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Microenvironment acidity as a major determinant of tumor chemoresistance: Proton pump inhibitors (PPIs) as a novel therapeutic approach

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ABSTRACT

Despite the major progresses in biomedical research and the development of novel therapeutics and treatment strategies, cancer is still among the dominant causes of death worldwide. One of the crucial challenges in the clinical management of cancer is primary (intrinsic) and secondary (acquired) resistance to both conventional and targeted chemotherapeutics. Multiple mechanisms have been identifiedthat underlie intrinsic and acquired chemoresistance: these include impaired drug uptake, increased drug efflux, deletion of receptors, altered drug metabolism, quantitative and qualitative alterations in drug targets, increased DNA damage repair and various mechanisms of anti-apoptosis. The fast efflux of anticancer drugs mediated by multidrug efflux pumps and the partial or complete reversibility of chemoresistance combined with the absence of genetic mutations suggests a multifactorial process. However, a growing body of recent evidence suggests that chemoresistance is often triggered by the highly acidic microenvironment of tumors. The vast majority of drugs, including conventional chemotherapeutics and more recent biological agents, are weak bases that are quickly protonated and neutralized in acidic environments, such as the extracellular microenvironment and the acidic organelles of tumor cells. It is therefore essential to develop new strategies to overcome the entrapment and neutralization of weak base drugs. One such strategy is the use of proton pump inhibitors which can enhance tumor chemosensitivity by increasing the pH of the tumor microenvironment. Recent clinical trials in animals with spontaneous tumors have indicated that patient alkalization is capable of reversing acquired chemoresistance in a large percentage of tumors that are refractory to chemotherapy. Of particular interest was the benefit of alkalization for patients undergoing metronomic regimens which are becoming more widely used in veterinary medicine. Overall, these results provide substantial new evidence that altering the acidic tumor microenvironment is an effective, well tolerated and low cost strategy for the overcoming of anticancer drug resistance.

1. Introduction

In 2012 approximately 32.6 million people were living with cancer (within 5 years of diagnosis), 14.1 million were adults newly diagnosed and 8.2 million of these cancer patients died. (Globocan, http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx). Hence, cancer is among the leading causes of death worldwide together with cardiovascular diseases, diabetes and chronic lung diseases (Source WHO, http://www.who.int/mediacentre/factsheets/fs310/en/). Despite the use of multiple drug combination protocols and the development of novel targeted anticancer strategies, chemoresistance remains a primary hindrance towards cancer treatment (Wilting and Dannenberg, 2012). A better understanding of tumor biology is of paramount importance in order to devise targeted and efficient therapies. In this regard, one of the hallmarks of cancer is the alteration of energy metabolism that occurs in hypoxic conditions, leading to lactate accumulation and

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intracellular acidification (Chen et al., 2007). Rapidly dividing cancer cells produce and release large amounts of protons into the extracellular compartment due to heightened glucose utilization, amino acid metabolism and ATP hydrolysis (Tredan et al., 2007; De Milito et al., 2010; McCarty and Whitaker, 2010; Solyanik, 2010; Bellone et al., 2013; Ackerman and Simon, 2014; Chen et al., 2014; Peppicelli et al., 2014; Lugini et al., 2015). One intriguing hypothesis is that the highly competitive microenvironment secondary to tumor proliferation, selects the cells best adapted to survive in these hostile conditions. As a result, cancer cells overexpress many growth factors that modulate glucose metabolism like IGF (Bach et al., 2005; Biernacka et al., 2013; Durzyńska, 2014; Elzein and Goodyer, 2014; Sax et al., 2014), TGFβ and EGF (Jiang et al., 2014; Philip et al., 2014). Uncontrolled tumor cell proliferation, acid production, and tissue hypoxia secondary to limited blood supply, all contribute to the generation of a highly hostile tumor microenvironment with conditions that are unsuitable for most cells. In these conditions, cancer cells have to undergo a thorough "reprogramming" that leads to the overexpression of gene products which are involved in hypoxia and glucose metabolism, such as hypoxia-inducible factor 1 (HIF-1), Myc, p53, Ras, Akt, Src, pyruvate kinase (PK) M2, lactate dehydrogenase A (LDHA) and PKM2 (Semenza, 2007; Christofk et al., 2008; Dang et al., 2008; Hsu and Sabatini, 2008; Le et al., 2010; Levine and Puzio-Kuter, 2010; McCarty and Whitaker, 2010; Huang et al., 2014; Wang et al., 2014; Ho Kim et al., 2015; Popov et al., 2015; Wu et al., 2015; Xiang et al., 2015). In order to thrive in such an unfavorable microenvironment, tumor cells must develop systems to actively extrude excess protons (Izumi et al., 2003). These mechanisms include V-ATPase, Na^+/H^+ exchanger (NHE), monocarboxylate transporters (MCTs) and carbonic anhydrase 9 (Spugnini et al., 2014a,b).

2. The acidic microenvironment of tumors

Tumor acidity is caused by increase in protons generation from the anaerobic metabolism of cancer cells through the overexpression of M2-PK, a dimeric isoenzyme of pyruvate kinase (Sonveaux et al., 2008). Consequently, tumors have a significantly lower extracellular pH (~6.5–7.1) (De Milito et al., 2010; Gillies et al., 2004; Smallbone et al., 2008) in comparison to normal tissues (7.4), due to the maintenance of proton efflux pumps (Spugnini et al., 2014a,b). The ultimate result of this active proton extrusion is a severe alteration of pH gradients, mainly characterized by a reversed pH gradient between the acidic extracellular microenvironment and the alkaline cytosol (Spugnini et al., 2014a,b). The resulting increase in acidity induces the selection of tumor cells which are capable of withstanding these extreme conditions leading to a more aggressive phenotype. Indeed, proton export leads inevitably to a low extracellular pH and a low endosomal and lysosomal pH (Adar et al., 2012; Ellegaard et al., 2013; Gotink et al., 2011; Jansen et al., 1999; Kallunki et al., 2013; Zhitomirsky and Assaraf, 2015), thus protecting the cell from cytosolic acidity (Martinez-Zaguilan et al., 1996; Rofstad et al., 2006). Recently it was shown that increased number of intracellular acidic vesicles like lysosomes per cell, is a determinant of resistance to hydrophobic anticancer drugs (Zhitomirsky and Assaraf, 2015; Gotink et al., 2011; Jansen et al., 1999). Moreover, hydrophobic weak base anticancer drugs can induce lysosomal biogenesis in various carcinoma cells were found to undergo lysosomal biogenesis. Hence, sequestration of hydrophobic anticancer drugs in acidic lysosomes away from their drug targets is an important mechanism of multidrug resistance. This has been exploited to target lysosomes *via* a pharmacologic Trojan horse to eradicate multidrug resistant cancer cells (Adar et al., 2012).

In carcinomas, melanoma and sarcomas, the extracellular low pH has been reported to promote invasiveness, metastatic behavior, abnormal phagocytic behavior, nanovesicle release and drug resistance (Barar and Omidi, 2013; Daniel et al., 2013; Lugini et al., 2006; Fais and Fauvarque, 2012; Ochotny et al., 2013; Parolini et al., 2009). As mentioned above, resistance to antineoplastic drugs is also achieved by the trapping of hydrophobic weak base drugs (Adar et al., 2012; Bailey et al., 2012; Gillies et al., 2004; Gotink et al., 2011; Kallunki et al., 2013; Ndolo et al., 2012; Zhitomirsky and Assaraf, 2015) or enhancement of membrane rigidity (Drori et al., 1995; Rauch, 2009a, 2009b). Several interdisciplinary studies have been conducted which combine mathematical simulations and laboratory investigation and these indicate that the acidic microenvironment plays a key role in achieving both tumor invasiveness and metastatic potential (Gatenby et al., 2006; Martin et al., 2010; Smallbone et al., 2008).

3. Tumor acidity and chemoresistance

A major obstacle that must be overcome during treatment of solid tumors is the frequent emergence of drug resistance to chemotherapy (Assaraf, 2007; Gonen and Assaraf, 2012; Gottesman, 2002; Liu, 2009; Livney and Assaraf, 2013; Luqmani, 2005; Nobili et al., 2006; Shapira et al., 2011). As stated above, a major contributor to the development of chemoresistance is the acidic tumor microenvironment (pO_2 and pH) that attenuates and frequently abrogates therapeutic efficacy (Bailey et al., 2012; Gillies et al., 2004; Ndolo et al., 2012; Shekhar, 2011; Wojtkowiak et al., 2011). Hypoxia (Fig. 1) and altered glycolytic activity, the so called "Warburg Effect", contribute to the establishment of the acidic extracellular milieu characteristic to malignant tumors (Gillies et al., 2004; Smallbone et al., 2008; Wojtkowiak et al., 2011; De Milito et al., 2010). Several investigations have shown that the acidic extracellular pH in tumors is combined with a neutral or alkaline intracellular environment (Hashim et al., 2011; Wojtkowiak et al., 2011); the pattern of acidic extracellular environment and the alkaline cytosol is considered a hallmark of malignant cancers and is referred to as a "Reversed pH Gradient" (Damaghi et al., 2013). This pattern is induced and maintained through the overexpression of proton transporters and through the modulation of intra-cytoplasmic and extracellular pH sensors (Damaghi et al., 2013; Frantz et al., 2008). Interestingly, the switch to an acid-producing metabolism seems to occur in the early phases of tumor growth during the avascular stage (Barathova et al., 2008; Damaghi et al., 2013). These hostile conditions result in selection that promotes tumor invasion and distant metastasis (Fais et al., 2014). The extracellular and endosomal and lysosomal acidic pH contribute to drug resistance both *in vitro* and *in vivo* (Azzarito et al., 2015; Bailey et al., 2012; Gillies et al., 2004; Luciani et al., 2004; Ndolo et al., 2012; Shekhar, 2011; Wojtkowiak et al., 2011;). The reverse pH gradient occurring between the cytosolic and extracellular spaces, impacts negatively on the distribution, uptake and bioavailability of weak base antineoplastic drugs, leading to chemoresistance (Adar et al., 2012; De Milito and Fais, 2005; Ellegaard et al., 2013; Gerweck et al., 2006; Gotink et al., 2011; Kallunki et al., 2013; Jansen et al., 1999; Mahoney et al., 2003; Raghunand et al., 2003; Wojtkowiak et al., 2011; Zhitomirsky and Assaraf, 2015). The cell membrane is a semi-permeable barrier that mediates the exchange between the intra- and extracellular compartments, allowing free passage of small-uncharged molecules through the lipid phase, while charged molecules are incapable of crossing the cell membrane. Due to these properties, the acidic extracellular space of solid tumors hampers the cellular uptake of weak bases (Wojtkowiak et al., 2011; Zhitomirsky and Assaraf, 2015; Gotink et al., 2011; Jansen et al., 1999). This phenomenon is also known as "ion trapping" (Fig. 2). Ion trapping occurs when there is a large membrane permeability difference between ionized (non-permeant) and non-ionized

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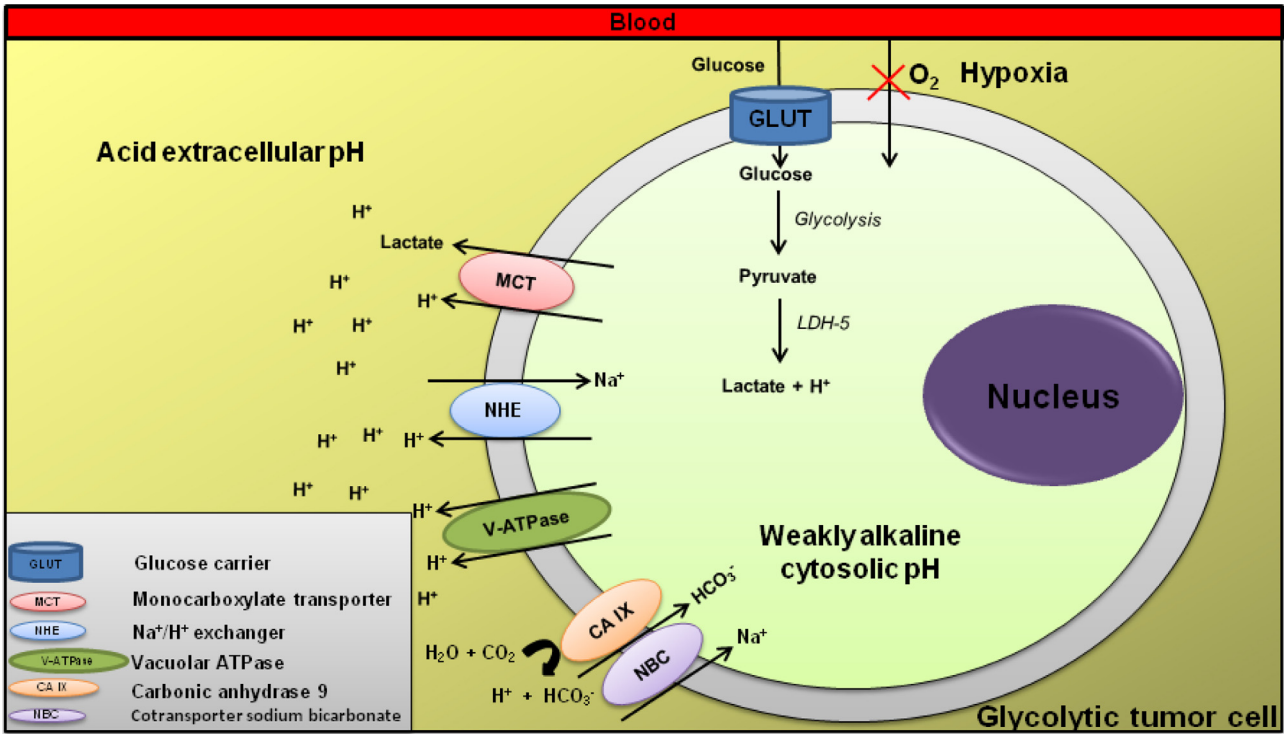

Fig. 1. Model of hypoxia-induced extracellular and extratumoral acidosis. After absorption of glucose by cancer cells, the sugar is metabolized in anaerobic condition, leading to the generation of 2 ATP for energy use and pyruvate as a byproduct. In an oxygen deprived environment, pyruvate is reduced to lactate, which is exported into the extracellular space in order to protect the tumor homeostasis. These processes release high amounts of protons (H+) in the extracellular space, causing extratumoral acidification. This figure summarizes the major proteins involved in the regulation of intracellular and extracellular pH in tumor: monocarboxylate transporter (MCT), which transport lactic acid derived from the anaerobic metabolism of glucose; Na+/H+ exchanger (NHE); the plasma membrane proton pump vacuolar ATPase (V-ATPase); carbonic anhydrase IX (CAIX); and Na+/HCO co-transporter (NBC). As a result of the extracellular acidification, tumor cells become refractory to chemotherapy agent as a consequence of their impaired acid-base balance.

(permeant) components of a drug (Bailey et al., 2012; Gillies et al., 2004; Ndolo et al., 2012); the resulting equilibrium is dictated by the Henderson–Hasselbach formula (Wojtkowiak et al., 2011). For weak bases, the ratio of ionized to non-ionized forms is highly sensitive to pH changes. As a result, the uptake and efficacy of weak base drugs such as anthracyclines, anthracenediones, campothecins, and *Vinca* alkaloids are reduced (Adar et al., 2012; Ellegaard et al., 2013; Gerweck et al., 2006; Gotink et al., 2011; Jansen et al., 1999; Kallunki et al., 2013; Mahoney et al., 2003; Raghunand et al., 2003; Wojtkowiak et al., 2011; Zhitomirsky and Assaraf, 2015). Complex drugs such as cisplatin that are considered weak acids in their primary structures can also be affected by a low pH. While cisplatin is partially ionized at pH 6.5 the percentage of ionized cisplatin greatly increases at a pH ranging from 3 to 5 and a recent report has shown that the majority of cisplatin remains outside malignant melanoma cells due to low acidic conditions (Federici et al., 2014). It has long been postulated that the multidrug efflux transporter P-glycoprotein (ABCB1) mediates the main mechanism of resistance within cancer cells (Luqmani, 2005) and only recent evidence has shown a key role of cytoskeleton participation to this phenomenon (Luciani et al., 2002; Brambilla et al., 2012). However, an alternative explanation is that this phenomenon may not be an essential mechanism when compared to the key role of the pH gradient that exists in cancer cells. It appears that there are two existing mechanisms by which weak bases may be excluded from entering cancer cells: either due to drug ionization in the acidic interstitial compartment which leads to a reduced ability to cross the plasma membrane of cancer cells (Mellor and Callaghan, 2008, 2011) or due to an alteration in the interaction between phospholipids from the inner leaflet making the bilayer membrane much stiffer with regards to drug penetration into cells (Rauch, 2009a,b). Accordingly, it was also shown that such membrane rigidity driven by the pH gradient across the membrane permits the interaction between P-glycoprotein and antineoplastic drugs when the former is overexpressed (Rauch and Pluen, 2007; Panagiotopoulou et al., 2010).

If weak bases are protonated and trapped extracellularly in the acidic microenvironment, then the uptake of weak acidic chemotherapeutics such as chlorambucil should be enhanced when the extracellular pH is low. Chlorambucil has a dissociation constant of 5.78 (*i.e.* p*K*a < 7.5), allowing it to readily cross the plasma membrane of cultured cells resulting in a 2.3 fold increase in the efficacy compared to the weak base drugs (such as doxorubicin) (Gerweck et al., 2006). In addition, direct tumor alkalinization with sodium bicarbonate severely hampers chlorambucil efficacy in *in vitro* and *in vivo* studies (Wojtkowiak et al., 2011). Similar observations have been made with another alkylating agent melphalan, whose uptake and efficacy are enhanced by low extracellular pH, in several studies using tumor xenograft in mice (Siemann et al., 1991; Wood et al., 1995) or enhancing hypoxia and acidosis by using the technique of isolated limb perfusion (Coventry et al., 2014; Jakob et al., 2014; van Broekhoven et al., 2014; Rashid et al., 2014).). These results suggest that the consideration of the "ion trapping" concept may facilitate improved therapeutic strategies. In this regard, Table 1 summarizes the chemical features of some of the most commonly used chemotherapeutic agents. Intriguing results were obtained with proton pump inhibitors (PPIs), in which MDR-positive chemoresistant cells were re-sensitized to the antineoplastic effects of vinblastine (Luciani et al., 2004) by exposure to omeprazole or lansoprazole, suggesting that counteracting the acidity of the tumor microenvironment may overcome classical mechanisms of chemoresistance.

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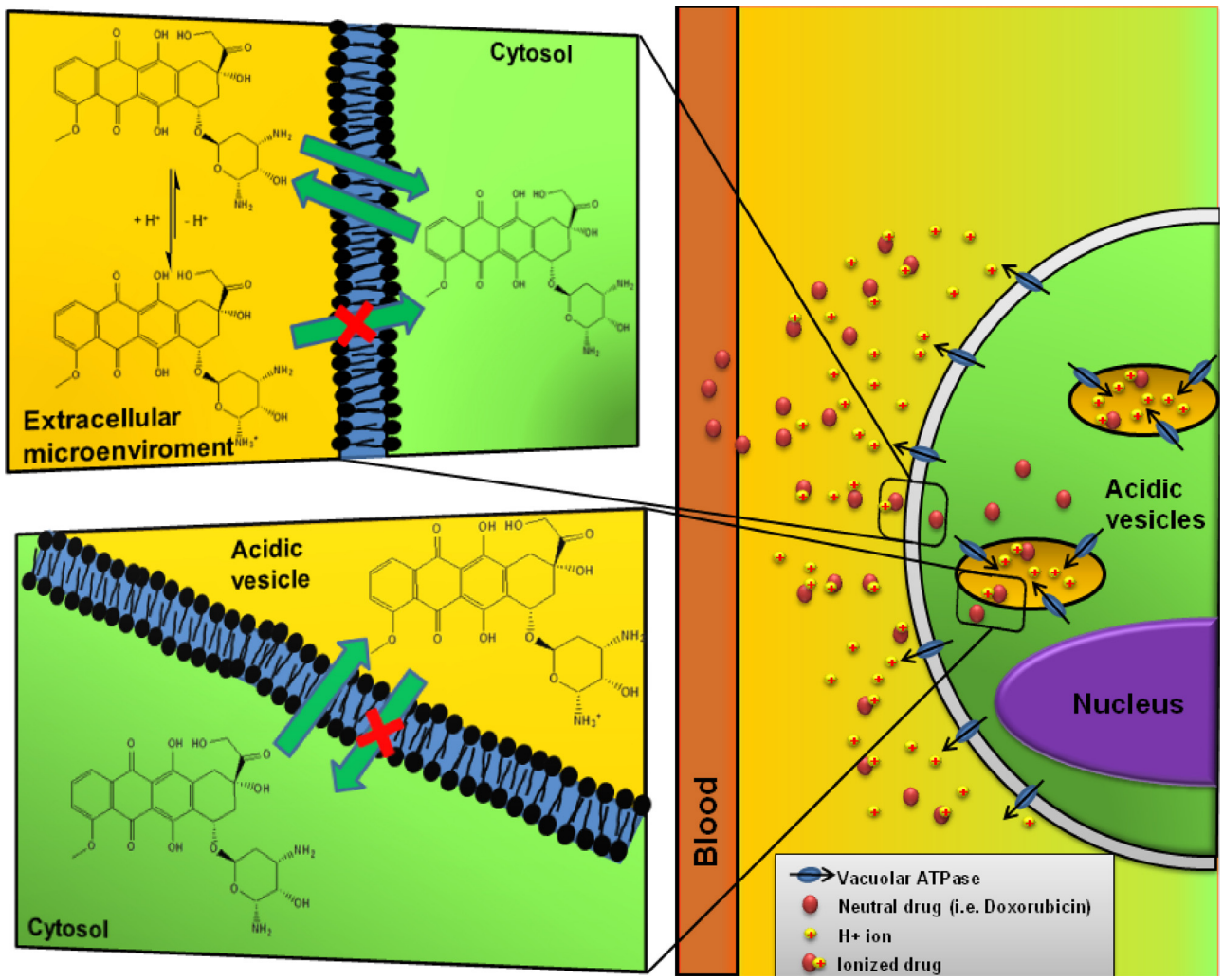

Fig. 2. Ion trapping theory. This figure shows that in solid tumors the extracellular H+ concentration is greater than the intracellular H^+ concentration as a consequence of the anaerobic metabolism. Weak base antineoplasic drugs (Table 1) can be protonated hereby reducing their ability to cross the membrane of cells. While unable to traverse the membrane protonated drugs can still be transported in in intracellular acidified compartments *via* fluid phase endocytosis (see Rauch, 2007, 2009a, 2009b). An external pH as low as ~6.7 promotes adaptive changes in tumor cells resulting in cytoskeleton remodeling, local invasion, apoptosis evasion and metastasis.

4. Mathematical and physical models of MDR

As already alluded to, a common finding in cancer cells is a change in the pH gradient across the membrane. The first model that was put forward is the drug protonation model (DPM). The DPM stems from pharmacokinetic studies demonstrating that the bioavailability of a drug differs largely depending on its p*K*a status. Classical examples include codeine that is a weak base (p*K*a ~ 7.9) and that is poorly absorbed in acidified milieu (as in the stomach) compared to aspirin (a weak acid p*K*a ~ 3.4). The main reason why such drug absorption differs is related to the electrostatic charge of the drug. A drug with no extra electrostatic charge can cross the membrane efficiently. Mathematically, the relationship between pH and p*K*a as well as the protonated and non-protonated concentrations of the substance is written as:

$$\mathrm{pH} = \mathrm{p}K\mathrm{a} + \mathrm{Log}\frac{[B;A^-]}{[BH^+;AH]} \quad (1)$$

In Eq. (1), B, BH^+, A^- and AH are, the non-protonated base, the protonated base, the non-protonated acid and the protonated acid, respectively. To comprehend how Eq. (1) was thought to be involved in MDR, let us focus on a base (the same development can be done for an acid) and assume the existence of two compartments, the cytosol (termed "cyt") and the extracellular milieu (termed "ext"). Eq. (1) is valid in the case of cancer with regards to the differing pH microenvironments in both cytosol and the extracellular spaces. This leads to $\mathrm{pH}_{ext} = \mathrm{p}K\mathrm{a} + \mathrm{Log}[B]_{ext}/[BH^+]_{ext}$ and $\mathrm{pH}_{cyt} = \mathrm{p}K\mathrm{a} + \mathrm{Log}[B]_{cyt}/[BH^+]_{cyt}$. At equilibrium, only the neutral form of the weak base will be able to cross the membrane meaning that its concentration should be similar between the two above mentioned compartments, in other words: $[B]_{cyt} \sim [B]_{ext}$. Using this assumption together with the relationships defined in each compartments leads to

$$\mathrm{pH}_{ext} - \mathrm{pH}_{cyt} = \mathrm{Log}\frac{[BH^+]_{cyt}}{[BH^+]_{ext}} \quad (2)$$

Eq. (2) provides what should be the ratio of weak base concentrations trapped in each compartment as a function of their own luminal pH. This formula is often applied in the cancer field to demonstrate how weak base drug redistribution across compartments is possible (Altan et al., 1998). It is noteworthy here that a small pH difference of one unit leads to a ratio drug concentration of ~10.

Although Eq. (2) and related experimental studies have highlighted the pH determinant as an important modulator of MDR; MDR in cancer has not been exclusively related to pH. For example, MDR has also been related to the molecular weight of drugs

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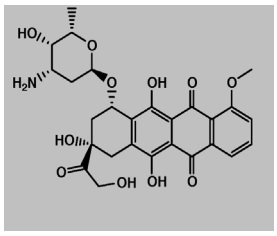
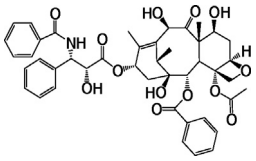
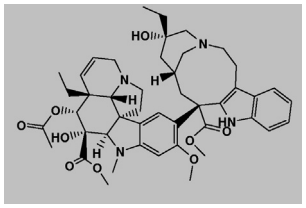
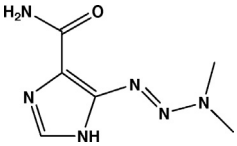
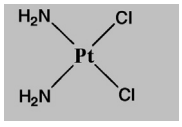
Table 1
Chemical features of some anti-tumor drugs. The table presents an overview of the p*Ka* of some of the most commonly used chemotherapy agents, showing their nature of weak basis or weak acids that affects resistance to therapy.

Drug	Structure	pKa	Hydrogen acceptor count	Hydrogen donor count	References
Doxorubicin		9.53	12	6	www.drugbank.ca/drugs/DB00997
Paclitaxel		10.36	10	4	http://www.drugbank.ca/drugs/db00997
Vinblastine		10.87	9	3	http://www.drugbank.ca/drugs/DB00570
Dacarbazine		5.89	5	2	http://www.drugbank.ca/drugs/DB00851
Cisplatin		5.06	2	2	http://www.drugbank.ca/drugs/DB00515

or the expression of drug transporters (see Daniel et al., 2013 and references within). These discoveries led to the postulation that MDR is multifactorial. The multifactorial nature of MDR generated an important controversy in the field of cancer until it was pointed out that the molecular weight and drug-transporter theories rely necessarily on an alteration of the biophysical properties of the cellular membrane (Rauch and Pluen, 2007) and that, these membrane alterations can be brought about by a change in cytosolic pH (Rauch, 2009b).

This attempt to unify the different viewpoints/theories in the field of MDR using Physics principles has given the pH gradient theory (and altered cellular metabolism) a role that is fundamental in cancer that must be considered in cases of chemotherapy treatments.

5. *In vitro* studies

Low pH has a role in the inhibition of gap junctions, thus inhibiting tissue formation and promoting malignant transformation (Ruch et al., 1990; LeBoeuf et al., 1992). Multiple genetic aberrations, including down-regulation of tumor suppressor genes, or up-regulation of tumor promoters have been described in association with the acid tumor microenvironment (Fukamachi et al., 2014; Marchiq et al., 2015; Song et al., 2015). Further studies inferred that tumor acidity led to the overexpression or expression *per se* of vascular endothelial growth factor (VEGF), carbonic anhydrase, interlukin-8, cathepsin B, matrix metalloproteinases and tumor necrosis factor receptor, IL-2 (interleukin 32), AREG (schwannoma-derived growth factor), ErbB3, involved in enhanced tumor drug resistance, local invasion and distant dissemination (Rozhin et al., 1994; Shi et al., 2001; Swietach et al., 2010; Fukamachi et al., 2014). Tumor cells, capable to thrive within an acidic extracellular pH, developed p53 mutations and increased the expression of multidrug efflux transporters, resulting in enhanced drug resistance through inhibition of apoptosis and increased drug efflux (Williams et al., 1999; Lotz et al., 2007; Gerweck et al., 2006; Luciani et al., 2004; Vukovic and Tannock, 1997; Wojtkowiak et al., 2011; Matsuyama and Reed, 2000). One of the strategies to overcome the acidity-based chemoresistance was based on the inhibition of a class of proton transporters, the vesicular ATPases (V-ATPases), by means of treatment with bafilomycin A1 in *in vitro* studies (Kawaguchi et al., 2011). Whilst bafilomycin A is toxic to humans and therefore is of limited use in a clinical setting, our group showed that pretreatment with PPIs (by *e.g.* omeprazole, esomeprazole, or pantoprazole) of melanomas, adenocarcinomas, and lymphoma cell lines resulted in an increased uptake and prolonged retention of several chemotherapeutic agents resulting in a better response to cytotoxic drugs (Luciani et al., 2004). These results have been further substantiated by experiments in laboratory animals carrying tumor xenografts, where pretreatment with PPI led to a marked decrease in tumor growth in a melanoma model and a concomitant increase in animal survival (Luciani et al., 2004).

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More recently, the efficacy of the PPI lansoprazole in rendering human melanoma susceptible to suboptimal doses of paclitaxel has been demonstrated (Azzarito et al., 2015), thus supporting the use of PPIs in reducing the level of systemic toxicity of antineoplastic drugs. As PPIs can be sold without prescription their tolerability is well established, resulting in their rapid transfer to preclinical *in vivo* and clinical studies, as discussed in the following sections.

6. *In vivo* studies: Implications for reversal of chemoresistance and immune tolerance

Several investigators have focused their attention at restoring a normal p53 function in order to "re-establish" the impaired apoptotic pathway in mouse models (Gottschalk and Klein, 2013; Zhang et al., 2013). Unfortunately, these attempts, although resulting in improved responses, failed at completely overcoming the malignant phenotype (Gottschalk and Klein, 2013). Other approaches, while not conclusive results, included either the use of nano-delivery systems to enhance the efficacy of chemotherapeutic agents (Kim et al., 2015), or attempts at blocking gene expressions linked to the acidic metabolism of cancer cells (Weiler et al., 2014). In other investigations in human tumor xenografts attempts at reversing chemoresistance through the blockade of P-gp were not successful, and no significant correlation was observed between the response rates and mRNA and protein expression levels of P-gp (Merk et al., 2011; McCarthy et al., 2014). However, pretreatment of SCID mice carrying orthotopic melanoma xenografts with omeprazole resulted in an improved response to either cisplatin or paclitaxel, associated with a marked delay in tumor growth (Luciani et al., 2004; Azzarito et al., 2015). Comparable results were obtained in mice carrying hepatocellular carcinoma by targeting tumor V-ATPase through RNA interference strategy against the subunit ATP6L of V-ATPase, with inhibition of both tumor growth and metastasis (Lu et al., 2005). Actually, PPIs showed clear efficacy in controlling human tumor growth in xenograft models of both human B-cell tumors (De Milito et al., 2007) and human melanomas (De Milito et al., 2010). Moreover treatment with PPI has significantly increased both the efficacy of adopted immune therapy and the spontaneous anti-tumor immune reaction (Calcinotto et al., 2012). This suggested that acidity may represent a target for future anti-tumor strategies aimed at improving tumor response to immune therapies but also to increase the body's immune reaction against tumors (Bellone et al., 2013). All in all, the ensemble of these data suggests that an antacid therapy could be instrumental to trigger a tumor response to conventional antineoplastic drugs and to allow the immune system to react against the tumors (De Milito et al., 2012; Huber et al., 2010; Fais et al., 2014). Targeting proton pumps may represent one approach to harness a class of existing drugs to buffer either the body or the tumors in order to render the tumors more accessible to either cytotoxic drugs or targeting therapies, but a systemic buffer approach is presently considered an alternative approach of molecules specifically directed against proton pumps (Fais et al., 2007, 2014; Fais, 2010). However, only very recently, it has been shown that the anticancer effect of PPIs extends beyond their ability to block proton efflux, probably related to their lipophilic properties (Lugini et al., 2015).

7. Clinical investigations

Paradoxically, progress in molecular cancer investigations has outpaced their clinical applications. The future is not blooming as, from an economical and clinical point of view, 10–12 years as well as ~$800 million dollars are needed to develop new compounds or diagnostic agents (Kola, 2008; Tobinick, 2009; Chit et al., 2015; DiMasi et al., 2013; DiMasi, 2014; Pamo Larrauri, 2014). This extremely lengthy and costly drug development process is partly due to the limited predictive value for drug toxicity and efficacy of established preclinical models (Porrello et al., 2006; Wypij, 2013). Companion animals with spontaneous neoplasms are still an underexploited approach that may speed up cancer treatment in humans and pets by testing new strategies that have been previously evaluated *in vitro* and in laboratory animals (Cekanova and Rathore, 2014; Khanna et al., 2006; Paoloni and Khanna, 2008). Our group has conducted two studies in companion animals with spontaneous neoplasms to assess the feasibility of modulating the acidic tumor microenvironment (Spugnini et al., 2011, 2014a,b). The first one evaluated the capacity of high doses of lansoprazole to reverse chemoresistance in dogs and cats with malignant cancers that have proven either refractory or chemoresistant (Spugnini et al., 2011). Lansoprazole is a PPI that is routinely used at low doses (1 mg/kg) for the treatment of gastric hyperacidity. In this study, the drug was used off-label with a three days loading dose followed by a maintenance period to prevent a rebound of gastric acidity due to drug withdrawal. Adopting a schedule of 5 mg/kg Monday through Wednesday and then 1 mg/kg Thursday through Sunday, 23 out of 34 pets had a measurable response (67% response rate) with the most striking positive outcomes being those from animals affected by either non-responsive multicentric lymphomas or appendicular osteosarcomas (Spugnini et al., 2011). The treatment was well tolerated with side effects limited to sporadic cases of mild gastro-intestinal toxicity (vomiting, diarrhea, flatulence). A second study has shown the potentiation of metronomic chemotherapy through patient's alkalinization *via* the combination of PPIs with a water alkalinizer that brought the water pH to a value close to 9 (Spugnini et al., 2014a,b). In this study, the alkalinized cohort showed improved tumor response (both in terms of numbers and response duration) when compared to the control group receiving metronomic chemotherapy alone. Side effects were again negligible. Notably, tumors known for their refractoriness to chemotherapy, such as inflammatory carcinoma and hemangiosarcoma, showed long lasting responses. Alkalinization of patients will probably become an adopted strategy in veterinary oncology due to its low cost, increased tumor control and limited toxicity.

In humans, the addition of PPIs to standard anticancer agents is currently ongoing. An article has been recently published considering this strategy with osteosarcoma patients (Ferrari et al., 2013). This malignancy carries a high percentage of chemoresistance (40%) in patients receiving neo-adjuvant chemotherapy that ultimately results in relapse and/or metastases. The standard neo-adjuvant protocol involves the administration of a multi-drug therapy using high doses of methotrexate, cisplatin, doxorubicin and ifosfamide. The percentage of tumors that undergoes necrosis upon neo-adjuvant chemotherapy is predictive of response rate and long-term survival (Hogendoorn et al., 2010; Whelan et al., 2012). Osteosarcoma patients with different histotypes were pretreated with the PPI esomeprazole before neo-adjuvant treatment. The analysis of the resected tumors after neo-adjuvant therapy revealed that, while PPI increases the effectiveness of polychemotherapy at the tumor level, it was the chondroblastic subtype that particularly benefited from the combined therapy with PPI, raising the response rate from 25% (chemotherapy alone) to 61%. The results of this pilot study suggested that high doses of PPI might be extremely beneficial in highly hypoxic tumors such as chondroblastic osteosarcoma (Matsubara et al., 2013). In fact, chondrocytic differentiation is mediated by hypoxia and a glycolytic metabolism (Rankin et al., 2011) and the maintenance of an acidic tumor microenvironment may retain the malignant/undifferentiated phenotype and their poor responsiveness to therapies. Therefore, it appears conceivable that PPI may affect tumor cells thriving in an acid/hypoxic

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microenvironment, thus leading to chemosensitization and a marked tumor burden reduction.

8. Summary and conclusions

There is a growing body of evidence proposing the acidic microenvironment of tumors as a major player in progression, invasion, dissemination and chemoresistance (Fais et al., 2014; Hulikova et al., 2014; McIntyre et al., 2014; Spugnini et al., 2014a,b; Swietach et al., 2012, 2014). As previously mentioned, cancer cells release protons into the extracellular milieu, thus rendering the tumor microenvironment extremely permissive to local invasiveness, progression and metastatic dissemination. However, tumor acidity highly compromises the efficacy of both chemotherapy and targeting approaches, also affecting the anti-tumor immune response (Fais et al., 2014). From a clinical point of view, tumor microenvironment-based acidity-based chemoresistance is major obstacle to overcome. Despite the many molecular mechanisms that have been found be involved in chemoresistance, the reversal of the pHe/pHi gradient appears to have a pivotal role in interfering with both the passage of drugs through the cell membrane and the drug retention within the tumor target cells (Luciani et al., 2004). Various anticancer drugs (vinblastine, 5-fluoruracil, doxorubicin and mitoxantrone) are weak bases which are sequestered and hence neutralized and sometimes inactivated by acid-base reactions within the microenvironment surrounding the cells, or alternatively sequestered into intracellular acidic vesicles such as lysosomes (Adar et al., 2012; Ellegaard et al., 2013; Fais et al., 2007, 2014; Gotink et al., 2011; Jansen et al., 1999; Kallunki et al., 2013; Spugnini et al., 2014a,b; Zhitomirsky and Assaraf, 2015). A recently discovered pH-dependent mechanism of cancer resistance to cisplatin involves both extracellular sequestration and exosomes-mediated elimination of the drug from melanoma cells (Federici et al., 2014). Moreover, it has been shown that a low pH promotes exosome release from tumor cells (Parolini et al., 2009). Several strategies are currently being developed to use tumor acidity against its "perpetrator", including inhibition of de-protonation mechanisms using drugs such as PPI, Cariporide (a transport inhibitor of NHE-1), inhibitors of carbonic anhydrases, and inhibitors of monocarboxylate transporters (Fais et al., 2014; Spugnini et al., 2014a,b). Another strategy involves the use of tumor acidity as an attractant for anti-tumor agents, such as cyclooxygenase inhibitors (COX-1 and COX-2) that are normally used as non-steroidal anti-inflammatory agents for arthritis and other inflammatory conditions due to their tropism for acid environments, where they are activated. Due to their anti-tumor properties, they are also successfully employed in several drug combinations, including metronomic chemotherapy (Spugnini et al., 2014a,b). Another drug that is attracting the attention of clinicians is acridine orange (AO), a weak base dye that has shown a marked sequestration in acidified intracellular compartments such as lysosomes and that can become cytotoxic following photoexcitation at particular wavelengths (Matsubara et al., 2006). These properties have been exploited to achieve local control of tumors in patients with aggressive and advanced sarcomas (Kusuzaki et al., 2005; Matsubara et al., 2010). Similarly, Assaraf and colleagues have demonstrated that imidazoacridinones, a class of photoactivatable cytotoxic agents can be highly sequestered in lysosomes of multidrug resistant cells and then activated by light thereby resulting in lysosomal phodestruction-based tumor elimination (Adar et al., 2012). This novel approach was successfully adopted to achieve angiostatic treatment of cancers; specifically, photoactivation of lysosomally sequestered sunitinib after angiostatic treatment was shown to cause vascular occlusion and enhanced tumor growth inhibition *in vivo* (Please add the following reference: http://www.ncbi.nlm.nih.gov/pubmed/25675301). The unraveling of the "chemoresistance riddle" has prompted basic and clinical researchers to pursue other avenues of investigation leading to the re-discovery of molecules such as PPI, NSAIDs and AO that are being used off-label, thus improving the outcome of several malignancies. It is foreseeable that in the future, novel molecules will be devised and engineered to take advantage of these insights, thus improving the efficiency of delivery and the tumoricidal effect of anticancer agents, and at the same time sparing unnecessary toxicities to healthy tissues (Fais et al., 2014; Yu et al., 2014).

The results above indicate that the investigation of tumor acidity and gene (over)expression due to the Warburg effect, can be readily harnessed for the development of novel cancer markers, diagnostic tools and/or chemotherapeutic targets. However, therapies aimed at buffering the tumor microenvironment either through PPI or real buffers, may enhance the efficacy of existing therapies, which are poorly effective or ineffective against a wide variety of cancers. Moreover, this approach may lead to the use of suboptimal and consequently less toxic doses of anticancer drugs. The development of molecules able to act as both tracers (diagnostic) and therapeutic agents (theranostics) will allow in the near future to diagnose, stage and treat cancer patients hence resulting in enhanced therapeutic efficacy.

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