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TUMOR ACIDOSIS IN MALIGNANT PROGRESSION AND THERAPY

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Tumor acidosis in malignant progression and therapy

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To my father

“The most important thing that parents can teach their children is how to get along without them.”

Frank A. Clark

ABSTRACT

Cancer still represents one of the leading causes of death despite the advances achieved during the past years. Improving the outcome of cancer patients requires the identification of more affective therapeutic strategies and a better understanding of the molecular mechanisms involved in the progression of the disease. Recently, the tumor metabolic reprogramming has been included in the hallmarks of cancer and one features associated with tumor metabolism is acidosis, which represents an environmental pressure contributing to the selection of malignant cells. For its contribution to tumor progression and therapy-resistance, the acidic tumor environment is being investigated as a target for cancer therapy.

In this study, we have characterized the catabolic autophagic process as a fundamental survival mechanism acting in cancer cells exposed to acidic conditions in order to adapt to the harsh environment. This finding, coupled to the role of autophagy in drug-resistance, suggests the use of autophagy inhibitors in cancer treatment as a strategy to better target malignant cells localized in a metabolically stressed environment and considered responsible for tumor progression, invasion, chemoresistance and disease relapse. We have observed that the autophagy inhibitor Chloroquine used in clinical studies is not the optimal drug for this purpose since it fails to inhibit the autophagic process and to induce toxicity on cancer cells in acidic conditions. Therefore, we aimed at identifying more effective compounds also active in conditions of acidosis. Salinomycin, also known to specifically kill cancer stem cells showed a preferential cytotoxic activity in cells under acidosis, a phenomenon associated with its ability to inhibit autophagy also at low pH. Finally, we developed a model of drug screening performed on cancer cells chronically adapted to pH 6.8 in order to identify compounds targeting cells in metabolic stress conditions. We identified Verteporfin as a promising new anticancer drug able to target colon carcinoma cells adapted to low pH better than cells in physiological conditions through a mechanism that still need to be further investigated.

We can conclude that a better understanding of the cellular mechanisms involved in the cell adaptation to tumor acidosis is important for the identification of new therapeutic targets and selective anticancer drugs overcoming acidosis-mediated drug-resistance.

LIST OF SCIENTIFIC PAPERS

I. Maria Lucia Marino, **Paola Pellegrini**, Giuseppe Di Lernia, Mojgan Djavaheri-Mergny, Slavica Brnjic, Xiaonan Zhang, Maria Hägg-Olofsson, Stig Linder, Stefano Fais, Patrice Codogno, Angelo De Milito. Autophagy is a protective mechanism for human melanoma cells under acidic stress.
J Biol Chem. 2012 Aug 31;287(36):30664-76.

II. **Paola Pellegrini**, Angela Strambi, Chiara Zipoli, Maria Hägg-Olofsson, Maria Buoncervello, Stig Linder, Angelo De Milito. Acidic extracellular pH neutralizes the autophagy-inhibiting activity of chloroquine: implications for cancer therapies. Autophagy. 2014 Apr;10(4):562-71.

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PAPERS NOT INCLUDED IN THE THESIS

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LIST OF ABBREVIATIONS

3-MA	3-methyladenin
ABC	ATP-binding cassette
AMBRA1	autophagy/beclin-1 regulator 1
AMPK	5' adenosine monophosphate-activated protein kinase
ATG	autophagy-related genes
ATP	adenosine triphosphate
BafA1	bafilomycin A1
BBB	blood-brain barrier
Bcl-2	B cell lymphoma 2
BIF1	Bax-interacting factor 1
BRCA1/2	breast cancer susceptibility genes 1/2
CA	carbonic anhydrases
CAFs	cancer associated fibroblasts
CAIs	carbonic anhydrases inhibitors
CHOP	C/EBP homologous protein
CQ	chloroquine
CMA	chaperone mediated autophagy
CSCs	cancer stem cells
CXCR4	C-X-C chemokine receptor type 4
DUBs	deubiquitinating enzyme
EGFR	epidermal growth factor receptor
EIFs	EMT-inducing factors
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
F2,6BP	fructose-2,6-bisphosphate
FDG	^{18}F-fluorodeoxyglucose
FGF	fibroblast growth factor
FIP200	FAK family kinase-interacting protein of 200KDa

GLUT1	glucose transporter 1
GLUT3	glucose transporter 3
GRP78	glucose-regulated protein 78
HCQ	hydroxychloroquine
HIF1α	hypoxia-inducible factor 1 α
HK-2	hexokinase 2
HOPS	homotypic fusion and protein sorting
Hsc70	heat shock cognate 70
HSP70	heat shock protein 70
IFP	interstitial fluid pressure
LAMP2	lysosome-associated membrane protein
LDH	lactate dehydrogenase
MAP1LC3	microtubule-associated protein 1A/1B-light chain 3
mHtt	mutant Huntingtin
MCS	multicellular spheroids
MDR	multidrug resistance
MLST8	mammalian lethal with SEC13 protein 8
MMP	matrix metalloproteinase
MRP-1	multi drug resistance-associated protein 1
mTORC1	mechanistic target of rapamycin Complex 1
NF-kB	nuclear factor kappa B
PB1	Phox and Bem1
PET	positron emission tomography
P-gp	P-glycoprotein
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PFK-2	phosphofructokinase 2
PI3P	phosphatidylinositol-3-phosphate
PLEKHM1	Pleckstrin homology domain containing protein family
PPI	proton pump inhibitors
pRb	protein retinoblastoma

PtdIns3K	phosphatidylinositol 3-kinase class III
Rab7	Rab GTPases protein
ROS	Reactive oxygen species
SAL	salinomycin
SMA	α-smooth muscle actin
SQSTM1 (p62)	sequestosome 1
TGF- α	transforming growth factor-α
TIGAR	TP53-induced glycolysis and apoptosis regulator
TSC1/2	tuberous sclerosis complex 1/2
Ub	Ubiquitine
ULK1/2	Unc-51 like autophagy activating kinase 1/2
UPR	unfolded protein response
UPS	ubiquitin proteasome system
UVRAG	ultraviolet irradiation resistance associated gene
VEGF	vascular endothelial growth factor
VP	verteporfin
VPS34	vacuolar protein sorting 34

1 INTRODUCTION

1.1 CANCER

Cancer is one of the leading causes of death in the world. According to the most recent World Health Organization report, 8.2 million people die for cancer every year, the 13% among all deaths, and the incidence of new cases is expected to increase by about 70% in the next years. Over the past years, significant progresses have been made in the field of cancer prevention, diagnosis and therapy but there is a major need to improve the outcome of cancer patients. A better understanding of the tumor biology combined with the development of new, specific and effective therapeutic strategies are fundamental to achieve better results.

Cancer is a spread multifactorial disease characterized by high heterogeneity. However, cancer cells share several hallmarks that make them different from normal cells. In 2000, Weinberg and Hanahan first described enabling replicative immortality, evasion of cell death and growth suppression, activating proliferation, invasion and angiogenesis as the six main principles driving the transformation process from a normal to a malignant cell [1]. Cancer cells develop from functional normal tissues and they give origin to malfunctioning and disorganised new tissues that interfere and impair the function of organs in which they arise. Tumors can be classified according to site of origin. The most common type of tumors arise from epithelial cells and they are named carcinoma while sarcomas, blood cells tumors and nervous system tumors develop from mesenchymal, hematopoietic and neuroectodermal tissues, respectively [2]. Tumors are defined as benign when they are non-invasive and localized in a determined area, however they become malignant once they acquire the ability to invade different organs and metastasize [3]. Metastasis is a peculiar characteristic of malignant tumors and it consists in the seeding of tumor colonies in distant sites of the body. The multistep process - local invasion, intravasion, blood circulation, extravasion and colonization - is mediated by metastatic cancer cells acquiring malignant features like adaptation to environmental changes, motility and invasiveness [4,5].

About the 90-95% of tumors are correlated to environmental issues while only the 5-10% are due to hereditary factors [6]. Different genetic mutations are closely linked to specific types of cancer: alterations in breast cancer susceptibility genes 1/2 (BRCA1/2) are associated to breast and ovarian cancer [7], c-Myc modifications cause Burkitt lymphoma [8], mutation in the gene for the retinoblastoma protein (Rb) is responsible for retinoblastoma [9] and p53 mutations cause the Li-Fraumeni syndrome [10]. However, every type of cancer is characterized by many different alterations that are direct consequence of environmental factors. Among these factors, lifestyle, chemical and physical agents, infections and radiations have been the most studied and described [11,12,13]. Diets rich in fats and proteins are correlated to the production of carcinogens but on the other hand a proper intake of fruit and vegetables as well as a correct physical activity seem to have a preventive effect on cancer development [14,15]. In obese people activation of the insulin/IGF-1/Akt pathway leads to inflammatory processes correlated to cancer onset [16]. In 1915, Yamagiwa treated rabbits with coal tar, a known chemical carcinogen which induced skin carcinoma in the animal ears. Since then, many molecules have been classified as mutagenic and/or tumorigenic: biocides and pesticides, dioxine and

oganochlorines, metals and asbestos have been shown to induce cancer through their ability to induce genetic mutations [17]. Infections are considered one of the major causes of cancer. For instance, *Helicobacter pylori* infection can cause stomach cancer, Hepatitis B and C viruses lead eventually to liver cancer, Epstein-Barr virus is associated to Burkitt's lymphoma and human papillomavirus is correlated to cervix uterine cancer [12,18]. The mechanisms involved are still under investigation but chronic inflammation seems to drive the tumorigenic process, after viral infection, through the inflammatory marker nuclear factor kappa B (NF-kB) [19-21]. Finally, a clear correlation between radiation and cancer has been described already long time ago since physicians and health personnel working with X-rays are subjected to higher risk of developing cancers [22,23]. X-ray, gamma rays and UV are carcinogenic and lead to several type of tumors such as leukemia, lymphoma, thyroid cancers, skin cancers, sarcomas, lung and breast carcinomas [6].

1.2 CANCER TREATMENT

The field of cancer treatment has made great progresses during the last decades. In fact, until 70 years ago **surgery** was the only approach used in cancer therapy. Since then, other methodologies have been developed and improved such as radiation therapy, chemotherapy, targeted therapy, hormone therapy and immunotherapy [24]. The surgical removal is still the first line approach that is considered in cancer patients with a solid tumor as long as the localization and the grade are advantageous [25]. However, in most cases surgical intervention is not sufficient due to technical limitations and to the aggressive nature of the tumor. In fact, cells that are not removed keep growing and spreading in surrounding tissues. For this reasons, surgery is often combined with other forms of treatment [26].

Radiation therapy exploits the ability of ionizing radiation to provoke DNA damages that cancer cells are often not able to repair due to alterations in their DNA repair systems [27,28]. Normal cells surrounding the tumor are also affected by radiations but they hold functional repairing system to cope with the damage [29]. Radiotherapy is combined with chemotherapy for a better outcome but different results are obtained depending on different type of tumors [30]. For instance, melanoma cells are really resistant to high doses of radiation while low doses are sufficient to successfully target other types of tumors such as leukemia [31,32].

Chemotherapy is the mainstream therapeutic approach used in cancer patients. In 1940, the first chemotherapeutic drug, a nitrogen mustard was used to treat lymphomas [33]. Since then many other molecules have been developed, characterized, approved and used for treatment of cancer patients. Most of the drugs belong to a specific class depending on molecular structure and mechanism of action: *alkylating agents, antimetabolites, topoisomerase inhibitors, antibiotics, anti-microtubule agents and hormones.* In general, chemotherapeutic agents induce DNA damage and impairment in the cell cycle, which lead to cell death through apoptotic pathway [34,35].

Alkylating agents mainly inhibit DNA replication but also alter RNA transcription and protein synthesis. The mechanism of action is based on their ability to insert alkyl groups (C_nH_{2n+1}) in nucleobases, especially in position N7 of guanine [36]. The consequences are cross-links within the double strand helix followed by inhibition of the DNA replication. Alkylating drugs are not phase-

specific and they affect cancer cell DNA but also normal proliferating cells, like hematopoietic cells, resulting in high toxicity. There are different classes of alkylating agents [37]:

- Nitrogen mustards include mechlorethamine, cyclophosphamide, ifosfamide, busulfan, and melphalan.
- Nitrosoureas include carmustine, lomustine, fotemustine, semustina, and N-nitroso-N-methylurea.
- Tetrazines include temozolomide, mitozolomide and decarbazine.
- Aziridines include mytomycin and thiotepa.
- Platinum drugs include cisplatin, oxaliplatin and carboplatin.
- Non-classical alkylating agents include procarbazine.

Antimetabolites are chemical analogues of nucleotides constituents that are necessary for the synthesis of DNA and RNA. They can either interfere with the formation of the pyrimidine and purines rings or directly substitute the biological molecules. They differ from alkylating agents for their specific inhibitory activity of the S-phase of the cell cycle: they block DNA synthesis and so mitosis, leading the cell to apoptosis [38]. There are different classes of antimetabolites:

- Purines analogues are thioguanine and mercaptopurine.
- Pyrimidine analogues are 5-fluoroacil, capecitabine and 5-bromouridin.
- Anti-folates are methotrexate and pemetrexed.

Topoisomerase inhibitors interfere with the function of important enzymes involved in many processes of DNA transcription, replication and recombination. In fact, these enzymes are involved in the unwinding of the double strand DNA that as single strand can enter mitosis and continue the cell cycle. The inhibition of these enzymes leads to apoptosis [39]. There are two classes of topoisomerase inhibitors:

- Topoisomerase I inhibitors include camptothecin, topotecan and irinotecan.
- Topoisomerase II inhibitors include, mitoxantrone, novobicin and etoposide.

Antibiotics are used in cancer therapy as cytotoxic drug due to their ability to intercalate the DNA. They form covalent bonds with nucleic acids interfering with DNA replication and synthesis and inhibiting progression of the cell cycle. Moreover, some antibiotics lead to the production of reactive species that induce DNA damage [40].

- Antibiotics used are puromycin anthracyclines, doxorubicin, daunorubicin, actinomycin, epirubicin and plicamycin.

Anti-microtubule agents often derive from natural products and they interfere with the microtubule function resulting in blocking of cell division. In fact, microtubules are fundamental for mitotic process and the inhibition of their assembly or disassembly cause the block of the cell cycle and apoptosis [41,42]. Two main groups of anti-microtubule agents can be distinguish:

- Vinca alkaloids include vinblastine and vincristine.
- Taxanes include paclitaxel and docetaxel.

Targeted therapy affects cancer cell growth by targeting specific molecules that are overexpressed and/or hyperactive in tumor cells. Most of the conventional chemotherapeutic drugs have low specificity, resulting in high toxicity and reduced efficacy but new biomedical technologies have led to the development of more specific strategies [43-45]. For instance, cancer cells synthetize and expose on the plasma membrane specific antigens that are normally recognized by the immune system and specific monoclonal antibodies for particular antigens are used in order to activate immune cells against the tumor [32]. Moreover, small molecules have been identified as specific and effective inhibitors of certain proteins overexpressed in tumor cells, such as growth factor receptors or protein kinases [46,47].

- Some examples of targeted cancer therapy agents are erlotinib and imatinib targeting tyrosine kinases, bortezomib targeting the proteasome and cetuximab, targeting the epidermal growth factor receptor (EGFR) [48-51].

1.3 CANCER RESISTANCE

The major limitation in cancer therapy is chemoresistence. Chemotherapy represents the most common treatment for cancer patients but in many cases malignant cells do no respond to drugs and/or develop resistance [52]. In particular, slow proliferating and quiescent cancer cells are not ideal targets of chemotherapeutics since these drugs usually target proliferating cells by affecting DNA replication [53]. Moreover, metastatic cells are genetically instable and heterogeneous due to multiple mutations acquired over time resulting in survival of chemoresistant subpopulations. Two types of drug resistance can be distinguished, a primary (or intrinsic) and an acquired drug-resistance which can be correlated to treatment failure and disease relapses, respectively [54]. Primary resistance is correlated to intrinsic features that make cancer cells insensitive to the treatment [53]. Several mechanisms have been studied and considered responsible such as mutations or expression of proteins that prevent drug activity. The resistance that arises after one or more complete cycles of chemotherapy is defined as

acquired and the mechanisms involved are a consequence of an adaptive response to drugs; for instance, mutations affecting the apoptotic pathway are considered responsible for acquired resistance as well as altered expression of ATP-binding cassette (ABC) transporters [55,56]. Drug-resistance is mediated by both cellular events and the complex tumor physiology [57]. At cellular level the following mechanisms have been described: reduction of drug uptake and increase of drug efflux, alteration of apoptotic pathways, mutations/alterations of the drug targets, enhanced DNA repair and upregulation of autophagy. However, hypoxia, altered perfusion, reduced drug diffusion as well as the high interstitial fluid pressure (IFP) are more correlated to the most general anatomy and physiology of the tumor [57].

Ion trapping

The ion trapping is a mechanism through which anions accumulate in a particular cell compartment depending on their charge and the pH gradient across the compartment membrane. Normally, weak acids and weak bases ionize differently, depending on the pH of the solution, in the charged species that exist in equilibrium with the uncharged form. For instance, basic drugs that enter cells and cross the lysosomal membrane become protonated due to the acidic lysosomal pH. The positive charged molecules are not able to cross back the membrane and they are trapped in the organelles preventing them to reach their molecular target [58].

Drug transport

Hydrophobic and small molecules can easily cross the plasma membrane by diffusion but charged and big compounds need transporters to enter the cells. Mutations of these transporters lead to reduction of the drug uptake, as in the case of methotrexate resistance [59,60]. Moreover, cancer cells overexpress membrane transporters whose biological role is to extrude toxins and xenobiotics outside cells. Among these, P-glycoprotein (P-gp) and multi drug resistance-associated protein 1 (MRP-1), belonging to ABC transporters [61,62], control the transport of many chemotherapeutic agents [52,63] and their overexpression is responsible for multidrug resistance (MDR), a phenomenon consisting in the cross-resistance to several drugs with different properties and mechanisms of action [64].

Mutations

Cancer cells undergo many mutations which may confer the resistant phenotype. Mutations in apoptotic pathway can prevent cancer cell death induced by a proapoptotic stimulus. In particular, reduced expression of proapoptotic proteins such as Bax and p53 and increased activity of antiapoptotic proteins like B cell lymphoma 2 (Bcl-2) impair the ability of the cells to undergo programmed cell death [65]. Malignant cells exposed to targeted therapy can also mutate the specific drug targets, thus evading the cytotoxic effects [66,67]. Moreover, mutations responsible for overexpression of genes involved in DNA repair make cancer cells able to overcome chemotherapy-mediated mutations, thus enabling cancer cells to escape from apoptosis [68].

Tumor structure and physiology

Outgrowth of high proliferating tumor cells lead to the development of an abnormal or absent vascularization within the tumor mass. A direct consequence is a defective perfusion resulting in a lower delivery of nutrients and oxygen but also of drugs. Therefore, therapeutic compounds are not able to reach at the tumor site the cytotoxic doses necessary to kill cells [69]. This is also correlated to the localization of some tumors in areas which are difficult to reach, such as the central nervous system, due to the high selective permeability of the blood-brain barrier (BBB) [70,71]. Moreover, some of the chemotherapic agents induce cytotoxicity through reactions that produce reactive oxygen species and cancer cells located in area with oxygen shortage are resistant to such compounds [33 72].

Chemoresistant cancer cells are considered a major determinant of tumor relapses that in most cases will lead to death of the patient. Due to the onset of mutations, cancer cells easily develop resistance mechanisms even against the most promising targeted therapies [69]. For these reasons, therapeutic strategies need to be developed and/or improved in order to overcome limits of conventional therapies. Cancer is considered a multifactorial disease after all and more than one type of treatment is necessary to deal with it. During the last decades, the scientific community has accepted the idea of the tumor as a complex system characterized not only by cancer cells but also by the surrounding and supporting milieu. Therefore, new studies are aimed at developing drugs able to target the *tumor microenvironment* in order to more efficiently kill cancer cells and possibly prevent and/or overcome chemoresistance.

1.4 TUMOR MICROENVIRONMENT

The tumor microenvironment of solid tumors is a complex and heterogeneous system characterized by different type of cells, **cells of the tumor microenvironment**, such as cancer cells, cancer stem cells, endothelial cells, fibroblasts and immune cells [73,74]. Moreover, several physical and biochemical factors such as metabolites, protons, oxygen and nutrients contribute to characterize the tumor microenvironment [75]. In particular, the high proliferative tumor mass growing far from blood vessels is characterized by low perfusion and poor vascularization, correlated to a decrease in nutrients and O_2 delivery and accumulation of metabolic acids leading to **tumor hypoxia, tumor acidosis** and **nutrient deprivation**, important features that affect **tumor metabolism** [76-78].

1.4.1 CELLS OF THE TUMOR MICROENVIRONMENT

Cancer cells and cancer stem cells (CSCs)

Cancer cells represent the main cellular population of the tumor mass. Different and heterogeneous populations of malignant cells are distinguished and localized in different area of the tumor. They mainly differ for the differentiation status, the genetic profile and for the metabolic profile (discussed in the next paragraphs), preferring a more glycolytic or oxidative metabolism. The multiregional genetic analysis of samples derived from tumor biopsies has revealed a large intratumor heterogeneity characterized by a regional distribution of mutations. In particular, about 70% of all somatic mutations

observed in the sequencing analysis were heterogeneous and not detected in every tumor region analyzed [79].

More recently, CSCs have been described as a new, although small, sub-population of cancer cells. They were isolated from different types of hematopoietic tumors first [80,81] and later also in solid cancers, like breast, prostate, colon, brain, and pancreatic cancers [82-86]. CSCs differ from other cancer cells due to the expression of stemness markers [83]. They are functionally defined by their strong ability to promote tumor formation in immunocompromised mice and they are characterized by self-renewal ability and capacity to differentiate into non-stem cancer cells forming the tumor. The mechanisms involved in CSCs origin are still unclear and they might depend on the type of tumor. However, the gaining of CSCs features might be the consequence of epithelial to mesenchymal transition (EMT) or mutations of oncogenes and/or tumor suppressor genes in normal adult stem cell. In particular, EMT-inducing factors (EIFs) could induce EMT transforming epithelial cells in to mesenchymal, fibroblast-like cells that in turn become CSC as a result of mutations [87,88]. The possible connection with the EMT, which is a reversible program [89], may indicate that the switch between CSCs and non-CSCs could be also reversible and dynamic [90]. Due to their strong ability to differentiate and their low proliferating rate, chemotherapy often fails to target CSCs which are considered responsible for metastasis, chemoresistence, tumor relapse and poor prognosis [91]. For instance overexpression of CSCs markers such as CD133, CD24, CD34 and CD44 have been correlated to resistance to conventional drugs and poor prognosis in some types of tumors [92,93].

Stromal cells – Pericytes, Cancer-Associated Fibroblasts (CAFs), Endothelial cells and immune cells

Pericytes are mesenchymal cells which support the endothelium of blood vessels. They have a paracrine function since they regulate homeostasis of endothelial cells by secreting molecules such as Ang-1 and vascular endothelial growth factor (VEGF) [94,95]. It has been shown that pericytes have important role in supporting the tumor endothelium during the formation of new blood vessels through the platelet-derived growth factor (PDGF) signaling. In fact, the specific inhibition of the PDGF receptor alters and disrupts blood vessels responsible for cancer cells growth, proliferation and dissemination [74,95].

Fibroblasts belong to the connective tissue and structurally support epithelial cells. In case of chronic inflammation, such as in cancer, myofibroblasts predominate on normal tissue-derived fibroblasts causing pathological fibrosis. Myofibroblasts produce and release the α-smooth muscle actin (SMA), and promote cancer cell proliferation, angiogenesis, invasion and metastasis [74,96]. Tumor stromal cells differentiate from progenitor cells existing in the normal tissue surrounding the tumor or from stem cells deriving from the bone marrow [97].

Endothelial cells are fundamental constituent of blood and lymphatic vessels. The formation of the new tumor vasculature is associated to the development and differentiation of new endothelial cells. Several pathways are involved in tumor-associated angiogenesis such as VEGF and fibroblast growth factor (FGF) signals [98]. It has been shown that endothelial cell associated to tumor express different markers as compared to normal endothelia [99].

The immune system plays an important role in tumor development and tumor progression. In particular, it displays a double activity in cancer due to the fact that it is involved in processes which

can inhibit but also promote tumor growth and progression. Several different types of infiltrating immune cells have been observed in tumors: macrophages, T and B-lymphocytes, neutrophils, mast cells and NK cells [100]. During transformation from a normal cell toward a malignant phenotype, immune cells like NK cells and cytotoxic T lymphocytes intervene in order to eliminate transformed cells. However, in many cases the chronic inflammatory status characterizing the tumor tissue eventually triggers biological processes promoting tumor progression, like angiogenesis and fibrosis [101,102].

The interaction between cancer and stromal cells is complex and dynamic, they communicate continuously through signaling molecules that are released from both type of cells [74]. It has been suggested that cancer cells actively recruit the stromal cells that are fundamental to support tumor growth and progression. Stromal cells, in turn are able to provide signals back to cancer cells which enhance the malignant phenotype until they can invade different tissue and metastasize. Therefore, metastasizing cells, arrived at the new site, start again the cell interactions with the local stroma in order to settle in the new organ [103].

1.4.2 TUMOR METABOLISM

In 2011, Weinberg and Hanahan revised their theories on hallmarks of cancer (previously described) and added four more features characterizing cancer cells. Among these, the cancer cell capability to regulate the energy metabolism is nowadays raising a lot of interest, especially about the possibility of developing new therapeutic targets [104].

Adenosine triphosphate (ATP) is the energy source for cells and it is essential for the functioning of all cellular processes. In normal conditions, at physiological levels of oxygen, cells rely on oxidative phosphorylation as main source of ATP, with a yield of 38 molecules of ATP per molecule of glucose oxidized. First, glucose is oxidized into pyruvate that translocate into the mitochondria and it is transformed in acetyl-CoA. Acetyl-CoA enters the Krebs cycle and its oxidation leads to reduction of the redox cofactors NAD+ and FADH into NADH and FADH2, respectively. These two reduced cofactors are further oxidised in mitochondria in the electron transfer respiratory chain leading to the production of ATP through the activity of the ATP synthase. In conditions of low oxygen levels, oxidative phosphorylation cannot be accomplished and cells produce ATP only through the glycolytic pathway, defined as anaerobic glycolysis. Hypoxia triggers the stabilization and upregulation of the hypoxia-inducible factor 1 α (HIF1α), a transcription factor which upregulates the transcription of more than 60 genes regulating angiogenesis, apoptosis and metabolism [105,106]. In fact, HIF1 α is involved in the transcriptional activation of proteins involved in biological processes aiming at increasing oxygen supply to tissues, such as the VEGF, a cytokine promoting angiogenesis [107], the insulin-like growth factor-2 (IGF2) and transforming growth factor-α (TGF-α), correlated to cell proliferation and survival [108,109]. Moreover, HIF1 α regulates genes involved in glycolysis which is the pathway on which cells rely on in case on lack of oxygen: glucose transporters like GLUT1 and GLUT3 are upregulated as well as several enzymes involved in the glycolysis, such as hexokinase (HK-II) and 6-phosphofructo-2-kinase (PFK-2) [110-114].

Otto Warburg was the first one describing a deregulated cancer energy metabolism in 1924 [115]. Malignant cells as well as highly proliferative cells switch their metabolism from the more efficient (in term of ATP production) oxidative phosphorylation to glycolysis, even in presence of a physiological oxygen pressure (*Warburg effect*), resulting in *aerobic glycolysis.* Cancer cells counterbalance the lower yield of ATP by upregulation of glucose uptake and by increasing the glycolytic rate. In fact, overexpression of glucose transporters and glycolytic enzymes has been correlated to malignant cells [116]. This feature of cancer cells has been successfully used for diagnostic purposes since radiolabeled glucose analogue ^{18}F-fluorodeoxyglucose (FDG) has been exploited in positron emission tomography (PET) to detect tumor lesions characterized by an increased avidity for glucose [117].

Mechanisms involved in the metabolic switch have been investigated and mutations in oncogenes and tumor suppressor gens are correlated to reprogramming of cell metabolism (Fig. 1) [110,118,119]. For instance, the tumor suppressor transcription factor p-53 has been found mutated in more than 50% of cancers and its alteration is correlated to upregulation of glucose transporters and down regulation of TP53-induced glycolysis and apoptosis regulator (TIGAR), which lead to an increased glucose uptake and increased glycolysis through regulation of fructose-2,6-bisphosphate (F2,6BP) levels, respectively [119-121]. C-Myc is an oncogene which is correlated to increased glucose uptake and glycolysis, glutamine uptake and metabolism as well as biogenesis of mitochondria [119,122-124].

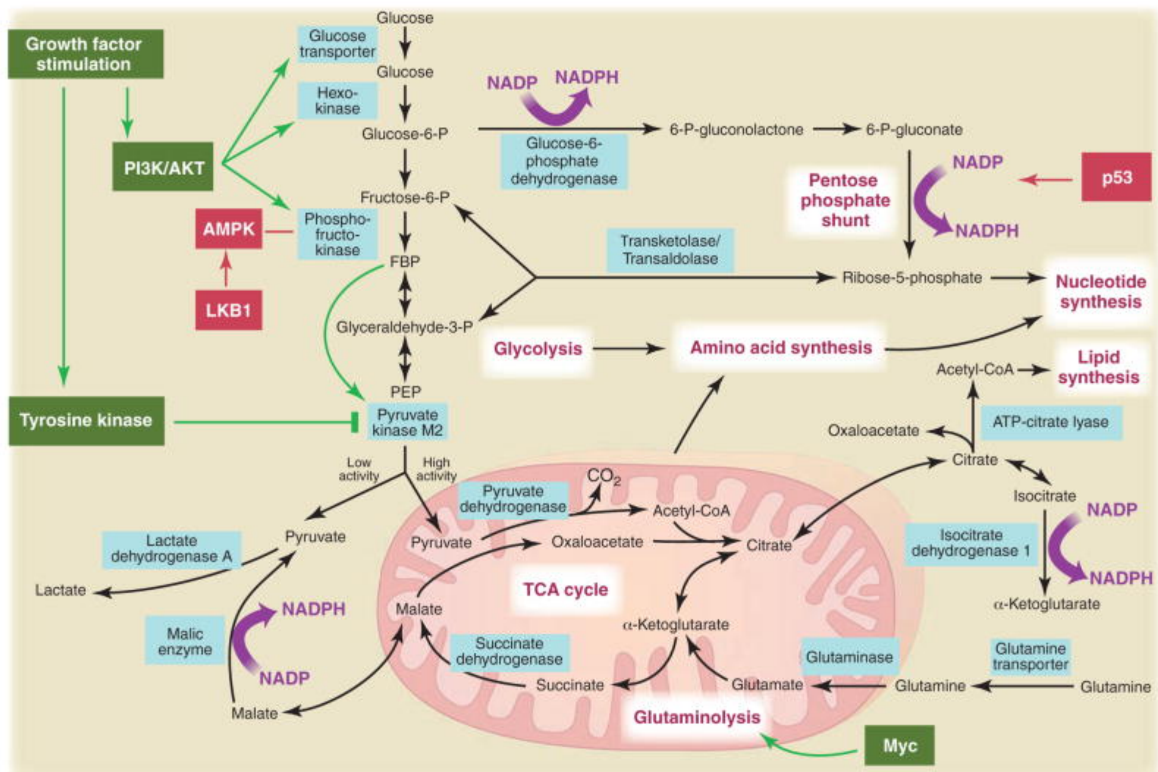

Figure 1. Oncogenes and tumor suppressors directly control cell metabolism. Various signalling pathways and genes regulate different metabolic pathways. Glycolysis, oxidative phosphorylation, pentose phosphate pathway and glutamine metabolism are interconnected and controlled by signalling pathways that are commonly altered in cancer (Figure by Vander Heiden et al. 2009) [118].

Molecular mechanisms involved in the metabolic reprogramming have been well described and potential explanations of the phenomena have been provided. For several years it has been unclear why cancer cells that have a great need of ATP would switch from a high productive energetic pathway, the oxidative phosphorylation, to glycolysis, faster but less efficient. In 2009, Vander Heiden provided a reasonable explanation claiming the metabolic switch as an advantage for cancer cells to quickly synthetize new cellular components. Highly proliferative cells, like cancer cells, constantly need to generate new nucleic acids, proteins and membranes for the new forming cells. Oxidative phosphorylation produces more ATP molecules that are eventually employed in to biosynthetic anabolic pathways but the whole process takes long time and it is not compatible with the needs of high proliferative cells. Conversely, increased glycolysis produces low amount of ATP but glycolytic intermediates and reducing agents (through the oxidative pentose phosphate pathway) can be fast and directly recycled for macromolecules biosynthesis [117,118,125]. The scientific community has agreed on considering the metabolic reprogramming as a adaptive mechanism to the dynamic stressful tumor environment[126].

Finally, tumors are really heterogeneous systems characterized by cells with different phenotype that coexist and cooperate for a better chance to survive. Different models of cooperating cells have been described. Sonveaux and colleagues described a cooperative model between oxidative and glycolytic tumor cells. According to this model glycolytic tumor cells metabolize glucose producing and releasing lactate, which is taken up by oxidative tumor cells and enters the TCA cycle after conversion to pyruvate [127,128]. Lisanti and colleagues suggested a reverse Warburg effect in which lactate acts as a mediator. In fact, they showed that high glycolytic cancer CAFs of the tumor stroma produce lactate that in turn is used by oxidative tumor cells. In both cases, the glycolytic-dependent cells and lactate-dependent cells work symbiotically achieving a more malignant phenotype. In fact, lactate production is not anymore considered as a collateral and toxic by-product of an augmented glycolysis but rather as a metabolic fuel and signaling molecule fundamental for tumor growth and progression. High levels of lactate have been associated to metastasis, chemoresistance, recurrence and poor clinical outcome in different human cancers [129,130].

1.4.3 TUMOR ACIDOSIS

The reprogramming of tumor metabolism has been associated with tumor acidosis and the upregulated glycolysis is considered to contribute to acidification of the extracellular tumor microenvironment along with other correlated molecular mechanisms [131]. During glycolysis one molecule of glucose is oxidized to two molecules of pyruvate and two protons (H^+). In normal cells pyruvate enters in the mitochondria and undergoes the TCA cycle while in cancer and high proliferative cells pyruvate is reduced to lactate by lactate dehydrogenase (LDH). Moreover, most human metabolic pathways end with production of CO_2 that is hydrated with water by carbonic anidrases (CA) and transformed in carbonic acid, one molecule of HCO_3- and one H^+, thereby contributing to protons production. In normal cells, intracellular pH is equal to 7.2, slightly more acidic than the physiological extracellular pH 7.4. However, in cancer cells, high metabolic rates and protons accumulation may lead to a further acidification which negatively affects enzymatic activities and protein structures. In order to cope with the toxic cytosolic acidification and maintain the proper intracellular pH, tumor cells activate transport systems that lead to alkalinisation of cytosolic pH and to acidification of the extracellular pH. There

are several proteins contributing to pH buffering (Fig. 2). The monocarboxylate transporters (MCT) intervene in the co-transport of one H^+ and one monocarboxylate such as lactate across the plasma membrane [132]; the Na^+/H^+ exchangers is a transmembrane sodium-hydrogen antiporter that becomes active during cytosolic acidification in order to extrude one proton for each sodium that is taken up, contributing to extracellular acidification [133]; the vacuolar-ATPase is a transmembrane proton ATPase localized on endo-lysosomal and plasma membranes involved the intraluminal and extracellular acidification through protons translocation [134]; CA are metalloenzymes able to reversibly catalyze the hydration of carbon dioxide into bicarbonate and protons, thereby regulating both intracellular and extracellular pH [135]. The upregulation of these proteins by cancer cells along with a disorganized vasculature and inefficient perfusion and clearance is responsible for acidification of extracellular pH [136]. Indeed, cancer cells show a reversed plasma membrane pH gradient since the intracellular pH is slightly more alkaline (from 7.2 up to 7.7) while the extracellular pH is acidic (as low as 6.0) [131].

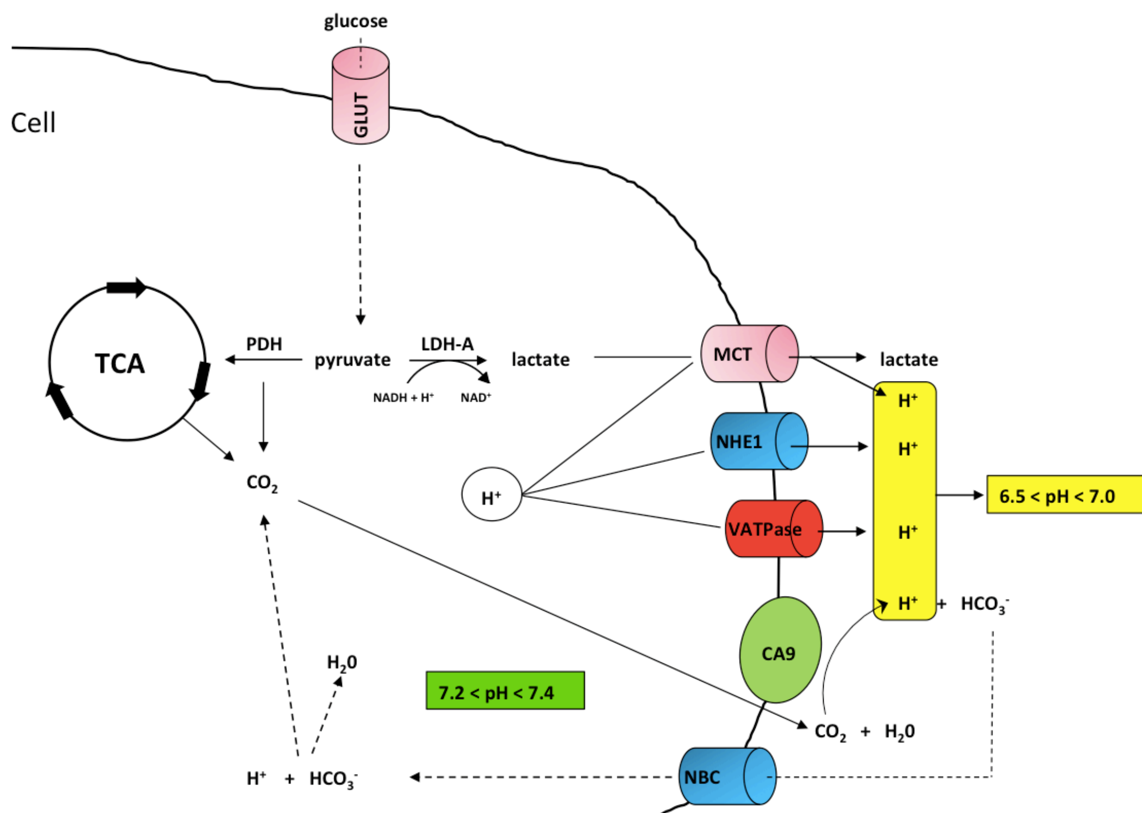

Figure 2. Regulation of pH homeostasis in tumor cells. The main players involved in pH regulation in tumor are: monocarboxylate transporters (MCTs), Na^+/H^+ exchangers (NHEs), the plasma membrane proton pump vacuolar ATPase (V-ATPase), Na^+/HCO_3^- co-transporters (NBCs) and carbonic anhydrase 9 (CA9). Tumor cells present a reversed pH gradient, with a pH_i slightly alkaline and a pH_e slightly acid (Figure by Angelo De Milito).

Tumor acidosis is not just the final result of tumor metabolism reprogramming but it is important for cancer cells to acquire selective advantages necessary for the achievement of a more aggressive phenotype [137,138]. In fact, the toxic environment can enhance invasiveness, chemoresistence, mutagenesis and neo-angiogenesis in those malignant cells located in hypoxic and acidic areas of the

tumor mass, leading to a selective advantage facilitating escape from apoptosis, unlike normal cells (Fig. 3) [128]. In fact, it has been showed that acidic extracellular pH induces the secretion of proteases, such as cathepsins and matrix metalloproteinases (MMP) which drive the degradation of the ECM. The disruption and remodeling of the matrix facilitates the migratory and invasive behavior of cancer cells toward a metastatic and more aggressive phenotype [139].

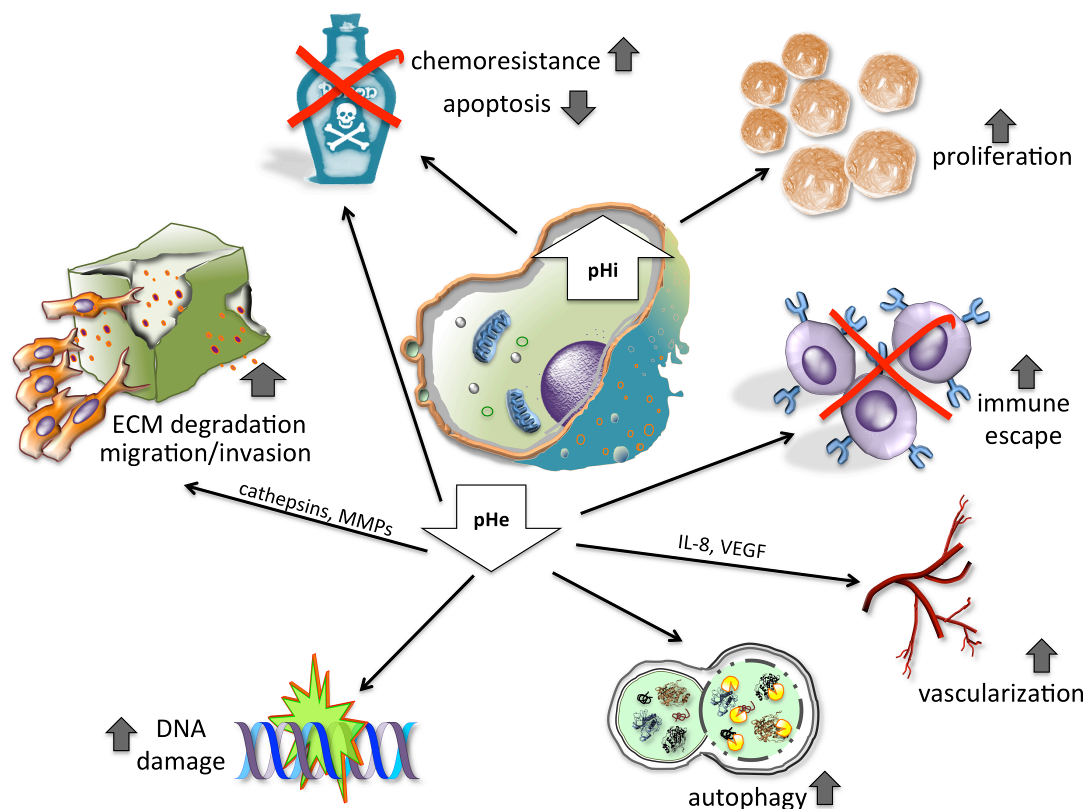

Figure 3. The altered tumor pH is correlated to malignant features. (Figure by Strambi and De Milito, 2015)

This ability is further enhanced by acidosis-induced release of proangiogenic factors, such as VEGF and IL-8, which trigger the formation of new blood vessel used by cancer cells to spread to new different sites [140,141]. Acidosis is also correlated to a reduced capability of DNA repair leading to a further increase of genomic mutations [142]. Acidic extracellular pH has been also correlated to inhibition of expression of several inflammatory markers and induction of anergy in T cells, thereby inactivating the immune system and promoting immune escape [143,144]. Moreover, acidosis has been described as one important factor that induces chemoresistance for the *"ion trapping"* phenomenon and upregulation of p-glycoprotein, as previously described [145,146]. Eventually, tissue acidosis also modulates autophagy as a survival and adaptive mechanism (this part will be better discussed in this thesis) [147-149].

Resistance to chemotherapy is one of the several benefits acquired by malignant cells during acidosis and it leads to treatment failure and relapse. In fact, one factor modulating drug efficacy is the ability of compounds to cross the plasma membrane, which, in turn, is correlated to the chemical properties of molecules and to interstitial/intracellular pH gradients [36]. Small and uncharged molecules can easily pass through the lipid bilayer of biological membranes. However, many conventional anticancer drugs are weak bases and weak acids, protons acceptors and proton donors respectively, which exist as equilibrium of the uncharged and the protonated charged forms [150]. This implies that weak bases accumulate in acidic compartments while weak acids concentrate in alkaline compartment for the *"ion trapping"*, responsible for chemoresistance in acidic tumors [58,145,151]. As we have previously described, solid tumors are characterized by a reversed pH gradient with a more acidic extracellular pH and slightly more alkaline intracellular pH as compared to normal cells [152]. In this condition, weak bases (such as doxorubicin), in their ionized form, cannot easily enter the cells and perform their cytotoxic activity while weak acids (such as 5-FU), in their neutralized form, easily enter the cells [145]. Tumor acidosis has been considered a new therapeutic target in cancer therapy and several drugs may target pathways involved in the regulation of the tumor pH. Targeting and inhibiting buffering systems have shown promising results in preclinical studies showing the ability of such drugs to alter pH homeostasis in the tumor mass and to induce apoptosis. Proton pump inhibitors (PPIs), such as omeprazole and esomeprazole, are pro-drugs activated only in acidic conditions and already used in clinic for the treatment of peptic diseases due to the fact that they inhibit the gastric proton-ATPase [153]. Moreover, PPIs have been associated to reverse the chemoresistence of several conventional chemotherapeutic drugs. In fact, the pre-treatment of human tumor xenografts with PPIs led to alkalinization of the tumor microenvironment and increased the sensitivity of cells to treatment with cisplatin, 5-FU and vinblastine [151,154]. Furthermore, PPIs showed tumor growth inhibition of human melanoma and human B lymphoma in xenograft mice model [155,156]. These promising results represented the proof of principle that PPI can be used as anticancer drugs and the preclinical studied opened the possibility to introduce these drugs in clinic on breast cancer patients [157]. The sulphonamide Indisulam (E7070) showed a potent anticancer activity through the CA inhibition inducing cell cycle arrest *in vitro* and tumor suppression *in vivo* [158-160]. During the past years several classes of CA inhibitors (CAIs) with different mechanism of action have been described and some enter the phase I/II of clinical trials for the treatment of metastatic solid tumors [161]. Finally, oral sodium bicarbonate treatment in mice carrying metastatic breast cancer showed the reduction of metastasis formation as a consequence of pH alkalization and it has been considered a promising system to overcome the chemoresistance characterizing weak bases through the buffering of tumor pH [162].

The acidic tumor microenvironment is a feature of solid tumors that provide a selective pressure to cancer cells. Only cancer cells able to adapt, through the several mechanism previously described, can acquire a selective advantage becoming more resistant to stress conditions and by aiming at invasiveness and metastasis [126]. Therefore, targeting tumor acidosis aiming at the restoration of more physiological pH leads to alteration of tumor cell homeostasis and to loss of fundamental survival mechanisms, resulting in cell death [137]. However there is a great need to identify and better characterize survival mechanisms involved during adaptation to acidosis in order to identify new potential therapeutic targets and to discover new anticancer compounds selectively active under acidic conditions.

1.5 CELLULAR DEGRADATION SYSTEMS

Cells are dynamic systems constantly renewing membranes and cytosolic components through the recycle of existing molecules. The turnover of macromolecules, necessary for new biosynthesis and to avoid accumulation of damaged/aberrant macromolecules, is mostly driven by two degradation systems: autophagy, which is a lysosomal degradation pathway and the ubiquitin proteasome system (UPS) which relies on protein degradation through the proteasome [163]. Degradation systems are important for cellular homeostasis in physiological and pathological conditions and impairment of such pathways may lead to cell death. For these reasons, autophagy and the UPS represent new targets for therapies in different pathological conditions, including cancer [164,165].

1.5.1 AUTOPHAGY

The word autophagy is the combination of two Greek words, *auto* and *phagein* which combined mean *self-eating* and it refers to the highly conserved and regulated catabolic process that cells use for the recycle of cellular components [166]. Christian De Duve first discovered and described lysosomes in 1955 as granules containing acid phosphatase and in 1963 he coined the name "Autophagy" [167]. Moreover, Ashford and Porter observed autophagic process already more than 50 years ago with electronic microscopy. In fact, they localized cytoplasmic components, such as endoplasmic reticulum and damaged mitochondria, in lysosomes from rats hepatic cells after exposure to glucagon [12]. Since then, 3 different types of autophagy have been mainly characterized: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA), that mainly differ for the level of complexity regarding the delivery process of the cargo to lysosomes [168]. Microautophagy is the simplest non-selective type of autophagy and it is characterized by a straight invagination of the lysosomal membrane intended to engulf directly the cytoplasmic cargo into lysosomes [169]. Macroautophagy (referred from now on as autophagy) is the most studied pathway and alterations in autophagy are associated with a series of human diseases, and in particular with tumor biology. Autophagy is characterized by membranes reorganization and it starts with the formation of an autophagosome, a double-membrane vesicle that engulfs cytoplasmic components and damaged organelles in unselective or selective manner. The membrane is known as autophagophore and it can derive from different sources, including the plasma membrane, the endoplasmic reticulum and the mitochondrial outer membrane [170]. After formation, the autophagosome fuses with lysosome forming the autolysosome [166,170]. The CMA is the most complex and specific type of autophagy. In fact, only specific proteins with the CMA targeting pentapeptide motif KFERQ are recognized by chaperone protein Hsc70 (heat shock cognate 70) and translocated to the lysosome for degradation [171]. The lysosome-associated membrane protein (LAMP-2A), a receptor on the lysosome membrane, recognizes the target protein and mediates the translocation into the vesicle for degradation [172]. The fate of the cargo is the same regardless the type of autophagy involved. In fact, the cargo is degraded by lysosomal enzymes and the resulting molecules (amino acids, nucleotides, fatty acids) are then released into the cytoplasm and recycled as building blocks in different anabolic pathways, such as protein synthesis, nucleic acid synthesis, gluconeogenesis and fatty acid synthesis [163]. Autophagy can be classified as selective when specific targeted cargo are delivered for degradation and as non-selective, that is responsible for turnover of bulk cytosolic components [173].

In normal conditions, cells maintain a basal constitutive level of autophagy which ensures the proper intracellular elimination of aberrant organelles and protein aggregates, thus preserving cell homeostasis [174]. However, cells can further activate autophagy under stress conditions such as nutrient deprivation, oxidative stress, hypoxia and drug treatment in order to prevent stress-induced death [175-177]. The relevance of the process for the intracellular quality control is emphasized by the fact that autophagy is an evolutionarily highly conserved process, that occurs also in other eukaryotes such as the yeast *Saccharomyces Cerevisiae*. In fact, more than 30 autophagy-related genes (Atg) have been identified in yeast and most of them are conserved also in multicellular eukaryotes such as mammals [178]. Most of the ATG genes discovered have been well characterized during the past years and they are necessary for the regulation of the 4 different phases of this dynamic process: induction, autophagosome nucleation, autophagosome elongation and completion (Fig. 4).

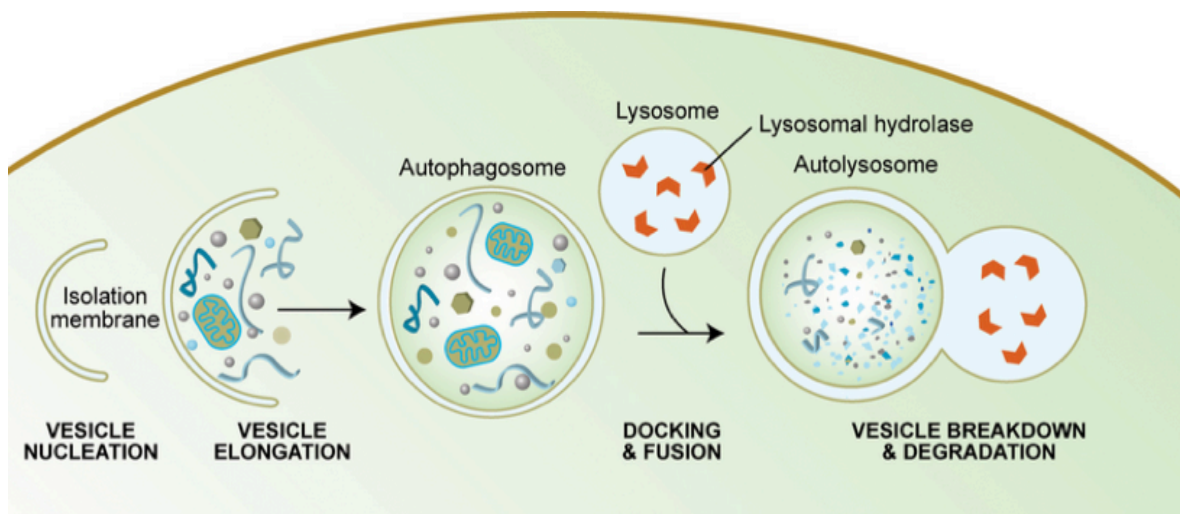

Figure 4. **Autophagy phases.** Vesicle nucleation: formation of the phagophore. Vesicle elongation: expansion of the phagophore into an autophagosome which engulf the cargo. Docking and fusion step: autophagosome fuses with a lysosome forming the autolysosome. Vesicle breakdown and degradation: the sequestered material is degraded inside the autolysosome and recycled (Figure by Meléndez and Levine, 2009) [179].

Induction of autophagy. *In this phase nutrient sensors drive the recruitment of multiple ATG proteins in order to enable autophagosome formation.*

The ULK protein complex is responsible for the initiation of the process and it is composed of Unc-51 like autophagy activating kinase 1/2 (ULK1/2), autophagy-related gene 13 (ATG13), autophagy-related gene 101 (ATG101) and FAK family kinase-interacting protein of 200KDa (FIP200) [180]. The initiating complex activation is regulated by stress factors which influence the 5' adenosine monophosphate-activated protein kinase (AMPK) and the mechanistic target of rapamycin Complex 1 (mTORC1) pathways, both sensors of the energy status of the cell. AMPK is a nutrient sensor activated by allosteric binding of AMP to its activating sites. MTORC1, the major negative regulator of autophagy, is a complex characterized by mTOR, regulatory-associated protein of mTOR (raptor),

mammalian lethal with SEC13 protein 8 (MLST8) and other non-core components. It is regulated by tuberous sclerosis complex 1/2 (TSC1/2) that is a GTPase activating factor and it is active in normal nutrient conditions. Low energy levels in the cell lead to reduction of intracellular ATP and increase of AMP, leading to activation of AMPK. Active AMPK phosphorylates TSC2 and activates the TSC2/TSC1 complex that in turn inactivates mTORC1. In fact, in normal nutrient condition mTORC1 is active and directly binds (through raptor), phosphorylates and inactivates ULK1/2, leading to autophagy inhibition. However, during nutrient deprivation mTORC1 is inhibited and released from the complex, therefore ULK1/2 can be activated by dephosphorylation. Active ULK1/2 phosphorylate ATG13 and FIP200, which translocate the complex to the pre-autophagosomal membrane thereby inducing the autophagic process [170,181,182].

Vesicle nucleation and cargo sequestration. *In this phase lipids and proteins necessary for the autophagosome membrane formation are recruited in order to initiate the phagophore formation* [148].

The protein complex Beclin1 and vacuolar protein sorting 34 (VPS34) are involved in the vesicle nucleation.

The class III phosphatidylinositol 3-kinase (PtdIns3K) protein VSP34, regulated upstream by the ULK complex, leads to production of phosphatidylinositol-3-phosphate (PI3P), essential for elongation and recruitment of other ATG downstream proteins involved during vesicle elongation. Beclin-1 and VPS34 interaction is responsible for VSP34 activation and for increased levels of PI3P. However, more regulatory proteins are involved [181,183]. Autophagy/beclin-1 regulator 1 (AMBRA1), ATG14L, ultraviolet irradiation resistance associated gene (UVRAG) and Bax-interacting factor 1 (BIF1) induce autophagy: BIF1 interacts with Beclin-1 through UVRAG and enhances class III PI3K enzymatic activity while AMBRA1 is phosphorylated and activated by ULK1 to promote the recruiting and activation of VPS34 [184]. On the other hand, Rubicon and Bcl-2 inhibit autophagy. For instance, Bcl-2 BH3 domain interacts with Beclin-1 destroying the interaction with VSP34 and leading to the inhibition of the autophagic process [185].

Vesicle maturation. *In this phase the phagophore expands and closes forming a mature double membrane autophagosome* [175].

The vesicle elongation is controlled by two ubiquitin-like conjugation systems that play a fundamental role in the phagophore formation [182]. The first conjugation is characterized by the covalent bond of the ubiquitin-like protein ATG12 and ATG5 through the activity of ATG7 (E1 like-enzyme) and ATG10 (E2 like-enzyme). Subsequently, the ATG12–ATG5 conjugate interacts non-covalently with ATG16-like 1 and promotes the second conjugation reaction acting as E3-ubiquitin like ligase [181,183]. The second conjugation occurs between phosphatidylethanolamine (PE) and microtubule-associated protein 1A/1B-light chain 3 (MAP1LC3 or simply LC3) through the activity of different enzymes [182]. LC3 protein is synthetized as precursor pro-LC3 and converted by protease ATG4 to the cytosolic and soluble form LC3-I while LC3-I is lipidated to insoluble and membranes associated LC3-II form through ATG7 and ATG3 (E2 like-enzyme) activity. LC3-II is associated to autophagosomes

membrane and necessary for maturation and cargo sequestration [186]. Eventually, LC3-II associated to the membranes is degraded along with the cargo during the last step of the process [187].

The recognition, sequestration and recycling of specific ubiquitinated proteins through the autophagic process are partially controlled by sequestosome 1 (SQSTM1 or p62) [188]. SQSTM1 is a multifunctional scaffold and adaptor protein with three different domains: a carboxy-terminal ubiquitin-associated domain for interaction with ubiquitinated proteins[168], a Phox and Bem1 (PB1) domain for self-oligomerization [168] and an LC3-interacting region for the binding with LC3-II on autophagosome membranes [189].

Lysosome fusion. *In this phase the mature autophagosome fuses with lysosome to form an autolysosome, a single membrane vesicle that degrades the inner autophagosome membrane and the cargo through the activity of lysosomal enzymes such as lysosomal acid hydrolases and cathepsins* [182].

The fusion of autophagosomes and lysosome is driven by a small Rab GTPases protein (Rab7), by the homotypic fusion and protein sorting (HOPS) complex and by lysosome-associated membrane protein 2 (LAMP-2). LAMP-2 is a membrane glycoprotein associated to lysosome, it exists in 3 forms (LAMP-2A, LAMP-2B and LAMP-2C) and it plays an important role in CMA [172,190]. Moreover it has been reported that Pleckstrin homology domain containing protein family member 1 (PLEKHM1) directly interacts with HOPS and LC3 on autophagosome membranes and mediates the binding necessary for the fusion. In fact, the alteration of PLEKHM1 blocks the lysosomal degradation of the cargo [191,192].

1.5.1.1 AUTOPHAGY IN CANCER

Autophagy is important in physiological conditions like aging and stress adaptation but it plays fundamental roles also in several pathological conditions. Due to its important role in regulation of cell homeostasis, defects in the autophagy machinery have been associated to the onset of different disorders [193]. For instance, in neurodegenerative processes impaired autophagy leads to toxic accumulation of mutated protein forms such as mutant Huntingtin (mHtt) and α-synuclein, respectively responsible for Huntington´s and Parkinson's disease [194,195]. Therefore, functional autophagy exerts a protective role against neurodegenerative processes [196]. However, in cancer, autophagy shows a controversial involvement due to a context-dependent role with cancer-promoting and cancer-suppressing functions [197]. In fact, in early stages of tumorigenesis autophagy may prevent cancer development (for instance by limiting genome mutations and chronic inflammation) while in established tumors it promotes tumor cell survival and tumor growth, likely by aiding cells to cope with stress conditions such as hypoxia, oncogenic stress and anticancer treatment [198].

Several evidences confirm the **tumor-suppressive function** of basal levels of autophagy and its correlation with cancer in case of deregulation [199]. Many autophagy related genes with a tumor suppressor function have been correlated to cancer: UVRAG deletion is common in colon carcinoma [200], LC3 gene locus is frequently founded deleted in liver, breast and ovarian cancer [201] and ATG5 and ATG7 deletion cause liver tumor in mice [202]. The onset of DNA mutations and the immune response

are two mechanisms that explain how impaired autophagy may promote tumor initiation. In fact, autophagy is responsible for degradation of cellular components such as damaged mitochondria, thereby regulating the production of ROS which are a threat for the cells due to their mutagenic capabilities. Therefore, a defective catabolic process leads to accumulation of the cellular waste and altered DNA which in turn trigger an inflammatory response and altered gene expression and so promoting tumor formation [198].

On the other hand, autophagy holds a **tumor-promoting** function in already established tumors since it provides a selective advantage during stress conditions. In fact, as previously described, high proliferating tumors are characterized by increased metabolism which combined with low vascularization and perfusion contributes to the formation of a toxic microenvironment: hypoxic, acid and devoid of nutrients [203]. In such a hostile condition, cancer cells with a functional and upregulated autophagy are able to adapt and survive escaping apoptosis. HIF1α activation, following the oxygen deprivation, activates autophagy through AMPK kinase activity and BNIP3/BNIP3L induction [204-207]. Increased levels of ATG5, a key regulator of autophagosome development, were found in breast cancer cells cultured in low pH conditions, suggesting autophagy as an adaptive mechanism in acidic conditions [148]. Finally, nutrient deprivation is a main positive regulator of autophagy [208]. Increased levels of autophagy have been observed and correlated to poor prognosis in some types of cancers [209,210]. Furthermore, the involvement of autophagy in drug resistance is of great clinical relevance. Several studies have shown the ability of cancer cell to induce the autophagic response as an adaptive mechanism to respond to cytotoxicity of chemotherapy and radiation [211]. In fact, the activation of the catabolic process is necessary to get rid of drug-induced damaged molecules, allowing tumor cell survival and proliferation. In this context, inhibition of autophagy has been correlated to an increased sensitivity of cancer cells to chemotherapies, in fact the use of autophagy inhibitors improved the therapeutic effects of several conventional chemotherapic drugs such as 5-FU [212-215], docetaxel and cisplatin [176,193,205,216].

The dual role played by autophagy in tumor biology provides the possibility to consider new approaches for cancer therapy and prevention [197]. From one hand the tumor suppressor activity of autophagy might be considered as a strategy for cancer prevention [212]. On the other hand, more interesting and likely feasible is the possibility to use autophagy inhibitors to reduce the ability of cancer cells to adapt to stressful conditions, like hostile microenvironment and drug cytotoxicity, reducing the resistance mechanisms responsible for tumor relapse [217]. Therefore, the combination of conventional chemotherapeutic drugs and autophagy inhibitors has been considered as a new therapeutic strategy to improve therapeutic efficacy and induce regression of established tumors.

1.5.1.2 TARGETING AUTOPHAGY IN CANCER

Several autophagy inhibitors are available and they can be classified according to the process phase that they can target. Early-stage autophagy inhibitors are 3-methyladenin (3MA), LY294002 and wortmannin which target PI3K, thereby interfering with the induction of the process [218-220]. Moreover, NSC185058 and NSC377071 target ATG4 suppressing the activation of LC3, the small molecule SBI-0206965 can selectively inhibit ULK1 kinase and several different small molecules such as SAR405 are able to inhibit VPS34 [221-224]. Late-stage inhibitors include bafilomycin A1 (BafA1) and

concancamycin as inhibitors of lysosomal vacuolar H^+-ATPase, E64D and Pepstatin A as inhibitors of lysosomal proteases [212,225,226] and Chloroquine (CQ) as inhibitor of the lysosomal activity.

CHLOROQUINE

CQ and its derivative Hydroxychloroquine (HCQ) are the only autophagy inhibitors used in clinical trials for treatment of solid tumors [227]. Both molecules belong to the 4-aminoquinoline class of antimalarial drugs exploited for 70 years to prevent and treat infection of *Plasmodium falciparum*, the parasite responsible for malaria [228]. When the parasite digests the hemoglobin into the digestive vacuoles, it releases the toxic ferriprotoporphyrin IX that is removed in normally conditions. However, CQ accumulates into acidic cellular compartment (after protonation), binds the porphyrin and prevents its degradation, leading to plasmodium cell death [229]. More recently, CQ has been used for treatment of autoimmune diseases such as rheumatoid arthritis and lupus erythematosus because of its immune suppressive activity [230,231]. Finally, CQ is also able to inhibit autophagy due to its lysosomotropic activity which increases lysosomal pH and alters lysosomal function [232,233].

CQ is a weakly basic tertiary amine that can exert an anticancer activity with multiple mechanisms of action still under investigation. Different studies have described its ability to block cell cycle and induce apoptosis but anticancer activity might be more correlated to its lysosomotropic activity which mediates radio-sensitization and chemosensitization [233,234]. According to the type of tumor, microenvironment and immune system, CQ generally can have different effects: autophagy inhibition, apoptosis induction, interaction with nucleotides, multidrug resistance pumps inhibition, improving drug uptake, and interfering with the immune system [233].

CQ inhibits autophagy. As a weak base with pKa 8.2, CQ is found mostly in the protonated form in acidic compartments [235]. Therefore, when it enters the lysosome, it is trapped within the lysosomal lumen, blocking lysosomal function and the degradation of the cargo of autolysosomes. The major limitation for CQ as autophagy inhibitor in clinical oncology is correlated to the dose. Low CQ concentrations inhibit autophagy in cancer cell lines in vitro but not on primary cell lines [236]. For malaria the therapeutic dose is 5 mg/kg and it could increase up to 10 mg/kg. However, above 20 mg/kg, CQ can cause serious toxic effects like retinopathy and doses higher than 86 mg/kg are lethal [237]. Several *in vivo* studies showed that high CQ doses are required to inhibit tumor growth and autophagy in mice but for instance, it has been also shown that the co-treatment of low doses of CQ (3,5 mg/kg) and bevacizumab delays tumor growth in human xenograft model [233,238]. The high variability of the efficacy observed in mice xenografts might be correlated to the different capacity of various tumor models to accumulate CQ at the effective concentration to inhibit autophagy [217,238]. Therefore, CQ is not used alone in cancer treatment but in combination with conventional chemo and radiotherapies in order to reach synergic affects [239,240]. CQ and HCQ showed ability to induce apoptosis at doses higher than 30 µM in different cell lines [241,242]. Treatment with HQ showed lysosome membrane permeabilization followed by release of lysosomal enzymes, such as cathepsins. As a consequence of the lysosomal membrane disruption, mitochondrial membrane permeabilization occurs and it triggers the mitochondrial apoptotic pathway through Cytochrome C release [238]. It has been also shown that CQ stabilizes p53 and induced p53-dependent pathway apoptosis [233]. CQ kills cancer stem cells. CSCs are considered the most difficult cell population to kill because of their

resistance to chemotherapy. However, low doses of CQ inhibited the in vitro formation of mammospheres of breast and pancreatic cancer while the same doses were not toxic on non-cancer stem cells [236,243]. A potential role of CQ on survival pathways in CSCs has been suggested, such as impairment of C-X-C chemokine receptor type 4 (CXCR4) signaling or inhibition of hedgehog pathway [236]. CQ interacts with nucleotides and at high doses interfering with DNA and RNA synthesis. In fact, CQ is able to bind nucleotides, especially purines, forming complexes which prevent the integration on the nucleotides itself into the acids nucleic [244,245]. CQ is a pleiotropic drug due to its ability to induce or inhibit cancer cell growth according to different doses and cell lines [246]. CQ has also indirect effects in cancer, by interfering with drugs uptake and the immune system. Many anticancer drugs, such as doxorubicin and mitoxantrone, are weak bases as well as CQ and they are likely protonated in the acidic extracellular tumor microenvironment and in acidic compartments, due to the " ion trapping" effect, responsible for low distribution and reduction of pharmacological effects. Therefore the CQ treatment may raise lysosomal pH and facilitate drug retention. Moreover, CQ directly binds to MRP reversing the MRP-mediated doxorubicin resistance [247,248]. CQ showed also ability to stimulate the immune system. The combination therapy of CQ and chemo-radio-therapy leads to an increased expression and presentation of MHC-I on tumor cell surface enhancing malignant cell death mediated by cells of the immune system [249]. On the other hand, high doses of CQ showed ability to inhibit cytokine production, such as TNF-α, IL-6, IL-1β, inhibiting the immune response [250].

However, at not toxic concentration (below 20 mg/kg) CQ can only inhibit MRP, interfere with immune system and kill cancer stem cells but it does not affect autophagy, apoptosis and nucleotides. Therefore, it is suggested that CQ is used in clinic an adjuvant to conventional chemo and radiotherapy and that the treatment scheme is specific for each chemotherapeutic regimen [233].

Preclinical studies on CQ provided conflicting results. In murine c-Myc-induced lymphomas, inhibition of autophagy by Chloroquine enhanced the ability of DNA alkylating agent to induce tumor cell death and tumor regression [238]. Although modest effects were observed on tumor growth in xenograft model of pancreatic cancer, a better efficacy was observed in other pancreatic ductal adenocarcinomas (PDAC) patient derived xenografts (PDXs) treated with HCQ [239,251-253]. Several ongoing phase I/II clinical trials are exploring the toxicity of CQ and HCQ in combination with conventional chemotherapeutic drugs in different tumors (Tab. 1). Despite some studies have shown clinical safety and promising efficacy of HCQ used in combination with chemotherapies, the results of different trials showed poor efficacy and several limitations such as the effective achievable doses for autophagy inhibition in human tumors [254,255]. The study and the development of more potent and specific autophagy inhibitors suitable for clinical application in combination with conventional cytotoxic agents is warranted because of the limitations observed with the use of CQ and HCQ [217,256].

Tumor type	Therapeutic combination
Breast cancer	HCQ only
	HCQ + ixabepilone
Lung cancer	HCQ + gefitinib
	HCQ + erlotinib
Pancreatic cancer	HCQ only
	HCQ + gemcitabine
Prostate cancer	HCQ only
	HCQ + docetaxel
Multiple myeloma	HCQ + bortezomib
Colorectal cancer	HCQ + regorafenib or vorinostat
Hepatocellular carcinoma	HCQ only
Renal cell carcinoma	HCQ only
	HCQ + aldesleukin
Lymphangioleiomyomatosis	HCQ + sirolimus
Melanoma	HCQ + gefitinib
Sarcoma	HCQ + sirolimus
Leukemia	HCQ + mitoxantrone or etoposide
Advanced solid tumors	HCQ + sirolimus or vorinostat

Table 1. Preclinical and ongoing clinical studies using CQ an HCQ in cancer treatments. Phase I/II clinical trials using combinations of CQ with different cytotoxic agents are currently being conducted in various tumor types.

SALINOMYCIN

Salinomycin (SAL) is a carboxylic polyether potassium ionophore with antibiotic activity against Gram-positive bacteria and it is used in veterinary medicine as anti-cocciostat and as growth promoting agent [257]. In 2009, Gupta et al. performed a high-throughput screening and they found that SAL was able to specifically kill breast CSCs. SAL was found to be 100-fold more potent than paclitaxel, the taxol commonly used in breast cancer chemotherapy. Moreover, SAL-treated mice showed inhibition of tumor growth and promotion of epithelial differentiation in breast cancer stem cells [258]. Since then, many studies have focused in understanding the mechanisms by which SAL kills cancer cells and CSCs, showing that different pathways are involved. In fact, SAL is able to induce apoptosis and overcome MDR in cancer cells expressing ABC-transporters that are responsible for the gain of the resistant phenotype [259]. SAL showed inhibition of the Wnt/B-catenin pathway, fundamental in stem cell development, through inhibition of a Wnt co-receptor phosphorylation in chronic lymphocytic leukemia and in hepatocellular carcinoma, thereby leading to apoptosis in cells that are dependent on Wnt activity [260,261]. SAL reduced the subpopulation of CSCs and induced their differentiation in colon carcinoma cells [262]. Moreover, SAL is able to sensitize cancer cells to

conventional chemotherapeutic drugs, such as doxorubicin and etoposide by promoting DNA damage and inhibiting the expression of cell cycle regulators [263]. All these findings identify SAL as a new promising compound to develop for cancer therapy. Despite it is clear how SAL is toxic to coccidia, there is the great need to better understand specific mechanisms of action that make this compound toxic on tumor cells [264].

More recently, SAL showed promising ability to inhibit the late stages of autophagy in breast CSCs and in hepatocellular carcinoma promoting cancer cell death through apoptosis [265,266]. The explanation might be correlated to the critical role of autophagy in promoting the cancer cell phenotype and tumor development. In fact, fundamental autophagy related genes, such has Beclin1 and ATG4A are necessary for the maintenance of cell stemness and tumorigenicity and the inhibition of the autophagic process is correlated to the differentiation on CSCs and the loss of their features [265,267,268]. However, it has also been reported that SAL is able to induce caspase dependent apoptosis through activation of the autophagic process in prostate, colon and breast cancer cells [269-271].

The major limitation for the potential clinical use of SAL for cancer therapy is the very high toxicity [264]. Despite the specific mechanisms that mediate this toxic effect are unknown, it was reported that SAL is highly toxic on dorsal root ganglia and Schwann nervous cells which die through apoptosis [272]. Clinical pilot studies of ovarian, neck and breast cancer patients treated with 200-250 mg/kg of SAL every second day for 3 weeks showed neither acute side effects nor long-term complication but a promising inhibition of the cancer progression [273]. Nevertheless, studies aiming at the synthesis of SAL derivatives are ongoing with the prospective to develop less toxic molecules with a better biological activity [274-276].

1.5.2 THE UBIQUITIN PROTEASOME SYSTEM (UPS)

The fundamental role of the UPS pathway in proteins turnover was discovered 30 years ago and provided a mechanism that explains how cells maintain a balance between protein synthesis and degradation [277]. The UPS is involved in the specific degradation of most of damaged proteins, such as product of oncogenes and tumor suppressors, that need to be removed in order to keep proper intracellular homeostasis [278]. Therefore, an altered UPS pathway is correlated to the onset of different disorders such as neurodegenerative disease and cancer [165,279,280].

The proteasome is a highly evolutionarily conserved complex able to recognize proteins that need to be degraded and the substrate identification is highly specific due to the ubiquitination process. Only proteins tagged with poly-ubiquitin chains are delivered to the proteasome for removal [281]. Ubiquitin (Ub) is a small and highly conserved regulatory protein of 76 amino acid including 7 lysine residues. Ubiquitination is a multi-step process regulated by 3 different enzymes: the Ub-activating (E1), the Ub-conjugating (E2) and the Ub-ligating (E3) which catalyse the Ub activation and the binding to substrates [278]. The process is reversible and the Ub removal is controlled by specific deubiquitinating enzymes (DUBs), such as USP14 and UCHL5 [282]. Three different types of ubiquitination exist: mono-ubiquitination (one Ub is attached to substrate), poly-monoubiquitination (several different mono-ubiquitin are bound to proteins) and poly-ubiquitination (substrates are linked to chains formed by several units of Ub). According to the different types of ubiquitination proteins undergo to different

cellular process and a chain of at least 4 Ub is necessary in order to obtain an effective degradation [278,283]. The linkage among the several Ub units involves the lysine residues and according to the specific lysine involved the tagged proteins are destined to different cellular fate. The Lys-K48 poli-ubiquitination is very specific target for protein degradation through the proteasome while Lys-K63 poli-ubiquitination is correlated to DNA repair and replication as well as protein degradation through proteasome pathway or autophagy [284-286].

The UPS is an important pathway for many physiological processes like cell proliferation and cell death, protein quality control, protein aggregation and response to oxidative stress. The proteasome controls the destine of several fundamental players of cell cycle (cyclins), pro-apoptotic proteins (Bax), NF-kB, and tumor suppressor protein (p53), deciding whether the cell has to die or proliferate [287]. Moreover, the UPS is responsible for the degradation of damaged and non-functioning proteins modified by oxidation due to ROS accumulation [288]. Thus, deregulation of the UPS and, in particular its upregulation have been observed in cancer cells that are thereby more sensitive to its blockade due to the fact that they rely more on proteasome function than normal cell [289]. For these reasons, the UPS is a new therapeutic target in cancer therapy and Bortezomib (trade name Velcade®), a synthetic dipeptide boronic acid is the first proteasome inhibitor approved by FDA for the treatment of multiple myeloma [50].

1.6 SCREENING MODELS FOR ANTI-CANCER DRUG DISCOVERY

The drug development process consists of several phases. Potential compounds are identified exploiting different types of screening models and introduced into clinical use after proper pre-clinical studies aimed at assessing the safety of the molecule. However, the number of drugs that eventually enter the clinic for cancer treatment is much lower than the total numbers of compounds obtained from initial screening phases and a main raison is correlated to the failure of *in vitro* preclinical models in representing the complex physiological *in vivo* conditions of the tumor mass.

The possibility to consider human tumor cell lines a useful tool for large-scale screening dates back to 1985, whereas human tumor xenografts had been the mainstream strategy exploited in anti-cancer drug discovery. The advantages of the new system consisted in the possibility to asses a larger number of compounds on a broader panel of human tumor cell lines in a shorter lapse of time. Subsequently only hit molecules would have been tested on mouse model for the preclinical evaluation [290,291]. Drug-screenings for anti-cancer drug discovery have been classified into empiric, based on cellular cytotoxicity assays, and mechanistically, exploiting specific molecular targets for cancer cells. The first type has been providing a great number of different hit compounds but with a mechanism of action limited to the non-specific ability to induce DNA damage. Conversely, new drug screenings models considering specific features of cancer cells have been developed and exploited leading to more successful compounds due to their higher selectivity towards malignant cells [292]. For instance, the screening for protein kinase inhibitors led to the identification of the lead compound that subsequently was modified into Imatinib, nowadays used for cancer treatment [293]. Despite their specificity, target-based screenings are not predictive of the effect of the compound in the more complex cellular system where other physiological factors can interact with drug activity.

Therefore, there is a need for drug screening strategies that can take into account the complexity of the whole tumor tissue, including for example hypoxia, starvation and metabolic factors. For instance the screening of drug libraries exploiting the multicellular tumor spheroids (MCS) model, tumor stem cells or glucose starved tumor cells has contributed to the identification of promising therapeutic molecules particularly active on cancer cells responsible for tumor relapses [294]. Unlike classical monolayer cell cultures, MCS is *in vitro* 3D model better resembling the tumor mass properties, such as hypoxia, acidosis and nutrient deprivation as well as the limited drug penetration [295]. MCS have been used as a promising tool for drug screening. In fact, it has been suggested that compounds showing cytotoxicity on MCS will also be effective on solid tumor *in vivo* [296-298]. Moreover, MCS are characterized by external layers of more proliferating cells and by a central core of quiescent slow proliferating cells. These latter are considered responsible for chemoresistance and compounds able to kill them in MCS model are most likely capable to target them *in vivo* [299,300].

2 AIM OF THE THESIS

The project of this thesis mainly focuses on the role of acidic tumor microenvironment on malignant progression and therapy. The major aims are to better understand which molecular mechanisms some cancer cells exploit to overcome stress induced by acidosis and to identify and possibly develop therapeutic strategies to target cancer cells in acidic environment.

Particularly, a part of the project aimed at investigating the **role of autophagy in acidic stress** (Paper I). It has been shown that tumour acidity and autophagy are both correlated somehow to malignant progression and poor outcome. Therefore, the purpose was to define whether exposure of cancer cells to acidic culture conditions affected the autophagic process. We found that autophagy is a survival mechanism for tumor cells in acidic conditions, further strengthening the idea of autophagy inhibition as therapeutic strategy. However, we found that **Chloroquine**, the only autophagy inhibitor used in clinical trials **does not inhibit autophagy** and is not cytotoxic in cancer cells in acidic conditions (Paper II). Therefore, we aimed at identifying other compounds able to inhibit autophagy during acidosis. We found that **Salinomycin is a potent autophagy inhibitor** in the acidic tumor microenvironment (Paper III).

The second part of the project is focused on the identification of compounds with anticancer activity through a **novel model of drug screening** (Paper IV)**.** In fact, most of the drug-screening assays for discovering of anticancer drugs are performed in neutral pH and normoxic culture conditions while cancer cell microenvironment is known to be acidic and hypoxic. This limitation could be one of the reasons for lack of efficacy for many drugs used in clinical oncology. So a drug screening performed in acidic and hypoxic conditions might lead to identify new and more effective drugs able to target acidic and hypoxic cells potentially responsible for tumor relapses after therapy.

3 RESULTS AND DISCUSSION

3.1 PAPER I: AUTOPHAGY IS A PROTECTIVE MECHANISM FOR HUMAN MELANOMA CELLS UNDER ACIDIC STRESS.

Background

Solid tumors are complex systems of different type of cells and several physical-biochemical factors that together characterize the tumor microenvironment. Cancer cells switch their metabolism from the oxidative phosphorylation to glycolysis even in normal oxygen pressure, leading to an increased production and accumulation of metabolic acids. To avoid cytosolic acidification, cells activate proton extrusion mechanisms leading to extracellular acidification, which correlates with chemoresistence, proliferation, angiogenesis and ECM degradation. Autophagy is a fundamental cellular process induced as a survival mechanism by different stress factors, such as starvation, hypoxia, ER stress and DNA damage.

Results

1. Human Melanoma cells survive and proliferate in acidic pH

We assessed cell viability over 72 hours of five different Melanoma cell lines and observed that all cell lines survived and showed a decreased proliferation when cultured at low pH (pH 6.8 and 6.5). Among the cell lines analysed, Me30966 maintained a rather normal proliferation rate even in acidic conditions.

2. Human melanoma cell up-regulate autophagy and maintain a functional autophagic flux in acidic pH (acute and chronic exposition)

Melanoma cells exposed to acidic stress showed increased level of LC3+ puncta, suggesting up-regulation of basal autophagy. However, increased levels of LC3+ vesicles could be also correlated with a defective degradation of autophagosomes. Therefore, in order to confirm that cells cultured under acidic stress could really up regulate autophagy, we looked at the autophagic flux using autophagy inhibitors like BafA1. BafA1 is able to block the latest phase of autophagy preventing the degradation of the autolysosomes, which therefore accumulates within the cell. Fluorescence and Western Blot analyses confirmed increased level of autolysosomes and LC3-II, indicating the presence of a functional autophagic flux. These findings were observed both in case of transient exposure of parental cancer cells to acidic pH (8 hours) as well as in case of chronic exposure (using a cell line which has been adapted to pH 6.8).

3. Human melanoma cell decreases nutrients uptake and ATP content in acidic pH

In acidic stress, melanoma cells reduced the uptake glucose and of the amino acid leucine. Lower levels of intracellular glucose were confirmed by the reduction of the cell ATP content, probably due to the reduction of glucose consumption and inhibition of glycolysis.

4. Acidic pH induces cytosolic acidification and inhibition of mTOR signalling

Melanoma cells showed a reduction of the cytosolic pH within few minutes after exposure to acidic medium. MTOR is a nutrient sensor and it is inhibited during stress conditions (like starvation), leading to the stimulation of autophagy**.** We observed that at low pH culture conditions the mTOR pathway is inhibited. Western Blot data showed a reduction of phosphorylated p70S6K and 4EBP1, two mTOR downstream effectors.

5. Autophagy is crucial for survival of melanoma cells under acidic stress

We investigated the effects of autophagy inhibition on cell death at different pH conditions. The knockdown of ATG5 induced augmented cell death in those cells that were exposed to low pH culture conditions as compared to cell cultured at pH 7.4. These data confirm that proficient autophagy is needed for melanoma cells to survive during acidic stress.

Significance

We showed that autophagy is an important protective mechanism for cancer cell to adapt and survive under acidic pH. Therefore, autophagy and acidity both represent potential therapeutic targets in cancer and combination therapies of autophagy inhibitors with conventional chemotherapy represent a feasible therapeutic strategy.

3.2 PAPER II: ACIDIC EXTRACELLULAR PH NEUTRALIZES THE AUTOPHAGY-INHIBITING ACTIVITY OF CHLOROQUINE.

Background

Tumor acidosis is a fundamental mechanism correlated to malignant progression and drug resistance. Others and we have shown that autophagy is an important mechanism that cancer cells use in order to adapt and survive to the stress induced by acidosis. Therefore, targeting the autophagic process is envisaged as promising new strategy in cancer therapy to kill cells adapted in acidic pH. CQ is an antimalarial medication and it has been the only anti-autophagic drug so far used in combination anticancer therapies in clinical trials. However, *in vivo*, CQ has not reproduced the antitumor effects that have been obtained in models *in vitro* and in some cases it has been associated to a slightly increase of tumor growth. Therefore, we assessed the capability of CQ and Lys-01, a novel CQ derivative, to block autophagy under acidic conditions.

Results

1. CQ is not cytotoxic *in vivo* and *in vitro* on colon carcinoma cells

We assessed the toxicity of CQ in *in vivo* models of human colon carcinoma xenografts (HCT116 and HT29) and we observed that tumor growth was not affected in mice treated with CQ. Moreover, we evaluated CQ cytotoxicity *in vitro* on colon carcinoma and melanoma cells and we found that cells cultured at low pH were completely insensitive to CQ.

2. CQ does not block autophagic flux in cells under acute acidic stress

We evaluated the actual ability of CQ to block autophagy in transient acidic conditions by assessing the changes in expression of the autophagic marker LC3-II. In presence of CQ, high levels of LC3-II were detected in cells cultured at physiological pH as predicted but the levels of LC3-II were unchanged in different cell lines cultured at pH 6.8 and pH 6.5 for 24 hours. These findings suggested that CQ does not block the autophagic process in different cancer cell lines during acidic stress.

3. CQ does not block autophagic flux in cells under chronic acidosis

We found that CQ does not block autophagy also in melanoma and colon carcinoma cells that have been adapted to growth a pH 6.8 (chronic acidosis). However, when neutral pH conditions were restored in these cells, CQ treatment was able to lead again to autophagy inhibition, meaning that pH is a main factor affecting CQ activity. Acid-adapted cells are also resistant to the cytotoxicity of CQ since we did not detect any reduction in cell viability after treatment even with high CQ doses. However, when physiological pH was re-established in culture even acid-adapted cells showed to be sensitive to the toxicity of CQ.

4. Autophagy inhibition by Lys-01 is detectable at acidic conditions

Lys-01 is a CQ dimer reported to be a better autophagic inhibitor. We found that Lys-01 is able to inhibit autophagy even at pH 6.8, unlike CQ. However, the LC3-II accumulation was reduced in cells treated with Lys-01 at lower pH, suggesting a pH-dependent activity of this compound. We wondered whether this was related to a different cellular uptake of the molecules, especially at acidic conditions, due to the different chemical properties as a basic or acid. We performed HPLC in order to quantify the cellular content of the 2 compounds after treatment and found that at pH 7.4 Lys-01 accumulates at higher levels as compared to CQ, thus possibly explaining its better activity. At pH 6.8, a CQ concentration was 7-fold lower than at normal pH while Lys-01 content was about 2-fold lower but still significantly higher than CQ.

5. CQ effect on autophagy *in vivo*

In vivo, CQ administration to human colon carcinoma xenografts did not lead to reduction of tumor growth. Thus, we performed IHC analysis on HCT116 tumor sections from mice untreated and treated with CQ in order to check any possible change in the autophagic process that could explain the lack of efficacy. We performed staining with CA9 to identify putative acidic regions (carbonic anhydrase 9 is highly expressed in hypoxic regions characterized by extracellular acidification) and LC3 to detect modulation of autophagy. In untreated mice, we observed that normoxic areas close to blood vessel showed low level of CA9 expression and LC3 while hypoxic areas with high CA9 expression showed an increased LC3 expression, suggesting that autophagy is more activated. This set of data confirmed that autophagy might be upregulated in hypoxic and acidic areas. Effective CQ treatment is expected to induce a further increase in the LC3 signal intensity due to a block of LC3-II degradation. In fact, CQ treatment leads to a 5-fold increase, as compared to the untreated tumors in LC3 expression in normoxic areas close to blood vessels but only a 1.4 increase in the hypoxic and acidic areas.

Significance

Autophagy inhibition is being tested as a new strategy in combination cancer therapy. However, we showed here that the only autophagy inhibitor currently used in clinical oncoloy does not actually inhibit autophagy in acidic conditions. Our findings may partly explain the modest CQ efficacy shown in the first clinical trials and underline the need to find and characterize more effective autophagy inhibitors.

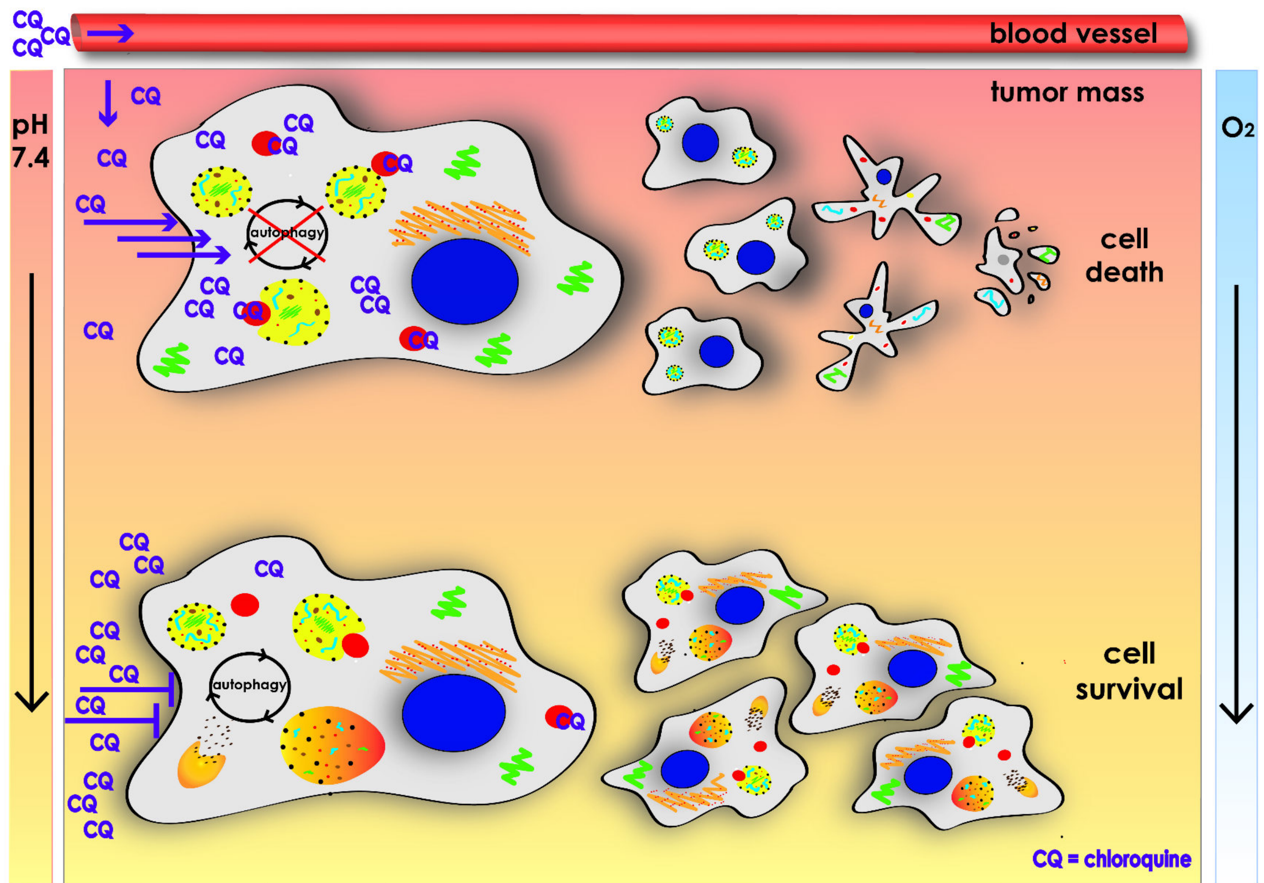

Figure 5. CQ does not inhibit autophagy in the acidic tumor microenvironment resulting in the loss of its cytotoxic activity.

3.3 PAPER III: TUMOR ACIDOSIS ENHANCES AUTOPHAGY INHIBITION BY SALINOMYCIN ON CANCER CELL LINES AND CANCER STEM CELLS.

Background

Cancer cells exploit autophagy during acidosis in order to adapt and survive to the hostile microenvironment that characterizes solid tumors. Despite autophagy inhibition has been considered a therapeutic strategy in cancer, so far only CQ and HCQ are approved in clinical trials. Unfortunately,

it has been shown that CQ is effective only in some models while it has no effect in others, sometimes even increasing tumor growth. Moreover, CQ may have autophagy-independent effects. In paper II we showed that CQ does not inhibit autophagy in tumor cells exposed to acidic environment, probably due to its chemical properties as a weak base. The need to find new autophagy inhibitors led us to identify Salinomycin as a potent compound that is active also during acidosis not only in cancer cell lines but also in breast cancer stem cells.

Results

1. Salinomycin is a potent autophagy inhibitor in acidic conditions (transient and chronic acidosis)

HOS cells stably transfected with a GFP-LC3 vector were treated with Sal in presence and absence of lysosomal inhibitor BafA1 and in medium buffered at pH 7.4 and 6.8. The accumulation of GFP-LC3-positive autophagosomes was assessed by flow cytometry and showed that when cells were cultured at pH 6.8 Sal was able to block completely the autophagic flux.

We confirmed the autophagy inhibiting activity of SAL on AA-HCT116 and AA-Me30966 cell lines adapted to pH 6.8. The co-treatment with BafA1 did not further increase the amount of LC3-II in SAL-treated cells, demonstrating that SAL blocks the autophagic flux. The strong autophagy inhibiting activity was also detected in HCT116 and Me30966 that were transiently exposed to medium at pH 6.8 and 6.5.

2. Salinomycin inhibits autophagy in the core of multicellular spheroids (MCS)

MCS are a 3D culture model that more closely reproduce *in vitro* some features that characterize a solid tumor mass. It has a central acidic and hypoxic core and a peripheral layer of cells growing in oxygen- and nutrients-rich environment. We obtained MCS from HCT116 and we treated them with BafA1, CQ, Lys-01 and Sal and performed IHC evaluating the LC3 pattern distribution. We observed that MCS treated with CQ and Lys-01 showed LC3 accumulation only in the peripheral layer but not in the core while MCS treated with Sal revealed a more homogeneous LC3 distribution as also occurring in the positive control treated with BafA1.
Viability and clonogenic assays done on MCS indicated that Sal has a strong cytotoxic effect.

3. SAL blocks the autophagic flux in cancer stem cells

CD24+ (non-CSC) and CD24low (CSC) HMLER cells were transfected with tandem probe RFP-GFP-LC3 that allows the identification and quantification of autolysosome (GFP- RFP+) and autophagosomes (GFP+ RFP+). We found that Sal treatment induced accumulation of autophagosomes without significant changes in autolysosomes in both cell lines, especially in those cultured at pH 6.8, suggesting a decreased autophagic flux. Moreover, WB analysis showed that lower doses of SAL are sufficient to block the autophagic flux in CD24low cells as compared to CD24+ cells both at pH 7.4 and pH 6.8.

4. Acidosis enhances the ability of salinomycin to kill cancer cells and cancer stem cells

We performed viability assays on HCT116 and Me30966 cultured at pH 7.4 and the respective

sublines adapted at pH 6.8 to understand whether the autophagy inhibiting activity of SAL at low pH is correlated to an increased cytotoxicity. SAL showed an increased cytotoxicity in low pH-adapted cells with a 10-fold difference in IC_{50} values. Moreover, we observed that cells cultured at acidic pH treated with SAL show a reduced clonogenic cell growth as compared to cells treated at pH 7.4.

Viability assays also showed that CD24low cells are more sensitive to both Lys-01 and SAL as compared to CD24+ cells in physiological culture conditions (pH 7.4), confirming that breast CSCs are more sensitive to autophagy inhibition. Moreover, the IC_{50} of SAL for CD24low cells cultured in medium at pH 6.8 was 10-fold lower than that for CD24+ cells, suggesting an even more selective effect of SAL on these cells at acidic conditions.

5. Acidic pH enhances the ability of SAL to inhibit mammospheres formation from breast cancer tissue derived stem cells.

It has been shown that a CSCs property is the ability of cancer cells to form mammospheres in vitro [168]. Therefore, we investigated whether this CSCs feature is affected by the pH-dependent activity of SAL. CD24low cells cultured and treated with Sal formed a lower number of mammospheres as compared to the untreated cells at pH 7.4. This effect was increased in CD24low cells treated with SAL at pH 6.8.

We confirmed the results obtained with CD24low cells with patient-derived breast CSCs in order to understand the potential clinical relevance of our findings. SAL inhibited the formation of mammospheres in cells cultured at pH 7.4 in a dose-dependent manner and the ability of SAL to inhibit mammosphere formation was dramatically increased at pH 6.8, confirming that SAL has a pH-dependent activity also on CSC derived from patients.

6. Salinomycin accumulates at higher concentrations in cells under acidic conditions

As previously described, CQ is a weak base and in acidic conditions protonated CQ does not easily enter cells, thus affecting the autophagy inhibiting activity of the drug itself. However SAL, as a weak acid, is protonated in acidic condition and may better cross the plasma membrane. UPLC-MS/MS analysis showed that in HCT116, HMLER CD24+ and HMLER CD24low cells the amount of SAL accumulating in the cells is significantly higher when cells are cultured in acidic conditions.

Significance

We identified SAL as a potent cytotoxic and autophagy-inhibiting agent whose activity on cancer cells and CSCs is further increased by acidosis.

3.4 PAPER IV: A DRUG-SCREENING ASSAY ON CANCER CELLS CHRONICALLY ADAPTED TO ACIDOSIS IDENTIFIES A NOVEL ANTICANCER ACTIVITY FOR VERTEPORFIN.

Background

Most of drug screenings for identification of anticancer drugs are performed in vitro in culture conditions at pH and oxygen levels very different from those observed in the tumor microenvironment *in vivo*. This limitation might be one of the reasons behind the lack of drug efficacy often observed in clinical oncology. In order to increase the chances of identifying more effective anticancer compounds, we optimized a drug screening performed in acidic and hypoxic conditions, with the aim to discover effective drugs targeting quiescent and acidic/hypoxic cancer cells, reasonably considered responsible for tumor relapses after therapy.

1. Characterization of the acid adapted colon carcinoma cell line AA-HCT116

The screening has been performed with HCT116 cells that have been adapted to grow at pH 6.8. We found that AA-HCT116 cells are different from the parental cells. They have a different phenotype being characterized by a bigger cell size and a more mesenchymal trait. RNA sequencing analysis showed also that parental HCT116 and AA-HCT116 cells have a different transcription profile and preliminary data also indicate that these cells differ in their metabolism with changes in mitochondrial respiratory capacity and glycolytic rates.

2. Screening of the Prestwick library screening

The drug screening has been performed using AA-HCT116 cells in conditions of hypoxia and normoxia and viability was used as a read out. For both conditions we optimized the assay with regard to number of cells and serum concentration. Subsequently, we proceeded with the screening of the Prestwick Chemical Library, a small library of 1280 compounds already approved by FDA and already used in clinical applications for the treatment of different pathologies. Cells were treated with compounds at 10 uM and cell viability was assessed after 48 hours with enzymatic acid phosphatase assay. Hit compounds were confirmed in dose-concentration assays and 11 out of 1280 compounds showed a substantial reduction of cell viability in the range 1-10 mM. Furthermore, the cytotoxicity of the selected 11 hits was tested in parallel on parental HCT116 cells at physiological pH and on hTERT-RPE1 cells (immortalized epithelial cells). Among the 11 hits, only Verteporfin (trade name Visudyne®) showed preferential cytotoxicity in AA-HCT116 cells as compared to parental HCT116 cells and epithelial cells, suggesting a more specific activity towards cancer cells in stressed conditions and the possibility of a therapeutic window.

3. VP is more cytotoxic on AA-HCT116

We confirmed that VP had a stronger cytotoxicity on AA-HCT116 as compared to parental HCT116 cells using viability assay, time-lapse microscopy and clonogenic assays. Moreover, we observed that ambient light activation of VP during treatment lead to an enhanced cytotoxicity of the compound. Finally, we evaluated VP intracellular accumulation by fluorimetric analysis and

observed that VP accumulates 3-fold more in AA-HCT116 than in the parental cells, likely because of its chemical properties as an acidic molecule (a similar finding was confirmed in melanoma cells).

4. VP interferes with polyubiquitinated proteins

VP is a benzoporphyrin derivative, clinically used in photodynamic therapy for the treatment of macular degeneration. It has been previously shown that VP is able to inhibit the early phase of autophagy and for this reason we studied whether VP-mediated autophagy modulation was associated with higher cytotoxicity in AA-HCT116 cells. Western blot analysis did not show any significant change in the turnover of LC3-II, indicating a normal autophagic flux in presence of VP. However, we observed the disappearance of the SQSTM1, another autophagy marker, in VP-treated samples together with the appearance of correspondent high molecular weight bands. SQSTM1 is a protein that shuttles ubiquitinated proteins to autophagosomes for degradation and modifications of its structure may alter its function and its role in the UPS pathway. Therefore, we evaluated the effects of VP on polyubiquitinated conjugates. In VP-treated samples we detected a shift from K48-poly-ubiquitination to K63-poly-ubiquitination with a concomitant decrease in the pool of free ubiquitin. This alteration was observed at early time points and it was enhanced by light exposure. RNA sequencing data showed a clear alteration of UPS related genes in AA-HCT116 cell line as compared to parental cells and this might be correlated to the increased sensitivity of AA-HCT116 cells to the drug. However, we showed that VP does not inhibit the proteasome function in these cells.

5. VP and unfolded protein response

The accumulation of high MW cross-linked proteins, such as SQSTM1, might induce endoplasmic reticulum (ER) stress and the activation of the unfolded protein response (UPR) which is a survival mechanism involved in the removal of toxic protein aggregates through the activity of glucose-regulated protein 78 (GRP78). Despite ER stress induced proteins such as C/EBP homologous protein (CHOP) and heat shock protein 70 (HSP70) were not altered, GRP78 expression was affected upon VP treatment. In fact, GRP78 was induced in HCT116 treated with VP with the appearance of high MW bands but not in the respective acidic adapted cell lines.

Significance

The drug-screening model that we describe may lead to identify new drugs and/or repurposing existing drugs active on cells under metabolic stress. Verteporfin has been already used in a clinical trial for pancreatic cancer and we show that it is more cytotoxic on cancer cells adapted to acidic pH. However, the potential mechanism of action needs to be further investigated. The screening of larger drug-libraries will hopefully lead to find and characterize more compounds able to target slow proliferating and therapy resistant acid-adapted cancer cells.

4 CONCLUSIONS

Cancer cells up-regulates autophagy as a survival mechanism in order to cope with the toxic acidic tumor microenvironment.

During the past years the metabolic reprogramming of tumor cells has acquired an important role in the field of cancer progression and therapy. One consequence of altered metabolism coupled with abnormal tumor tissue structure is the development of acidosis. The exposure of cancer cells to acidosis has been considered responsible for the acquisition of a more malignant and aggressive phenotype for the cells able to adapt, by aiding migration, invasion, metastasis and resistance to therapy. Different mechanisms are involved in the adaptation process of the cells to the harsh acidic environment and the upregulation of the autophagic process showed to play a fundamental role. We showed that human melanoma cells exploit autophagy in order to survive to acidic conditions. Therefore, our finding provides an additional support to the use of autophagy inhibitors as a therapeutic strategy to target tumor cells that have acquired the ability to adapt to acidosis. However, there is a need to identify other molecular mechanisms than autophagy involved during the cancer cell adaptation process to acidosis in order to be able to find new targets and develop different therapeutic strategies.

The only autophagy inhibitor available in clinic fails to inhibit the autophagic process in acidic conditions raising the need for the identification of more effective compounds.

The autophagic process has been considered a promising therapeutic target under clinical investigation. Until now, little results have been achieved in clinical settings regarding the use of autophagy inhibitors. In fact CQ and its derivative HCQ are the only drugs used in cancer therapy for this purpose, in combination with conventional chemotherapy. However, we show that CQ and HCQ fail to inhibit autophagy during acidosis possibly explaining their low cytotoxic effect in some tumor models. The acidic tumor microenvironment is responsible for the lower drug penetration through the plasma membrane due to protonation of weak base molecules, resulting in a subsequent reduction of the therapeutic effective dose *in vivo*. However, additional molecular mechanisms may be involved in the pH-dependent acquired resistance other than the d chemical properties of drugs. Our finding provides an explanation for a poor efficacy often observed in CQ and HCQ clinical trials and it suggests the need to identify new inhibitors effective during acidosis. Lys-01, a dimeric analogue of CQ has been recently claimed as a more potent compound *in vitro* and *in vivo* and we correlated this finding with its better accumulation in the cells in acidic conditions, therefore resulting in a more effective autophagy inhibition. However the use of Lys-01 showed limitations due to a pH dependent activity, suggesting that it is not the perfect candidate in such conditions. Conversely, we showed that the antimalarial compound SAL inhibits autophagy at low pH conditions, displaying a promising toxicity on cancer cell lines and CSCs. The stronger effects observed in acidic conditions might be correlated to SAL pharmacological properties as a weak acid that is responsible for an increased intracellular accumulation at low pH as compared to cells cultured in physiological pH. However, off target effects different than autophagy inhibition need to be further investigated to understand whether

there are different mechanisms explaining the stronger activity of this drug in acidic conditions. Moreover, less toxic SAL derivatives need to be developed in order to assess its efficacy on cancer patients.

A new drug-screening model leads to the identification of new compounds effective in cells under acidic stress.

It is known that most of the anticancer drugs that show a good anticancer activity *in vitro* show lack of efficacy on cancer cells *in vivo*. One explanation is that cancer cell microenvironment is different and more complex than the one reproduced in the laboratory settings. Others and we previously described tumor acidosis as cause of drug resistance. The possibility to exploit the hypoxic and acidic conditions that characterize solid tumor *in vivo* for the screening of drug libraries may lead to the identification of new anticancer compounds. A further investigation of specific molecular mechanisms of action of the hits identifies new therapeutic targets characterizing therapy-resistant cancer cells adapted to acidic stress. An attractive strategy that has been considered in the past years to identify new effective anticancer drugs is to propose the use of existing compounds already used in clinic with a different purpose. Using this approach through the screening of drug libraries expensive and time-consuming phases of drug development can be omitted due to the fact that most of the informations required are available. In fact, the screening of the Prestwick library, a small library of compounds already exploited in clinical applications, led us to the identification of the photosensitizer VP as a promising anticancer drug toxic on cells adapted to acidosis. We observed that VP accumulates more in cells under acidic conditions but this is not strictly correlated to its cytotoxicity. Despite it has been used since many years in clinic for different purposes, VP shows a promising ability to kill cancer cells through molecular mechanisms that still need to be fully understood. Preliminary studies indicate alterations of the ubiquitinated proteins and activation of the unfolded protein response after VP treatment but further studies are needed for its potential use in clinical oncology.

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