, lactate production from tumour metabolism and the low blood and nutrient supply all contribute to a tumour microenvironment with many molecules that are extremely toxic for either normal or more differentiated cells. It is therefore possible that those cells that survive in this unfavourable microenvironment possess the means for avoiding intracellular accumulation of toxic molecules. Some of the most efficient mechanisms that allow cancer cell survival in adverse environmental conditions include several proton extruders [5, 6]. Amongst the proton flux regulators are the V-ATPase [7], the Na^+/H^+ exchanger (NHE) [8] and carbonic anhydrase 9 (Table 1). These hyperactive proton pumps create a disturbance of pH gradients that is typical of malignant cells. In fact, malignant tumour cells are characterized by a disturbed pH gradient between the acidic extracellular and the alkaline intracellular compartments (reversed pH gradients; Fig. 1). This is a condition that quickly kills normal or more differentiated cells. Proton pumps efficiently extrude H^+ out of the cell (Fig. 2a) and/or into internal vacuoles (Fig. 2b), thus avoiding potentially highly cytotoxic acidification of the tumour cell cytosol. In many tumours, the chronic exposure to acidic pH has been reported to promote invasiveness, metastatic behaviour and resistance to cytotoxic agents [9–12]. Moreover, some evidence suggests that abnormal pH may be involved in some tumour-associated cellular functions, such as driving acidic vesicular traffic [13], drug resistance [14], lytic enzyme activation [13] and aberrant phagocytic activity [15, 16]. Detoxifying mechanisms may thus represent both a key survival option and a mechanism that progressively selects individual cells armed to survive against all odds rather than collaborate with other cells for a common end-point. This makes malignant cells very similar to microorganisms [17]. In fact, proton pumps, as cellular mechanisms that counteract the accumulation of toxic agents, are very active in microbes and yeasts too [18–20]. We propose that an efficient approach to the fight against cancer would be to block or inhibit as many mechanisms involved in cell detoxification as possible, to deprive cancer cells of a key survival option and thus trigger tumour cell death. During the last decade, several lines of evidence have suggested that the majority of human cancers may potentially be responsive to therapies based on inhibition of mechanisms underlying tumour acidification [3–14, 21]. In fact, extracellular acidity is a hallmark of tumour malignancy and proton pumps seem to be the major mechanism responsible for acidification of the tumour extracellular environment [4–8, 21]. Acidity has a key role in increasing the metastatic behaviour of tumours [9, 10]. In sum, to overcome tumour acidity by inhibiting mechanism of

Table 1 *Efflux pumps described as hyperexpressed and/or hyperfunctional in malignant tumours or cell lines*

Type of pump	Cellular localization	Function	References
H^+-ATPase	Plasma membrane and acidic organelles	Acidification of extracellular microenvironment and endo-lysosomal compartment	[7, 14, 76]
Na^+/H^+-ATPase (NHE)	Plasma membrane	Alkalinization of cytosol and acidification of extracellular microenvironment	[59]
MCT1 (H^+/ lactate symporter)	Plasma membrane	Elimination of lactate from glucose catabolism, and acidification of extracellular milieu	[77]
Carbonic anhydrase 9	Plasma membrane	Regulation of intracellular pH and pH gradients	[21]
H^+/K^+-ATPase	Gastric epithelial cell line	Regulation of extracellular pH	[78]
ATP-binding cassette	Plasma membrane	Extrusion of chemotherapeutic drugs	[31,79,80]

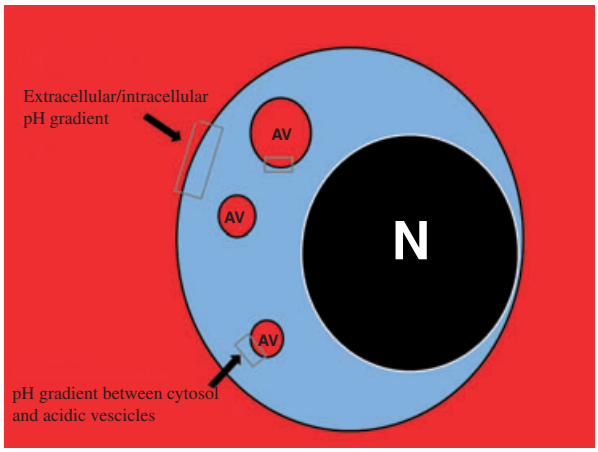

Fig. 1 *Cellular pH gradients. At least two pH gradients are present both in normal and tumour cells: one between the extracellular space and the cytosol (big grey rectangle) and the other between the cytosol and the acidic vesicle (small grey rectangle). In a tumour cell, the extracellular pH is acidic (ranging between 5.5 and 6.8), the intracellular pH is weakly acidic (ranging between 7.3 to 7.8) and the pH of the acidic vesicle (AV) is very acidic. N, nucleus.*

acidification may represent the future path of antitumour strategies.

In this review, we present data to support the idea that a future anticancer approach should include molecules that counteract proton pump activity as a key mechanism of tumour cells homeostasis.

Failure of a general cytotoxic approach

Many human solid tumours are difficult to treat with conventional therapies, even though the majority of anticancer drugs are cell poisons that have the potential to kill all cells, alone or in combination. At least two main mechanisms may be involved in this poor responsiveness of malignant tumours to the cytotoxic approach. One is acquired resistance to chemotherapeutic drugs, a phenomenon known as multidrug resistance (MDR), which is still considered a major cause of treatment failure in cancer patients. However, it specifically refers to tumour cells or tumours that respond to chemotherapy in the first-line setting, but after recurrence do not show sensitivity to a wide spectrum of anticancer drugs [22, 23]. In classical MDR, tumour cells exhibit resistance to a wide range of structurally and functionally unrelated compounds, such as vinca alkaloids, anthracyclines, taxoids and other antimitotic drugs [24, 25]. MDR cells

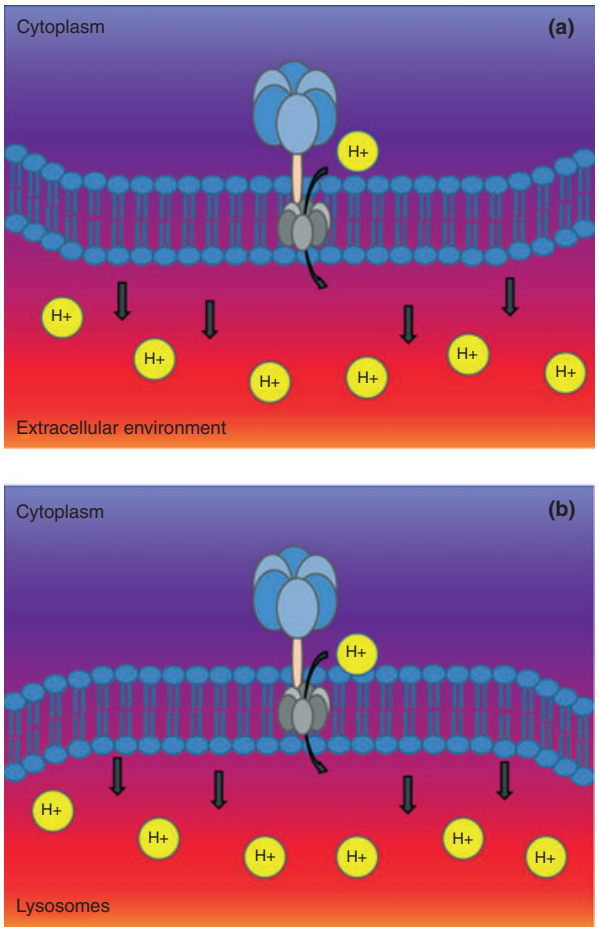

Fig. 2 *Subcellular localization of V-ATPases. (a) V-ATPase is so called because of a preferential expression on the membrane of internal acidic vesicles where it pumps H^+ from the cytosol to the intravesicle space. The localization and function of V-ATPase are common to a variety of cells. The role of this enzyme is to maintain lysosomes or phagosomes in an acidic state, suitable for lytic enzyme activation. (b) V-ATPase may also be expressed on plasma membranes. This is the case for cells that require extracellular acidification to function, such as osteoclasts. These cells need a low pH microenvironment to directly remodel bones and activate proteolytic enzymes in young children and adolescents. V-ATPases pump H^+ ions outside the cell with a net consumption of ATP.*

overexpress a variety of transmembrane drug efflux pumps belonging to the family of ATP-binding cassette transporters, which includes P-glycoprotein (P-gp or MDR-1) [23], the multidrug resistance-associated protein-1 [26], the lung resistance protein [27, 28] and the breast cancer resistance protein [29]. These proteins extrude drug molecules against a concentration gradient into the extracellular

compartment, leading to a significant decrease in intracellular drug retention. Unfortunately, whereas the increase in the expression and activity of these proteins was directly related to MDR *in vitro*, the same relationship was not found for *in vivo* chemoresistance of solid tumours, raising considerable doubts about the clinical relevance of this phenomenon [30]. In fact, the precise mechanism/s of lack of response to chemotherapy in the vast majority of cancers is unknown. Most proposed mechanisms involve biochemical and microenvironmental factors. A very rough but extremely efficient mechanism of lack of responsiveness to chemotherapy is the change in pH gradient between the extracellular environment and the cell cytoplasm, and between the cell cytoplasm and lysosomal compartments [31, 32]. Drug entry into the cell is highly dependent on the pH gradient between the external and internal compartments of the cell. Malignant tumours are characterized by reverse pH gradients (Fig. 1) and this may severely affect the entry of drugs [4, 33]. Indeed, low extracellular pH reduces the uptake of weakly basic chemotherapeutic drugs through direct protonation, thus preventing drug intracellular targeting [25]. It is therefore conceivable that a very efficient mechanism of tumour unresponsiveness to chemotherapeutics would be the direct protonation and neutralization of drugs by the H^+-rich extracellular compartment [4].

Moreover, an important mechanism for chemoresistance involves acidic vesicles and the altered pH gradient between the cytoplasm and intracellular organelles. Many chemotherapeutic drugs are membrane permeable in their neutral form and relatively membrane impermeant when protonated and charged. Upon entry, they are protonated and sequestered within the acidic compartments of the cell (lysosomes, recycling endosomes, trans-Golgi network and secretory vesicles). Furthermore, lysosomal-like vesicles of MDR cells are more acidic than vacuoles of drug-sensitive cells [34]. This suggests that a common mechanism used by tumours to neutralize cytotoxic drugs is sequestration within acidic vesicles possibly followed by elimination outside the cells by vesicle degranulation. Proton pumps and pH changes exert a key role in the traffic of microvesicles within the tumour microenvironment [35]. Indeed, agents that disrupt or normalize the pH gradient in tumours may reverse MDR and/or directly inhibit tumour growth. This effect may be obtained by either a direct buffering by sodium bicarbonate treatment [25] or lysosomotropic agents inducing both pH gradient modification and alkalinization of acidic vesicles [36]. Key evidence supporting the role of proton pumps and acidity in chemoresistance has been provided by experiments with proton pump inhibitors (PPIs). Inhibition of proton pumps by drugs belonging to the PPI family (e.g. omeprazole and lanzoprazole) induced reversion of drug resistance in chemoresistant human cancer cells, consistent with increase of extracellular pH, inhibition of acidic vesicle secretion and increased intracellular drug retention [37]. These data support a new approach for sensitizing chemoresistant tumours to cytotoxic drugs through specific inhibition of a mechanism that actively maintains the tumour microenvironment in a state of low pH. However, PPIs also have another very important feature: they are pro-drugs that need acidity for full activation [38]. This chemical property of PPIs allows both specific delivery of these drugs to the acidic body compartments and specific transformation into the active compound (tetracyclic sulphenamide) within the acidic microenvironment [39]. This feature of PPIs is key for reducing potential side effects of PPI. These enzymes are widely expressed within the human body and there is evidence that mutations of some proton pumps may lead to serious and often lethal conditions. Genetic defects in the expression of V-ATPases in osteoclasts and intercalated cells may lead to development of osteopetrosis and renal tubule acidosis, respectively [40]. Recent data also show that inhibition of V-ATPase subunit (ATP6L expression) by small interfering RNA in MDR human tumour cells sensitizes the cells to doxorobicin, 5-fluorourocil and vincristine. This effect was mediated by a significant increase in lysosomal pH and retention of anticancer drugs in the cell nucleus, thus further supporting a role of proton pumps and tumour acidity in resistance to chemotherapy [41].

Involvement of proton pumps in tumour cell invasion and metastasis

V-ATPases are not merely a harmful mechanism leading to extracellular acidification. They pump protons into the extracellular environment or lumen of some membrane-bound organelles to avoid accumulation of H^+ within the cell cytosol [39, 40, 42, 43] (Fig. 2). Moreover, overexpression of V-ATPases is related to invasion and metastasis [6, 42]. However, these enzymes, as well as maintaining cytoplasmic pH homeostasis, are also involved in many cellular functions such as receptor- and non-receptor-mediated endocytosis and intracellular transport [43]. Moreover, V-ATPases have a key role in the regulation of more complex functions of normal organs and tissues, such as renal acidification, pH maintenance in mechanosensory hair cells and bone reabsorption [40]. V-ATPases are very active in malignant cells,

correlating with a high expression at both the plasma membrane and acidic vesicle level [42] (Fig. 3), probably because of a large amount of H^+ ions from the acidic extracellular space. 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 (Fig. 3). Extracellular acidification may represent a real advantage for cancer cells, inasmuch as it is the best condition for secretion and activation of many proteases involved in the digestion of the extracellular matrix (ECM). Proteases that need a low extracellular pH for optimum activation include matrix metalloproteinases (MMPs), bone morphogenetic protein-1-type metalloproteinases, tissue serine proteases and adamalysin-related membrane proteases [44, 45]. MMPs are proteases essentially involved in degradation and remoulding of the ECM, owing to their ability to collectively degrade all the structural components of the ECM. Knockdown of ATP6L (c-subunit gene) inhibits cancer metastasis by decreasing proton flux and, consequently, inhibiting protease activation [46]. Cancer metastasis ultimately leads to the failure of clinical treatment for patients with malignant tumours [44]. Remodelling and degradation of the ECM is a constant feature of cancer invasion and metastasis [45]. It is therefore conceivable that blocking tumour acidification may represent a future therapeutic approach for inhibiting cancer invasion and metastasis, through loss of a key requirement for lytic enzymes activation. Tumour cells with different metastatic behaviour may be distinguished through their different use of ion exchangers [6]. Some data suggest that cancer cells with low metastatic potential preferentially use NHEs and bicarbonate-based H^+-transporting mechanisms, whereas highly metastatic cells preferentially use plasma membrane V-ATPases [6]. The expression of V-ATPases is increased in chemoresistant cancer cells and can be induced by chemotherapeutic agents [47, 48]. These data have indicated that, for various reasons, V-ATPases may represent a valuable target for new anti-cancer strategies [4, 39, 42, 43].

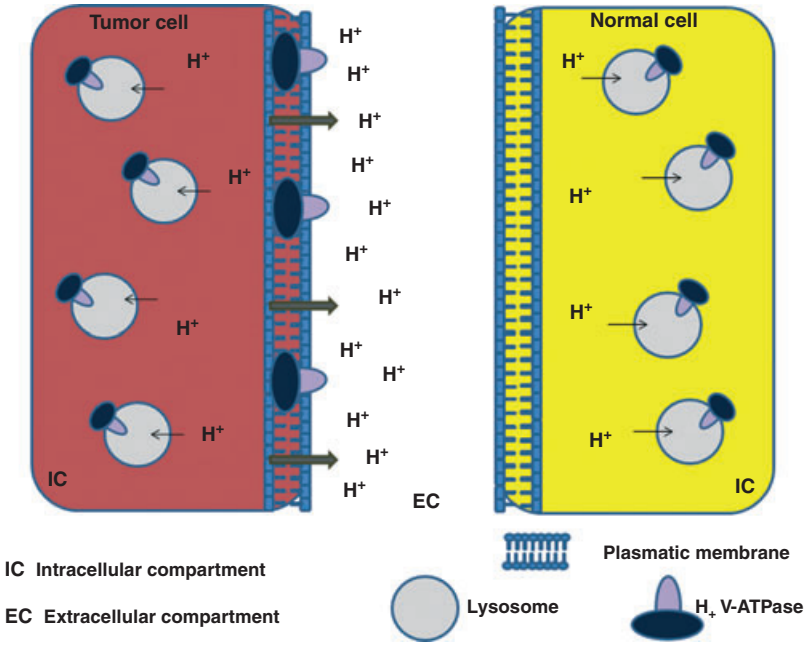

Fig. 3 *Differences in pH gradients and V-ATPase activity between normal and tumour cells. Tumour cells are characterized by an alkaline cytosolic pH and an acidic extracellular pH; this gradient is involved in tumour progression and malignancy, resulting in chemoresistance, metastasis and increased rate of proliferation. Such pH gradients are maintained by the upregulated activity of V-ATPases that extrude protons outside the cell and acidify intracellular vesicles. However, a footprint of tumour cells is a marked V-ATPase expression on the cell plasma membrane, which in normal conditions occurs only in very specialized cells (such as osteoclasts). Extracellular acidification through V-ATPase occurs normally in acid-secreting compartments of the stomach. Indeed, proton pump inhibitors need an H^+-rich microenvironment, as occurs in the stomach and in the tumour mass, for protonation and activation. Within the tumour, the result of V-ATPase hyperactivity is the so-called 'pH reverse gradient', with an acidic extracellular space and an alkaline cytosol.*

Inhibition of proton pumps: a new strategy against cancer

V-ATPase inhibitors have shown a high level of efficacy *in vitro*, but their potential application in the clinical setting has been hampered by likely toxicity in normal cells [4, 39]. However, molecular silencing of some V-ATPase subunits has shown delayed cancer growth even with only a cytostatic effect, and no clear cytotoxicity against cancer cells [39, 43]. The approach of using PPIs, such as omeprazole and esomeprazole, to inhibit tumour proton pumps without systemic toxicity seemed to be most promising for cancer therapy. After protonation in the acidic spaces of the stomach, PPIs irreversibly bind to the proton pumps, inhibiting proton translocation and acidification of the extracellular environment. The specific targets of PPIs are H^+-ATPases that are normally contained within the lumen of gastric parietal cells. PPIs have been used for decades as first-line treatment of peptic diseases, with minimal side effects [38, 49] even when administered at high dose and using chronic schedules as required for patients with Zollinger–Ellison syndrome [50, 51]. However, PPIs also inhibit the activity of V-ATPases, thus blocking proton transport across membranes. As a result of their broad action on various proton ATPases, pretreatment with PPIs reversed drug resistance in a variety of human tumour cells [37, 52]. Results of the safety profiles of PPIs [38, 49] together with our preclinical data [37] presented the rationale for two clinical trials currently ongoing at the National Tumor Institute (Milan) and the Orthopedic Institute Rizzoli (IOR; Bologna, Italy), with the end-point of evaluating the chemosensitizing effect of PPIs in patients with melanoma or osteosarcoma. However, it has been shown that *in vivo* inhibition of V-ATPase activity by RNA interference significantly delays human cancer growth by decreased proton extrusion [46], suggesting that pH regulation may have a key role in tumour homeostasis [39]. On the basis of these results, a possible direct antitumour activity of PPIs has recently been explored. The antitumour activity of PPIs was first investigated in human B-cell tumours in which, despite a good response rate to polychemotherapies [53, 54], a proportion of patients show chemoresistance and/or clinical relapse. Moreover, recent improvements in therapy have often been achieved by dose intensification, which can cause severe toxicity and secondary malignancies [53, 54]. Results showed that PPIs induced pro-apoptotic effects in various human B cell tumour-derived lines and in blasts of children with preacute lymphoblastic leukaemia [55]. Of interest, the PPI effect was increased by unbuffered culture medium [55], suggesting the need for free acidification of the medium for optimum PPI activation. In fact, the great potential of PPIs as V-ATPase inhibitors is that they require protonation to be transformed into the active drug [4, 38, 39] and cell culture conditions in the absence of buffering molecules allows spontaneous acidification by human tumour cells, and conceivably a better activation of PPIs. Moreover, PPIs induced a clear inhibition of B-cell tumour growth following repeated oral treatment, in the absence of any additional standard or nonconventional treatment [55], thus supporting the idea of PPI as a potential treatment of cancer [56, 57]. These findings also suggested that PPIs may be specifically targeted to cancer cell acidic compartments, generated by aberrant tumour metabolism, where they are activated inducing tumour growth inhibition, without detectable systemic toxicity. However, B-cell tumours were not the best model to measure PPI antineoplastic effect with respect to the level of acidity in the microenvironment, inasmuch as these cells do not tolerate baseline low pH conditions. To fully address this point, a more suitable model of human melanoma cells derived from metastatic lesions has been exploited. In fact, these human melanoma cells may be cultured at low pH, without any evidence of intolerance [16]. Using melanoma cells, PPIs showed a clear pH- and dose-dependent antitumour cytotoxic effect [58]. *In vivo* experiments confirmed that PPIs induce tumour growth inhibition, consistent with changes in pH gradients (with increase in extracellular pH and decrease in cytosolic pH), thus abolishing the typical tumour-dependent reversal of pH gradients [59, 60]. Moreover, repeated *in vivo* treatments with high-dose PPIs markedly increased the survival of xenografts, without any evidence of systemic toxic effects. These data provided the proof of concept that PPIs may be used as antineoplastic agents, and that they target tumour mass by acting on intracellular and extracellular pH regulators and thus cause tumour cell death. The underlying mechanism may involve acidification of the cell cytosol by depriving tumour cells of a pivotal survival option to dispose of H^+ and probably of acidic metabolites [39, 57, 60]. However, experimental data suggested that the PPI effect differed between tumour cell histotypes.

PPI-induced tumour cell death: apoptosis or nonconventional mechanism of cell death

Preclinical data suggested that PPIs induce cell death of many human malignant tumour-derived cell lines [52, 55, 58], with targeting of tumour cells because of their acidic pH [39, 57, 60], but apparently without targeting of any specific tumours. However, impor-

tant information regarding the mechanism that PPIs exploit to kill tumour cells came from further experiments. PPIs induced selective cytotoxicity in B-cell tumours that had undergone early massive reactive oxygen species (ROS) activation and lysosomal membrane perturbation, leading to a caspase-independent cell death [55]. In line with the expected inhibition of pH regulation, PPIs caused alkalinization of acidic vesicles and acidification of the cytosol, suggesting that PPI-mediated antitumour activity may undergo strong inhibition of a crucial mechanism that allows tumour cells to efficiently eliminate toxic molecules, including protons and ROS [55]. ROS accumulation is an early event in the PPI-mediated antineoplastic effect and permeabilization of acidic vesicles is a crucial part of this apoptotic cascade [55]. The subsequent acidification of the cytosol may create the optimum conditions for massive activation of protease and other lytic enzymes, thus leading to cell death through a type of autodigestion (or at least cessation of metabolic function). This was also consistent with an inhibition of protease needing acidity to be active in their ECM degradation [61, 62]. The results obtained with melanoma cells were in many respects comparable with those obtained with B-cell lymphoma, but the two cell death pathways appear to differ in the participation of caspases. Indeed, whereas PPIs induce caspase-independent cell death with involvement of mitochondrial membrane depolarization in human B-cell lymphomas [55], PPI-induced cell death is caspase dependent in human melanoma [58]. Moreover, PPI-induced melanoma cell death also involves typical features of caspase-mediated cell death, such as poly (ADP-ribose) polymerase cleavage and DNA fragmentation but, in contrast to B-cell lymphomas, it did not occur through mitochondrial alteration [58]. Furthermore, the cytotoxic effect of PPIs was not related to a particular mutational profile or activated signalling pathways, suggesting that PPIs may encompass molecular hallmarks of human malignant tumours such as melanoma.

The preclinical data on possible treatment doses and schedule of PPIs, suggest doses compatible with those administered daily to patients with Zollinger–Ellison syndrome [49–51] who receive up to 240 mg day^{-1} of esomeprazole for several days without major side effects. Magnetic resonance imaging data suggested also that a possible schedule for PPI treatment of patients could be discontinuous administration, because PPIs need an acidic tumour microenvironment to be transformed into the active molecule and a continuous treatment regimen may buffer the tumour and hence prevent full activation of PPIs [58].

Tumour acidity: a new target for anticancer molecules

Taken together, these data suggest for the first time that PPIs may represent a prototype model of anti-tumour pro-drugs that exploit the acidic extracellular pH of the tumour both as a therapeutic target and as a selective delivery system [39, 55, 58]. In fact, orally administered PPIs target the tumour site, shifting the baseline tumour extracellular pH from acidity to neutrality and reducing tumour intracellular pH, thus globally affecting the pH gradient within the tumour mass [58]. This may have important consequences not only on the capacity of malignant cells to proliferate and survive in acidic conditions but also on the distribution and penetration of chemotherapeutic drugs (and perhaps many other classes of old and new antitumour molecules) in the tumour microenvironment and within tumour cells [3–5, 39, 59, 60]. Different proton pumps may be inhibited by different drugs; for example, inhibition of the NHE by amiloride [59, 60] or of V-ATPases by PPIs [39] (Fig. 4). PPI-induced disturbance of tumour pH gradients is consistent with inhibition of tumour growth and an increased survival of melanoma-bearing animals [58], thus suggesting that PPIs may also positively affect the general status probably by acting on tumour-dependent cachexia, which is thought to be related to acidosis of many organs and tissues [63].

It is interesting that the concept of targeting or exploiting the acidic tumour pH as an antitumour therapeutic strategy has been supported by several studies using different molecules. For example, acridine orange (AO) and pH-low insertion peptides have been proposed as novel antitumoural strategies that employ tumour acidity as a delivery system. In a model of osteosarcoma, AO selectively accumulates in the tumour tissue owing to reversed pH gradients and, following photodynamic therapy, AO is activated and selectively kills cancer cells [64]. Recently, interesting nanotechnology data identified peptides that are 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 tumour sites [65]. Another important example is the inhibition of both tumour growth and metastasis formation induced by alkalinization of tumour pH via systemic bicarbonate treatment [66, 67]. pH-sensitive polymeric micelles and nanogels, which have recently been developed to target the slightly acidic extracellular pH environment of solid tumours, also

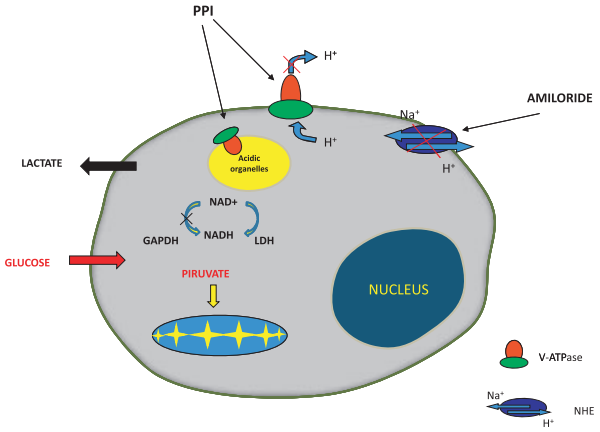

Fig. 4 *Mechanism of action of novel antitumour agents targeting pH regulation. The mechanism of action of proton pump inhibitors (inhibit V-ATPaases) and amiloride (inhibit the Na^+/H^+ exchanger) is shown. However, these drugs do not directly interfere with tumour acidification via lactate accumulation. It is thought that lactic dehydrogenase (LDH) competes with GAPdH for NAD+, resulting in cytosolic accumulation of lactate; the anaerobic condition upregulates lactate metabolism and reduces or eliminates glycolytic metabolism.*

provide an interesting and novel approach. The pH-targeting approach is preferred to conventional specific tumour cell surface targeting, because the acidic tumour microenvironment is considered to be a common feature of solid tumours [68]. The acidic environment created by solid tumours has been exploited to activate anticancer lytic peptides by making them cationic only at low pH, thus increasing their selectivity and pH-dependent activity [69].

These tumour acidity-dependent approaches include PPIs in a list of future strategies against cancer based on tumour acidity, taking advantage of the low pH of tumours as both a target and a way to specifically activate drugs within the tumour mass.

Concluding remarks

V-ATPase is expressed in all eukaryotes, from yeast to man, and in both normal and abnormal cells [40, 43]. Our results and those of other recently published studies indicate a new path to anticancer treatment, and important potential therapeutic options emerge from these new data. The mechanisms controlling the abnormal pH gradients in tumours may represent a selective and specific target for new anticancer strategies. V- ATPase is perhaps the most important of these targets because of its crucial role in determining the acidification of the tumour microenvironment and consequently the elimination of toxic molecules (such as H^+ or ROS). It is of great interest as malignant tumour cells, like unicellular organisms, use proton pumps for detoxification. It is reasonable in fact that PPIs may exert antibacterial activity against *Helicobacter pylori* [70], but it is not so immediate when the same evidence comes from studies on leishmaniasis [71] or salmonella [72]. We know that proton pumps are key enzymes for homeostasis of some infectious agents, such as malaria [73] and yeasts [20]. These diverse data on the importance of proton pumps in tumour cells and microorganisms strengthen the hypothesis that the main aim of both is to survive in a hostile microenvironment. Of note, tumour acidity is related to another important tumour feature, cannibalism, which is also considered to be a survival mechanism of malignant tumours [16]. Tumour cannibalism is a function through which metastatic tumours feed on 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 [16]. It is therefore reasonable that PPIs or V-ATPase inhibitors may reduce cannibalism of tumours, thus inhibiting a mechanism by which tumours feed in conditions of low nutrient supply, such as a growing tumour mass. This hypothesis is supported by the recent finding of a gene that is common to human melanoma cells and amoebae and is involved in phagocytic feeding activity [74].

These findings remind us of the Nobel laureate Otto H. Warburg who, nearly a century ago, pointed out that 'We can only cure what we can understand first'. In this respect, we still need to learn much more about the mechanisms of cancer development and survival, including the strategies that cancer cells adopt to cope with an adverse microenvironment, and how they deal with host defenses and toxic molecules. We need to understand why cancer cells select and generate a microenvironment that allows them to survive [75], whilst killing normal cells that are not capable of surviving in these toxic tumour-produced conditions of low pH, low oxygen levels and limited nutrient supply. In this context, a possible new approach may be to try to further elucidate the mechanisms that allow tumour cells to survive in the hostile microenvironment created during their growth. Examples of these survival mechanisms are the several proton pumps and transporters that allow cancer cells to expel H^+ ions, thus avoiding intracellular acidification and, therefore, cell death. By treating tumours with PPIs, cancer cells can be specifically killed by a cell death mechanism selectively induced by intracellular H^+

Table 2 *Human tumour cells and tumours sensible to inhibition of proton pumps*

Human tumour histotype	Chemosensitization	Antitumour effect	References
Melanoma	Cisplatin/5-FU/vinblastin/ doxorubicin	Cytotoxicity and caspase dependent	[37, 58, 81]
B-cell lymphoma	Vinblastin/doxorubicin	Cytotoxicity and caspase independent	[37, 55]
T-cell lymphoma		Cytotoxicity and caspase dependent	[55]
Breast cancer	Cisplatin/5-FU/vinblastin		[14, 37]
Colon cancer	Cisplatin/5-FU/vinblastin		[37]
Gastric cancer	Cisplatin/5-FU/doxorubicin	Cytotoxicity and apoptosis	[52, 82]
Ovarian cancer	Cisplatin/5-FU/vinblastin		[37]
Osteosarcoma	Cisplatin		De Milito A *et al.*, personal observation
Liver cancer		Inhibition of proliferation	[46, 83]
Colangio carcinoma		Cytotoxicity and apoptosis	[84]

accumulation. Data have also suggested that almost all human tumours may respond to PPI with chemosensitization and/or cytotoxicity, as summarized in Table 2. It is therefore likely that PPI may represent a new therapeutic strategy for all human cancers.

Conflict of interest statement

No conflict of interest was declared.

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References

1 Welch DR. Biologic considerations for drug targeting in cancer patients. *Cancer Treat Rev* 1987; **14:** 351–8.
2 Gatenby RA. A change of strategy in the war on cancer. *Nature* 2009; **459:** 508–9.
3 Trédan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst* 2007; **99:** 1441–54.
4 De Milito A, Fais S. Tumor acidity, chemoresistance and proton pump inhibitors. *Future Oncol* 2005; **1:** 779–86.
5 Izumi H, Torigoe T, Ishiguchi H *et al.* Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. *Cancer Treat Rev* 2003; **29:** 541–9.
6 Sennoune SR, Bakunts K, Martı′nez 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.
7 Martinez-Zaguilan R, Lynch RM, Martinez GM, Gillies RJ. Vacuolar-type Hα-ATPases are functionally expressed in plasma membranes of human tumor cells. *Am J Physiol Cell Physiol* 1993; **265:** C1015–29.
8 Lee AH, Tannock IF. Heterogeneity of intracellular pH and of mechanisms that regulate intracellular pH in populations of cultured cells. *Cancer Res* 1998; **58:** 1901–8.
9 Martinez-Zaguilan R, Seftor EA, Seftor RE, Chu YW, Gillies RJ, Hendrix MJ. Acidic pH enhances the invasive behaviour of human melanoma cells. *Clin Exp Metastasis* 1996; **14:** 176–86.
10 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.
11 Wachsberger PR, Landry J, Storck C. Mammalian cells adapted to growth at pH 6.7 have eleveted HSP27 levels and are resistant to cisplatin. *Int J Hyperthermia* 1997; **13:** 251–5.
12 Raghunand N, Mahobey 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.
13 Glunde K, Guggino SE, Solaiyappan M, Pathak AP, Ichikawaz Y, Bhujwalla ZM. Extracellular acidification alters lysosomal trafficking in human breast cancer cells. *Neoplasia* 2003; **5:** 533–45.
14 Martinez-Zaguilàn R, Raghunand N, Lynch RM *et al.* pH and drug resistance. I. Functional expression of plasmalemmal V-type H+-ATPase in drug resistant human breast carcinoma cell lines. *Biochem Pharmacol* 1999; **57:** 1037–46.
15 Lugini L, Lozupone F, Matarrese P *et al.* Potent phagocytic activity discriminates metastatic and primary human malignant melanomas: a key role of ezrin. *Lab Invest* 2003; **83:** 1555–67.

16 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.
17 Fais S. Cannibalism: a way to feed on metastatic tumors. *Cancer Lett* 2007; **258:** 155–64.
18 Huynh KK, Grinstein S. Regulation of vacuolar pH and its modulation by some microbial species. *Microbiol Mol Biol Rev* 2007; **71:** 452–62.
19 Ferguson SJ, Ingledew WJ. Energetic problems faced by microorganisms growing or surviving on parsimonious energy sources and at acidic pH: I. *Acidithiobacillus ferrooxidans* as a paradigm. *Biochim Biophys Acta* 2008; **1777:** 1471–9.
20 Martínez-Muñoz GA, Kane P. Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. *J Biol Chem* 2008; **283:** 20309–19.
21 Robertson N, Potter C, Harris AL. Role of carbonic anhydrase IX in human tumor cell growth, survival, invasion. *Cancer Res* 2004; **64:** 6160–5.
22 Nielsen D, Skovsgaard T. P-glycoprotein as multidrug transporter: a critical review of current multidrug resistant cell lines. *Biochim Biophys Acta* 1992; **1139:** 169–83.
23 Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 1993; **62:** 385–427.
24 Dalton WS. Is P-glyccoprotein a potential target for reversing clinical drug resistance? *Curr Opin Oncol* 1994; **6:** 595–600.
25 Raghunand N, He X, van Sluis R *et al.* Enhancement of chemotherapy by manipulation of tumor pH. *Br J Cancer* 1999; **80:** 1005–11.
26 Cole SPC, Bhardwaj G, Gerlach JH *et al.* Overexpression of a transporter gene in a multidrug-resistance human lung cancer cell line. *Science* 1992; **95:** 1650–8.
27 Scheper RJ, Broxterman HJ, Sceffer GL *et al.* Overexpression of a M 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. *Cancer Res* 1993; **53:** 1475–9.
28 Izquierdo MA, Scheffer GL, Flens MJ, Schroeijers AB, van der Valk P, Scheper RJ. Major vault LRP-related multidrug resistance. *Eur J Cancer* 1996; **32A:** 979–84.
29 Doyle LA, Yang W, Abruzzo LV *et al.* A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* 1998; **95:** 15665–70.
30 Leonard GD, Fojo T, Bates SE. The role of ABC transporters in clinical practice. *Oncologist* 2003; **8:** 411–24.
31 Simon S, Roy D, Schindler M. Intracellular pH and the control of multi-drug resistance. *Proc Natl Acad Sci USA* 1994; **91:** 1128–32.
32 Mahoney BP, Raghunand N, Baggett B, Gillies RJ. Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents *in vitro*. *Biochem Pharmacol* 2003; **66:** 1207–18.
33 Raghunand N, Gillies RJ. pH and drug resistance in tumors. *Drug Resist Updat* 2000; **3:** 39–47.
34 Altan N, Chen Y, Schindler M, Simon SM. Defective acidification in human breast tumor cells and implications for chemotherapy. *J Exp Med* 1998; **187:** 1583–98.
35 Parolini I, Federici C, Raggi C *et al.* Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 2009; **284:** 34211–22.
36 Ouar Z, Bens M, Vignes C *et al.* Inhibitors of vacuolar H+-ATPase impair the preferential accumulation of daunomycin in lysosomes and reverse the resistance to anthracyclines in drug-resistant renal epithelial cells. *Biochem J* 2003; **370:** 185–93.
37 Luciani F, Spada M, De Milito A *et al.* Effect of proton pump inhibitor pre-treatment on resistance of solid tumors to cytotoxic drugs. *J Natl Cancer Inst* 2004; **96:** 1702–13.
38 Mullin JM, Gabello M, Murray LJ *et al.* Proton pump inhibitors: actions and reactions. *Drug Discov Today* 2009; **14:** 647–60.
39 Fais S, De Milito A, You H, Qin W. Targeting vacuolar H+-ATPases as a new strategy against cancers. *Cancer Res* 2007; **67:** 10627–30.
40 Hinton A, Bond S, Forgac M. V-ATPase functions in normal and disease processes. *Pflugers Arch* 2009; **457:** 589–98.
41 You H, Jin J, Shu H *et al.* Small interfering RNA targeting the subunit ATP6L of proton pump V-ATPase overcomes chemoresistance of breast cancer cells. *Cancer Lett* 2009; **280:** 110–19.
42 Sennoune SR, Luo D, Martinez-Zaguilan R. Plasmalemmal vacuolar-type H+-ATPase in cancer biology. *Cell Biochem Biophys* 2004; **40:** 185–206.
43 Nishi T, Forgac M. The vacuolar (H^+)-ATPases – nature's most versatile proton pumps. *Nat Rev Mol Cell Biol* 2002; **3:** 94–103.
44 Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med* 2006; **12:** 895–904.
45 Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell* 2006; **127:** 679–95.
46 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.
47 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.
48 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.
49 Der G. An overview of proton pump inhibitors. *Gastroenterol Nurs* 2003; **26:** 182–90.
50 Metz DC, Forsmark C, Lew EA *et al.* Replacement of oral proton pump inhibitors with intravenous pantoprazole to effectively control gastric acid hypersecretion in patients with Zollinger–Ellison syndrome. *Am J Gastroenterol* 2001; **96:** 3274–80.
51 Ramdani A, Mignon M, Samoyeau R. Effect of pantoprazole versus other proton pump inhibitors on 24-hour intragastric pH and basal acid output in Zollinger–Ellison syndrome. *Gastroenterol Clin Biol* 2002; **26:** 355–9.
52 Chen M, Zou X, Luo H *et al.* Effects and mechanisms of proton pump inhibitors as a novel chemosensitizer on human gastric adenocarcinoma (SGC7901) cells. *Cell Biol Int* 2009; **33:** 1008–19.
53 Kasamon YL, Swinnen LJ. Treatment advances in adult Burkitt lymphoma and leukemia. *Curr Opin Oncol* 2004; **16:** 429–35.
54 Nilsson A, De Milito A, Engstrom P *et al.* Current chemotherapy protocols for childhood acute lymphoblastic leukemia induce loss of humoral immunity to viral vaccination antigens. *Pediatrics* 2002; **109:** e91.
55 De Milito A, Iessi E, Logozzi M *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.
56 Pérez-Sayáns M, García-García A, Reboiras-López MD, Gándara-Vila P. Role of V-ATPases in solid tumors: importance of the subunit C. *Int J Oncol* 2009; **34:** 1513–20.

57 Pérez-Sayáns M, Somoza-Martín JM, Barros-Angueira F, Rey JM, García-García A. V-ATPase inhibitors and implication in cancer treatment. *Cancer Treat Rev* 2009; **35:** 707–13.
58 De Milito A, Canese R, Marino ML *et al.* pH-dependent antitumor activity of proton pump inhibitors against human melanoma is mediated by inhibition of tumor acidity. *Int J Cancer* 2009; Oct 28.
59 Cardone RA, Casavola V, Reshkin SJ. The role of disturbed pH dynamics and the Na+/H exchanger in metastasis. *Nat Rev Cancer* 2005; **5:** 786–95.
60 Harguindey S, Arranz JL, Wahl ML, Orive G, Reshkin SJ. Proton transport inhibitors as potentially selective anticancer drugs. *Anticancer Res* 2009; **29:** 2127–36.
61 Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002; **295:** 2387–92.
62 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.
63 Drochioiu G. Chronic metabolic acidosis may be the cause of cachexia: body fluid pH correction may be an effective therapy. *Med Hypotheses* 2008; **70:** 1167–73.
64 Kusuzaki K, Murata H, Matsubara T, *et al.* Acridine orange could be an innovative anticancer agent under photon energy. *In Vivo* 2007; **21:** 205–21.
65 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 USA* 2007; **104:** 7893–8.
66 Robey IF, Baggett BK, Kirkpatrick ND *et al.* Bicarbonate increases tumor pH and inhibits spontaneous metastases. *Cancer Res* 2009; **69:** 2260–8.
67 Silva AS, Yunes JA, Gillies RJ, Gatenby RA. The potential role of systemic buffers in reducing intratumoral extracellular pH and acid-mediated invasion. *Cancer Res* 2009; **69:** 2677–84.
68 Lee ES, Gao Z, Bae YH. Recent progress in tumor pH targeting nanotechnology. *J Control Release* 2008; **132:** 164–70.
69 Makovitzki A, Fink A, Shai Y. Suppression of human solid tumor growth in mice by intratumor and systemic inoculation of histidine-rich and pH-dependent host defense-like lytic peptides. *Cancer Res* 2009; **69:** 3458–63.
70 Sjøstrøm JE, Kühler T, Larsson H. Basis for the selective antibacterial activity *in vitro* of proton pump inhibitors against *Helicobacter* spp. *Antimicrob Agents Chemother* 1997; **41:** 1797–801.
71 Kochar DK, Saini G, Kochar SK *et al.* A double blind, randomised placebo controlled trial of rifampicin with omeprazole in the treatment of human cutaneous leishmaniasis. *J Vector Borne Dis* 2006; **43:** 161–7.
72 Puiac S, Negrea A, Richter-Dahlfors A, Plant L, Rhen M. Omeprazole antagonizes virulence and inflammation in *Salmonella enterica*-infected RAW264.7 cells. *Antimicrob Agents Chemother* 2009; **53:** 2402–9.
73 Marchesini N, Vieira M, Luo S, Moreno SN, Docampo R. A malaria parasite-encoded vacuolar H(+)-ATPase is targeted to the host erythrocyte. *J Biol Chem* 2005; **280:** 36841–7.
74 Lozupone F, Perdicchio M, Brambilla D *et al.* The human homologue of *Dictyostelium discoideum phg1A* is expressed by human metastatic melanoma cells. *EMBO Rep* 2009; **10:** 1348–54.
75 Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 2004; **4:** 891–9.
76 Marquardt D, Center MS. Involvement of vacuolar H(+)-adenosine triphosphatase activity in multidrug resistance in HL60 cells. *J Natl Cancer Inst* 1991; **83:** 1098–102.
77 Semenza GL. Tumor metabolism: cancer cells give and take lactate. *J Clin Invest* 2008; **118:** 3835–7.
78 Shimokawa O, Matsui H, Nagano Y, *et al.* Neoplastic transformation and induction of H^+,K^+-adenosine triphosphatase by N-methyl-N'-nitro-N-nitrosoguanidine in the gastric epithelial RGM-1 cell line. *In Vitro Cell Dev Biol Anim.* 2008; **44:** 26–30.
79 Gervasoni JE Jr, Fields SZ, Krishna S, *et al.* Subcellular distribution of daunorubicin in P-glycoprotein-positive and -negative drug-resistant cell lines using laser-assisted confocal microscopy. *Cancer Res* 1991; **51:** 4955–63.
80 Klohs WD, Steinkampf RW. The effect of lysosomotropic agents and secretory inhibitors on anthracycline retention and activity in multiple drug-resistant cells. *Mol Pharmacol* 1988; **34:** 180–5.
81 Wahl ML, Owen JA, Burd R, *et al.* Regulation of intracellular pH in human melanoma: potential therapeutic implications. *Mol Cancer Ther* 2002; **1:** 617–28.
82 Yeo M, Kim DK, Park HJ, Cho SW, Cheong JY, Lee KJ. Blockage of intracellular proton extrusion with proton extrusions with proton pump inhibitor induces apoptosis in gastric cancer. *Cancer Sci* 2008; **99:** 185.
83 Morimura T, Fujita K, Akita M, Nagashima M, Satomi A. The proton pump inhibitor inhibits cell growth and induces apoptosis in human hepatoblastoma. *Pediatr Surg Int* 2008; **24:** 1087–94.
84 Di Sario A, Bendia E, Omenetti A, *et al.* Selective inhibition of ion transport mechanisms regulating intracellular pH reduces proliferation and induces apoptosis in cholangio carcinoma cells. *Dig Liver Dis* 2007; **39:** 60–9.

Correspondence: Stefano Fais, Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità (ISS) (Italian National Institute of Health), Viale Regina Elena 299, Rome 00161, Italy.
(fax: +39 06 49902436; e-mail: stefano.fais@iss.it).