ACIDIC TUMOR pH:
IMPLICATIONS FOR THERAPY AND
MALIGNANT PROGRESSION

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Parts of this thesis have been published and the original papers are referred to arabic numerals 1-3 in the Results section:


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PH-DEPENDENT ANTITUMOR ACTIVITY OF PROTON PUMP INHIBITORS AGAINST HUMAN MELANOMA IS MEDIATED BY INHIBITION OF TUMOR ACIDITY

Metastatic melanoma is associated with poor prognosis and still limited therapeutic options. An innovative treatment approach for this disease is represented by targeting acidosis, a feature characterizing tumor microenvironment and playing an important role in cancer malignancy. Proton pump inhibitors (PPI), such as esomeprazole (ESOM) are prodrugs functionally activated by acidic environment, fostering pH neutralization by inhibiting proton extrusion. We used human melanoma cell lines and xeno-transplanted SCID mice to provide preclinical evidence of ESOM antineoplastic activity. Human melanoma cell lines, characterized by different mutation and signaling profiles, were treated with ESOM in different pH conditions and evaluated for proliferation, viability and cell death. SCID mice engrafted with human melanoma were used to study ESOM administration effects on tumor growth and tumor pH by magnetic resonance spectroscopy (MRS). ESOM inhibited proliferation of melanoma cells in vitro and induced a cytotoxicity strongly boosted by low pH culture conditions. ESOM-induced tumor cell death occurred via rapid intracellular acidification and activation of several caspases. Inhibition of caspases activity by pan-caspase inhibitor z-vad-fmk completely abrogated the ESOM-induced cell death. ESOM administration (2.5 mg kg\(^{-1}\)) to SCID mice engrafted with human melanoma reduced tumor growth, consistent with decrease of proliferating cells and clear reduction of pH gradients in tumor tissue. Moreover, systemic ESOM administration dramatically increased survival of human melanoma-bearing animals, in absence of any relevant
toxicity. These data show preclinical evidence supporting the use of PPI as novel therapeutic strategy for melanoma, providing the proof of concept that PPI target human melanoma modifying tumor pH gradients.

PROTON PUMP INHIBITION INDUCES AUTOPHAGY AS A SURVIVAL MECHANISM FOLLOWING OXIDATIVE STRESS IN HUMAN MELANOMA CELLS

Proton pump inhibitors (PPI) target tumor acidic pH and have an antineoplastic effect in melanoma. The PPI esomeprazole (ESOM) kills melanoma cells through a caspase-dependent pathway involving cytosolic acidification and alkalinization of tumor pH. In this paper, we further investigated the mechanisms of ESOM-induced cell death in melanoma. ESOM rapidly induced accumulation of reactive oxygen species (ROS) through mitochondrial dysfunctions and involvement of NADPH oxidase. The ROS scavenger N-acetyl-L-cysteine (NAC) and inhibition of NADPH oxidase significantly reduced ESOM-induced cell death, consistent with inhibition of cytosolic acidification. Autophagy, a cellular catabolic pathway leading to lysosomal degradation and recycling of proteins and organelles, represents a defence mechanism in cancer cells under metabolic stress. ESOM induced the early accumulation of autophagosomes, at the same time reducing the autophagic flux, as observed by WB analysis of LC3-II accumulation and by fluorescence microscopy. Moreover, ESOM treatment decreased mammalian target of rapamycin signalling, as reduced phosphorylation of p70S6K and 4EBP1 was observed. Inhibition of autophagy by knockdown of Atg5 and Beclin-1 expression significantly increased ESOM cytotoxicity, suggesting a protective role for autophagy in ESOM-treated cells. The data presented suggest that autophagy
represents an adaptive survival mechanism to overcome drug-induced cellular stress and cytotoxicity, including alteration of pH homeostasis mediated by proton pump inhibition.

**AUTOPHAGY IS A PROTECTIVE MECHANISM FOR HUMAN MELANOMA CELLS UNDER ACIDIC STRESS**

Cyclic hypoxia and alterations in oncogenic signalling contribute to switch cancer cell metabolism from oxidative phosphorylation to aerobic glycolysis. A major consequence of upregulated glycolysis is the increased production of metabolic acids responsible for the presence of acidic areas within solid tumors. Tumor acidosis is an important determinant of tumor progression and tumor pH regulation is being investigated as a therapeutic target. Autophagy is a cellular catabolic pathway leading to lysosomal degradation and recycling of proteins and organelles, currently considered an important survival mechanism in cancer cells under metabolic stress or subjected to chemotherapy. We investigated the response of human melanoma cells cultured in acidic conditions in terms of survival and autophagy regulation. Melanoma cells exposed to acidic culture conditions (7.0<pH<6.2) promptly accumulated LC3+ autophagic vesicles. Immunoblot analysis showed consistent increase of LC3-II in acidic culture conditions as compared to cells at normal pH. Inhibition of lysosomal acidification by Bafilomycin A1 further increased LC3-II accumulation, suggesting an active autophagic flux in cells under acidic stress. Acute exposure to acidic stress induced rapid inhibition of mTOR signalling pathway detected by decreased phosphorylation of p70-S6K, associated with decreased glucose and leucine uptake. Inhibition of autophagy by knock-down of the autophagic gene Atg5 consistently reduced melanoma
cell survival in low pH conditions. These observations indicate that induction of autophagy may represent an adaptation mechanism for cancer cells exposed to acidic environment. Our data strengthen the validity of therapeutic strategies targeting tumor pH regulation and autophagy in progressive malignancies.
1. INTRODUCTION

The traditional model of carcinogenesis is based on a sequence of genetic mutations and epigenetic alterations which results in abnormal expression and/or activity of oncogenes, protooncogenes, tumor suppressor genes or apoptotic pathways [Feron ER and Vogelstein B, 1990; Gillies RJ and Gatenby RA, 2007]. It has been taken for granted for several years that cancer was caused by the accumulation of a limited number of genetic mutations which allowed cancer cells to indefinitely proliferate. However, it is now clear that genetic alterations in cancer are extremely numerous and heterogeneous [Folkman J et al, 2000]. Indeed, the high genetic complexity and variability of cancer cells constitutes a major barrier for effective and successful gene-based therapies. For example, despite the efficacy of Gleevec (imatinib mesylate, an inhibitor of the BCR-ABL kinase) in controlling (but not curing) progression of chronic myeloid leukemia, most of approved targeted therapies have failed to improve overall survival in different cancer types. Moreover, the hope for a successful personalized therapy with the RAF inhibitor PLX4032 has been crushed due to the frequent occurrence of drug resistant cells responsible for tumor relapses [Aplin AE et al, 2011; Wagle N et al, 2011]. The use of chemotherapy and cytotoxic drugs has been successful in the treatment of some cancers (mostly hematopoietic tumors) but it has largely failed in the
treatment of most solid tumors. Chemotherapy preferentially targets cellular replication mechanisms and a fundamental principle is to use drugs that are more toxic to tumor cells than to healthy cells, as many tumors replicate faster than the host tissue (except for fast replicating tissues such as epithelia). However, proliferation rates greatly differ among cellular populations within a tumor tissue. Typically, cells localized in the outer regions and/or closer to blood vessels are characterized by a high proliferation rate while cells located in the hypoxic, scarcely perfused and acidic deep tumor parenchyma are in a quiescent/dormant state, thus not a suitable target for this type of approach. Moreover, if intrinsic drug-resistance is not clinically observed, many cytotoxic drugs often induce drug-resistance, have long-term and severe side effects, and may indeed cause cancers [Chabner BA and Roberts TG Jr, 2005]. Similar and predictable drawbacks have also been reported with the use of anti-angiogenic drugs. These drugs exert an antitumor effect but at the same time may elicit malignant progression by favoring local invasion and distant metastases [Ebos JM et al, 2009; Paez-Ribes M et al, 2009]. It appears conceivable that the anti-angiogenic effect leads to selection of cancer cells whose metabolism does not depend on blood supply.

As stated by the World Health Organization (WHO), cancer mortality has not significantly changed during the last decades. Actually, the decreased mortality
observed in some types of cancer may often be consequence of early diagnosis and treatment, and prevention actions via screening programs, rather than successful therapeutic interventions in advanced disease (WHO). Today, scientists and clinical oncologists start realizing that a complete eradication of cancer may be an utopist and so far unreachable goal, and that alternative therapeutic strategies aimed at controlling rather than curing cancer are to be considered [Gatenby RA, 2009]. This may be possible if cancer is not simply treated as a genetic disease but as a disease in which selected phenotypic changes (the hallmarks of cancer) and genetic mutations evolve in a complex physical and biochemical microenvironment. These hallmarks may be related to genetic alterations but are also, directly or indirectly, associated or linked to alterations in cancer cell metabolism [Cairns RA et al, 2011; Kroemer G and Pouyssegur J, 2008].

The Nobel Prize in Physiology/Medicine 1931 Otto Heinrich Warburg was the first to describe the peculiar metabolism of tumors [Warburg O, 1956]. Warburg discovered that tumors use a glycolytic metabolism even in the presence of normal oxygen tensions (the Warburg effect), that from the bioenergetics point of view would not be convenient since glycolysis is much less efficient than oxidative phosphorylation (OXPHOS) to produce energy (ATP). He also found that cancer cells were able to live in an acidic
microenvironment, thought to be a consequence of elevated lactic acid production in poorly oxygenated areas where glycolysis is upregulated.

Whatever the triggering pathways, acidity is a common feature of all solid tumors and contributes to both tumor malignant progression and drug-resistance [Cardone RA et al, 2005; De Milito A and Fais S, 2005; Gatenby RA and Gillies RJ, 2004; Raghunand N and Gillies RJ, 2001; Raghunand N et al, 2003]. For this reason, targeting tumor pH homeostasis is being considered as a valid and feasible strategy against cancer [Fais S et al, 2007; Gerweck LE, 1998; Harguindey S et al, 2009; Izumi H et al, 2003; Robey IF et al, 2009; Tannock IF and Rotin D, 1989].

1.1 THE TUMOR METABOLIC MICROENVIRONMENT

Tumor cells localized in proximity of blood vessels are supplied with oxygen and nutrients which greatly contribute to support growth and proliferation. As tumor mass increases, dysplastic growth will result in the formation of scarcely oxygenated areas with reduced perfusion capacity, due to the lack of an organized and sufficient vasculature. Tumor cells located in these regions are exposed to low oxygen tension (hypoxia), lack of nutrients and accumulation of extracellular acids, which all contribute to the establishment of an extremely toxic microenvironment (the tumor metabolic microenvironment).
Such microenvironment exerts an important selective pressure for the further progression of malignant cancer cells, characterized by increased invasive capacity and increased genetic instability. In fact, although carcinogenesis has so far been explained by the clonal expansion of genetically mutated cells, the growth and selection of malignant cells do occur in a complex physical and biochemical local microenvironment determining the fate of developing cancers [Gillies RJ et al, 2008]. A challenge regarding cancer cell metabolism has been so far to understand the advantages for tumors to metabolize glucose by aerobic glycolysis rather than by oxidative phosphorylation. The paradox relies on the net amount of ATP molecules produced by the two different metabolic pathways [Vander Heiden MG et al, 2009]. In cells using OXPHOS, glucose is converted into pyruvate and acetyl-CoA, which is oxidized in mitochondria through the tricarboxylic acid (TCA) cycle producing 36 ATP molecules with the formation of CO₂. In cancer cells, the glycolytic pathway ends up with the conversion of glucose into pyruvate and lactate, which produces only 2 ATP molecules. Moreover, acetyl-CoA may enter a truncated TCA cycle, with the result that acetyl-CoA is exported into the cytosol and contributes to the synthesis of fatty acids and cholesterol, important membranes’ components of proliferating cells. Today we know that metabolic transformation does not only involve aerobic glycolysis. In fact, in addition to
glucose it has recently been recognized that also glutamine metabolism may as well play a pivotal role for the synthesis of macromolecules needed to meet the demand of highly proliferating cells \[\text{DeBerardinis RJ et al, 2008}\]. The activity of hypoxia-inducible factor 1 (HIF-1) and oncogene activation are considered major pathways governing the metabolic changes occurring during cancer progression (metabolic reprogramming) \[\text{Hsu PP and Sabatini DM, 2008; Pouyssegur J et al, 2006}\]. The ability of tumor cells to adapt their metabolism to a complex and hostile microenvironment provides cancer cells a selective advantage to further progress towards malignancy. However, critical metabolic pathways for tumor cell survival may also represent a selective therapeutic target to be considered in new treatment strategies \[\text{Hersey P et al, 2009; Tennant DA et al, 2010}\].

A major consequence of the altered glycolytic tumor metabolism is the extracellular acidity with a selection of tumor cells armed to avoid intracellular accumulation of acids. One of the major mechanisms tumor cells exploit to counteract cytosolic acidification is an active proton extrusion by proton pumps. Such activities result in an aberrant pH homeostasis critical for cancer cell survival and may be considered an important target for therapeutic purposes. Maintenance of the intracellular pH (pHi) is crucial to normal cell function because many cellular biochemical processes have a narrow pH
optimum. The major types of pH regulatory mechanisms operating in tumor cells are Na+/H+ exchangers (NHE) [Harguindeguy S et al, 2009], carbonic anhydrases (CA) [Supuran CT, 2008], bicarbonate transporters [Izumi H et al, 2003], H+-linked monocarboxylate transporters (MCT) [Porporato PE et al, 2011] and proton pumps like the vacuolar ATPase (VATPase) [Fais S et al, 2007] and the F1F0-ATP synthase [Chi SL and Pizzo SV, 2006]. However, these transporters represent feasible therapeutic targets currently under investigation both in the preclinical and clinical settings.

1.1.1 ROLE OF PROTON PUMPS IN MALIGNANCY OF TUMORS

The Pivotal Role of V-ATPase

The V-ATPase is an ubiquitous proton pump expressed on the membrane of endolysosomal organelles and also on the plasma membrane of specialized cells (like renal intercalated cells and osteoclasts) [Nishi T and Forgac M, 2002]. It is a multimeric protein consisting of a catalytic complex (V1) facing the cytosol where ATP hydrolysis occurs, and a transmembrane complex (V0) where proton translocation occurs. V-ATPase expression and activity play a crucial role in the regulation of vesicular trafficking both in normal and tumor cells [Nishi T and Forgac M, 2002; Marshansky V and Futai M, 2008]. V-ATPases are functionally expressed in plasma membranes of human tumor
cells and may have specialized functions in cell growth, differentiation, angiogenesis, invasion and metastasis [Martinez-Zaguilan R et al, 1993; Martinez-Zaguilan R et al, 1999]. In tumor cells the extrusion of protons by V-ATPases causes intracellular alkalinization and extracellular acidification which are important mechanisms favouring growth, resistance to apoptosis and invasion [Martinez-Zaguilan R et al, 1999; Hinton A et al, 2009; Martinez-Zaguilan R et al, 1996]. Such a mechanism contributes to the maintenance of an aberrant pH gradient between the alkaline cytosol and the acidic extracellular microenvironment. The low pH of tumor extracellular microenvironment may influence the increased secretion and activation of proteases. Moreover, low extracellular pH (pHe) may promote the degradation and remoulding of extracellular matrix (ECM) through the activation of proteolytic enzymes, including matrix metalloproteinases (MMPs), bone morphogenetic protein-1-type metalloproteinases, tissue serine proteases, and adamalysin-related membrane proteases, thus contributing to cancer invasion and metastasis [Martinez-Zaguilan R et al, 1996; Rofstad EK et al, 2006]. Some data suggest that different use of ion exchanger may help to distinguish tumor cells with different metastatic behavior [Sennoune SR et al, 2004]. In fact, while breast cancer cells with low metastatic potential preferentially utilize Na+/H+ exchange and bicarbonate-based H+-
transporting mechanisms, highly metastatic cells preferentially use plasma membrane V-ATPases, suggesting that V-ATPases are important in the acquisition of a more metastatic and invasive phenotype [Sennoune SR et al, 2004]. Furthermore, V-ATPases have been involved in the acquisition of the multidrug resistance phenotype [Martinez-Zaguilan R et al, 1999; Raghunand N et al, 1999] and treatment with inhibitors of the V-ATPases (most of which are highly toxic) may reverse tumor resistance [Hizumi H et al, 2003; Altan N et al, 1998; Luciani F et al, 2004; Ouar Z et al, 2003].

**Invasion and Metastasis**

The acidic pH of the tumor microenvironment has been recognized as an important component driving tumor invasive capacity, neoangiogenesis, anchorage-independent growth and genetic instability [Morita T et al, 1992; Gillies RJ et al, 2002; Gatenby RA and Gillies RJ, 2008; Orive G et al, 2003; Yuan J et al, 2000], which in combination contribute to malignant progression. The acidic extracellular pH has been shown to enhance the metastatic behavior and invasive capacity of breast cancer cells and melanoma [Martinez-Zaguilan R et al, 1996; Rofstad EK et al, 2006; Moellering RE et al, 2008; Smallbone K et al, 2005]. Both the V-ATPase and the NHE-1 have been implicated in the pH-dependent degradation of the ECM and support invasive growth and
metastasis [Cardone RA et al, 2005; Fais S et al, 2007]. Acidic pH not only enhances the pH-dependent activity of proteolytic enzymes involved in ECM degradation (like cathepsin B, matrix metalloproteases, gelatinase and collagenase) but also induces their secretion [Hinton A et al, 2005; Lu X et al, 2005; Bourguignon LY et al, 2004; Busco G et al, 2010]. Moreover, the release of pro-angiogenic factors like VEGF and IL-8, both in soluble form and in tumor microvesicle-associated form, is greatly enhanced in tumor cells cultured in acidic pH conditions and may therefore contribute to neoangiogenesis and tumor cell dissemination [Rofstad EK et al, 2006; Fukumura D et al, 2001; Giusti I et al, 2008; Taraboletti G et al, 2006; Xu L and Fidler IJ, 2000; Xu L et al, 2002]. Thus, inhibition of tumor acidic pH may represent a tool in limiting dissemination of cancer cells and formation of metastases, as also recently suggested by studies using NaHCO₃ to reduce tumor pH [Robey I et al F, 2009, Silva AS et al, 2009].

**Proton Pumps and Drug-resistance**

As mentioned above, an important consequence of the accumulation of acids following upregulated glycolysis in cancer cells is the increased proton efflux through the plasma membrane, which causes a shift in pH towards more alkaline values. This has been reported for several human tumors in vitro
including breast carcinoma [Raghunand N et al, 1999], B and T cell leukemia [Rich IN et al, 2000], melanoma [Wahl ML et al, 2002] and further confirmed by MRI studies in vivo in several animal models [Gillies RJ et al, 2008; Gillies RJ et al, 2002; Gillies RJ et al, 1994; Hjelmeland AB et al, 2011]. Changes in the pH gradient between the extracellular microenvironment and the cell cytoplasm and/or in the pH gradient between the cell cytoplasm and the lysosomal compartments are common features of many tumors [Mahoney BP et al, 2003; Simon S et al, 1994]. Since the mechanisms of entry of drugs into the cell are dependent on both concentration gradients and pH gradients, the reversed pH gradients of tumors may severely affect drug distribution, uptake and activity [De Milito A and Fais S, 2005; Gerweck LE et al, 1998; Altan N et al, 1998; Gerweck LE et al, 2006; Raghunand N and Gillies RJ, 2000; Tredan O et al, 2007]. Weakly basic chemotherapeutic drugs (like doxorubicin, mitoxantrone, vincristine, vinblastine) are protonated in the acidic tumor stroma and/or in the intracellular acidic organelles (such as the lysosome, recycling endosomes, trans-Golgi network, or secretory vesicles) with the consequence of decreased cellular uptake and/or sequestration and extrusion within acidic vesicles trafficking. Alkalinization of tumor pH has been shown to increase the uptake and the cytotoxic activity of weakly basic drugs [Raghunand N et al, 2003; Raghunand N et al, 1999; Luciani F et al, 2004]. Conversely, weakly acidic
compounds like camptothecin, cyclophosphamide and chorambucil showed an increased activity at low pH [Mahoney BP et al, 2003; Gerweck LE et al, 2006; Tredan O et al, 2007; Gabr A et al, 1997] and further tumor acidification by glucose intravenous administration may increase the activity of these compounds [Gerweck LE et al, 2006; Adams DJ et al, 2005]. Moreover, prostate carcinoma cells exposed to low pH showed enhanced P-glycoprotein (P-gp) activity, which may further contribute to the reduced cytotoxicity of chemotherapeutic agents in hypoxic/acidic tumors [Thews O et al, 2006]. An additional contribution to drug-resistance comes from the effects of pH on drug permeability [Rauch C and Pluen A, 2007]. Indeed, it has been shown that cytosolic pH fixes the molecular weight of drugs entering cells, by altering the mechanical packing of lipids in cell membrane and that, both cytosolic pH and membrane mechanical properties are needed to explain doxorubicin resistance levels in cancer cell lines [Rauch C, 2009; Rauch C, 2011]. Agents that disrupt or normalize the pH gradient of tumor cells may improve sensitivity to cytotoxic agents and/or inhibit tumor growth. For example, sodium bicarbonate treatment causes alkalinization of MCF-7 tumor xenografts and increases the therapeutic efficacy of doxorubicin [Raghunand N et al, 1999]. Lysosomotropic agents that induce pH gradient modification and alkalinisation of acidic vesicles may reverse anthracycline resistance in
multidrug-resistance cells [Ouar Z et al, 1999]. In addition, severe disturbance of pH cellular homeostasis following treatment with proton pump inhibitors (PPI) induced reversion of drug-resistance in chemo-resistant human carcinomas and increased sensitivity to cytotoxic drugs in multidrug resistant (MDR) cell lines [Luciani F et al, 2004]. Drug-resistant cancer cell lines display a more alkaline pH than drug-sensitive cells [Simon S et al, 1994; Belhoussine R et al, 1999; Miraglia E et al, 2005]. Besides the role of alkaline pH in determining the fate of weakly basic drugs, such conditions also affect the activity of pro-apoptotic compounds. In fact, an early event induced by several agents inducing apoptotic cell death is the rapid acidification of pH [Goossens JF et al, 2000; Gottlieb RA et al, 1996; Marches R et al, 2001; Nilsson C et al, 2006]. Acidification of the intracellular microenvironment may also enhance the effect of pro-apoptotic drugs [Li J and Eastman A, 1995; Park HJ et al, 1996]. Drug-induced hydrogen peroxide production may induce intracellular acidification and release of cytochrome C, thereby creating an intracellular microenvironment permissive for caspase activation [Hirpara JL et al, 2001].

**Inhibition of Proton Pumps**

Specific inhibitors of V-ATPase like Bafilomycins have been reported to induce apoptosis in human cancer cells [Nakashima S et al, 2006] and other molecules
have been identified and are being evaluated for their antineoplastic activity [Perez-Sayan M et al, 2009]. However, all the V-ATPase inhibitors were highly cytotoxic for normal cells and tissues as well, due to the ubiquitous expression and the key role of these proton pumps in many cells and organs [Hinton A et al, 2009]. Proton Pump Inhibitors (PPI) have been successfully used for the treatment of peptic diseases, due to their anti-acid 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 microenvironment. The specific targets of PPI are H⁺/K⁺-ATPases contained within the lumen of gastric parietal cells and, though to a lesser activity, they inhibit the activity of V-ATPases, thus blocking proton transport across membranes. Recent data obtained by our group [De Milito A et al, 2007] and by Lu and collaborators [Lu X et al, 2005] demonstrated that inhibition of the V-ATPase has an antineoplastic activity [Fais S et al, 2007]. In fact, the inhibition of V-ATPase function via knockdown of ATP6L (c subunit gene) expression using siRNA suppresses cancer metastasis by decreased proton extrusion and downregulated protease activity [Lu X et al, 2005]. Our group has shown that proton pump inhibitors induce cell death in human B cell tumors by a mechanism involving oxidative stress and lysosomal membrane perturbation [De Milito A et al, 2007]. Interestingly, these two different types
of antineoplastic strategies resulted in delayed tumor growth \textit{in vivo} as shown by i) inhibition of B cell lymphoma growth in PPI-treated SCID mice and ii) retarded tumor growth and suppressed cancer metastasis in nude mice. These data suggest that V-ATPases and its activity regulating the reversed tumor pH gradients may represent a potential target for cancer therapy.

\textbf{1.2 PROTON PUMP INHIBITORS: A CLASS OF PRO-DRUGS}

As mentioned before, disruption of tumor pH homeostasis is a feasible therapeutic strategy already at an advanced stage of development with different biological targets and therapeutic approaches [\textit{Harguindeguy S et al, 2009; Supuran CT, 2008; Chiche J et al, 2009; Fais S, 2010}]. Besides targeting the gastric proton pump, PPI have been shown to also inhibit the activity of V-ATPase, although at much higher concentrations [\textit{Mattsson JP et al, 1991; Moriyama Y et al, 1993}]. Based on the involvement of V-ATPase in both metastatic spread and chemo-resistance we reasoned that treatment with PPI might be convenient to provoke disturbance of pH homeostasis in cancer cells to be exploited for therapeutic applications. Before describing the different biological effects that PPI may have on cancer cells we will make a summary of the chemical and pharmacological properties of PPI. Proton pump inhibitors as blockers of gastric acid secretion were discovered at the beginning of 1970. In
1975 the Hassle AB Swedish drug company synthesized two compounds that inhibited acid secretion independently of the nature of the stimulus: Timoprazole and Picoprazole [Fellenius E et al, 1981; Wallmark B et al, 1983]. However, administration of these compounds in rats was associated with thyrotoxicity and thymotoxicity.

Based on examination of the structure and the characteristic of inhibitory effect it was deduced that these two compounds were acid-activated pro-drugs [Shin JM, 2008]. Subsequently, omeprazole and other compounds with similar chemical structure were synthesized and introduced in clinical practice since 1989 for treatment of diseases related to gastric acid hypersecretion. PPI are administered as enteric-coated tablets or capsules that are absorbed in the proximal small bowel [Vanderhoff BT and Tahboub RM, 2002]. Through the blood flow PPI reach the canaliculus of secreting parietal cell. This acidic microenvironment allows the protonation of PPI and consequently the activation into the sulphenamide form. Cysteine residues of the luminal face of H+/K+-ATPase are blocked by the sulphenamide form of PPI. The cysteine residues involved in this reaction are located in the M5, M6 domain of the α-subunit of proton pump. The blockade of the enzyme occurs through a covalent bond and the restoration of acid production needs the de novo synthesis and translocation of new pumps [Brandstrom A et al, 1985; Kroemer
W, 1995]. PPI dispatch their anti-secretory activity through the inhibition of gastric proton pumps H+/K+ ATPase. PPI in their acid-activated form, the sulfonamide derivatives, bind through a disulfide bond the cysteine CYS813 residues located in the luminal vestibule of the H+/K+ ATPase that is accessible from the extracytoplasmic region of the protein. In the disulfide “bridge” one sulfur atom will come from a CYS residues on the ATPase and the other one will come from the sulfonamidic form of PPI. The covalent disulfide bond leads to an irreversible inhibition of the gastric pump [Roche VF, 2006]. Recovering of gastric acid secretion requires de novo biosynthesis of the enzyme, which normally occurs within 96 hours in dog and man. The main bond-site of PPI is CYS813 but PPI can also interact with a second cystein residue, the CYS822. The rate of conversion to the thiophilic intermediate may determine access to this cysteine. PPI with rapid conversion react rapidly with CYS813 which prevents access to CYS822. For PPI with slow conversion rate (like Pantoprazole) CYS822 can be accessed by the protonated compound [Shin JM et al, 2004]. Moreover, Omeprazole is also able to bind to cysteine CYS892 and Lansoprazole to cysteine CYS321. In addition to CYS813 and CYS822, two anionic residues at position 820 and 824 are believed to be important in the positioning of the drug for irreversible interaction with the CYS residue [Roche VF, 2006].
Chemistry of PPI

The PPI pharmacophore is 2-pyridyl-methyl-sulfinyl-benzimidazole. The PPI products on the market (Omeprazole, Esomeprazole, Lansoprazole, Pantoprazole and Rabeprazole) contain this basic structure and differ only in the nature of substituents in the aromatic rings [Roche VF, 2006; Horn J, 2000]. Additionally, the PPI Tenatoprazole chemically differs from other PPI because it has an imidazopyridine ring in place of the benzimidazole moiety [Hunt RH et al, 2010]. The structure of PPI contains a chiral center on the sulfur atom. All PPI, with exception of Omeprazole are produced as racemic mixture while Esomeprazole is the S-isomer of Omeprazole. The chemical structure consists of three parts, a substituted pyridine ring, a substituted benzimidazole ring and a sulfoxide-containing chain (CH$_2$SO) which connects the 2-position of these ring system, all three are essential for activity. The electro-chemical properties of the substituent (electron donating or withdrawing groups) have a significant impact on chemical reactivity and biological effects. Electron donating groups on the pyridine ring will push electrons to the pyridine N and increase the percentage existing in ionized form at acidic pH. Moreover, this substituent increases the nucleophilic character of any PPI pyridine nitrogen atoms in the unionized conjugate base form enhancing the rate of formation of the active sulfenic acid/sulfonamide rearrangement compound. The
substituents in the benzimidazole ring are also important: electron donating substituents on C5 position of the benzimidazole ring push electrons to N3 and increase the percentage existing in cationic form at acidic pH, thus enhancing the formation rate of active product (sulfenic/sulfonamide derivative) [Roche VF, 2006].

PPI are acid activated pro-drugs. The sulfinyl moiety connecting the pyridine and benzimidazole rings is not sufficiently reactive to form the disulfide bridge with cysteine residues of H+K+/ATPase and need to be activated through two protonations and spontaneous rearrangement in the active sulfonamide or sulfenic acid derivatives. In acidic condition, PPI undergo two protonation reactions. The nitrogen atoms involved in these reactions are the pyridine nitrogen and the doubly bonded benzimidazole nitrogen. The pKa of the pyridine nitrogen (pKa1) ranges from 3.83 to 4.53 and ensure that the pyridine nitrogen of all PPIs will be completely ionized at acidic pH of parietal cells (pH 1.3), trapping the drug at the right site of action. The pKa value of the benzimidazole N3 (pKa2) is much lower than that of the pyridine nitrogen and ranges from 0.11 to 0.79. These lower pKa values mean that the benzimidazole ring protonates after the pyridine ring and the extent of protonation will be significantly lower. The higher the pKa2 value the more willingly the benzimidazole nitrogen accepts proton and becomes cationic. In fact, the
second reaction affects the doubly bounded benzimidazole nitrogen which once protonated leads the intramolecular nucleophilic attack at the C2 position of the benzimidazole [Roche VF, 2006; Horn J, 2000]. The possibility to release the proton from NH group of benzimidazole ring confers a weak acidity that is useful to prepare more stable alkali salts for pharmaceutical preparations [Brandstrom A et al, 1985]. PPI are chiral drugs and are administered as racemate (mixture of 50/50 of its enantiomers) with exception of Omeprazole, whose S-enantiomer (Esomeprazole) is also widely used in clinical applications. In fact, Esomeprazole is metabolized to a lesser degree and at lower rate than Omeprazole by CYP450 enzymes, resulting in higher plasma levels and as a consequence, in a more effective acid suppression and better therapeutic efficacy [Andersson T et al, 2001]. For other PPI there are not clinical advantages to justify the development of any enantiomers of the drugs [Andersson T and Weidolf L, 2008].

**Metabolism of PPI**

PPI extensively bind to serum proteins (about 95%) and are subject to first-pass metabolism [Vanderhoff BT and Tahboub RM, 2002]. PPI are metabolized by the microsomial enzymes CYP450. In particular, the isoform 2C19 is responsible of PPI conversion to inactive metabolites, although 3A4 isoform is
involved in PPI biotransformation. The major metabolic degradation pathways for PPI provide the hydroxylation reaction to aromatic ring, for Omeprazole/Esomeprazole the benzylic hydroxylation produce the inactive derivative 5 hydroxyomeprazole. The 5-hydroxy-derivate is enzymatically converted in Omeprazole hydroxysulfone which is the final metabolite of the degradation pathway. The minor degradation pathway involves mainly the CYP3A4 and provides the reaction of O-dealkilation, S-oxidation and hydroxylation at different sites, leading to the formation of inactive metabolites. The metabolism of PPI is stereoselective, and for Omeprazole the metabolism rate of S-isomer is lower and less variable than R-isomer, resulting in higher plasma concentration following administration of the same dose [Abelo A et al, 2000; Andersson T et al, 2001].

An important aspect that may influence the pharmacodynamics of PPI is the polymorphism of CYP2C19 isoform. The genotypes of CYP2C19 are classified into two groups, extensive metabolizer (EM) and poor metabolizer (PM). PM do not express a functional CYP2C19 enzyme and accordingly the metabolism is shifted to CYP3A4 which leads to lower metabolic clearance of PPIs [Andersson T and Weidolf L, 2008; Fock KM et al, 2008]. The direct consequence for these patients is a greater response to all PPIs (except Rabeprazole) but also a higher risk of toxicity, especially during administration
of Omeprazole and Esomeprazole because these not only are metabolized by CYP2C19 but are able to inhibit them. In contrast, EM can result as “non responsive” to common therapeutic doses of PPI, except Rabeprazole which works independently of CYP2C19 phenotype. Despite this general behavior, PPI show marked differences about the bioavailability that is about 30 to 40% for Omeprazole, 80 to 85 % for Lansoprazole, 52% for Rabeprazole and 77% for Pantoprazole. Upon repeated dosing (both intravenous and oral doses) the bioavailability of Omeprazole increases to 65%. This trend indicates a non-linear pharmacokinetic that can be explained by assuming a saturation process of first pass metabolism [Kromer W, 1995]. The plasma halflife of PPI is between 1 and 2 hours.

Adverse Effects of PPI

PPIs are among the most widely prescribed medications worldwide. They are well tolerated and safe drugs. Serious adverse events are extremely rare with a percentage of 1-2% [Thomson AB et al, 2010]. The most common symptomatic adverse effects with PPIs include headache, diarrhea, nausea (similar profiles as with cimetidine and ranitidine). Interstitial nephritis, neutropenia and hypomagnesaemia have been reported as serious adverse effects of PPI therapy. PPI use is associated with the development of funding glande polyps
and with parietal cells hyperplasia \cite{Thomson2010}. The evidences regarding the use of PPI and the occurrence of gastrointestinal cancer are divergent. Studies reported that the long-term use of PPI in animals infected with \textit{H. pylori} promotes development of adenocarcinoma \cite{Hagiwara2001} and neuroendocrine tumor in the stomach \cite{Tsukamoto2011}. Other studies reported that PPI use may be modestly associated with increased risk of Colorectal Cancer (CRC) \cite{Chubak2009}. A recent case–control study reported that current PPI users with more than 3 years of use experienced a three-fold increased risk of non-cardia gastric cancer, whereas current long-term users of H2RA had no excess risk \cite{Garcia-Rodriguez2006, Poulsen2009}. In contrast, other studies indicate no association between PPI use and the risk of colorectal cancer \cite{Robertson2007, vanSoest2008}. Moreover, PPIs may alter the absorption of nutrients as minerals and vitamins, and alter the effectiveness or bioavailability of drugs \cite{Vanderhoff2002}. The most common example about the interaction with the drugs is the association with clopidogrel, for which PPI use, with the exception of Pantoprazole has been associated with reduced effectiveness of clopidogrel and a resulting 40% increased risk of coronary stent occlusions \cite{Juurlink2009}. Moreover, some studies have reported that PPI administration delays elimination of methotrexate
[Santucci R et al, 2010; Santucci R et al, 2010; Suzuki K et al, 2009] while this was not observed in other settings [Vakily M et al, 2005; Whelan J et al, 1999]. Omeprazole was shown to reduce bone resorption likely by inhibiting the osteoclastic proton pump [Mizunashi K et al, 1993]. In case controlled studies, long-term PPI use was associated with an increased risk of bone fractures, and this increased risk depended on the duration and dose of chronic use of PPI [Targownik LE et al, 2008]. In conclusion, the collective body of information suggests an increased risk of infectious complications and nutritional deficiencies. Data regarding any increased risk in gastric or colon malignancy are less convincing [Ali T et al, 2009].

1.3 AUTOPHAGY

Autophagy is a word derived from Greek: “auto” means oneself and “phagy” means to eat, so it indicates a process in which cell digest part of its self. Autophagy is a catabolic process aimed at eliminating protein aggregates, misfolded protein, damaged organelles and intracellular pathogens or recycle long-lived proteins and oxidized lipids [Mehrpour M et al, 2010; Yang Z and Klionsky DJ, 2010; Rabinowitz JD and White E, 2010]. Autophagy is the only mechanism to degrade large structures such as organelles and protein aggregates. This process occurs in normal condition to maintain cellular
homeostasis. It can be upregulated under physiological condition such as nutrient starvation and growth factor deprivation, or in response to a variety of stress stimuli. In these conditions, autophagy leads to the generation of free amino and fatty acids, which can become part of the Krebs cycle for ATP production or used for the de novo biosynthesis of proteins and lipids required to adapt to stress, thus promoting cell survival [Meschini S et al, 2011; Wang CW and Klionsky DJ, 2003; Mejier AJ and Codogno P, 2004]. Under other conditions, autophagy may represent a non-apoptotic form of programmed cell death. Autophagy is also important to maintain DNA stability [Mathew R et al, 2007]. Indeed, defective autophagy causes failure of recycling of damaged protein, insufficient energy for proper DNA replication and repair, inefficient removal of damaged mitochondria with consequent accumulation of reactive oxygen species (ROS). All of these factors may contribute to genomic instability in autophagy-defected cells [Levine B and Kroemer G, 2008; Jin S and White E, 2007; Mathew R et al, 2007].

The autophagic process starts by the formation of a double-membrane bound autophagosome dependent on the sequential intervention of Atg (AuTophaGy-related) proteins [Mizushima N and Komatsu M, 2011; Weideberg H et al, 2011]. In yeast about 30 Atg genes involved in autophagic process have been identified [Tsukada M and Oshumi Y, 1993; Thumm M et al 1994]; most of
these genes have homologs in mammals, indicating that the molecular mechanism of autophagy is highly conserved [Klionsky DJ and Emr SD, 2000].

In nutrient rich conditions the ULK1 (ATG1) binds to mTORC1 and inhibits autophagy. In nutrient deprivation, mTORC1 dissociates from the ULK1 complex, freeing it to trigger autophagosome nucleation and elongation [Kuma A and Mizushima N, 2010]. Autophagosome nucleation requires a complex containing Beclin 1 (Atg6) [Funderburk SF et al, 2010]. Expansion of autophagosome membranes involves two ubiquitin-like molecules, Atg12 and Atg8 (called LC3 in mammals), and two associated conjugation systems that covalently link Atg12 with Atg5, which together bind Atg16L1 to form pre-autophagosomal structures [Rabinowitz JD and White E, 2010]. In the second ubiquitin-like reaction, LC3 is cleaved, conjugated with Phosphatidylethanolamine (LC3-II) and associated with newly forming autophagosome membranes. LC3-II remains on mature autophagosomes until after fusion with lysosomes and is commonly used to monitor autophagy. The process beginning with the Beclin 1 complex gives rise to nascent autophagosome membranes. These membranes assemble around cargo, encapsulating the cargo in a vesicle that subsequently fuses with a lysosome, generating an auto-lysosome [Rabinowitz JD and White E, 2010]. To target proteins for autophagic degradation, ubiquitin on modified proteins are
recognized and bound by autophagy receptors, such as p62, which interact with LC3 to deliver cargo to autophagosomes [Kuma A and Mizushima N, 2010]. The contents are then degraded by proteases, lipases, nuclease, and glycosidases. Lysosomal permeases release the breakdown products—amino acids, lipids, nucleosides, and carbohydrates—into the cytosol, where they are available for synthetic and metabolic pathways [Rabinowitz JD and White E, 2010].

One of the key regulators of autophagy is the target of rapamycin, TOR kinase, which is the major inhibitory signal that shuts off autophagy in the presence of growth factors and abundant nutrients. The class I PI3K/Akt signaling molecules link receptor tyrosine kinases to TOR activation and thereby repress autophagy in response to insulin-like and other growth factor signals [Levine B and Kroemer G, 2008; Lum JJ et al, 2005]. Some of the other regulatory molecules that control autophagy include 5′-AMP-activated protein kinase (AMPK), which responds to low energy; the eukaryotic initiation factor 2α (eIF2α), which responds to nutrient starvation and endoplasmic reticulum (ER) stress; the tumor suppressor protein, p53; death-associated protein kinases (DAPk); the ER-membrane-associated protein, Ire-1; the stress-activated kinase, c-Jun-N-terminal kinase; the inositoltrisphosphate (IP3) receptor (IP3R); GTPases; Erk1/2; ceramide; and calcium [Levine B and Kroemer G, 2008; Criollo
A et al, 2007; Maiuri MC et al, 2007; Meijer AJ and Codogno P, 2006; Rubinsztein DC et al, 2007].

Less well-characterized mTOR-independent regulators of autophagy also exist. One is ammonia, a by-product of amino acid catabolism, which stimulates autophagy, likely in poorly perfused tissues and tumors [Eng CH et al, 2010]. Glucagon, a predominant hormone of the fasted state, also triggers autophagy in the liver. Adrenergic receptor activation, which like glucagon activates adenylate cyclase and cyclic adenosine monophosphate (cAMP) production, also stimulates liver autophagy. [Rabinowitz JD and White E, 2010]. Recent studies have revealed the importance of autophagy in development [Mizushima N and Komatsu M, 2011; Cecconi F and Levine B, 2008], ageing [Cuervo AM, 2008; Rubinsztein DC et al, 2011], immune response [Deretic V, 2011; Levine B et al, 2011] and pathophysiology such as neurodegenerative disease, obesity, infectious disease and cancer [Mizushima N and Komatsu M, 2011; Levine B et al, 2011; Harris H and Rubinsztein DC, 2011; Mizushima N et al, 2008; White E and DiPaola RS, 2009].

**Autophagy and Cancer**

The relationship between autophagy and cancer is subject of many controversial studies. Several genetic links support the concept that autophagy is, at least of the first phase of carcinogenesis, a tumor suppressor pathway
Several signaling pathways are common to autophagy and tumorigenesis. For example, tumor suppressor genes inhibiting TOR signaling (like PTEN) stimulate autophagy and conversely oncogene products activating TOR (class I PI3K and Akt) inhibit autophagy. It should be clarified whether the modulation of autophagy through tumor suppressor and onco-genes is a functionally part in their mechanism of action. The first link between autophagy and cancer was demonstrated from Liang and colleagues [Liang XH et al, 1999], who discovered that the ATG gene Beclin 1 promotes autophagy in human breast carcinoma MCF-7 and this process is associated with inhibition of MCF-7 proliferation and tumorigenesis in nude mice. Expression of endogenous Beclin 1 is low in human breast carcinoma (cell lines and tissue) but Beclin 1 is expressed at high level in normal breast epithelia, suggesting that decreased expression of autophagy proteins may contribute to the development or progression of breast and other human malignancies [Liang XH et al, 1999]. Other Atg genes are linked to cancer: upregulation of Atg5 expression increases sensitivity to antitumor drugs in vitro and in vivo [Yousefi S et al, 2006]; knockout of Atg4C increase chemically-induced fibrosarcoma [Marino G et al, 2007]. Tumor suppression effect of autophagy seems to be independent of both pro-death and pro-survival effects [Mathew R et al, 2007]; in fact,
epithelial tumors derived from cells with deletion of Beclin 1 or Atg5, do not are more resistant to cell death and under metabolic stress they undergo cell death as expected [Mathew R et al, 2007]. The capacity of autophagy to direct cells to survival or death assumes great importance in cancer treatment.
2. BACKGROUND AND RATIONALE

A major mechanism for drug-resistance in cancer is the reversed pH gradient characterizing tumor tissues. An acidic extracellular microenvironment represents a chemical and physical barrier for weakly basic chemotherapeutics, which are easily protonated and once neutralized are unable to cross the plasma membrane of tumor cells [Raghunand N and Gillies RJ, 2001; Tannock IF and Rotin D, 1989; Raghunand N and Gillies RJ, 2000]. Manipulation of tumor pH has long been suggested to increase efficacy of chemotherapeutics. Weakly acid drugs should be able to exploit low pH to better target cancer cells while strategies aiming at increasing tumor pH would result in stabilization and better penetration of weakly basic drugs (e.g. doxorubicin) in cancer cells.

For their known capacity to act as blockers of acid extrusion Dr. Fais’ group reasoned that PPI might shift tumor pH towards more alkaline values, thus creating a microenvironment more suitable to increase the efficacy of chemotherapy. This was first demonstrated on a large panel of drug-resistant human melanoma cell lines. Treatment with physiologically achievable concentrations of Omeprazole induced alkalinization of pH in human melanoma and gastric cancer cells in vitro, rendering these cells sensitive to the cytotoxic activity of several chemotherapeutics [Luciani F et al, 2004].
effect was associated not only with an increased intracellular retention of the drugs but also with inhibition of secretion of acidic vesicles. Indeed, another important mechanism for resistance to cytotoxic agents in cancer is their protonation and neutralization into lysosomes and acidic intracellular organelles, which are then secreted by the cells causing elimination of the drugs. Luciani et al, showed that PPI may cause alkalinization of the lumen of acidic vesicles acting as lysosomotropic agents. In fact, following treatment with PPI human melanoma cells display a less acidic lysosomal compartment which may decrease drugs compartmentalization and elimination through the secretory pathway [Luciani F et al, 2004].

Treatment with PPI causes acidification of pH$_i$ and alkalinization of pH$_e$ in human gastric adenocarcinoma cells (SGC7901) [Chen et al, 2009]. In their report, Chen and colleagues describes that treatment with PPI induces a dose and time-dependent decrease in the expression of V-ATPase in human gastric cancer cells, which the authors consider instrumental for the effects on pH gradients. Interestingly, in vivo administration of Pantoprazole (PPZ) in a model of murine T cell lymphoma was reported to reduce expression of V-ATPase [Vishvakarma NK and Singh SM, 2011]. An interesting observation was the fact that Pantoprazole increased the brain penetration of imatinib in a model of glioblastoma due to its inhibitory effect on breast cancer resistance
protein (BCRP) [Breedveld P et al, 2005], belonging to the family of ATP-Binding Cassette (ABC) transporters. In fact, imatinib was shown to be a BCRP substrate and this may limit penetration and distribution of this drug into the brain since the blood–brain barrier contains several efflux transporters. The authors demonstrated that Pantoprazole inhibits BCRP activity and through such inhibition PPZ contributes to an improved delivery of imatinib to the CNS. These findings suggest that PPI may increase the efficacy of chemotherapy through mechanisms other than a direct pH modulation. Additional evidence for the complex relationship between PPI and ABC-transporters comes from the finding that Omeprazole, Lansoprazole and Pantoprazole act as P-gp substrates and directly inhibit P-gp activity [Pauli-Magnus C et al, 2001]. Since P-gp activity depends on the cell metabolic state [Gatlik-Landwojtowicz E et al, 2004] and may to some extent contribute to extracellular acidification, the relationship between P-gp activity and PPI deserves further investigation in the context of drug-resistance.

PPI are able to induce cell death in human tumor cells. The first evidence for the anticancer activities of PPI was reported using a model of gastric cancer [Yeo M et al, 2004]. The authors elegantly showed that gastric cancer cell lines were able to maintain high viability when cultured at acidic pH conditions while non-cancer gastric cell lines lost viability at pH below 5.9, suggesting that
cancer cells adapt to low pH conditions better and more promptly than normal cells. Interestingly, Pantoprazole induced loss of viability and increased apoptosis in gastric cancer cells only at pH<6.9, suggesting the potential selectivity of PPZ in targeting tumor cells and tumor tissues. Gastric cancer cells showed an increased plasma membrane expression of H+/K+-ATPase with respect to normal cells, suggesting that overexpression of gastric proton pumps and therefore increased proton efflux may sustain the viability of cancer cells in the acidic tumor microenvironment. Administration of PPZ (40 mg/kg) to human gastric cancer xenografts significantly inhibited tumorigenesis and induced apoptosis of tumor cells. The ability of PPI to induce apoptosis in cancer cells has been since then repeatedly reported in a large panel of human tumors, including hematopoietic cancers and solid tumors. Omeprazole at very high concentrations (>400 μM) induced a caspase- and cathepsins-dependent apoptosis in human lymphoblastic T cells (Jurkat) [Scaringi L et al, 2004]. Similarly, De Milito et al, reported that Omeprazole and Esomeprazole induced apoptosis in three human B cell lines [De Milito A et al, 2007]. In the experimental settings, they observed that B cell lines kept in medium without buffering capacity (without sodium bicarbonate and Hepes) spontaneously acidified the culture medium within 24 hours, thus reproducing an acidic extracellular milieu suitable for efficient protonation of the PPI used.
In these conditions, they observed that PPI inhibited proliferation of human B cell lines in a dose-dependent manner. Notably, this effect was much more pronounced when cells were cultured in unbuffered medium rather than in medium buffered at pH 7.4. Analysis of the pro-apoptotic activity showed that PPI induced apoptosis in the B cell lines and also in Jurkat cells. Such effects were detectable with 150 μM PPI but only with PPI 200 μM about 30-50% of the cells displayed apoptotic features. Interestingly, they found that PPI-induced apoptosis was clearly caspase-dependent in Jurkat cells. Conversely, despite the presence of active caspases in PPI-treated cells treatment with pancaspases inhibitors had no effects on PPI-induced cell death, suggesting that caspases are not involved in the cytotoxic effects of PPI on human B cell tumors. Treatment with PPI was associated with early lysosomonal membrane permeabilization, release of cytochrome c from mitochondria and increased accumulation of ROS. Indeed, treatment with the ROS scavenger NAC partially inhibited the pro-apoptotic effects of PPI. In this study they also showed that bone-marrow derived leukemic blasts isolated from patients with ALL were sensitive to the dose-dependent apoptosis-inducing effects of Omeprazole. Conversely, human macrophages cultured in the same conditions were totally resistant to the cytotoxic activity of PPI, suggesting that PPI may selectively target cancer cells. To test the potential antitumor effects of PPI in vivo, they
used SCID mice engrafted with the human B cell line Raji. Omeprazole-treated animals received six doses of Omeprazole (2.5 mg/kg) with 1-day interval starting at the time the tumor mass appeared. In these conditions, Omeprazole induced a 60% reduction in tumor growth, suggesting that PPI may have an antineoplastic activity in vivo. Similar data have recently been reported in a study evaluating the tumor growth retarding effect of Pantoprazole on the murine Dalton's T cell lymphoma (DL) [Vishvakarma NK and Singh SM, 2011].

As mentioned above, autophagy has a complex role in tumor development and progression. Autophagy is a tumor suppressor pathway at the initiation stage of tumors by limiting the production of ROS and limiting DNA damaged [White E and DiPaola RS, 2009; Janku F et al, 2011; Kimmelman AC, 2011]. However, autophagy is a survival pathway for tumor cells to overcome metabolic stress and to resist cell death in response to radiation and chemotherapeutic agents [Mathew R and White E, 2011; Rabinowitz JD and White E, 2010; Song J et al, 2011]. Autophagy plays a major role in metabolic adaptation in Ras-transformed cells [Guo JY et al, 2011; Yang S et al, 2011]. Thus, autophagy has a fundamental and driving role in developing tumors and their malignant progression. Notably, recent studies performed on tissue sections of human
tumors suggest the association of upregulated autophagy with tumor hypoxia and acidity [Giatromanolaky A et al, 2010; Koukourakis M et al, 2010; Sivridis E et al, 2011].

Autophagy can promote cell adaptation and survival, but sometimes it may trigger cell death. Accumulating evidence revealed that some anticancer agents can lead to autophagy or autophagic cell death in different types of cancers (derived from breast, colon, prostate and brain tissues, to name a few). For example tamoxifen, which targets the estrogen receptor, triggers cell death through autophagy in breast cancer cells [Bursch W et al, 1996], whereas the DNA alkylating agent temozolomide (TMZ) induces autophagy, but not apoptotic cell death, in malignant glioma cells [Kanzawa T et al, 2004]. Moreover, various natural compounds are able to promote autophagy in cancer cells. In fact, arsenic trioxide (As$_2$O$_3$), resveratrol [Opipari AW Jr et al, 2004] — a phytoalexin that is present in grape nuts and red wine — , and the soybean B-group triterpenoid saponins, have been reported to induce autophagy in malignant glioma cells [Kanzawa T et al, 2003; Kanzawa T et al, 2005], ovarian cancer cells and colon cancer cells [Ellington AA et al, 2005], respectively. Rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), seems not only to promote autophagy but also to suppress the proliferation of malignant glioma cells [Takeuchi H et al, 2005]. Unfortunately,
the activation of autophagy in cancer cells in response to anticancer
treatments may result in cell death, but it can also function as a protective
mechanism, which allows the recycling of proteins and cellular components
[Ogier-Denis E and Codogno P, 2003; Gozuacik D and Kimchi A, 2004]. In fact,
inhibitors of autophagy can lead either to cell growth or to cell death. It will be
important for clinical oncologists and cancer researchers to determine which
cancer cell types most commonly undergo autophagy in response to therapy,
and whether increased autophagy is a sign of drug responsiveness or
resistance.
Whether cancer cells are ultimately destroyed by autophagy remains a
controversial issue. The presence of autophagic vacuoles in dying cancer cells
after treatment indicates that they undergo an autophagy-related cell death,
but there is not always a cause-effect association between autophagy and cell
death. When anticancer treatments damage organelles in cancer cells,
autophagy might initially be triggered to protect the cells by sequestering and
degrading the damaged organelles. However, once a certain level of
intracellular damage is reached, autophagy might serve to remove the
damaged cells from a tissue by initiating cell death [Cuervo AM, 2004].
Apoptosis is morphologically and biochemically distinct from the cell death
through autophagy [Gozuacik D and Kimchi A, 2004; Bursch W et al, 2000;
Bursck W, 2001]. Although tumor cells have been observed to undergo both apoptosis and autophagic cell death in response to therapy, little is still known about the connection between these two. It seems that crosstalk between the apoptotic and autophagic pathways may exist. In fact, in many cases inhibition of apoptosis causes autophagy, and inhibition of autophagy triggers apoptosis. Moreover, induction of autophagy delayed apoptosis induced by sulindac sulphide (a non-steroidal anti-inflammatory drug) in colon cancer cells by sequestering mitochondrial death-promoting factors such as cytochrome C [Bauvy C et al, 2001], whereas inhibition of autophagy by the H⁺-ATPase inhibitor Bafilomycin A1 increased apoptotic cell death in various cancer cells treated by irradiation or chemotherapy [Kanzawa T et al, 2003; Paglin S et al, 2001; Kanzawa T et al, 2005]. These observations indicate that apoptosis and autophagy could be interconnected. However, electron microscopy analysis showed that a tamoxifen treatment in breast cancer cells led to apoptosis or autophagy in some cells, whereas other cells display signs of both [Bursch W et al, 1996]. The mechanisms of this phenomenon remain to be better elucidated. One possible explanation could be represented by the cell type and the stimulus. For example, the DNA-alkylating agent cisplatin induces apoptosis, but not autophagy, in malignant glioma cells, whereas another DNA-alkylating agent, TMZ, induces autophagy, instead of apoptosis, in the
same tumor cell lines [Kanzawa T et al, 2004]. Also, As$_2$O$_3$ induces apoptosis in leukaemia cells [Boya P et al, 2005], whereas it induces autophagy in malignant glioma cells [Kanzawa T et al, 2003].

Several autophagy inhibitors have been developed that can be used to study the role of autophagy in tumorigenesis and in response to therapy. When the class- III-PI3K inhibitor, 3-MA, was used to inhibit autophagy by blocking pre-autophagosome formation, the death of breast cancer cells treated with tamoxifen was prevented, indicating that the induction of autophagy might be one antitumor mechanism of this drug [Bursch W et al, 1996]. 3-MA can also inhibit γ-irradiation-induced autophagy in breast, prostate and colon cancer [Paglin S et al, 2001], and TMZ-induced autophagy in malignant glioma cells [Kanzawa T et al, 2004]. When autophagy was inhibited by 3-MA, these cancer cells did not undergo apoptosis, whereas treatments with bafilomycin A1, hydroxychloroquine and monensin, which inhibit autophagy [Boya P et al, 2005] by preventing the fusion of autophagosomes and lysosomes [Yamamoto A et al, 1998], led to cell death by apoptosis [Paglin S et al, 2001]. These results indicate that autophagy could be a defense mechanism of cancer cells against radiation-induced apoptosis. A similar effect of bafilomycin A1 was observed in As$_2$O$_3$- [Kanzawa T et al, 2003] and hyperthermia- induced autophagy [Komata T et al, 2004]. So if autophagy protects cells from drug-
induced apoptosis, agents that block autophagy might strengthen the antitumour efficacy of cytotoxic agents.

Cells also undergo apoptosis when they are induced to begin autophagy and this process is then disrupted. For instance, in malignant glioma cells, induction of autophagy by TMZ following by bafilomycin A1 treatment led to cell death by apoptosis, through activation of caspase-3 along with mitochondrial and lysosomal membrane permeabilization [Kanzawa T et al, 2004]. Similar effects were observed under conditions of nutrient depletion. When autophagy was inhibited in HeLa cells by the addition of bafilomycin A1, hydroxychloroquine and monensin, cells underwent apoptosis, as determined by assays for caspase activation and a disrupted mitochondrial transmembrane potential. Lastly, autophagy seems to be a cytoprotective mechanism that enables the cells to survive under conditions of nutrient depletion, because inhibition of autophagy with 3-MA has been reported to sensitize HeLa cells to starvation-induced cell death [Boya P et al, 2005]. These observations indicate that the outcome of autophagy inhibition depends on the autophagy initiator, the type of inhibitors used, and the extent of cellular damage.
3. AIMS of the THESIS

In order to demonstrate that PPI may have an antineoplastic effect, we have used human melanoma cell lines which are known to be resistant to chemotherapy and are able to better tolerate long-term acidic culture condition. The aims of the thesis were:

- to investigate in a pre-clinical setting the activity of Esomeprazole (ESOM), a prototype of PPI, as antineoplastic agent for human melanoma;

- to identify the upstream mechanisms mediating the antineoplastic effect of ESOM in human melanoma cells;

- to evaluate the effects of acidic pH on the autophagic activity in human malignant melanoma cells.
4. MATERIALS AND METHODS

Drugs

Esomeprazole sodium salt (AstraZeneca, Mölndal, Sweden) was resuspended 2 mg/ml in 0.9% NaCl (saline) immediately before use.

Reagents

Bafilomycin A1, hydrogen peroxide, N-acetyl-L-cysteine (NAC), diphenylene-iodonium (DPI), E64d, and 4-Nitrophenyl phosphate disodium salt hexahydrate (for acid phosphatase viability assay) were from Sigma (Milan, Italy).

RPMI 1640, penicillin/streptomycin, phosphate-buffered saline (PBS), Trypsin/EDTA and fetal bovine serum (FBS) were from Lonza (Milan, Italy).

Pepstatin A and Fugene 6 Transfection Reagent were from Roche Applied Science (Milan, Italy). HiPerFect Transfection Reagent was from Qiagen.

ON-TARGETplus SMARTpool for human ATG5 and Beclin 1 was from Dharmaco (Thermo Scientific).

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was from Promega (Milan, Italy).

Annexin-V-FITC and propidium iodide staining kit, Z-VAD-fmk, Z-VDVAD-fmk, Z-DEVD-fmk, Z-IETD-fmk and Z-LEHD-fmk were from Alexis Biochemicals (Florence, Italy).
CaspGLOW-FITC kit was from MBL International (Japan).

Leucine, L-[3, 4, 5 – $^3$H(N)] was from Perkin Elmer.

CA-074 was from Calbiochem (Milan, Italy).

2-$(N$-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), dihydorhorhodamine 123 (DHR123), dihydroethidium (HE) lipofectamine 2000 and BCECF-AM were from Invitrogen - Molecular Probes (Milan, Italy).

Lactate Assay Kit was from BioVision.

The following antibodies were used: LC3 (Novus Biological); p70S6K, phospho-p70S6K, 4EBP1 and phospho-4EBP1 (Cell Signaling Technology); p62, active caspase-3 and PARP 1 (BD Biosciences); Beclin-1 and Atg5 (Santa Cruz Biotechnology, USA); V-ATPase subunits A and a (Molecular Probes); $\alpha$-actin (Cell Signaling Technology); anti-rabbit and anti-mouse-HRP-conjugated antibodies (Biorad Laboratories, Hercules, CA).

The GFP-LC3 plasmid and the mRFP-GFP-LC3 plasmid were a kind gift from Dr. Tamotsu Yoshimori (National Institute of Genetics, Mishima, Japan).

**Cell culture**

Human melanoma cell lines Me30966 and Mel501 were previously established from melanoma lesions of patients (Istituto Nazionale dei Tumori, Milan, Italy), WM743 (a gift from Dr. Meenhard Herlyn, Wistar Institute, PA), A375 and SK-
Mel-28 were a kind gift from Johan Hansson (Karolinska Institute, Sweden). All cell lines were cultured using RPMI 1640 culture media supplemented with 10% FCS and penicillin/streptomycin (100 µg/ml of each). All cell lines were grown in 5% CO₂ at 37°C. Experiments were performed in buffered medium (pH 7.4), unbuffered medium (w/o sodium bicarbonate) or buffered acidic medium as specified through the text.

**Cell viability**

Melanoma cells were plated at 2,000/5,000 cells/well in 96-well plates in buffered, unbuffered or acidic medium (pH 6.0) and 24 hours later ESOM was added. After 24 hours ESOM effect on cell viability was determined in cells cultured in unbuffered medium using MTS assay (Promega, Milan, Italy).

**Cell death determination**

Cells were seeded at 15,000-40,000 cells/well in 6 or 12 well-plates in duplicate wells. The next day ESOM was added at indicated concentrations and 24 hours later cells were collected for analysis. Determination of cell death was performed by Annexin-V-FITC and propidium iodide staining following the manufacturer’s instructions (Alexis Biochemicals, Florence, Italy). Cells showing positivity for Annexin-V binding were defined as apoptotic. Cells were sorted on a Becton Dickinson FACScan machine and analyzed using CellQuest.
software (Becton Dickinson Systems). At least 10,000 cells/sample were acquired.

**Caspases involvement determination**

The involvement of specific caspases in ESOM-mediated cell death was evaluated by using the specific caspase inhibitors Z-VDVAD-fmk (caspase-2), Z-DEVD-fmk (caspase-3), Z-IETD-fmk (caspase-8) and Z-LEHD-fmk (caspase-9) diluted 1:200 (Alexis Biochemicals, Florence, Italy). The pan-caspases inhibitor Z-VAD-fmk was used 10 μM. Cells were pre-treated with all caspases inhibitors for 2 hours, followed by ESOM. Pepstatin A (Roche Molecular Biochemicals, Milan), CA-074 (Calbiochem, Milan) and E64d (Sigma Aldrich) were respectively used 100 μM, 10 μM and 25 μM. Cell death was determined by Annexin-V-FITC/propidium iodide staining (Alexis Biochemicals, Florence, Italy). Cells were sorted on a Becton Dickinson FACScan and analyzed using CellQuestPro software (Becton Dickinson Systems).

**Caspase activity**

Caspases activity was measured using the caspases-specific CaspGLOW-FITC kit (MBL International, Japan). Briefly, 0.1 x 10^6 cells untreated or treated with ESOM were collected and washed once in PBS. The cells were then
resuspended into 100 μl Wash Buffer containing 0.33 μl of the caspase-specific FITC-conjugated peptide and incubated at 37°C for 60 minutes. After one wash in Wash Buffer the cells were analysed by flow cytometry evaluating the percentage of FITC-positive cells.

**Flow cytometry**

Analysis of V-ATPase subunits A and a expression was performed on 0.5 x 10⁶ cells fixed with 2% paraformaldehyde and permeabilised with 0.05% Triton-X. Following incubation with subunit-specific antibodies for 1 hours at 4°C (Molecular Probes) and AlexaFluor-488 secondary anti-mouse antibody for 30 minutes at 4°C, cells were analysed by FACS using CellQuestPro software and V-ATPase expression was quantified by mean fluorescence intensity.

**Intracellular pH determination by flow cytometry**

The effects of PPI on cytosolic pH was evaluated by flow cytometry using the pH-sensitive fluorescent probe BCECF-AM (Molecular Probes) as previously described [De Milito A et al, 2007; Nilsson C et al, 2003]. About 10 x 10⁶ cells were incubated at 37°C for 30 min in 1 ml RPMI 1640 containing 20 μmol/L BCECF-AM. The cells were then washed in HBSS, placed on ice, and analyzed with a FACSCalibur equipped with a 488-nm argon laser collecting the emission
of BCECF-AM in the FL1 and FL2 channels. The relative cytosolic pH of individual cells was displayed in the two-dimensional dot-plot showing their fluorescence intensity at 520 nm (FL1, base) and 640 nm (FL2, acid) as described in details previously [Nilsson C et al, 2003; Marches R et al, 2001]. The BCECF-AM loaded cells were incubated with different potassium phosphate buffers in a range of pH from 5.5 to 7.5 in the presence of nigericin (10 μmol/L) to obtain a calibration curve [Nilsson C et al, 2003].

**Western blot analysis**

Cells were collected and treated with RIPA buffer (150mM NaCl, 50mM Tris-HCl pH 7.4, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitors and protein concentration of cellular extracts was determined by Biorad Protein Assay (Biorad Laboratories, Hercules, CA). SDS-PAGE was performed on pre-casted acrylamide gels (12-15%) using the NuPage system loading 30 μg of proteins per lane. After electrophoresis, proteins were transferred to PVDF membranes and incubated for 1 hour at RT in TBS-Tween 20 with 5% non-fat dry milk to block nonspecific binding sites. Protein loading was assessed by Red Ponceau staining of the membranes. Membranes were then incubated with specific primary antibodies diluted in TBS-Tween overnight at 4°C by mixing, followed by appropriate
horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Antibody staining was visualized by the enhanced chemiluminescence method according to the manufacturer’s protocol (Pierce, Rockford, IL, USA).

**Analysis of oxidative stress**

Detection of reactive oxygen species (ROS) was evaluated on $0.25 \times 10^6$ cells at different time points after ESOM treatment. Hydrogen peroxide (100 $\mu$M) was used as positive control for induction of ROS. HE and DHR-123 were used to detect superoxide radicals and hydrogen peroxide, respectively. After treatment, cells were incubated with 10 $\mu$M HE or DHR-123 in FCS-free RPMI 1640 for 10 min at 37°C and immediately analysed by FACS collecting the fluorescence into FL3 and FL1 channels, respectively.

**Transmission Electron microscopy analysis**

Samples were fixed with 4% paraformaldehyde and 2% glutaraldehyde in phosphate buffer 0.12 M and post-fixed with 1% OsO$_4$ in cacodylate buffer, dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections (60 nm), obtained with ultra microtome Ultracut E Reichert-Jung, were doubly
stained with uranyl acetate and lead citrate and examined by transmission electron microscope CM 10 Philips (FEI, Eindhoven, the Netherlands).

**Laser Scanning Confocal Microscopy (LSCM) Analysis**

Cell culture monolayers were flattened over a glass slide, fixed with 4% paraformaldehyde and treated briefly with 0.1 M Glycine in PBS (pH 7.4) followed by 0.3% Triton X-100 buffer. Cells were later stained with anti-LC3 Ab (1:200) overnight at 4ºC and then with Alexa Fluor 488-conjugated anti-rabbit Ab (1:200) for 1 hour at room temperature. TOTO-3 iodide (642/660) (Invitrogen) was used for nuclei staining. Glass slides were washed and mounted with 95% glycerol in PBS. Confocal microscopy was carried out on a Radiance 2100 laser scanning confocal microscope (Biorad Laboratories) equipped with a Krypton/Argon laser and a red laser diode. In order to reduce bleed-through, confocal images were acquired sequentially. Noise reduction was achieved by “Kalman filtering” during acquisition.

**Evaluation of autophagy**

The peGFP-LC3 plasmid and the mRFP-GFP-LC3 plasmid were used. Twenty-four hours after plating, the cells were transfected with either of the plasmids by using lipofectamine 2000 or Eugene 6. Following 24 hours:
- cells were treated for 8-24 hours with ESOM in unbuffered medium (2)

- the medium was replaced with freshly prepared medium at different pH for 8 hours (3)

Cells were then fixed using 2% paraformaldehyde and autophagy was determined by quantification of the number of cells with LC3-positive organelles, counting at least 100 cells in triplicates per condition. The presence of autophagic vacuoles expressing endogenous LC3 was also assessed.

**RNA interference of autophagic genes**

Small interfering RNAs (siRNA) for Atg5 and Beclin-1 were synthesized by DharmacoRNA Technologies (siGENOME SMART pool). For siRNA transfection, Me30966 and WM793 cells were seeded in unbuffered medium at 30% confluence in 12-wells tissue dishes and transfected with 5 nM of scramble, Atg5 and Beclin-1 targeting siRNAs using HiPerFect Transfection Reagent (Qiagen) following the manufacturer’s protocol. The silencing efficacy of the various siRNAs was assessed by Western Blot analysis of Atg5 and Beclin-1 expression levels using specific polyclonal antibodies. Forty-eight hours after transfection:

- 160 μM ESOM was added
- the medium was replaced with medium at different pH (7.4, 6.5 and 6.2) or with EBSS and 24 hours later, cells were collected and evaluated for cell death by using the Annexin-V-FITC and propidium iodide staining (BD Pharmingen).

**Viability assay**

Growth curves of melanoma cell lines at different pH conditions were performed by using the acid phosphatase viability assay. Briefly, cells were plated in 96-wells plates at 2,000/5,000 cells/well in standard RPMI 1640 medium. Following 24 hours cell culture medium was replaced with media at indicated pH. Starting from time 0 the plates were collected every 24 hours and the acid phosphatase assay was run as previously described by Yang and colleagues [Yang TT et al, 1996].

**Glucose uptake**

Melanoma cells cultured at different pH conditions were incubated with 100 \(\mu\)M 2-NBDG, a fluorescent derivative of 2-deoxy-D-glucose, for 45 minutes. Cells were collected and washed with PBS and then analyzed by flow cytometry to collect green fluorescence.

**Extracellular lactate determination**
The concentration of extracellular lactate was determined by a fluorometric assay using Lactate Assay Kit (BioVision). Melanoma cells were plated in 96 wells plates at 50 % confluency. After 24 hours, cell culture medium was removed and replaced with fresh media at pH 7.4 and 6.5 for 3 hours. 50 µl of “Reaction Mix Preparation” (containing: Lactate assay buffer, Lactate probe and Enzyme mix) was then added to each well and incubated for 30 minutes. For calibration curve, serial dilution of Lactate Standard was used following the manufacturer’s instruction. After incubation time, fluorescence was measured at Ex/Em = 535/590 nm in microplate reader Tecan Infinite M1000 (Tecan, Männedorf, Switzerland). After background subtraction, the fluorescence values of sample were converted to extracellular lactate concentration using a calibration curve.

**Measurements of intracellular pH by fluorescence spectroscopy**

Cytosolic pH was evaluated by fluorescence spectroscopy using the pH-sensitive fluorescent probe BCECF-AM as previously described [Grant RL and Acosta D, 1997]. Briefly, cells were plated in black 96-wells plates (Costar) at 50% confluency. The next day, cells were incubated for 30 minutes with 10 µM BCECF-AM in isotonic saline solution (ISS: 150 mM NaCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 10 mM Hepes, pH 7.4), washed once in ISS and incubated at 37°C for an
additional 20 minutes. Immediately after exposing cells to ISS at different pH, BCECF fluorescence was recorded at 530 nm after excitation at 445 and 495 nm, in a fluorescence microplate reader Tecan Infinite M1000 (Tecan, Männedorf, Switzerland). After background subtraction, the fluorescence ratio of BCECF-AM (495/445) was converted to pH values by using a calibration curve with different potassium phosphate buffers in a range of pH (6.0 to 8.0) in presence of 10 μM nigericin.

**Amino acid uptake**

Cells were incubated in RPMI 1640 buffered medium at different pH (7.4 or 6.5). After two hours, [³H] leucine was added (1 mCi/ml) and incubated for two hours. As positive control, we used C2-ceramide (C2-Cer, 50 μM) which is known to inhibit amino acids uptake [Guenter GG, 2008]. Cells were washed twice with ice-cold RPMI 1640 medium, lysed in RIPA buffer, and tritium levels determined by scintillation counting.

**Statistical analysis**

Differences between groups were analysed by Mann-Whitney test, student T test or by ANOVA as appropriate. Data are expressed as mean ± SD and P values reported are two-sided.
5. RESULTS

5.1 Expression of A and a Subunit of V-ATPase: comparison between primary and metastatic melanoma (1)

Since PPI target subunit A of human V-ATPase [Moriyama Y et al, 1993] and subunit a expression is involved in tumor cell invasion [Hinton A et al, 2009], we evaluated the expression of subunit A (peripheral domain, V1) and subunit a (integral domain, V0) in the melanoma cell lines. Interestingly, the expression of V-ATPase subunit a was significantly higher in metastatic cells (111±13 MFI) as compared to primary cells (72±10 MFI, P < 0.05) (Data not shown).

5.2 Esomeprazole induces cytotoxicity in human melanoma cell lines (1)

Based on these observations, we evaluated the cytotoxic effects of ESOM on melanoma cells. Since PPI are pro-drugs which need acidity to be transformed in the active molecule, we reasoned that tumors should be sufficiently acidic to induce proper PPI activation. Thus, we investigated the antiproliferative effect of PPI in buffered and acidic culture conditions (i.e. unbuffered medium and medium at pH 6.0). ESOM treatment induced a dose-dependent inhibition
of cell proliferation in all culture conditions. Consistent with the increased expression of subunit a, metastatic melanoma showed increased sensitivity to ESOM as compared to cells derived from primary lesions. Analysis of cell viability by MTS assay performed on some cell lines showed that ESOM induced dose-dependent decrease in cell viability (Fig. 1A).

5.3 Esomeprazole induce apoptotic cell death in human melanoma cell lines (1)

In order to analyse the ESOM-induced cytotoxic effect, we cultured melanoma cell lines in unbuffered medium in the presence of 160 μM ESOM for 24 hours. Cell death was determined as the percentage of Annexin-V+ cells, including both PI- and PI+ cells. The analysis showed that ESOM caused cell death in all melanoma cells tested (Figure 1B). We then investigated the role of pH in ESOM-induced cell death by culturing melanoma cells in unbuffered medium and medium at pH 5.0. We observed that ESOM-induced cell death was significantly increased in cells cultured in medium at pH 5.0 (Figure 1C) while no cytotoxic effect was detectable at neutral pH. Moreover, as represented for Me30966 cells (Figure 1D), the increased activity of ESOM in low pH culture condition was dose-dependent.
Figure 1: Esomeprazole induces pH-dependent cell death in human melanoma cells.

A) ESOM induces dose-dependent loss of viability (MTS assay) in melanoma cells cultured in unbuffered medium for 24 hours. B) ESOM (160 μM) induces cell death in human melanoma cells cultured in unbuffered medium for 24 hours. Cell death was defined as percentage of Annexin-V+ cells. C) The cell death induced by ESOM (160 μM) is enhanced by acidic culture medium and undetectable in cells cultured in neutral pH. D) Quantification of cell death in presence of ESOM in different pH culture conditions is representatively shown for Me30966 cells. To note, cells cultured in buffered medium (pH 7.4) are not sensitive to ESOM-induced cytotoxicity.
5.4 **Esom induces acidification of cytosolic pH (1)**

An important mechanism of resistance to cytotoxic stimuli of malignant tumor cells is the ability to maintain an alkaline pH [Hirpara JL et al, 2001; Coss RA et al, 2003]. In fact, we measured the pH in the panel of melanoma cell lines and found that pH was clearly alkaline (7.43 ± 0.16). Thus, we cultured Me30966 cells in unbuffered medium in the absence or presence of ESOM (160 μM) and found that ESOM treatment induces a time-dependent intracellular acidification (Figure 2A), consistent with the inhibitory activity of ESOM on V-ATPase, which results in drastic break of proton extrusion into extracellular environment and H+ accumulation within cells.

5.5 **Esom induces caspase-dependent cell death (1)**

Intracellular acidification has been shown to modulate caspases activation [Matsuyama S et al, 2000; Marches R et al, 2001]. Interestingly, ESOM-induced cytotoxic effect was accompanied by a time-dependent activation of several caspases as shown for Me30966 and Mel501 cells cultured in unbuffered medium (Figure 2B). To analyze the role of caspases in ESOM-induced cell death the pan-caspases inhibitor Z-VAD-fmk was titrated and used 10 μM, a concentration known to specifically inhibit caspases (Figure 2C). We observed
by western blot that pre-incubation with Z-VAD-fmk inhibited the activation of caspase-3 and the caspase-mediated cleavage of PARP-1 in ESOM-treated Me30966 cells (Figure 2D). In line with these observations, pre-incubation with Z-VAD-fmk resulted in abrogation of cell death in Me30966 and Mel501 cells (Figure 2D) and inhibition of active caspase-3. Using caspase-specific inhibitors we could observe that while pan-caspase inhibition completely abrogated cell death, inhibition of caspases 3 and 8 partially reduced such effects (Figure 2E). Altogether, these data indicate that blockade of proton pumps through ESOM-induced caspase-dependent cell death in human melanoma cells. PPI-mediated cytotoxicity was dependent on extracellular acidity.
Figure 2: Esomeprazole induces acidification of cytosolic pH and caspase-dependent cell death in melanoma cells. A) ESOM treatment induces intracellular acidification in melanoma cells (Me30966). B) ESOM treatment of Me30966 cells induces a time-dependent activation of several caspases, including caspase 2, 3, 8 and 9. C) Effects of different concentrations of Z-VAD-fmk and various cathepsins inhibitors on ESOM-induced cell death in Me30966 cells. D) Western blot analysis of active caspase-3 and PARP-1 cleavage following ESOM treatment in Me30966 cells cultured in unbuffered medium in presence or absence of Z-VAD-fmk (10 μM).
Adherent and floating cells were used to make the protein lysates. As positive control, cells were treated for 18 hours with 0.1 μM staurosporine (). **E** ESOM-induced cell death in Me30966 and Mel501 cells is completely abrogated by the pan-caspase inhibitor Z-VAD-fmk and reduced by caspase-3 and -8 inhibitors. * indicates P < 0.05. All experiments were performed culturing cells in unbuffered medium and ESOM was used at 160 μM.

### 5.6 ESOM induces ROS-dependent cell death in human melanoma (2)

As demonstrated above (Fig 1), ESOM cytotoxic effects were detected in unbuffered culture conditions and dramatically enhanced in acidic culture conditions. Here, we evaluated which upstream events triggered ESOM-induced cell death in human melanoma. Treatment with ESOM rapidly induced accumulation of hydrogen peroxide and superoxide radicals in melanoma cells, shown by DHR123 and HE fluorescence, respectively (Figure 3A).

The major sources of intracellular ROS are mitochondria and NADPH oxidase and we analysed their possible involvement in driving ESOM-mediated ROS accumulation. First, we observed that DPI, a specific pharmacological inhibitor of NADPH oxidase, and the common ROS scavenger NAC significantly reduced ROS accumulation in cells treated with ESOM (Figure 3B). Moreover, TMRE staining after ESOM treatment showed depolarization of mitochondrial membrane (Figure 3C) while release of cytochrome c from mitochondria and its accumulation in the cytosolic fraction was observed by Western blot in a
time-dependent manner and within 6 hours after treatment (Figure 3D). These experiments indicated that both mitochondria and NADPH oxidase participate in ROS-induction by ESOM in human melanoma cells.
**Figure 3: Esomeprazole induces ROS and mitochondrial alterations in human melanoma.**

**A)** ESOM (160 mM) induces accumulation of hydrogen peroxide and superoxide radicals, as shown by DHR123 and HE fluorescence, respectively. In the histogram untreated Me30966 cells are indicated by the dotted line and cells treated with ESOM after 1, 2 and 4 hours are indicated with black line, light grey and dark grey histograms, respectively. **B)** ESOM-induced ROS-accumulation is inhibited by both the ROS scavenger NAC and the NADPH oxidase inhibitor DPI in Me30966 cells. **C)** Me30966 cells treated with 160 µM ESOM for 5 hours show mitochondrial membrane depolarization detected by TMRE probe. The black, red and green lines indicate untreated, ESOM-treated and staurosporine-treated cells, respectively. **D)** Me30966 cells treated with 160 µM ESOM for 2, 4 and 6 hours were processed to purify the cytosolic and mitochondrial fractions. Expression of cytochrome c was assessed by western blot and indicates a time-dependent release of the protein from the mitochondria into the cytosol. The Red Ponceau staining indicates equal loading of proteins while purity of the fractions was assessed by proibitin expression.

**5.7 ROS-scavenger N-Acetil-L-cysteine reverts Esom effect**

We evaluated the involvement of ROS in ESOM-induced apoptosis in Me30966 and Mel501 cells. These cells pretreated with 5 mM NAC were completely protected from cell death induced by 100 mM H₂O₂ (data not shown). As shown from morphological analysis of Me30966 cells (Figure 4A), pre-treatment with NAC completely abrogated ESOM-induced cell death in both Mel501 and Me30966 cells (Figure 4B). Since we reported that ESOM-induced apoptosis is caspases-dependent, we observed here that the inhibition of apoptosis in presence of NAC was associated with decreased percentage of cells with active caspase-3 and caspase-8 (Figure 4C).
PPI are known to induce cytosolic acidification in cancer cells [De Milito A et al, 2007] and we found that ESOM-induced acidification of cytosolic pH was significantly prevented by NAC in Me30966 cells (Figure 4 D, P<0.01), suggesting that ROS production is an early event upstream of ESOM effects on cytosolic pH and alteration of intracellular pH gradients.
5.8 ESOM induces the accumulation of autophagic vacuoles in melanoma cells (2)

It is known that ROS are involved in the autophagic response of cancer cells to nutrient starvation or metabolic stress [Scherz-Shouval R et al, 2007]. Moreover, PPI have been reported to cause alkalinization of lysosomal pH and disturbance of acidic vesicles traffic. As proper lysosomal function and ROS are crucial autophagy regulators, we investigated whether ESOM-induced cytotoxicity involved alteration in the autophagic pathway. ESOM-treated cells showed massive vacuolization (Figure 5A upper panel), which has been reported to occur in cells before apoptosis [Gonzales-Polo RA et al, 2005]. Electron microscopy experiments showed that treatment of Me30966 with ESOM leads to an increased number of autophagic vacuoles per cell as compared with untreated cells (Figure 5A, middle and lower panels). To evaluate autophagy, we looked at the cellular distribution of the microtubule-associated protein 1 light chain 3 (LC3), a marker of autophagy. In conditions
of autophagy activation, cytosolic LC3 is conjugated to phosphatidylethanolamine (LC3-II) and localizes to autophagosomes. Therefore, the number of LC3-positive vesicles and the amount of LC3-II are commonly used as a measure of autophagosomes formation [Klionsky DJ et al, 2008]. Following cell transfection with GFP-LC3-expressing plasmid, ESOM strongly enhanced the appearance of punctuate fluorescence in Me30966 (Figure 5B, lower panel) and WM793 cells, indicating redistribution of LC3 to autophagic vacuoles with respect to untreated cells. Quantitative evaluation of cells carrying GFP-LC3-positive dots showed that ESOM treatment significantly increased the percentage of cells carrying autophagosomes markers (Figure 5B, upper panel).

5.9 ESOM induces autophagosomes accumulation and slows down the autophagic flux (2)

Increased autophagosomes markers may be explained by increased autophagosomes formation due to induction of autophagic activity, or accumulation of autophagic organelles due to impaired degradation of autolysosomes content. Autophagic flux may be measured by the levels of LC3-II degraded in autolysosomes in which lysosomal hydrolases are functional.
Thus, the status of autophagic flux in Me30966 cells was analyzed in presence or absence of lysosomal protease inhibitors (E64d/Pepstatin A). We observed that ESOM induced a time-dependent accumulation of LC3-II in Me30966 cells (Figure 5C), which was further enhanced in the presence of E64d/Pepstatin A, supporting the notion that, in cells treated with ESOM, the autophagic flux is not completely blocked. However, the effect of lysosomal inhibitors on LC3-II accumulation in control cells is increased with respect to ESOM-treated cells, also indicating that ESOM reduces the autophagic flux. These findings suggest that ESOM likely causes the accumulation of autophagosomes and slows down the autophagic flux. In line with the accumulation of LC3-II, Me30966 cells treated with ESOM showed lack of p62 degradation as early as 4 hours after treatment (Figure 5D), further indicating that ESOM may slow down the degradation of autophagic cargo. These data were further confirmed by the analysis of cells transfected with an mRFP-GFP-LC3 expressing plasmid. This probe allows distinguishing between autophagosomes (GFP+RFP+ puncta) and autolysosomes (GFP-RFP+ puncta) since the GFP fluorescence is quenched in the acidic lumen of autolysosomes [Klionsky DJ et al, 2008]. In fact, ESOM-treated cells carried an increased number of dots with RFP+GFP+ fluorescence with respect to untreated cells (Figure 5E, 5F) whereas the number of RFP+GFP- dots was not affected,
suggesting that ESOM induces the accumulation of autophagosomes as it slows down the autophagic flux.
Figure 5: Effects of ESOM on autophagic flux.
A) Me30966 cells treated with ESOM (160 μM) for 8 hours are characterised by massive intracellular vacuolization. B) Treatment with ESOM for 8 hours induces accumulation of LC3-positive autophagic vacuoles in Me30966 and WM793 cells. C) ESOM induces accumulation of LC3-II form in autophagy-competent melanoma cells as shown by the further accumulation of LC3-II in cells pretreated with inhibitors of lysosomal proteases. D) ESOM-induced block of the autophagic flux is confirmed by the lack of p62 degradation. E, F) Analysis of mRFP-GFP LC3-plasmid shows the accumulation of autophagosomes in ESOM-treated Me30966 cells.

5.10 Role of ROS on ESOM effect on autophagy (2)

As ESOM-induced cell death was mediated by massive ROS production, we evaluated the effects of ROS inhibition on ESOM-induced alterations in the autophagic flux. The expression and localization of endogenous LC3 in Me30966 cells was studied by Laser Scanning Confocal Microscopy analysis. Basal levels of LC3-positive autophagic vesicles was significantly increased 8 hours after ESOM treatment, with a significant proportion of cells showing a clear punctuate fluorescence (Figure 6A). The presence of lysosomal protease inhibitors did not affect ESOM-induced accumulation of endogenous LC3 dots, confirming that ESOM partially reduces the autophagic flux (Figure 6A). Conversely, pre-treatment with NAC prevented the accumulation LC3-positive dots induced by ESOM (Figure 6A) and inhibited LC3-II (Figure 6B), suggesting that ROS are involved in ESOM-induced accumulation of autophagosomes in melanoma cells.
5.11 ESOM inhibits the mTOR signaling pathway (2)

An important pathway regulating autophagy is the signaling through the mammalian target of rapamycin (mTOR), whose inhibition activates autophagy in cells under nutrient starvation [Meijer AJ and Codogno P, 2009]. Decreased mTOR signaling can be evaluated by the reduced phosphorylation of p70-S6K and 4EBP1 which are two important downstream targets of mTOR. We observed that in Me30966 cells treated with ESOM the phosphorylation of both p70-S6K and 4EBP1 was decreased in a time-dependent manner and with similar time kinetics (Figure 6C). In line with the role of ROS in driving autophagy, we observed that the inhibition of the mTOR activity was prevented by ROS scavenging since the presence of NAC restored the phosphorylation of both 4EBP1 and p70 (data not show). These findings further confirm that ESOM induces the accumulation of autophagosomes and suggests that inhibition of mTOR signaling pathway may drive the autophagic response induced by ESOM in melanoma cells.
Figure 6: Role of ROS and mTOR in ESOM effect on the autophagic flux.

A, B) NAC pre-treatment prevents the accumulation of autophagosomes induced by ESOM (160 mM) in Me30966 cells as shown by confocal fluorescence analysis of endogenous LC3 and western blot analysis of LC3-I and LC3-II. C) ESOM treatment inhibits the mTOR pathway as shown by the time-dependent reduced phosphorylation of 4EBP1 and p70S6K proteins.
5.12 Inhibition of autophagy increases PPI-cytotoxicity in melanoma cells (2)

In order to analyse the role of autophagy in ESOM-induced cell death, we measured cell death in Me30966 and WM793 cells in which autophagy was inhibited by knock-down of Beclin-1 or Atg-5 gene expression or by inhibition of autolysosomes formation by Bafilomycin A1. First, we observed that pre-treatment of melanoma cells Me30966 with Bafilomycin A1 significantly increased the cytotoxicity of ESOM in melanoma cells (Figure 7A). In line with this result, we observed that in ESOM-treated Me30966 cells there is a further accumulation of LC3-II in presence of Bafilomycin A1. However, the effect of Bafilomycin A1 in ESOM-treated cells was reduced when compared to control cells, further confirming that ESOM also slows down the autophagic flux (Figure 7A). Then, we measured ESOM-induced cell death in Me30966 and WM793 cells in which the autophagic genes Atg5 and Beclin-1 were silenced. Interestingly, knock-down of Atg5 (and to a lesser extent Beclin-1) increased ESOM-induced cell death in both cell lines (Figure 7B) and also in Mel501 cells (not shown), strongly indicating that autophagy (and autophagosomes formation) may represent an adaptive survival mechanism used by melanoma cells in response to ESOM insult.
Figure 7: Autophagy is a cytoprotective mechanism in ESOM-treated melanoma cells.

A) Me30966 cells pre-treated with 40 nM Bafilomycin A1 show increased sensitivity to ESOM-induced apoptosis in association with a further increased LC3-II accumulation. B) Knock-down of Atg5 and Beclin-1 increases the cytotoxicity of ESOM in Me30966 cells.

5.13 Human melanoma cells survive and proliferate also at acidic pH (3)

An acidic pH environment is known to delay cell growth and increase spontaneous apoptosis in cell lines derived from colon cancer and malignant
glioma [Reichert M et al, 2002; Williams AC et al, 1999]. However, human melanoma cells are known to survive during acidic stress [De Milito A et al, 2010; Lugini L et al, 2006]. We cultured Me30966, WM793, A375, SK-Mel-28 and Mel501 cells at different pH conditions and evaluated cell proliferation over 72 hours. As indicated in Figure 8, melanoma cells cultured at pH 6.8 showed a reduction of 20-50% in proliferation as compared to cells cultured at pH 7.4, depending on the cell line. Interestingly, all cell lines cultured at pH 6.5 continue to slowly proliferate, at least up to 72 hours after exposure to acidic medium. Among the cell lines analysed, Me30966 showed the highest ability to grow even at pH 6.5 as compared to other cell lines, thus representing an appropriate model to study how melanoma cells adapt to survive and proliferate in very acidic conditions.
Figure 8: Human melanoma cells slowly proliferate under acidic stress.
The human melanoma cell lines WM793, A375, Me30966, Mel501 and SK-Mel-28 were cultured over three days at different pH conditions. Cell viability was measured every day with the acid phosphatase assay.
5.14 Acidic stress upregulates autophagy in human melanoma (3)

Increased autophagy linked to acidity has recently been reported as a marker of poor prognosis in melanoma and colorectal cancer [Giatromanolaki A et al, 2010; Koukourakis AI et al, 2010; Sivridis E et al, 2011]. Moreover, increased autophagy was shown to represent a marker for aggressive and metastatic melanoma [Ma XH et al, 2011]. Since autophagy is activated in cells under metabolic stress, we hypothesised that autophagy might be a protective mechanism for cancer cells acutely exposed to acidic stress. To test this hypothesis, we evaluated the effects of short-term exposure to acidic pH on basal autophagy in human melanoma cells. We used the human melanoma cell line Me30966 transiently transfected with a GFP-LC3 expressing plasmid. 24 hours after transfection the cell culture medium was replaced with medium at different pH (7.4-5.5) and GFP-LC3 fluorescence was monitored 8 hours later. As depicted in Figure 9A, cells cultured at neutral pH showed a mostly cytosolic distribution of GFP-LC3 fluorescence and around 30 LC3-positive dots/cell. With decreasing pH, the number of LC3-positive dots per cells significantly increased 2-fold (Figure 9A, 9B), suggesting that acidic culture conditions increase the number of autophagic vacuoles. The number of LC3+ autophagic vesicles per cell reached a plateau at pH 6.5 (Figure 9B).
To gain insight into the effects of acidic stress on basal autophagy we first cultured Me30966 cells in media at different pH and analysed the conversion of LC3-I into LC3-II after 8 hours. Interestingly, the LC3-II form was significantly increased as the medium pH decreased (Figure 9C). p62 is a cytoplasmic protein which shuttles ubiquitinated proteins to autophagosomes. Expression of p62 increases when autophagy is inhibited, thus the levels of p62 may reflect the autophagic flux. Analysis of p62 in the same samples indicated that p62 expression decreased in a pH-dependent manner after short-term acidic stress (Figure 9C). Quantification of protein expression showed that cell cultured at acidic pH have higher LC3-II levels and lower p62 levels, suggesting a functional autophagy in these conditions (Figure 9D). The accumulation of LC3-II and degradation of p62 observed in cells cultured at acidic pH was time-dependent and started as early as 2 hours after exposure to acidic medium (Figure 9E).
Figure 9: Autophagy response in melanoma cells upon acidic stress.
A, B) Me30966 cells transiently transfected with GFP-LC3 plasmid were exposed for 8 hours to media at different pH. The presence of GFP-LC3+ dots was observed by fluorescence microscopy and quantified. C) Me30966 cells were exposed to differently acidic media for 8 hours and LC3 and p62 expression was evaluated by western blot. D) The expression of LC3 was measured by gel densitometry using Adobe Photoshop Elements 9 and normalized to the pH 7.4 condition. E) Me30966 cells were plated overnight and culture medium was changed to pH 6.5 and 6.2. Cells were collected at time 0 and 2, 4 and 8 hours after medium change. Expression of p62 and LC3 was analysed by western blot.
5.15 Melanoma cells maintain a functional autophagic flux under acidic stress (3)

Since the accumulation of GFP-LC3+ vesicles was already detected at pH 6.5 and did not significantly change in lower pH culture conditions (Figure 9B) we restricted further analysis to a pH range of 7.4-6.5. Moreover, in vivo MRS data indicate that pH 6.5 represents the average tumor pH [Gillies RJ et al, 2002; Hjelmeland AB, 2011; De Milito A et al, 2010]. To analyse the status of the autophagic flux we performed experiments culturing Me30966 cells at pH 7.4 and 6.5 in the presence or absence of the lysosomal inhibitor Bafilomycin A1, using EBSS-treated cells as positive control. Me30966 cells cultured at pH 7.4 or 6.5 were equally sensitive to the effects of Bafilomycin A1 (Figure 10A). In fact, expression of LC3-II increased in the presence of Bafilomycin A1 in both pH conditions. Similarly, accumulation of p62 in the presence of Bafilomycin A1 was also increased in cells under acidic stress (pH 6.5). The same results were observed when different lysosomal inhibitors were used on Me30966 cells (not shown). Analysis of autophagic flux on WM793 (Figure 10B), A375 (Figure 10C) and on SK-Mel-28 cells (not shown) confirmed that under acidic stress conditions melanoma cells maintain a fully functional autophagic flux.

Another way to analyze the autophagic flux is to use the tandem probe RFP-GFP-LC3 (49). This probe differentiates autophagosomes (RFP+GFP+, yellow
puncta) from autolysosomes (RFP+GFP-, red puncta) as the GFP fluorescence is quenched in acidic compartment. The number of RFP+ GFP- puncta was significantly increased in Me30966 cultured in acidic pH, indicating that the autophagic flux is maintained at acidic pH. (Figure 10D)
Figure 10: Autophagic flux in melanoma cells under acidic stress

A

Baf
- + - + - +

LC3-I
- + - + - +

LC3-II
- + - + - +

p62
- + - + - +

β-actin
- + - + - +

p62/actin
1.0 1.3 0.8 1.2 0.8 1.2

pH 7.4  pH 6.5  EBSS

B

WM793
Baf
- + - + - +

LC3-I
- + - + - +

LC3-II
- + - + - +

p62
- + - + - +

β-actin
- + - + - +

p62/actin
1.0 1.3 0.8 1.2 0.8 1.2

pH 7.4  pH 6.5

C

A375
Baf
- - - - - -

LC3-I
- - - - - -

LC3-II
- - - - - -

p62
- - - - - -

β-actin
- - - - - -

p62/actin
1.0 1.3 0.8 1.2 0.8 1.2

pH 7.4  pH 7.1  pH 6.8  pH 6.5

D

pH 7.4

pH 6.5

DpH 7.4

DpH 6.5

RFP+GFP+

RFP+GFP-

Total

P=0.001

P=0.01

P=0.01
5.16 Acidic stress decreases nutrients uptake (3)

An important role of autophagy is to sustain cell survival in conditions of metabolic stress and limited nutrient availability. Therefore, we measured glucose and amino acids uptake in human melanoma cells after exposure to acidic medium. To measure glucose uptake we used 2-NBDG, a fluorescent non-metabolizable glucose analogue. Me30966 and WM793 cells were exposed to medium at pH 7.4 and 6.5 and the uptake of 2-NBDG was measured at different time points. Melanoma cells cultured at acidic pH (pH 6.5) showed a time-dependent reduction in the uptake of glucose compared to cells cultured at neutral pH (Figure 11A). The effects on glucose uptake were also pH-dependent as shown for Me30966 cells (Figure 11B). In line with this observation, the concentration of extracellular lactate was also reduced in cells cultured at acidic pH, likely as a result of decreased glucose consumption and inhibition of glycolysis (Figure 11C).
Autophagy may be triggered by reduced amino acids uptake [Meijer AJ, 2008] and we used $[^3]H$ leucine to investigate the effects of low pH medium on amino acids uptake in Me30966 and WM793 cells. As positive control, we used C2-ceramide (C2-Cer) which is known to inhibit amino acids uptake [Guenter GG et al, 2008]. Indeed, treatment with C2-Cer significantly reduced the uptake of leucine in both cell lines (Figure 11D). Similarly to the results from glucose uptake, short-term exposure to acidic pH induced a reduced uptake of the amino acid leucine in both Me30966 and WM793 cells (Figure 11D). Taken together, these findings show that acidic pHe may affect the capacity of cancer cells to uptake nutrients and suggest that exposure to acidic pH may reduce glucose consumption and amino acids uptake in human melanoma cells.
Figure 11: Effects of acidic stress on nutrients uptake and mTOR signaling.

A) WM793 and Me30966 cells were exposed to pH 7.4 or 6.5 and the uptake of 2-NBDG was analysed over time. The * shows a p<0.05 for differences between the acidic conditions with respect to cells cultured at pH 7.4. B) Flow cytometry analysis of 2-NBDG uptake in Me30966 cells exposed for 4 hours to pH 7.4 (black), pH 6.5 (green) and pH 6.2 (red). C) Me30966 cells were kept at pH 7.4 or 6.5 and lactate was measured in the medium after 3 hours. D) Me30966 and WM793 cells were exposed to medium at different pH (7.4 and 6.5) or to 50 μM C2-Cer for 4 hours and the uptake of [3H] leucine was measured. The bars represent the mean and SD of two experiments run in triplicate, where * shows a p<0.05.
5.17 Acidic pH induces cytosolic acidification and inhibition of mTOR (3)

Exposure to acidic pHe was previously shown to induce acidification of pHi and inhibit glycolysis in human cancer cells [Newell KJ and Tannock IF, 1989; Rotin D et al, 1986]. Therefore, we first sought to investigate the effects of acidic stress on pHi in melanoma cells. The pHi was measured in A375 cells by using a ratiometric fluorescence method after incubation of cells with the pH-sensitive probe BCECF and generation of a standard curve (Figure 12A). While pHi of cells exposed to pH 7.4 did not significantly change during the observation time, the pHi of cells exposed to different acidic media dropped to acidic values within minutes (Figure 12B).

The mammalian target of rapamycin (mTOR) has a crucial role in the regulation of metabolism and cell growth and acts as sensor for the availability of nutrients like amino acids and glucose. Inhibition of mTOR by rapamycin or other signaling pathways is known to stimulate autophagy [Meijer AJ, 2008; Mejer AJ and Codogno P, 2009] and it has been recently reported that mTOR may be regulated by pH [Balgi AD et al, 2011]. To evaluate whether the prompt acidification of pHi observed following acute acidic stress was associated with inhibition of the mTOR signaling, we first analysed the
phosphorylation status of p70-S6K following exposure of human melanoma cells to pH 6.5 and 6.2 culture conditions. As shown in figure 12C, the levels of phospho-p70-S6K decreased significantly during acute exposure to acidic medium. Inhibition of mTOR activity was detected as early as 1 hour after exposure to acidic pH. Long-term exposure of melanoma cells to acidic stress showed that the mTOR inhibition was stable over time and detected at least over 16 hours incubation (Figure 12D), suggesting that inhibition of the mTOR pathway occurs very early and may drive autophagy in melanoma cells cultured at acidic pH.
Figure 12: Effects of acidic stress on intracellular pH and mTOR signalling.

A) Representative standard curve for measuring pHi in A375 cells by a ratiometric fluorescence method using BCECF. B) The effect of medium at different pHs on intracellular pH was evaluated in A375 cells. Representative data of two independent experiments run in triplicate wells are shown. (C, D) The status of mTOR activation was analysed in Me30966 cells exposed to acidic media and at different time points by analysing the phosphorylation of p70 S6K. The expression of total and phosphorylated p70-S6K was evaluated by western blot and measured by densitometry using Adobe Photoshop Elements 9.
5.18 Authophagy rescues cells from acidic pH induced cell death (3)

Functional autophagy represents a survival strategy for cells under metabolic stress [Mathew R and White E, 2011; Rabinowitz JD and White E, 2010; Song J et al, 2011]. In order to determine the role of autophagy in acidic stress-induced cell death in human melanoma we used siRNA to knock-down the expression of Atg5, a crucial protein involved in the build-up of autophagosomes. Interestingly, the expression of Atg5 was increased in Me30966 and WM793 cells after 8 hours exposure to pH 6.5. Me30966 and WM793 cells were transfected with Atg5 siRNA and after 48 hours the medium was replaced with EBSS, pH 7.4, pH 6.5 and pH 6.2 medium, respectively. After exposure for 24 hours to these conditions cell death was evaluated and expressed as the percentage of apoptotic and necrotic cells. Knock-down of Atg5 reduced the expression of Atg5 protein and LC3-II in both cell lines (representatively shown in Figure 13A for WM793 cells), indicating functional inhibition of autophagy. Knock-down of Atg5 did not significantly affect survival of WM793 cells at neutral pH while it significantly increased the sensitivity to cell death induced by acidic medium (p<0.05) (Figure 13B). In fact, at pH 7.4 cell death was 8±1% in control cells while it was 15±5% in Atg5-KD cells. On the other hand, at pH 6.5 and 6.2 cell death was 11±2% and
24±6% in control cells and it increased to 25±5% and 48±3% in Atg5-KD cells, respectively. As expected, expression of Atg5 and induction of autophagy was instrumental for cell survival in conditions of nutrient starvation (Figure 13B). Similar results were observed on Me30966 cells (Figure 13C, D). In these cells, absence of functional autophagy induced up to 80% cell death in cells cultured at low pH (pH 6.2) while it did not significantly affect survival of cells cultured at pH 7.4. Interestingly, the cell death modes induced by acidic stress in these two cell lines seemed to be different: while in WM793 cells necrosis was the main cell death mode following autophagy inhibition, apoptosis was prevalent in Me30966 cells. These findings indicate that functional autophagy serves as a protective mechanism adopted by melanoma cells following acute exposure to acidic stress.
Figure 13: Autophagy protects melanoma cells following acidic stress.

A) Immunoblot analysis of Me30966 and WM793 cells exposed to pH 7.4 or 6.5 culture medium showed that expression of Atg5 is increased under acidic conditions. B, C) WM793 cells and Me30966 cells were transfected with siRNA for human Atg5 gene and cell death was measured 24 hours after exposing the cells to different pH conditions (pH 7.4, pH 6.5 and pH 6.2). Cells exposed to nutrients starvation (EBSS) were used as positive control. Cell death is expressed as the mean percentage of apoptotic (white bars) and necrotic (black bars) cells. D) Representative plots showing the percentages of Annexin-V-FITC and propidium iodide positive cells is shown for Me30966 cells transfected with control (siSCR) or Ag5 (siATG5) siRNA and cultured at pH 7.4 and 6.5 for 24 hours.
6. DISCUSSION

Tumor cell metabolism is being nowadays object of renewed consideration for better understanding of cancer biology and therapeutic strategies [Hsu PP and Sabatini DM, 2008; Kroemer G and Pouyssegur J, 2008]. The adaptive response to metabolic stress occurring in cancer cells includes both the up-regulation of proton extrusion [Hsu PP and Sabatini DM, 2008; Kroemer G and Pouyssegur J, 2008; Provent P et al, 2007], which represents a detoxification mechanism significantly contributing to tumor microenvironment acidification [Hsu PP and Sabatini DM, 2008], and autophagy as the most important mechanism to degrade long-lived protein, lipids and macromolecules to recover nutrient and overcome metabolic stress [Rabinowitz JD and White E, 2010; Levine B and Kroemer G, 2008].

This study was divided into two parts with different but complementary aims. The first aim was to demonstrate the antineoplastic activity of PPI and to study in detail the molecular pathway underlying the cytotoxic effect of PPI. The second aim was to evaluate if the acidity of extracellular milieu can modulate autophagy.

Our recent study demonstrated that inhibition of proton pumps by PPI impairs viability of human B cell tumors and sensitizes drug-resistant melanoma cells to chemotherapeutics [De Milito A et al, 2007; Luciani F et al, 2004]. Since PPI
are pro-drugs acting only at acidic pH, we hypothesized that altering tumor pH regulation through proton pump inhibition would selectively impair tumor growth without affecting metabolism of normal tissues. In fact, low pH culture condition highly potentiated the inhibition of proliferation and the induction of cell death by the PPI prototype ESOM in human melanoma cells. Cell lines derived from metastatic lesions showed increased expression of V-ATPase subunit a as compared to primary cells, together with an increased sensitivity to the antiproliferative effects of PPI at low pH, suggesting that proton pumps activity may represent a key mechanism for homeostatic adaptation of metastatic cells to adverse microenvironment conditions [Martinez-Zaguilan R et al, 1996; Lu X et al, 2005; You H et al, 2009; Forgac M, 2007; Lugini L et al, 2006; Torigoe T et al 2002]. This observation is also in line with recent reports on the crucial role of V-ATPase in invasion and metastasis of cancer cells [Martinez-Zaguilan R et al, 1996; Martinez-Zaguilan R et al, 1993; Lu X et al, 2005; Hinton A et al, 2009].

Reversal of pH gradient across plasma membrane and membranes of intracellular organelles is an important hallmark of malignancy [De Milito A and Fais S, 2005; Fais S et al, 2007; Rofstad EK et al, 2006; Gerweck LE et al, 2006], related to invasion, metastasis [Martinez-Zaguilan R et al, 1996; Rofstad EK et al, 2006], proliferation and resistance to chemotherapy [Hirpara JL et al,
In fact, we found that ESOM induced acidification of the pH thus creating the optimal conditions for caspase activation and killing melanoma cells through a caspase-dependent mechanism [De Milito A et al, 2010]. Studying the molecular and biochemical pathways activated in human melanoma cells treated with ESOM we observe that the first event is the accumulation of ROS. Both mitochondria and NADPH oxidase seemed to be involved in triggering ROS accumulation in ESOM-treated cells [Marino ML et al, 2010]. Besides their function in triggering apoptosis, ROS may act as important signaling molecules in starvation-induced autophagy [Scherz-Shouval R et al, 2007]. Indeed, we found a time-dependent induction of autophagy in ESOM-treated human melanoma cells and inhibition of ROS by a ROS scavenger N-Acetyl-L-cysteine, completely inhibited both apoptosis and the autophagosomes accumulation [Marino ML et al, 2010].

Targeting the autophagy regulation of cancer cells is a therapeutic strategy yet to be properly designed. A tumor suppressive role for autophagy was first suggested by the fact that mice with allelic loss of Beclin-1 are more prone to develop spontaneous cancers [Qu X et al, 2003]. Moreover, many currently used anticancer agents are able to induce the accumulation of autophagosomes in vitro, which likely represents a protective mechanism against the stress induced by cytotoxic agents [Levine B and Kroemer G, 2008;
It has been recently reported that autophagy is activated in tumor cells that survive apoptosis following therapeutic stress [Amaravadi RK et al, 2007]. In this model, chloroquine treatment inhibited autophagy-dependent survival of tumor cells, indicating that autophagy may contribute to the selection of cells surviving chemotherapy. As known, chloroquine is a lysosomotropic agent that raises lysosomal pH and induces accumulation of autophagosomes [Boya P et al, 2005]. Interestingly, several PPI (Omeprazole, Esomeprazole and Pantoprazole) are able to raise lysosomal pH and decrease intracellular pH, a feature associated also with their ability to sensitize cancer cells to chemotherapeutics. We found that in human melanoma cells in which autophagy was downregulated through gene silencing the pro-apoptotic effects of ESOM were significantly enhanced, supporting evidence that an intact autophagic pathway is necessary to overcome ESOM-cytotoxicity and that induction of autophagy in these cells represents an adaptive survival mechanism to ESOM-induced damage [Marino ML et al, 2010]. Recently, the polyphenol kaempferol has been reported to trigger a ROS-dependent and caspase-dependent cell death in HeLa cells and these cells activate autophagy as a survival mechanism to the drug-induced bio-energetics failure [Filomeni G et al, 2010]. In light of the reported ability of PPI to revert chemoresistance of
tumor cells [Luciani F et al 2004], it is conceivable that this effect may be mediated by the inhibition of autophagy.

Functional autophagy also contributes to reduce tissue damage, local inflammation and DNA damage, thus suppressing tumorigenesis and tumor progression [Mathew R et al, 2009; Mathew R et al, 2007] Moreover, constitutive activation of mTOR by oncogenic signaling via PI3K and Akt in cancer cells also supports the tumor suppressive function of autophagy [Cairns RA et al, 2011; Yang Z and Klionsky DJ, 2010]. On the other hand, autophagy sustains tumor growth and progression in aggressive tumors. In fact, cancer cells subjected to metabolic stress and chemotherapy upregulate the autophagic machinery to promote cell survival and/or avoid apoptosis [Rabinowitz JD and White E, 2010; Degenhardt K et al, 2006]. Moreover, high basal autophagy is required for growth of pancreatic cancer and human cancers carrying activated K-Ras [Guo JY et al, 2011; Karantza-Wadsworth V et al, 2007]. Intriguingly, upregulated autophagy linked to hypoxia and acidity has been reported as a marker of poor prognosis in melanoma and colorectal cancer [Koukourakis MI et al, 2010; Sivridis E et al, 2011]. In line with this, autophagy was shown to be mostly localized in hypoxic non vascularised tumor regions [Degenhardt K et al, 2006], suggesting that autophagy may be important to sustain tumor growth and survival in the developing of scarcely
perfused tumors. Elegant experiments performed in melanoma spheroids showed that inhibition of autophagy results in cell death mostly localized in the spheroids core [Ma X et al, 2011], suggesting that increased autophagy may predict invasiveness and drug-resistance in malignant melanoma [Ma X et al, 2011].

A major consequence of the abnormal tumor metabolism is the accumulation of metabolic acids within the tumor microenvironment, characterized by low pH heterogeneous distribution within the tumor mass [Gatenby RA and Gillies RJ, 2004; Gillies RJ et al, 2002]. Exposure of cancer cells to acidic environment plays a crucial function in promoting invasive capacity, metastasis and resistance to apoptotic-inducing agents.

We hypothesized that exposure of cancer cells to acidic environment would stimulate autophagy. We used human melanoma cell lines to investigate how exposure to acidic stress affected autophagy and to study the underlying mechanisms. As shown in our experiments human melanoma cells exhibit a peculiar capacity to easily adapt to low pH culture conditions. Moreover, autophagy was recently suggested as an important therapeutic target for treatment of human melanoma [Sheen JH et al, 2011]. The major finding of our study is that acidic stress stimulates a protective autophagic response in melanoma cells. We observed that melanoma cells upregulated the conversion
of LC3-I into LC3-II upon short-term exposure to acidic medium, with the appearance of numerous LC3-positive vesicles. Analysis of the autophagic flux indicated the presence of functional autophagy in melanoma cells exposed to acidic stress. These data are in apparent contradiction to recently reported observations suggesting that acidic stress may indeed inhibit autophagy in breast cancer cells [Xu T et al, 2011]. To exclude differences in experimental settings we analysed the response of MCF7 cells to acidic conditions and we confirmed that autophagic flux is in fact inhibited in MCF-7 cells under acidic stress (not shown). Interestingly, while all melanoma cell lines tested were able to slowly proliferate at pH 6.5, MCF-7 cells did not show this capacity. MCF-7 cells are known to have low basal autophagic activity likely because of the low expression of Beclin-1 [Liang XH et al, 1999] and this might explain differences in the capacity of maintaining a functional autophagic flux under acidic stress. Moreover, human melanoma cells rely on functional autophagy for survival and invasion [Ma X et al, 2011] and increased autophagy in several human cancers, including melanoma, is associated to metastasis and poor outcome [Lazova R et al, 2012]. In line with the clinical studies so far available, our data support a crucial role of autophagy in survival of melanoma cells under acidic stress.
Balgi and colleagues recently demonstrated that exposure to acidic pH causes a rapid inhibition of mTOR signaling, supporting a crucial role for pH in modulation of mTOR activity [Balgi AD et al, 2011]. These findings indicate that mTOR signaling may be sensitive to cytosolic pH variations. To further validate this hypothesis, we analysed the status of mTOR pathway in response to acute extracellular acidification in human melanoma by investigating the phosphorylation of p70-S6K, a downstream effector of the mTOR signaling pathway. We consistently found that acidic stress induces very early inhibition of mTOR activity which occurred within 1 hour. Acidic pH was reported to inhibit glycolysis via decreased glucose uptake in Ehrlich ascites tumor cells [Kaminskas E, 1978] and our data indicate that melanoma cells exposed to acidic pH show signs of glycolysis inhibition since they reduced glucose uptake and lactate release. Similarly, glycolysis inhibition by 2-deoxy-D-glucose was reported to induce cytoprotective autophagy in endothelial and melanoma cells [Wang Q et al, 2011; Giammarioli AM et al, 2011]. Since it is known that active mTOR stimulates glucose uptake via increased membrane expression of glucose transporters [Edinger AL and Thompson CB, 2002] it is likely that inhibition of mTOR by acidic pH may be the upstream event leading to reduced glycolysis and autophagy induction. In line with this, we reported that cytosolic acidification mediated by proton pump inhibitors and associated with mTOR
inhibition induces a protective autophagy in melanoma cells. Moreover, we also observed that acidic stress inhibited the uptake of the amino acid leucine. Intriguingly, mTOR signaling can regulate nutrient transporters, as shown in the case of leucine transporter [Roos S et al, 2007]. Since leucine is a potent inhibitor of autophagy [Meijer AJ and Codogno P, 2009], it is possible that acidic pH-mediated inhibition of mTOR stimulates autophagy in melanoma cells via decreased leucine uptake. With this regard, combination of leucine deprivation and autophagy inhibition has been recently suggested as anticancer strategy against human melanoma [Sheen JH et al, 2011].

To get insight on the functional role of autophagy under acidic stress, we analysed acidic stress-induced cell death in human melanoma after knock-down of Atg5, a crucial gene for the formation of autophagosomes. We observed that defective autophagy achieved by Atg5 knock-down significantly increased the sensitivity to acidic stress-induced cell death in human melanoma cells. Intriguingly, while in Me30966 cells apoptosis was the main cell death mechanism, WM793 cells died mostly through necrosis. Recently, increased autophagic index has been associated to the malignant behaviour of aggressive melanoma cells [Ma X et al, 2011]. An important observation made by Ma and colleagues was that autophagy inhibition via Atg5 knock-down induced selective killing of metabolically stressed cells localized in the core of
multicellular spheroids. Moreover, the authors suggest a direct link between upregulated autophagy and invasive potential of melanoma cells. Since acidic pH stimulates melanoma cell invasion, our findings support a role for autophagy in melanoma progression.
7. CONCLUSIONS

Human solid tumors display acidosis as a common biophysical feature that may be exploited as therapeutic target and delivery system. We identified in PPI safe and well tolerated drugs that at very high doses have an antineoplastic effect most likely mediated by deregulation of tumor pH homeostasis. Due to their chemical properties, PPI may specifically target the tumor lesions and exert their antitumor activities locally, without significant involvement of other tissues.

PPI may really represent a model of a new anti-cancer drug for at least two important properties: (1) their ability to inhibit a mechanism allowing tumor cells to survive in very hostile microenvironment and (2) their ability to target tumor acidity and only there they will be transformed in the active drug. This in turn, suggests that to inhibit mechanism pivotal for cancer homeostasis, to use pro-drugs and to target cancer acidity may represent three important features of new anti-cancer compounds. The increasing availability of agents able to target and disrupt tumor pH regulation may open novel possibilities for effective and safe therapies when PPI are combined with standard chemotherapy and/or other inhibitors of tumor pH regulation.

Moreover, we showed that melanoma cells under acidic conditions upregulate autophagy as a protective mechanism following treatment with proton pump
inhibitors. Inhibition of autophagy in these cells increased their sensitivity to PPI-induced cell death. Since targeting pH regulation in tumor cells represents a novel and attractive therapeutic strategy [Fais s, 2010; Robey IF et al, 2009; Supuran CT, 2008], the finding that functional autophagy may protect cells exposed to acidic stress, thus contributing to malignant progression, further supports the validity of therapeutic targeting autophagy and pH regulation in cancer.
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