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by

Janice M. Santiago-O'Farrill, MS

APPROVED:

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Eugenie S. Kleinerman, MD, Supervisory Professor

_____
Hesham Amin, MD

_____
Joya Chandra, Ph.D.

_____
Juan Fueyo, MD

_____
Varsha Gandhi, Ph.D.

APPROVED:

_____
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

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A

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Janice M. Santiago-O'Farrill, MS

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DEDICATION

This dissertation is dedicated to my loving parents, Jesusa O'Farrill and Ramón Santiago and to my wonderful husband, Steve Rodríguez.

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THE ROLE OF AUTOPHAGY IN THE SENSITIVITY OF OSTEOSARCOMA CELLS TO GEMCITABINE TREATMENT

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Supervisory Professor: Eugenie S. Kleinerman, MD

Despite treatment improvement for osteosarcoma (OS), overall survival has remained unchanged in the last 20 years. Pulmonary metastasis continues to be the main cause of death; novel therapeutic strategies are urgently needed to improve the survival rate of these patients. Previous data in our laboratory has demonstrated that aerosol gemcitabine (GCB) treatment has a significant therapeutic effect on metastatic OS. However, treatment efficacy is decreased due to acquired resistance by a population of tumor cells that fails to respond to treatment. Recent studies have implicated autophagy as a resistance mechanism in various types of cancer. The purpose of this study was to determine whether the process of autophagy plays a role in protecting OS cells from the cytotoxic effects of GCB, allowing them to survive and avoid drug-induced cell death.

We first evaluated the ability of GCB to induce autophagy using a panel of OS cells. Following GCB treatment Acridine orange staining revealed the formation of acidic vesicular organelles (AVOs), a hallmark of autophagy. Western blot analysis showed an increase in several autophagy markers including; increased conversion of microtubule-associated light chain 3 (LC3I/LC3II), increased Beclin expression and a decrease in p62/SQSTM1 protein expression. Formation of autophagic vacuoles was also demonstrated using electron microscopy. These results confirm that autophagy was induced following GCB treatment. Furthermore, western blot analysis and kinase

array assays demonstrated that GCB treatment decrease the phosphorylation of AKT, mTOR, p70S6K and PRAS40, suggesting that this signaling pathway is involved in the induction of autophagy by GCB.

To test whether autophagy contributes to the resistance of OS cells to GCB, we determined the impact of autophagy inhibition on the sensitivity of OS cells to GCB. Autophagy inhibition by either the pharmacologic inhibitor Hydroxychloroquine or genetic inhibition of BECN and ATG5 resulted in either increased or decreased sensitivity of OS cells to gemcitabine depending on the cell line tested. Specifically, the sensitivity of LM7 cells to GCB was greatly enhanced after autophagy inhibition, suggesting autophagy as a cytoprotective mechanism. However, inhibition of autophagy in CCH-OS-D and K7M3 cells decreased cell sensitivity to GCB, suggesting that GCB-induced autophagy contributed to cell death.

A more thorough understanding of the molecular pathways that govern the autophagy process may allow the identification of a biologic marker that can predict whether autophagy function to inhibit or promote GCB-induced cytotoxicity. Our preliminary data showed that phosphorylation of Heat Shock Protein 27 (HSP27) correlated with autophagy as a mechanism of cellular resistance in different OS cell lines. Taken together, these results suggest that GCB induces autophagy in OS cells through the AKT/mTOR pathway and that when HSP27 is induced inhibition of autophagy will increase sensitivity to GCB.

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ABBREVIATIONS

μg	micrograms
μL	microliters
μM	micromolar
AMBRA	autophagy/beclin-1 regulator 1
AMPK	AMP-activated protein kinase
AKT	protein kinase b
ATGs	autophagy related genes
ATP	adenosine triphosphate
AVO	acidic vesicular organelles
Barkor	Beclin 1- associated autophagy-related key regulators
BECN	Beclin
BCL2	B cell Lymphoma 2
Caspase	cysteine aspartate-specific protease
Cisplatin	*cis*-diaminedichloroplatinum(II)
CMA	chaperone-mediated autophagy
CO_2	carbon dioxide
CQ	Chloroquine
CT	computed tomography
Ctrl	control
DCK	deoxycytidine
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid

ABBREVIATIONS

EM	electron microscopy
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FIP200	focal adhesion kinase family interacting
GCB	Gemcitabine
HCQ	hydroxychloroquine
HDMTX	Methotrexate
Hsp27	heat shock protein 27
Hsp70	heat shock protein 70
IF	Immunofluorescence
IHC	Immunohistochemistry
JNK	C-Jun N-terminal kinase
LAMP2	lysosome-associated membrane protein
LC3	microtubule-associated protein light chain
L-MTP-PE	liposomal muramyl tripeptide
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
NCI	national cancer institute
OS	Osteosarcoma

ABREVIATIONS

p62/SQSTM1	sequestome1
PBS	phosphate buffer saline
PCD	program cell death
PE	phosphatidyl ethanolamine
PET	positron emission tomography
PI3K	phosphatidyl inositol 3 kinase
PRAS40	proline-rich AKT substrate of 40kDa
RB	Retinoblastoma
RIPA	radio immunoprecipitation assay
ShRNA	short-hairpin ribonucleic acids
SDS-PAGE	sodium dodecyl sulfate
ULK1/2	unc-51-like kinase ½
UVRAG	UV-radiation-resistance-associated
WB	western blot
WT	wild-type

Chapter 1: Introduction

Osteosarcoma

Osteosarcoma (OS) is the most common type of pediatric bone cancer with approximately 400 new cases diagnosed per year in the United States. It is characterized by formation of immature bone or defective osteoids by tumor cells [1]**.** OS mainly affects children and adolescents between the ages of 10-25, and also patients over the age of 50 [1, 2]**.** The most common sites of occurrence are in the metaphysis of long bones and rapid growth areas such as the knees, femur, tibia and humerus [1-3]**.** OS is a highly aggressive tumor with a tendency to spread to other organs, with the lungs as the most common site of metastasis [2, 4]. OS diagnosis is based on x-rays, x-ray computed tomography (X-ray CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) scan [1]**.** The etiology of OS is unknown. Studies have identified some epidemiologic, environmental and genetic causes associated with OS including ionizing radiation, alkylating agents, mutations in the retinoblastoma gene (RB), Li-Fraumeni syndrome (deletion in p53), Paget's disease (bone dysplasia), Rothmund-Thomson syndrome (mutation in RECQL4 gene) and Wermer Syndrome (mutation in the WRN ,RECQL3, gene) [1, 4, 5].

Available treatment options for Osteosarcoma

In the 1960s, treatment of OS consisted of surgical excision of all detectable disease [3]**.** However, despite good control of the primary tumor, most patients recurred with metastatic lung disease [3]. Since surgery alone is not effective for OS, the current therapeutic strategies consist of pre-operative chemotherapy followed by surgery, and then post-operative chemotherapy. Limb-salvage is the most common type of surgery for bone sarcoma treatment [3]. Chemotherapy includes high-dose methotrexate

(HDMTX), leucovorin, doxorobucin, cisplatin and ifosfamide, and has increased the 5-year survival rate of patients with localized disease from 20% to approximately 70% (See Table 1) [1, 3]**.**

Lung metastases represent the main cause of death of OS patients [3]**.** With the introduction of adjuvant therapy, the survival rate in patients with non-metastatic disease has significantly improved. However, in patients with pulmonary metastasis, little improvement has been observed and 5-year survival rate remain low at merely 25%-30% [3]**.** Thus, new strategies targeting primary osteosarcoma and lung metastasis are urgently needed.

Table 1. Current chemotherapy agents in OS [3, 4]

Surgery	
Limb-Salvage (Gold standard)	Remove the tumor and part of the surrounding tissue but preserve limb function.
Chemotherapy	**Mechanism of Action**
Methotrexate (MTX)	Folate anti-metabolite. Inhibits the function of dihydrofolate reductase, resulting in folic acid deficiency and cell death.
Leucovorin	Reduced folic acid. Used in combination with MTX to help decrease side effects.
Doxorobucin (Adriamycin)	Intercalates into the DNA and inhibits the function of Topoisomerase II stopping the process of transcription and replication.
Cisplatin	Forms intra-strand and inter-strands crosslinks with DNA and affects replication.
Ifosfamide	Analogue of cyclophosphamide and a nitrogen mustard-like alkylating agent. Crosslinks and inhibits the synthesis of DNA.

Emerging Therapies

Due to the lack of advancement in extending the survival rate of OS patients in the last 20 years; several groups are attempting to discover new therapeutic approaches. Some studies are focusing on testing immune system modulation strategies. For example, Kleinerman and colleagues demonstrated that the addition of Liposomal Muramyl Tripeptide (L-MTP-PE) to the therapeutic regimen targets and activates monocytes and macrophages, stimulating the immune system, resulting in the prolongation of relapse time among osteosarcoma patients [1, 6, 7]. L-MTP-PE has been approved by the European Union for the treatment of non-metastatic OS [8].

Additionally, there are ongoing clinical studies for the development of new small molecules that specifically target intracellular signaling pathways. The Sarcoma Alliance for Research through Collaboration (SARC) in conjunction with the Global Cooperative Network (GCN) is conducting a phase II clinical trial with saracatinib, a Src (tyrosine kinase involved in tumor cell motility and invasion) inhibitor, in OS patients with metastatic disease [3]. Preclinical work is also investigating the effectiveness of several mTOR inhibitors. mTOR is a serine-threonine kinase involved in regulation of protein synthesis, it has been related to metastatic OS and poor prognosis [1, 9, 10]. Combination of sorafenib and everolimus has exhibited complete inhibition of both mTORC1 and mTORC2 [3, 11]. Grignani et. al., reported that the combination of these two agents displayed some effect in a study of 38 patients with relapsed and non-resectable high-degree OS [12] .

Bisphosphonates, which inhibits the bone resorption of osteoclast, have been considered as a new therapeutic approach for OS [3]. To date, Zolendronate is being

tested in two different ongoing clinical trials for OS treatment, with the aim to determine optimal dose and disease-free survival, respectively.

Gemcitabine

Gemcitabine (GCB) (2'-Deoxy-2',2'-difluorocytidine, dFdC or GCBdFdC) is a deoxycytidine analogue that has been shown to have a broad range of antitumor effects in various types of tumors including pancreatic cancer, breast cancer, ovarian cancer and lung cancer [13-16]. GCB has three different mechanisms of action: 1) incorporates into the DNA and inhibits DNA synthesis; 2) prevents DNA repair and 3) through the inhibition of Ribonucleotide Reductase [17]**.**

GCB is a pro-drug that once incorporated into the cell membrane by nucleoside transporters is phosphorylated by deoxicytidine kinase (DCK) to GCB monophosphate and then subsequently phosphorylated to GCB diphosphate (dFdCDP) and GCB triphosphate (dFdCTP) by nucleoside monophosphate and diphosphate kinase, respectively (Figure 2) [17]**.** dFdCTP incorporates into the DNA and interferes with DNA synthesis and cell growth, causing cell death.

A second mechanism of action for GCB that has been identified is termed "masked chain termination". GCB being a nucleoside analogue is incorporated at the end of an elongating DNA strand, after which one more deoxunucleotide is added, thus ending the DNA strand. This process effectively locks the drug into the DNA chain as the exonucleases that edit DNA polymerization are unable to remove the GCB nucleotide from this specific position, preventing DNA repair [17].

GCB possesses a third mechanism of action. Through accumulation of dFdCDP, the action Ribonucleotide Reductase enzyme is inhibited, the levels of deoxycytidine

triphosphate (dCTP) are decreased; dCTP is involved in the inhibition of DCK (which phosphorylates GCB). Therefore, decreased cellular levels of dCTP results in GCB self-potentiation and incorporation into the DNA [17]. GCB also perpetuates its cytotoxic activity by inhibiting deoxycytidine monophosphate deaminase, which is involved in GCB degradation.

Preclinical studies in our laboratory have had success with the use of aerosol GCB in the treatment of metastatic OS lung disease. Decrease in both primary tumor and OS lung metastasis suggest GCB aerosol as a potential new treatment option [18, 19]. However, the efficacy of this drug in the treatment of OS lung metastasis is decreased by the acquired resistance of some OS cells to treatment, suggested by the residual tumors after treatment.

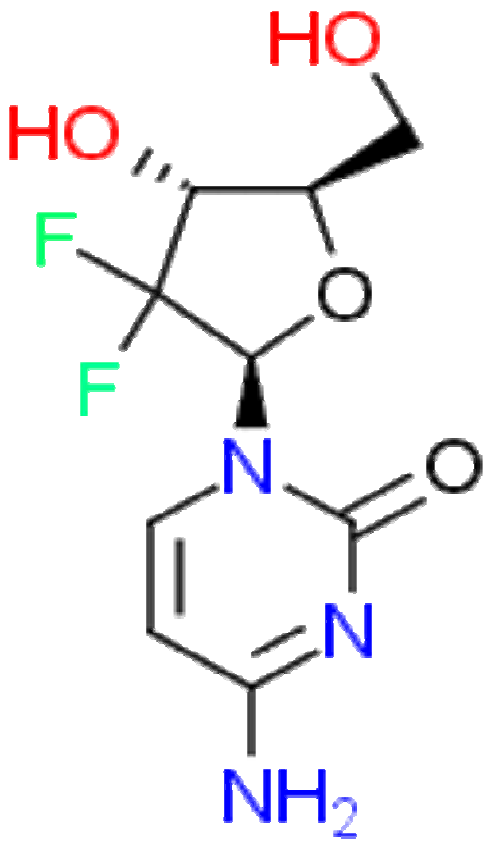


Figure 1. Molecular structure of Gemcitabine. (A) GCB (Gemzar; Eli Lilly and Company) is categorized as a nucleoside analogue with the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms. *Adapted from pubchem.ncbi.nlm.nih.gov*

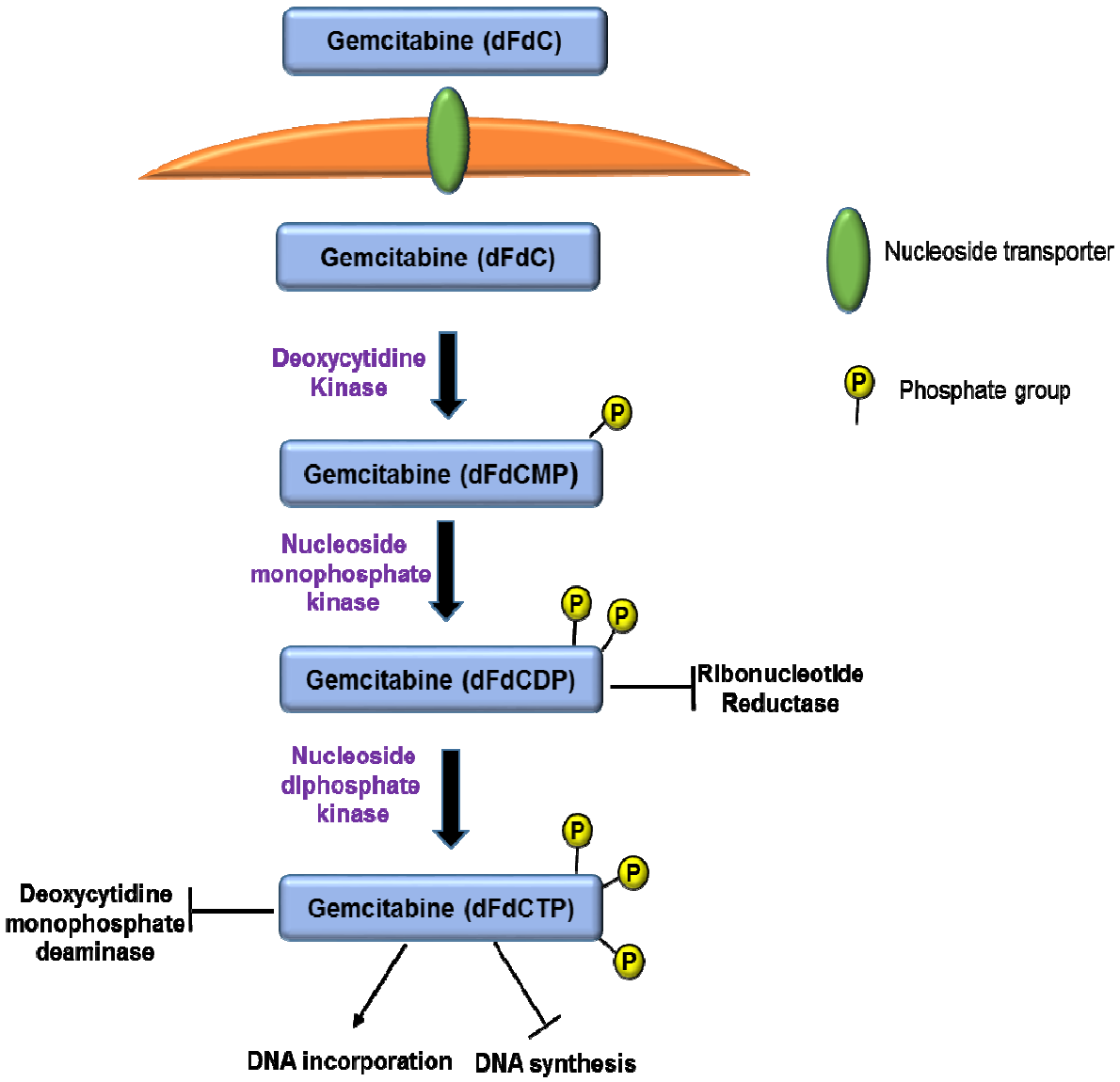


Figure 2. Mechanism of action of Gemcitabine. GCB is transported into the cell membrane by nucleoside transporters. Following influx into the cell, GCB must be phosphorylated by deoxycytidine kinase and converted into GCB monophosphate (dFdCMP). It is then phosphorylated to the active GCB diphosphate (dFdCDP) and GCB triphosphate (dFdCTP) by other kinases. The active form of GCB is then accumulated in the cells and incorporated into the DNA, leading to inhibition of DNA synthesis.

Autophagy

One of the major challenges in cancer treatment is acquired resistance by cancer cells to chemotherapy. Therefore, there is an increased interest in determining the molecular mechanisms implicated in cancer cell resistance to chemotherapy. Autophagy has been described in several cancers as a protective mechanism and thus it has recently become one of the most studied cell mechanisms in the cancer research community.

Autophagy, a Greek word derived from Auto or “self” and phagy or “eating” was coined by Christian de Duve in 1963 [20]. It is a catabolic mechanism that delivers unwanted cytoplasmic components such as long-lived proteins and damaged organelles to the lysosomes for degradation. Autophagy is activated as a defense mechanism in response to adverse conditions and a variety of stressors such as nutrient deprivation, hypoxia and cytotoxic effects, to promote cell survival [2, 21]. Even though studies have suggested that autophagy enhances cell survival, it also, at high levels, can lead to cell death, recognized as autophagic cell death or programmed cell death (PCD) type II [21].

There are three different types of autophagy that has been described based on the mechanism by which the material is delivered to the lysosome; they are: 1) micro-autophagy, 2) chaperone-mediated autophagy (CMA) and 3) macro-autophagy [22, 23].

Microautophagy entails the direct engulfment of cytoplasmic material into the lysosome. Cellular content is sequestered into the lysosome through invagination of the lysosomal membrane [20, 23]**.**

CMA is a process where cytosolic proteins that carry the KFERQ pentapeptide motif are recognized by a chaperone, such as heat shock protein 70 (HSP70). This facilitates the delivery of the protein to the lysosomal membrane protein 2A (LAMP2A), which functions as a receptor in the lysosomal membrane and aids the translocation of the protein into the lysosome for degradation [20, 23]**.**

Macroautophagy (hereafter referred as autophagy) is the most studied type of autophagy. It refers to an evolutionary conserved process that maintains cellular homeostasis through turnover and degradation of unwanted/damaged proteins and organelles. The cellular material is delivered into double membrane vesicles, called autophagosomes. The autophagosome then fuses with the lysosomes to form the autophagolysosomes, where the sequestered material is degraded by hydrolases (Figure 5). The degradation of this material produces amino acids and fatty acids that provide energy to the cell [23-25].

Molecular mechanism of autophagy

The process of autophagy comprises several steps that are regulated by more than 30 autophagy related genes (ATGs). These steps are divided into; induction, nucleation, elongation, maturation of the autophagosome, autophagosome-lysosome fusion and degradation (Figure 5) [26].

Phagophore Formation

The process of autophagy is initiated by the formation of the phagophore. Although the origin of the phagophore is not completely understood, it is thought that it is derived from endoplasmic reticulum, trans-Golgi and endosomes [20].Formation of

the phagophore in yeast is regulated by the ATG1/ATG13/ATG17 complex [20, 26]. In mammals, it requires the ULK1, ULK2 (unc-51-like kinase), mATG13 and FIP200 (focal adhesion kinase family –interacting protein of 200 kDa) complex. This complex is regulated by mTOR (Figure 3). Under stressful conditions, such as nutrient deprivation, mTORC1 dissociates from the ULK1/2 complex. This leads to partial dephosphorylation and activation of ULK1/2, mATG13 and FIP200, resulting in the activation and localization of the ULK complex to the phagophore and triggering of the downstream events that induce autophagy [20, 26].

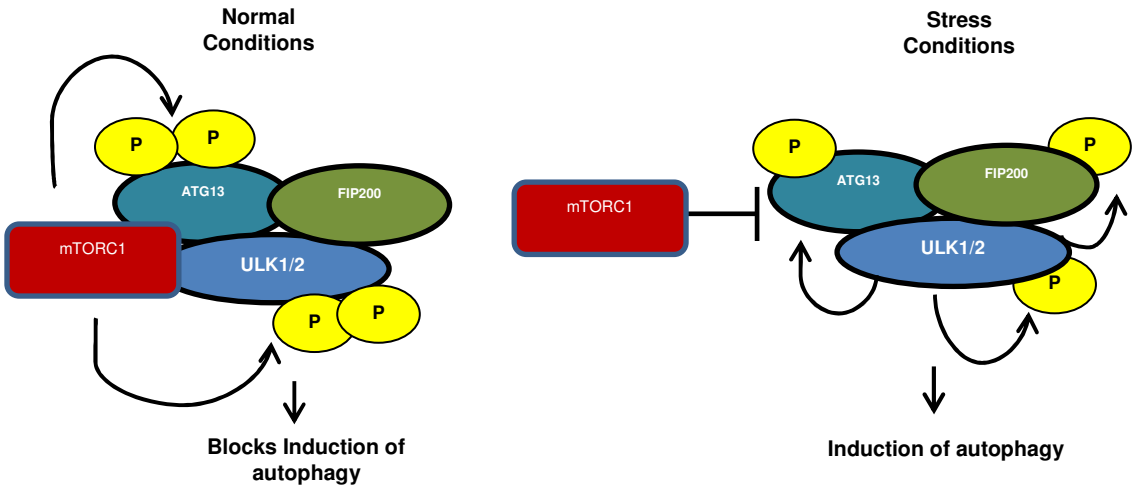


Figure 3. Regulation of the ULK1 complex by mTOR. Under normal conditions mTORC1 interacts with and phosphorylates ULK1/2 and ATG13. Under stressful conditions, mTORC1 is released from the complex, ULK1/2 is dephosphorylated and activated, which subsequently induces ULK to phosphorylate ATG13, FIP200 and itself, leading to induction of autophagy.

Class III phosphatidylinositol 3 kinase (PI3K) complex

The Class III phosphatidylinositol 3-kinase (PI3K) complex consists of the PI3K protein, Vps34, Beclin and p150 and ATG14/Barkor. Once Vps34 is activated it produces phosphatidylinositol-3-phosphate (PI-3P) [20]**.** This molecule is incorporated into the autophagosome and although its precise role is still unclear, it is suggested to be required for nucleation of the phagophore and recruitment of other ATGs [**19**]. Studies have reported additional regulators and binding partners of Beclin such as the ultraviolet (UV) radiation resistance-associated gene (UVRAG), AMBRA and Bif-1 that can also be part of the complex and positively regulate the formation of the phagophore [20]. The phagophore expands and recruits cytoplasmic proteins and organelles, thus sequestering the cargo in a double membrane called the autophagosome.

Elongation and autophagosome formation

The next step involves the formation and elongation of the phagophore membrane. There are two essential ubiquitin-like conjugation complexes involved in the formation of the autophagosome: the ATG12-ATG5 complex and the LC3-PE complex (Figure 4) [20, 26]**.**

ATG12-ATG5 Complex

The formation of the first complex requires the E1-like ATG7 to bind to the carboxyterminal glycine residue of ATG12.This binding activates ATG12 and allows the subsequent binding with ATG10 through a thioester bond. Finally, the ATG12-ATG5 complex binds to ATG16L, forming the ATG12-ATG5-ATG16L complex which is

required for the recruitment of microtubule-associated protein light chain 3-II (LC3II) [20]. The ATG12-ATG5-ATG16L complex associates with the phagophore and it is thought to assist with the curvature of the expanding phagophore. Once the autophagosome is formed, this complex dissociates from the membrane [20, 27].

LC3-PE Complex

The second complex is the LC3-phosphatidylethanolamine (PE) complex. LC3 is expressed as a cytosolic protein, upon autophagy induction; it is cleaved to LC3I by the action of ATG4. LC3I is activated by ATG7 in a similar way that the ATG12 and then transferred to ATG3 where it is phospholipidated, resulting in LC3II [20, 26]. LC3II is then translocated from the cytoplasm into the autophagosome membrane to assist in the elongation process. Simultaneously, p62 targets and binds to damaged proteins and organelles to be engulfed by the autophagolysosome [20].

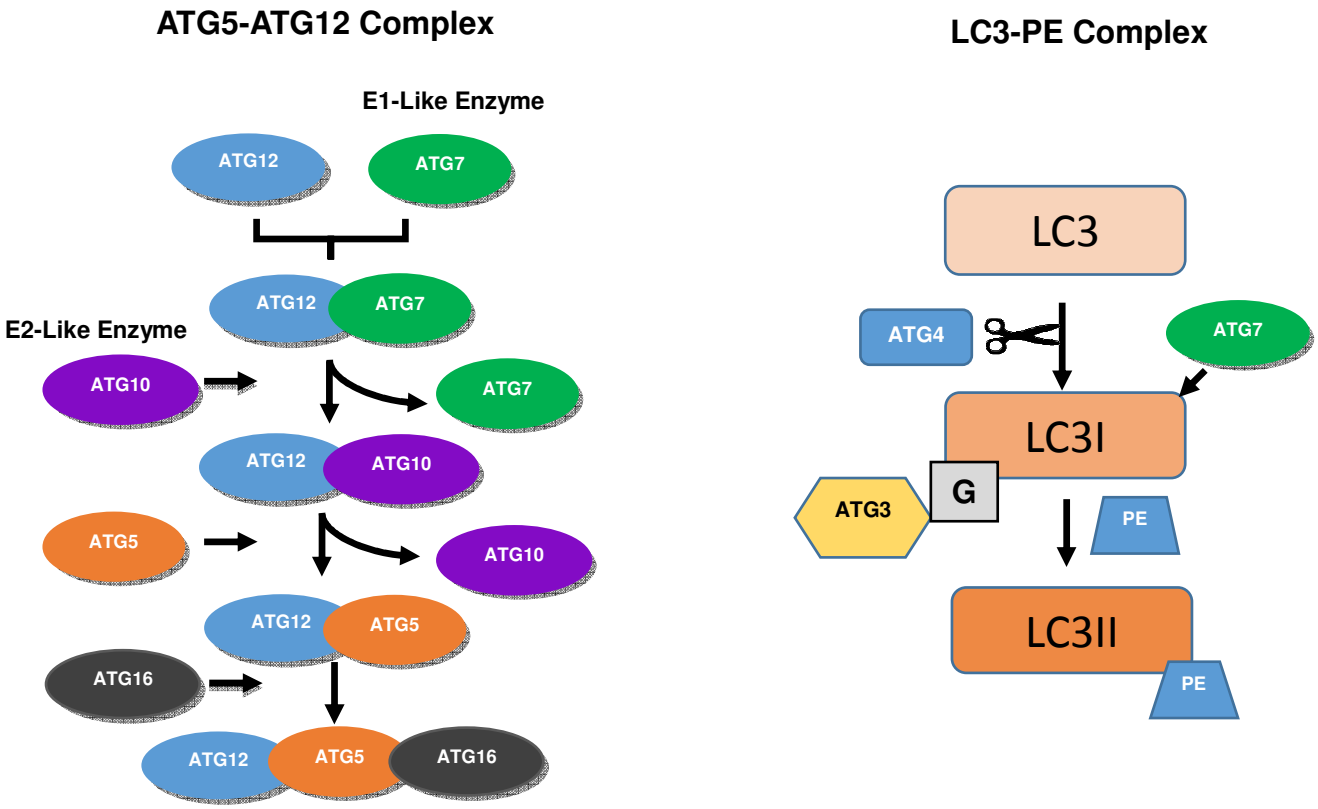


Figure 4. Conjugation of the ATG5-ATG12 and LC3-PE complexes. (A) ATG5 and ATG12 interact with each other as a result of a series of reactions mediated by ATG7 (E1-like enzyme) and ATG10 (E2-Like enzyme). The ATG5-ATG12 forms a complex with ATG16, forming a multimeric ATG5-ATG112-ATG16 complex that binds to the membrane of the phagophore, allowing its expansion and formation of the autophagosome. (B) The LC3 pathway involves the cleavage of LC3 by ATG4, resulting in LC3 which is then phospholipidated and converted into LC3II.

Maturation and fusion of the autophagosome with the lysosome

At this stage, the outer membrane of the lysosome fuses with the autophagosome, forming the autophagolysosome. Proteolytic enzymes proceed to degrade the content and the final product is recycled and reused to generate amino acids or to produce adenosine triphosphate (ATP) for use in cell survival [20].

Importantly, since LC3 binds to the membrane of the autophagosome, it is considered as a marker of the autophagosome and reflects induction of autophagy. Additionally degradation of p62 and formation of double membrane autophagic vacuoles are considered as the most common molecular and morphological markers of autophagy.

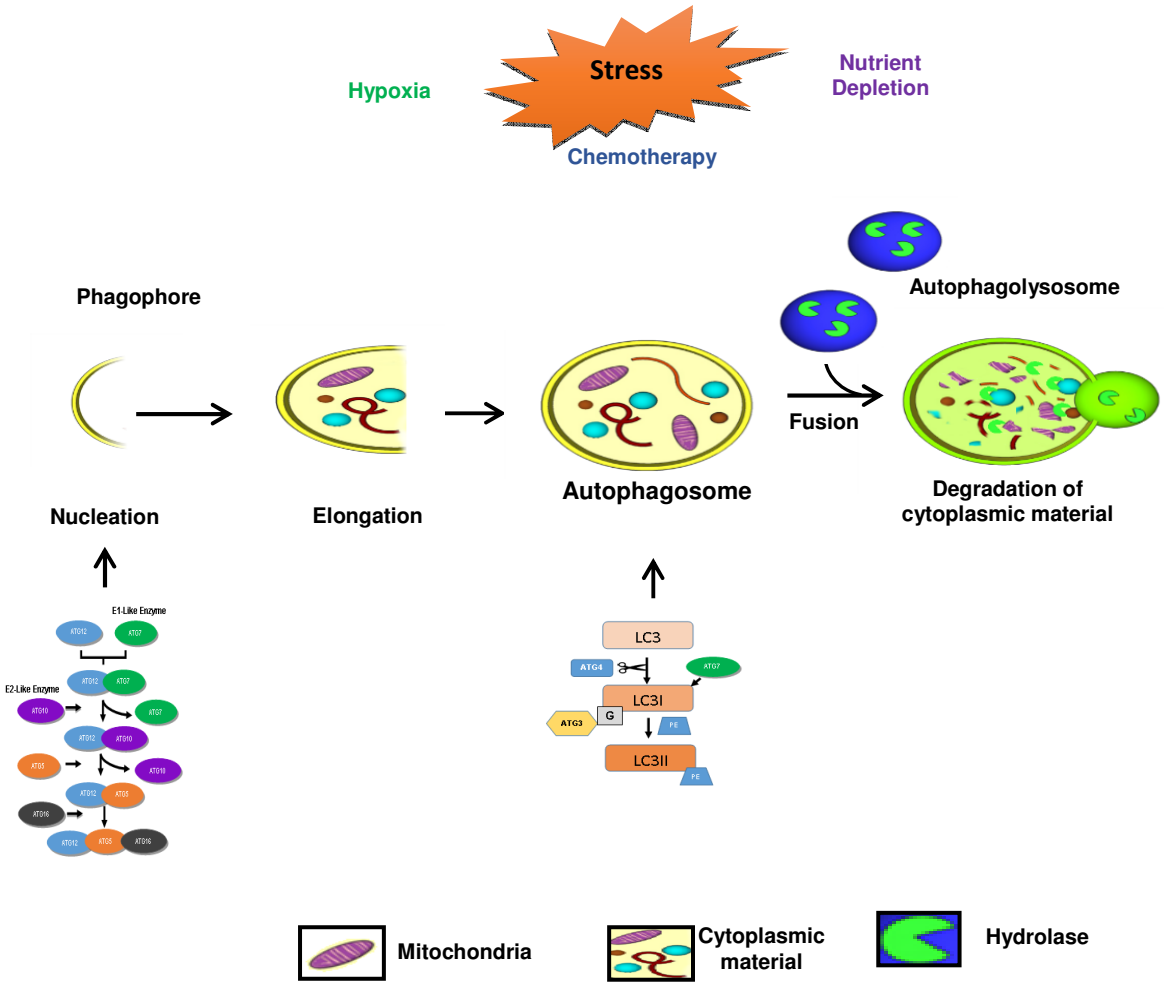


Figure 5. Process of autophagy. Autophagy begins with the formation of the phagophore membrane (nucleation), the phagophore membrane expands and sequesters cytoplasmic material becoming a double membrane called the autophagosome (elongation). The autophagosome membrane fuses with the lysosomes, forming the autophagolysosome where the sequestered material is delivered for degradation by hydrolases. Finally, this material is broken down into the basic components of proteins and recycled as a source of energy.

Regulation of autophagy

Induction of autophagy by mTOR

Several signaling pathways have been identified which regulate the process of autophagy. However, the most common negative regulator of autophagy is the mTOR pathway [27]. mTOR is a serine/threonine kinase involved in the regulation of different cellular functions such as; survival, protein synthesis, metabolism and autophagy [28]. mTOR forms two complexes: mTORC1 and mTORC2. Under normal conditions, when nutrients are available, mTORC1 serves as a sensor of nutrient signaling, and it is responsible for autophagy inhibition. mTORC1 inhibits the formation of the autophagosome by phosphorylation of ULK1 and ATG13 [29]. Under stressful conditions such as nutrient deprivation, mTOR is inhibited and autophagy is activated, providing the cells energy to sustain cell survival [26]**.**

Induction of autophagy by kinases

AMPK

Additional signaling pathways can regulate autophagy. Adenine monophosphate –activated protein kinase (AMPK) which is a cellular energy sensor; has been described as an essential regulator of the process of autophagy through different mechanisms [30]. It has been demonstrated that activation of AMPK in response to stress or low ATP, inhibits mTOR activity through phosphorylation of TSC2 (tuberous sclerosis complex-2) [2, 26, 30, 31]. It was recently discovered that, similar to mTORC1, AMPK can regulate autophagy through direct phosphorylation of ULK1. Egan et al. based on systemic mutagenesis analysis and *in vitro* phosphorylation came to the conclusion that there are two main phosphorylation sites in ULK1:Ser317 and

Ser777 [30-32]. Another potential target of AMPK is the transcription factor FoxO3a. Chiacchiera et al., demonstrated that activation of AMPK resulted in accumulation of FoxO3a and autophagy genes including ATG5, Beclin and ATG7 [33].

JNK

Activation of C-JUN N-terminal kinase (JNK) also leads to autophagy activation. One of the mechanisms by which JNK contributes to autophagy induction is through phosphorylation of the anti-apoptotic molecule BCL2 [26]. Phosphorylation of BCL2 prevents its binding to Beclin, allowing Beclin to form a complex with PI3K and induce autophagy. This has been demonstrated by the work of Klein et al., who identified JNK as essential regulator of oncolytic adenovirus-induced autophagy through the phosphorylation of BCL2 in glioblastoma multiforme [34]. Besides regulating BCL2, JNK can also regulates Beclin through phosphorylation of c-Jun. JNK can regulate the expression of autophagy genes (p62, ATG5 and ATG7) [26].

ERK

Extracellular signaling-regulated kinase (ERK) is an important serine/threonine kinase that regulates different cellular processes such as migration, proliferation and apoptosis [26]. Sivaprasad and colleagues demonstrated that TNKFα-induced autophagy in MCF-7 breast cancer cells is through activation of ERK [35]. In accordance with this, Ellington et al., reported that ERK induces autophagy in human colon cancer cells in response to soyasaponins [36]. Recent studies have shown that ERK is also involved in the formation and maturation of autophagic vacuoles [26].

Autophagy in Cancer

Autophagy functions to protect cells against various pathological and physiological diseases. For example in neurodegeneration; autophagy regulates the clearance of aggregate-prone proteins that cause neurodegenerative diseases such as Parkinson's disease [2]. Autophagy dysfunction or deregulation is linked to various diseases including cancer [31].The importance of autophagy in cancer has received increased interest within the scientific community in recent years, suggested by the numerous ongoing clinical trials in melanoma, myeloma , renal cell carcinoma and other cancer types, which involve manipulation of autophagy as potential cancer treatment [37-41]. The role of autophagy in cancer has been described as a complex process exhibiting both cell death and cell survival functions [42]**.** Currently, there is evidence to suggest the role of autophagy as a tumor suppressor in early stages of the disease and as a cell survival mechanism in advanced stage of cancers. It can exert this dual role in different ways and in a context-dependent manner, determined by stage of the disease, type of cell, tissue of origin and type of stress [29, 42-44]. The mechanisms underlying the regulation of this dual role are still unknown.

Autophagy as a cell survival mechanism

In normal cells, basal levels of autophagy are involved in cell homeostasis, protein quality control, survival and proliferation [45]. The pro-survival role of autophagy has been supported by studies demonstrating that organisms with loss of function of genes that are essential for the process of autophagy have been shown to die rapidly. Specifically, mice lacking Beclin die during embryonic development, while mice lacking ATG5 die during neonatal period [46-48].

In cancer cells, autophagy is upregulated under stressful conditions such as hypoxia, nutrient deprivation as well as chemotherapy. This up-regulation confers stress tolerance, which leads to cell survival and decreased cell death. The need for autophagy in cancer cells is supported in different studies. Qadir et al, showed that inhibition of different autophagy genes such as Beclin, ATG5 and ATG7, in tamoxifen – resistant MCF-7 breast cancer cells resulted in increased cell death, suggesting autophagy as a pro-survival mechanism [49]. Similarly, in pancreatic cancer cells, genetic and pharmacologic inhibition of autophagy was shown to inhibit cell growth and induce tumor regression *in vivo*. These results suggest that pancreatic cancer cells require autophagy to grow [50]. Additionally, recent studies show that inhibition of autophagy in cancer cell lines with activated Ras mutation resulted in suppression of cell growth. These results suggest that Ras-driven cancers require autophagy for tumor cell survival [50].These examples validate the idea that cancer cells utilize autophagy as a mechanism that sustains cell survival under stressful conditions such as chemotherapy.

Autophagy as a cell death-promoting mechanism

Chemotherapy is the standard and most effective cancer treatment. This type of treatment works by inducing cell death [25, 51]. Apoptosis (Type 1 cell death) and necrosis were the first two types of drug-induced cell death mechanisms to be discovered. Autophagy is now considered as type II programmed cell death (PCD) or autophagic cell death based on features and morphologic characteristics observed in dying cells [48]. High levels of autophagy or defective apoptosis leads to cell death due to the consumption and degradation of cellular proteins and organelles that exceed the capacity of the cell [2, 50]. Shen and Codogno have defined autophagic cell death as 1)

an apoptotic independent process ; 2) with increased levels of autophagic flux; and 3) suppression of autophagy by either genetic of pharmacologic approaches inhibits cell death [52].

In cancer cells, drug-induced autophagic cell death is cell and context dependent. In breast cancer, silencing ATG5 in MCF-7 cells with no Bcl2 (anti-apoptotic protein) inhibited doxorobucin induced autophagic cell death [53]. In lung cancer, Kim et al., have shown that H460 cells are more sensitive to radiation treatment when Z-DEVD, a caspase inhibitor and RAD001, an mTOR inhibitor were combined [54]. These data indicate that in addition to the survival role of autophagy there is also a cytotoxic function.

Autophagy in OS

The presence of autophagy in OS has been confirmed by various studies. However, the functional role of autophagy in OS is controversial. Shen and colleagues have demonstrated that cisplatin, one of the agents included in the standard treatment of OS, induces autophagy in SaOS-2 cells and promotes cell survival [55]. Inhibition of autophagy by chloroquine, resulted in cell death.

Conversely, Wang et al., reported that Riccardin D induces cell death through the activation of both apoptosis and autophagy mechanisms in different OS cells [56]. In addition, it has been reported that the plant alkaloid vocammine (VOA) induced autophagic cell death in doxorobucin-resistant U2OS human OS cells [57].

In OS, the Beclin-associated autophagy related key regulator (Barkor) has been shown to play an important role in autophagy induction. Knockdown of Barkor in U2OS cells resulted in decreased autophagy as evidenced by decreased autophagosome formation. In addition, the retinoblastoma (Rb) gene has been shown to repress E2 transcription factor 1 (E2F1), allowing the activation of the Beclin/Vps34 complex, resulting in autophagy induction [58].

Huang et. al., have demonstrated that the nuclear protein, high mobility group box 1 (HMGB1), induces autophagy by regulating the interaction of Beclin with the ULK1-ATG12-FIP200 complex. In accordance with this, knockdown of HMGB1 in three human OS cell lines (MG63, SaOS and U2OS) showed a significant increase in drug sensitivity [59, 60].

Overall, there is enough evidence to demonstrate autophagy induction in OS through different mechanisms. However, the role of autophagy in cancer, including in OS, is complex. It can either increase cell survival or induce cell death. Modulation of

autophagy by either increasing autophagic cell death or decreasing autophagic cell survival may be an alternative to improve the efficacy of chemotherapy.

Modulation of autophagy

Since the process of autophagy is essential for normal cell function and deregulation of autophagy has been associated with various pathological and physiological diseases, modulation of autophagy has recently received a great deal of attention by the scientific community and is an area of intensive study. This has resulted in the discovery of a variety of autophagy modulators.

Autophagy inhibitors

There are several different pharmacologic inhibitors that have been identified and are classified as early or late stage autophagy inhibitors. Some of the early stage inhibitors are 3-MA and wortmannin which interfere with Vps34. Late stage inhibitors include bafilomycin which inhibits vacuolar ATPase [50]. However, chloroquine (CQ) and its analogue hydroxychloroquine (HCQ), have been evaluated in humans, with HCQ the most commonly used. These two drugs prevent the acidification of the lysosomes which in turn blocks its fusion with the autophagosome, preventing the degradation of the cargo. The use of HCQ in a preclinical setting has demonstrated to augment the efficacy of various chemotherapeutic agents[[50]. However, one of the major concerns is that in order to block autophagy, high concentrations (micromolars) of HCQ are required. Therefore, Amaravadi and colleagues have developed Lys05, a more potent lysosomal autophagy inhibitor. Compared to HCQ, Lys05 has been shown to have a more potent anti-tumorigenic effect both *in vitro* and *in vivo* as a single agent [61].

In addition to pharmacological inhibition of autophagy, numerous studies have used genetic inhibition as another approach to ablate autophagy. Knockdown of ATGs such as ATG5 has been shown to effectively inhibit autophagy and exert an effect in cell death and tumor regression in various types of cancer. Amaravadi et al. demonstrated that shATG5 enhanced the sensitivity of lymphoma cells to cyclophosphamide in a similar way to CQ [50, 62].

Autophagy Inducers

On the other hand, excessive autophagy can lead to cell death. Thus, autophagy inducers have also been considered as potential anti-cancer treatment. It is well established that inhibition of mTOR induces autophagy. Therefore, mTOR inhibitors have been proposed as potential anti-cancer drugs. The most widely used autophagy inducers are Rapamycin and its analogues temsirolimus (CC-779), everolimus (RAD-001) and deforolimus (A3-23573), although these autophagy inducers have had limited efficacy in clinical trials [50]. The antidiabetic drug, metformin, has also been shown to enhance autophagy through the suppression of mTOR activity [50]. Shi et al., have shown that metformin increases the sensitivity of lymphoma cells and inhibits their growth via activation of AMPK which leads to the inhibition of mTOR and activation of autophagy [63].

Even though the use of these autophagy modulators have shown to exert an effect on enhancing the efficacy of chemotherapeutic agents, there is still a need for more research to define the complex functions of the autophagy process in cancer. As discussed above, autophagy can either induce cell death or cell survival. Thus. It is necessary to define the role of autophagy in order to introduce the manipulation of

autophagy as a potential strategy for cancer treatment. Currently, there are several ongoing clinical trials evaluating the effectiveness of these modulators. To date, there are no studies with a particular focus in OS.

Aim of the study

OS is the most common type of bone cancer, causing morbidity and mortality in children and adolescents. With the standard treatment regimens, the survival rate of patients with localized disease is approximately 70%. However, in patients with OS lung metastasis, the clinical outcome remains poor. A better understanding of key molecular pathways responsible for metastatic OS cells resistance to chemotherapy will lead to the development of novel therapeutic approaches.

Accumulating evidence suggests autophagy as a pro-survival and resistance mechanism against cancer therapeutics in different types of cancers. Therefore, this makes autophagy a valuable target in cancer cells. To date, little is known about the process of autophagy in OS. We undertook this project to evaluate autophagy as a process that protects OS cells from the cytotoxic effects of GCB allowing them to survive and circumvent drug induced-apoptosis. The first goal of our research was to confirm the induction of autophagy after GCB treatment in a panel of different OS cells. After establishing whether GCB induces autophagy in OS cell lines, we set to investigate the molecular mechanism underlying the induction of autophagy in GCB-treated OS cells. Further, we wished to determine the role of autophagy in the sensitivity of OS cells to GCB. Understanding the process of autophagy and the molecular pathways that regulates autophagy will allow identification of a mode to modulate these pathways in order to enhance the activity of chemotherapy. **Our central hypothesis is that GCB-induced autophagy leads to OS cell survival and decreased sensitivity to GCB.**

Chapter 2: Materials and Methods

Osteosarcoma cell lines and reagents

Human LM7 metastatic OS cell line was created in our laboratory by intravenous recycling of the parental cell line SAOS-2 through the lungs of nude mice. CCH-OS-D cells were kindly provided by Dr. Dennis Hughes (Pediatrics Research Department, The University of Texas MD Anderson Cancer Center). This cell line was derived from an 18-year-old male patient with lung metastases. The K7M3 cell line was derived from the K7M2 cell line by harvesting the lung metastasis of BALB/C mice that had been previously injected intravenously [64]. DLM8 is a highly metastatic cell line that was established by Asai and colleagues from LM8 and Dunn cell lines [65]. The established OS cell line MG63 was obtained from ATCC. All cell lines were tested for mycoplasma. A summary of each cell line is shown in Table 4. The list of reagents used in this project is presented in Table 2, while the list of antibodies is shown in Table 3.

Table 2. List of Reagents

Reagent	Company
2-mercaptoethanol	Fisher Scientific
Acridine Orange	Sigma-Aldrich
Alexa 488	Life Technologies
Ammonium persulfate	BioRad
Annexin V-FITC Apoptosis detection kit	Calbiochem
BCA Protein Assay	Thermo Scientific
Bromophenol blue	Sigma
DMEM	Hyclone GE
Ethanol	Decon Laboratories, Inc
Fetal Bovine Serum	Gibco
Gemcitabine	Eli Lilly
Glycine	GenDepot
Hematoxylin	Sigma-Aldrich
Hydroxychloroquine	Sigma-Aldrich
L-glutamine	Lonza
Methanol	Fisher Scientific
Nitrocellulose membranes	BioRad
PBS	Hyclone GE
RIPA Lysis Buffer	Santa Cruz Biotechnology
SDS-PAGE standards	BioRad
Sodium dodecyl sulfate	Sigma-Aldrich
Trypan blue	Beckman Coulter
Trypsin-EDTA	Gibco
Trizma base	Sigma- Aldrich
Visualizer Western Blot (ECL)	GE Healthcare Life Sciences
Xylene	Sigma-Aldrich

Table 3. List of antibodies

Antibody	Host	Company	Catalog Number
LC3	Rabbit	Novus Biological	NB600-1384
ATG5	Rabbit	Novus Biological	NB110-53818
p-HSP27 Ser78/82	Rabbit	R&D Systems	AF23141
Total HSP27	Mouse	Santa Cruz	sc-13132
Beclin	Rabbit	Santa Cruz	sc-11427
SQSTM1/p62	Rabbit	Cell Signaling	5114
AKT	Rabbit	Cell Signaling	4685
phospho-AKT	Rabbit	Cell Signaling	9271
mTOR	Rabbit	Cell Signaling	2983
Phospho-mTOR	Rabbit	Cell Signaling	2971
Phospho-p70S6k	Rabbit	Cell Signaling	9205
Cleaved-caspase3	Rabbit	Cell Signaling	9661

Cell culture

Each cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine serum (FBS), L-glutamine, sodium pyruvate, non-essential amino acids and minimal essential medium vitamin solution. Cells were maintained in a humidified incubator with 5% CO_2 at 37ºC. Each cell line was tested for mycoplasma contamination.

Table 4. Panel of Osteosarcoma cells

Cell Line	Type of Cell
K7M3	Mouse- Metastatic
DLM8	Mouse- Metastatic
LM7	Human - Metastatic
CCH-OS-D	Human Metastatic
MG63	Human- Metastatic

Gemcitabine preparation

Gemcitabine (Gemzar) was dissolved in phosphate buffer saline (PBS) to obtain a stock concentration of 38 mg/mL. It was then added to the cells to reach a final concentration of 0.5, 1, 5 or 10 µM and incubated for the desired period of time.

Autophagy modulation

Cells were treated after confluency with 15 or 20 µM of HCQ. For the dual treatment with GCB, cells were first pre-treated with 20 µM of HCQ for 30 minutes prior to GCB treatment.

Cell viability assay

Cells were seeded in 12-well plates and allowed to attach at 37^0C / 5% CO_2 overnight. Cells were then treated with 1 µM of GCB or GCB coupled with 20 µM of HCQ, cells with no treatment served as control. Cells were trypsinized and viability was analyzed by trypan blue using a cell counter (Vi-cell, Beckman Coulter). Trypan blue exclusion assay is based on the principle that in live cells, which have an intact membrane, the dye is not absorbed. However, in dead cells the dye can be absorbed through the damaged cell membrane. All experiments were performed three times. The incubation time and concentration are specified in each figure.

Western blot analysis

After the corresponding treatment, whole cell lysates were prepared by lysing the cells with RIPA lysis buffer for 30 minutes and centrifuged at 10,000 g for 10 minutes at 4ºC. Supernatants were collected and protein concentration was determined using Bio-Rad DC protein assay kit (500-0113-0115). Protein samples in SDS loading buffer were boiled to 100° F during 5 minutes. Equal amounts of protein were subjected

to SDS-poly acrylamide gels (SDS-PAGE). Samples were separated via electrophoresis at a constant voltage of 90 V for approximately 2 ½ hr. Protein samples were then transferred onto nitrocellulose membranes (85V for 1 hr). Membranes were blocked in 5% milk or BSA during 1hr at room temperature and then incubated overnight at 4ºC with primary antibody (1:1000) against p-AKT, Akt, Beclin, cleaved caspase-3, LC3, mTOR, p-mTOR, p-70S6K, p62, HSP27 or p-HSP27. After overnight incubation with the corresponding primary antibody, membranes were washed with TBS-T and incubated for one hour with secondary antibody (rabbit or mouse) at room temperature. Signal was detected using ECL reagents (GE Healthcare Life Science). Actin was used as loading control.

Western Blot Densitometry

Western blots were analyzed using NIH Image J software. The intensity of each band was obtained as percentages and copied to Excel; the data was then compared to the corresponding band intensity values of actin.

Acridine Orange (AO) Staining

Acidic Vesicular Organelles (AVO) were visualized using Acridine Orange (AO). Following treatment with GCB, cell culture media was removed and cells were stained with 1mg/ml AO and incubated during 15 minutes at 37ºC. After incubation, cells were washed twice with PBS and examined under a fluorescent microscope. The autophagic lysosomes appeared as orange vesicles in the cytoplasm of the cells, while nuclei were stained green. For quantification, cells were analyzed on a FACS Calibur flow cytometer using Cell-Quest software (Becton Dickinson).

Annexin V and PI staining

Annexin/PI staining was used to determine the percentage of apoptotic cells. After treatment with GCB and/or HCQ, LM7 cells were washed with PBS and trypsinized. Cells were then resuspended in binding buffer and incubated for 15 minutes with fluorescence isotiocianate (FITC) conjugated annexin V and propidium iodide (PI). After incubation, Annexin-binding buffer was added to the samples. Cells were analyzed using a FACS Calibur flow cytometer using Cell-Quest software (Becton-Dickinson).

Electron Microscopy

Cells were seeded in 6-well plates and treated with GCB. After treatment, samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.3 and stored at 4°C. After fixation, samples were submitted to the MD Anderson electron microscopy core facility. At the core facility, Mr. Kenneth Dunner Jr, processed the samples. Briefly, samples were washed in 0.1 M cacodylate buffer and treated with 0.1% Millipore-filtered buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 30 minutes, and stained with 1% Millipore-filtered uranyl acetate. The samples were washed several times in water, then dehydrated in increasing concentrations of ethanol, infiltrated, and

embedded in LX-112 medium. The samples were polymerized in a 60^0C oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc.) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp).

Human Phospho-kinase Antibody Array

The human phospho-kinase Antibody Array kit was purchased from R&D systems. CCH-OS-D and LM7 cells were treated with GCB for 48 and 72 hrs respectively. Untreated cells were used as a control. The cells were collected and lysed using Lysis Buffer 6 provided by the manufacturer. Protein concentration was determined by Bradford Protein Assay (Bio-Rad). Nitrocellulose membranes containing different phospho-specific antibodies to each kinase in duplicate were blocked during one hour and then incubated with 400 μg of protein at 4ºC. After overnight incubation, the membranes were washed and incubated with Detection Antibody Cocktails of biotinylated antibodies. Finally, protein was detected by using HRP-conjugated streptavidin antibodies and chemiluminescent detection reagents.

RNA interference

Lentiviral Beclin and ATG5 shRNA (Open Biosystem) was used to knockdown BECN and ATG5 protein expression. Control cells were infected with lentivirus

containing empty shRNA vector. Briefly, 293T cells were transfected with the 7ug/ml of the plasmid, 5 ug/ml psPAX2 and 4 ug/ml pMD2. After 48 hrs, 2 ml of supernatant containing lentivirus was collected and used for infection by adding it to each well of a 6-well plate containing $1x10^5$ cells. Cells were incubated for 12 hrs and transferred to a 75 mm flask. Confirmation of Beclin and ATG5 protein knockdown was determined by western blot analysis.

Mice studies

Animal studies were performed in accordance with the institutional guidelines, approved by the MD Anderson Institutional Animal Care and Use Committee (IACUC). Female nude mice were purchased from the National Cancer Institute (NCI).

Tumor implantation and GCB treatment

Nude mice were injected with 3×10^6 LM7 cells via the tail vein. Formation of lung metastases was verified after five weeks by hematoxylin and eosin (H&E) staining. Mice were randomly divided in 2 groups and received 0.5 mg/kg twice weekly of aerosol GCB or PBS (as control) for six weeks.

BALB/C mice were inoculated with 0.5×10^6 K7M3 cells via the tail vein. Formation of lung metastases was verified after one week by H&E staining. Mice were also randomly divided in 2 groups and received 0.5 mg/kg twice weekly of aerosol GCB or PBS (as control) for four weeks.

Immunohistochemistry

Paraffin samples (lung tissue sections) were warmed for 15 minutes at 60°C and de-paraffinized in xylene, rehydrated in serial dilutions using ethanol and antigen retrieval. Sections were then incubated with 3% hydrogen peroxide followed by 4% fish gelatin. The primary antibody, polyclonal rabbit anti-LC3B, was applied to the sections and left overnight at 4˚C. The secondary antibody labeled with horseradish peroxidase was then applied at room temperature for 1 hour. The slides were then developed with 3, 3'-diaminobenzidine and counterstained with hematoxylin.

Immunocytochemistry

K7M3, CCH-OS-D and LM7 cells were grown on glass coverslips. After treatment with GCB, the media was removed; cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 15 minutes. After fixation, cells were washed with PBS and permeabilized with Triton X in PBS containing BSA for 30 minutes. Cells were washed again with PBS and incubated with anti-LC3 specific antibody, overnight. Cells were incubated with the secondary anti- rabbit and Alexa Fluor 488 for one hour at room temperature. Finally the cells were washed with PBS and immediately observed using a Leica DM5500B Microscope.

Statistical analysis

Data were expressed as an average of three independent experiments. Experimental data were analyzed using GraphPad PRISM Software. P-values <0.05 were considered significant.

Chapter 3: Sensitivity of osteosarcoma cells to Gemcitabine

Rationale and Aims

Previous work from our laboratory has demonstrated the significant therapeutic effect of GCB against OS cells [18, 19]. However, after GCB treatment a subset of cells persist as small isolated lung metastases *in vivo.* Initial studies were designed to assess the sensitivity of a panel of OS cells to GCB treatment. An *in vitro* dose response study was performed using various concentrations of GCB during different time points, and the effects on cell viability were analyzed. These dose response experiments were conducted on three OS cell lines: K7M3, CCH-OS-D and LM7 cells, with the purpose of evaluating the response of these metabolically different cells and to compare their sensitivity to GCB treatment.

To further our knowledge about the sensitivity of OS cells to GCB we designed experiments to assess whether apoptosis was induced following GCB treatment. OS cells were treated with GCB and western blot analysis was performed for caspase-3 cleavage (activation), which is an essential marker of the apoptotic process.

Results

Gemcitabine cytotoxic effect varies within OS cell lines

To investigate the sensitivity of OS cells to GCB; K7M3, LM7 and CCH-OS-D OS cells were treated with increasing concentrations of GCB (0.5 to 10 µM) for up to 72 hrs. GCB cytotoxic effects and induction of apoptosis were determined by trypan blue exclusion assay and western blot for cleaved caspase 3, respectively.

As shown in figure 6, a significant decrease in k7M3 cell viability was observed as soon as 24 hrs. When compared to the control, the viability of K7M3 cells did not show a significant decrease when treated with 0.5 µM. However, when treated with 1 µM, the viability decreased to 47% ($p<0.05$). Doses of 5 and 10 µM did not induce any additional decrease in viability (5 µM: 51%; 10 µM: 48% $p<0.05$). At 48 hrs a significant decrease in viability (44.33% $p<0.05$) was observed with 0.5 µM of GCB. Cells treated with 1, 5 and 10 µM further decreased cell viability (1 µM: 28.3%; 5 µM: 23.4%; 10 µM: 30.0% $p<0.05$). At 72 hrs the viability was 45.8% ($p<0.05$) when cells were treated with 0.5 µM of GCB. Higher doses further decreased cell viability (1 µM: 34.8%; 5 µM: 23%; 10 µM: 22.5% $p<0.05$).

Next, we wanted to determine whether the cytotoxic effect of GCB is due in part to apoptosis. As shown by western blot analysis, increase cleaved caspase 3 protein levels were observed when K7M3 cells were treated with different doses of GCB and compared to control cells.

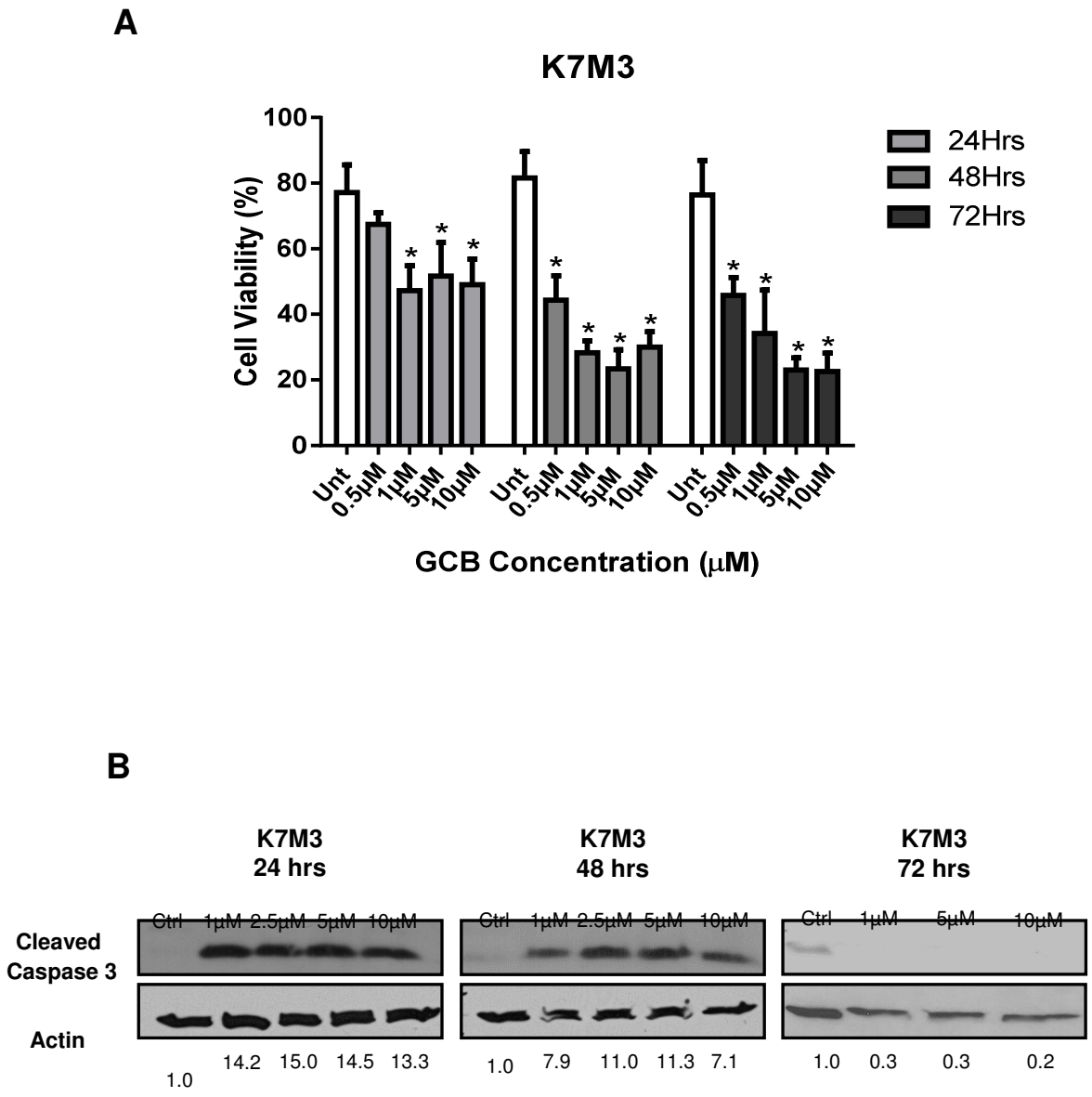


Figure 6. GCB decreased the viability of K7M3 OS cells and induced caspase 3 activation. Trypan blue exclusion assay was performed in order to assess the effect of different doses of GCB on cell viability over 24, 48 or 72 hrs period. (B) Analysis of GCB-induced OS cell death. Western blot analysis was performed using specific antibody for caspase 3. Actin was used as a loading control.

In response to increasing concentration of GCB, the viability of LM7 cells was significantly decreased over a period of 24 hrs when treated with 5 and 10 µM (5 µM: 56.0%; 10 µM: 53.8% $p<0.05$). No significant decrease in cell viability was observed when treated with 0.5 or 1 µM. After 48 hrs, cell viability significantly decreased for all GCB doses (0.5 µM: 68%; 1 µM: 70.5%; 5 µM: 67.2%; 10 µM: 56.05% $p<0.05$). After 72 hrs, cell viability was significantly reduced for all treatment doses (0.5 µM: 58.65%; 1 µM: 56.57%; 5 µM: 54.27%; 10 µM: 39.25% $p<0.05$).

Analysis of expression of cleaved caspase 3 by western blot, revealed that compared to untreated group, GCB induced cleaved caspase 3 starting at 24 hrs with a marked increased at 48 hrs, followed by a decreased at 72 hrs.

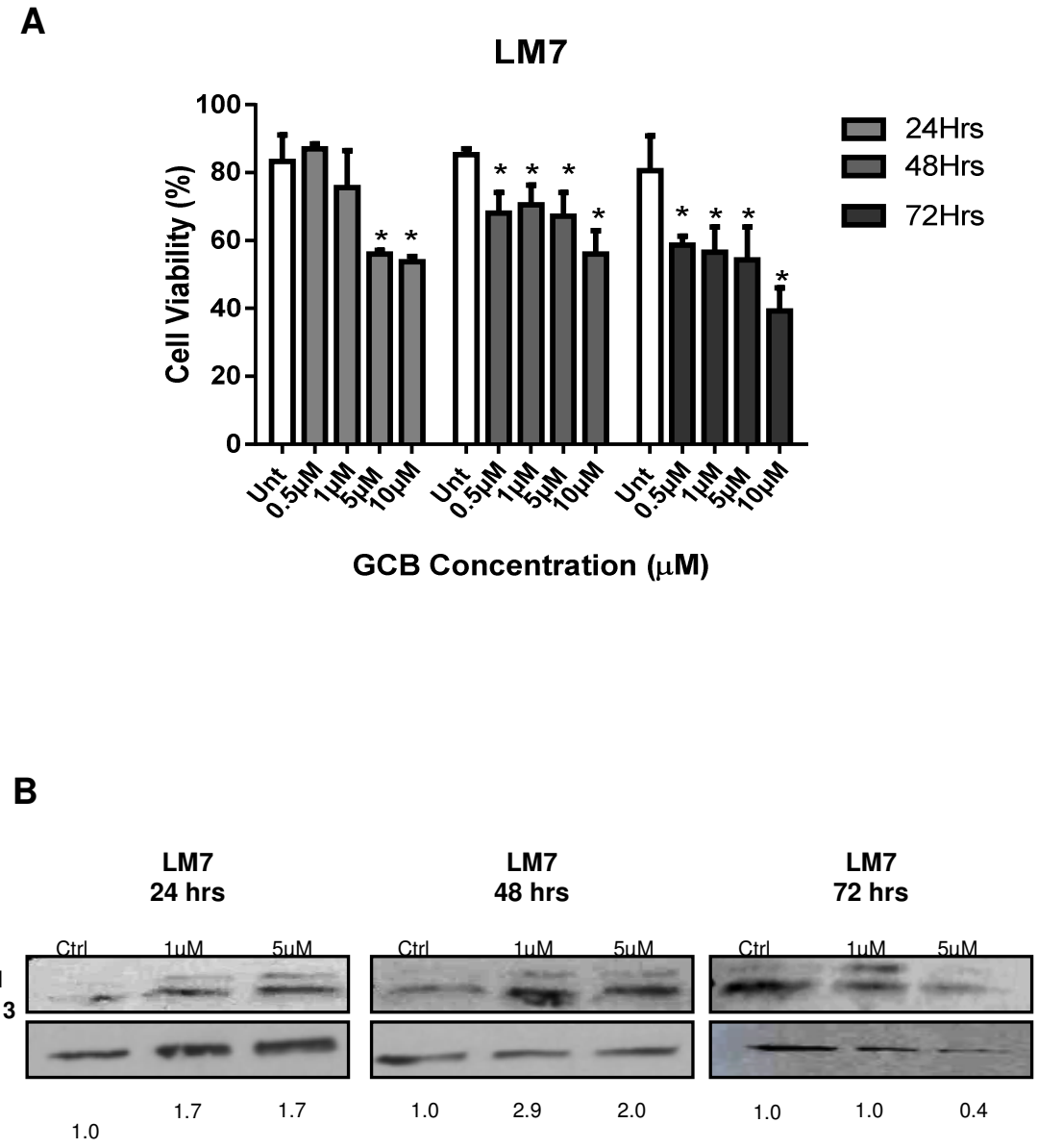


Figure 7. GCB decreased the viability of LM7 OS cells and induced caspase 3 activation. Trypan blue exclusion assay was performed in order to assess the effect of different doses of GCB on cell viability over 24, 48 or 72 hrs period. (B) Analysis of GCB-induced OS cell death. Western blot analysis was performed using specific antibody for caspase 3. Actin was used as a loading control.

After 24 hrs of GCB treatment the viability of CCH-OS-D cells was significantly reduced for the 5 and 10 µM doses (5 µM: 73.97%; 10 µM: 49.45% $p<0.05$). No significant reduction in viability was observed for the lower doses of 0.5 and 1µM (0.5µM: 73.23%; 1µM:72.73%). At 48 hrs, significant decrease in cell viability was observed for all treatment doses (0.5 µM: 56.33%; 1.0 µM: 50.5%; 5 µM: 52.67%; 10 µM: 45.1% $p<0.05$). Similar results were observed after 72 hrs of GCB treatment (0.5 µM: 26.73%; 1.0 µM: 26.03%; 5 µM: 22.67%; 10 µM: 19.53% $p<0.05$). In CCH-OS-D, expression of cleaved caspase 3 was also increased followed GCB treatment.

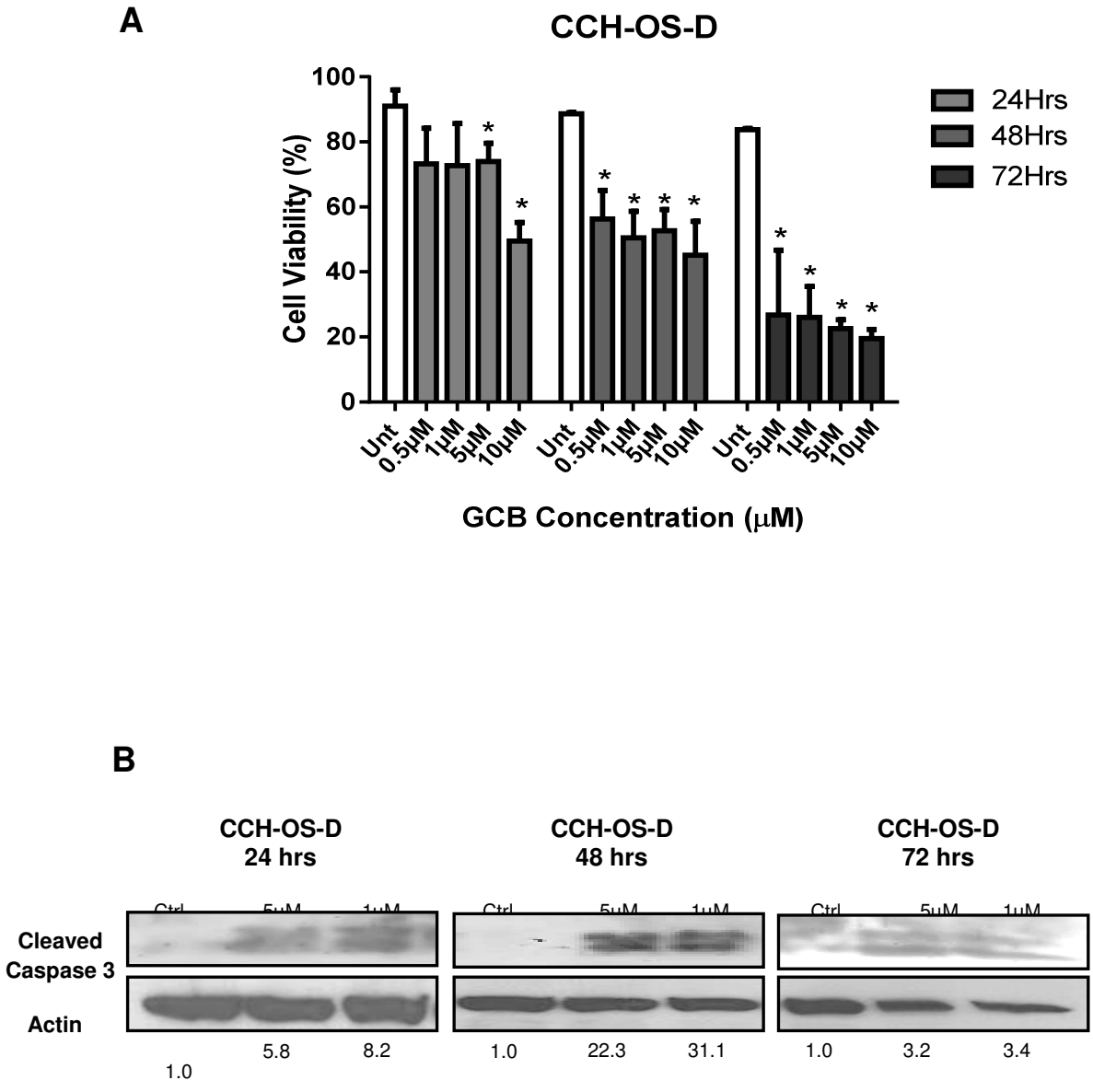


Figure 8. GCB decreased the viability of CCH-OS-D OS cells and induced caspase 3 activation. Trypan blue exclusion assay was performed in order to assess the effect of different doses of GCB on cell viability over 24, 48 or 72 hrs period. (B) Analysis of GCB-induced OS cell death. Western blot analysis was performed using specific antibody for caspase 3. Actin was used as a loading control.

Next, we compared the response of the three OS cell lines to GCB treatment after 72 hrs treatment. In Figure 9, comparison between the three cell line's viability when treated with 1 µM of GCB shows significant reduction in all three cell lines. We noticed that the viability of LM7 cells was significantly higher (57%) compared to the viability of K7M3 (26%) and CCH-OS-D (34%) cells. These results suggest, that LM7 cells were less sensitive to GCB treatment compared to the other two cell lines.

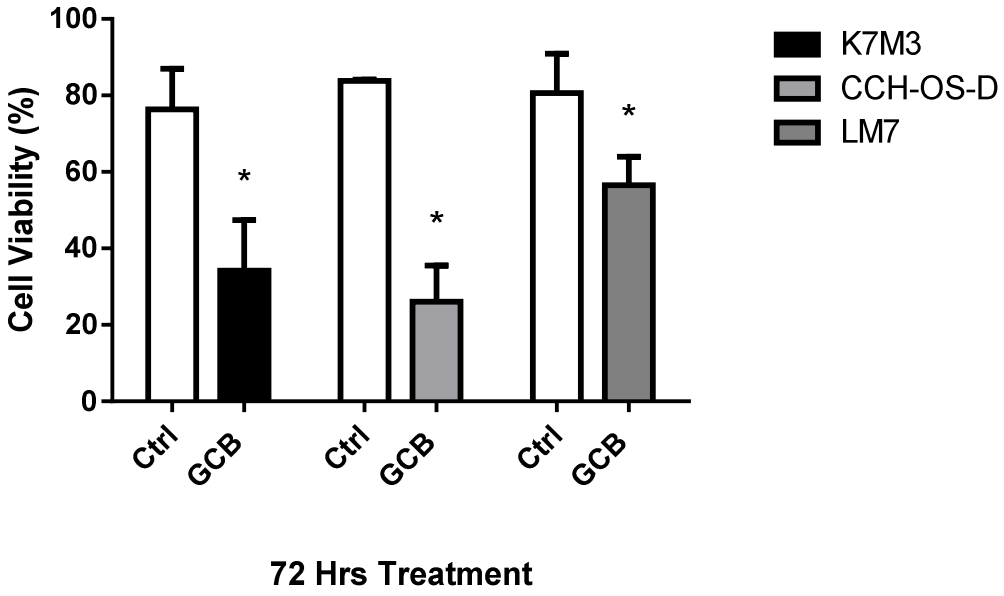


Figure 9. Viability of K7M3, CCH-OS-D and LM7 OS cells after 72 hrs treatment with GCB. Trypan blue exclusion assay was performed in order to assess the effect of of GCB (1µM) on cell viability over 72 hrs period.

Summary

Although we have previously shown that GCB has a significant therapeutic effect against OS cells, resistance remains a challenge for the treatment of OS. The aim of this chapter was first to perform an analysis of sensitivity on three different OS cell lines (K7M3, CCH-OS-D and LM7) and secondly assess whether apoptosis was induced.

For this purpose we performed a dose response analysis to evaluate the response of each OS cells to GCB treatment. The results obtained in the K7M3 cells showed that a significant decrease in cell viability was obtained with 0.5 µM of GCB as soon as 24 hrs. When analysis was performed in the CCH-OS-D cells, initial tolerance to 0.5 µM of GCB was observed. Significant decrease in cell viability was detected following treatment with 5 and 10 µM. These results suggest that CCH-OS-D cells were able to tolerate higher concentrations of GCB than K7M3 cells; viability was higher than 50% at 5 and 10µM doses. Similar results were observed in LM7 cells. As shown by the dose response graphs, a plateau was reached in cell viability indicating that higher doses offer no benefit in decreasing cell viability.

Figure 9 represents the viability of K7M3, CCH-OS-D and LM7 cells after 72hrs. When the viability was compared between the three cell lines, K7M3 and CCH-OS-D cells were the most sensitive cells. LM7 cells were relatively less sensitive to treatment when compared to the other cell lines.

The expression of cleaved caspase-3, a typical marker of apoptosis was assessed by western blot analysis. The expression of cleaved caspase 3 was increased in GCB treated OS cell lines compared to the control cells, suggesting apoptotic-induced cell death.

All together, these results indicate that treatment of OS cells with GCB decreased cell viability and induced apoptosis. This data supports the idea that GCB may be useful for the treatment of OS. However, evidence of cell resistance was suggested by a population of cells that remain viable after treatment.

Chapter 4: Gemcitabine induces autophagy in OS cells

Rationale and Aims

Several studies have shown that autophagy is induced in cancer cells following chemotherapeutic treatment with anti-cancer drugs and radiation [66]. It is reported that GCB can induce autophagy in pancreatic and breast cancer cells [67, 68]. However, GCB-induced autophagy has not been addressed in OS.

It is therefore relevant to study autophagy induction in OS after treatment with GCB, which may reveal autophagy as a possible target to improve GCB induced OS cell death. With the above mentioned rationale, the aim of this chapter is to determine whether there is autophagy induction after treatment with GCB on different OS cell lines.

In order to address this aim, we utilized different methods for studying autophagy induction in K7M3, LM7, CCH-OS-D, DLM8 and MG63 OS cells after GCB treatment. We utilized various cellular marker such as Acidic Vesicular Organelles (AVO), biochemical markers (Beclin, LC3 and p62) and ultrastructure analysis of double membrane autophagic vesicles.

In addition, we assessed the induction of autophagy after aerosol GCB treatment in two OS mouse models by evaluating the expression of autophagic markers in OS lung metastasis nodules and the formation of autophagic vesicules by electron microscopy.

Results

GCB induces formation of Acidic Vesicular organelles.

As part of the process of autophagy, the autophagosome fuses with the lysosome forming the autophagolysosomes (acidic structures). Accordingly, Acidic Vesicular Organelles (AVO) staining was performed to evaluate the formation of autophagolysosomes, as well as quantification using flow cytometry. For this, we used Acridine Orange (AO); a lysotropic dye that accumulates in acidic cellular compartments. The incorporation of AO was visualized by fluorescence microcopy in untreated and treated OS cells. As shown in figure 10, AVO induced by GCB stain orange-red, where the cytoplasm of cell stains green. Control cells show basal autophagy levels while GCB-treated cells exhibited increased AVOs formation when compared to the untreated cells.

Since the intensity of red fluorescence is proportional to the acidic vesicular compartments, we used flow cytometry to quantified AVO formation. On K7M3 cells, AVO formation increased after 24 hrs of treatment to 13.5%. At 48 hrs, AVO formation increased to 61.3%, and at 72 hrs it increased to 35.2%, all results compared to untreated cells. On LM7 cells, AVO formation increase to 3.3%, 8.0% and 21.96% at 24, 48 and 72 hrs respectively. CCH-OS-D cells also demonstrated increase AVO formation with increases to 2.3%, 10.0% and 18.07% at 24, 48 and 72Hrs respectively.

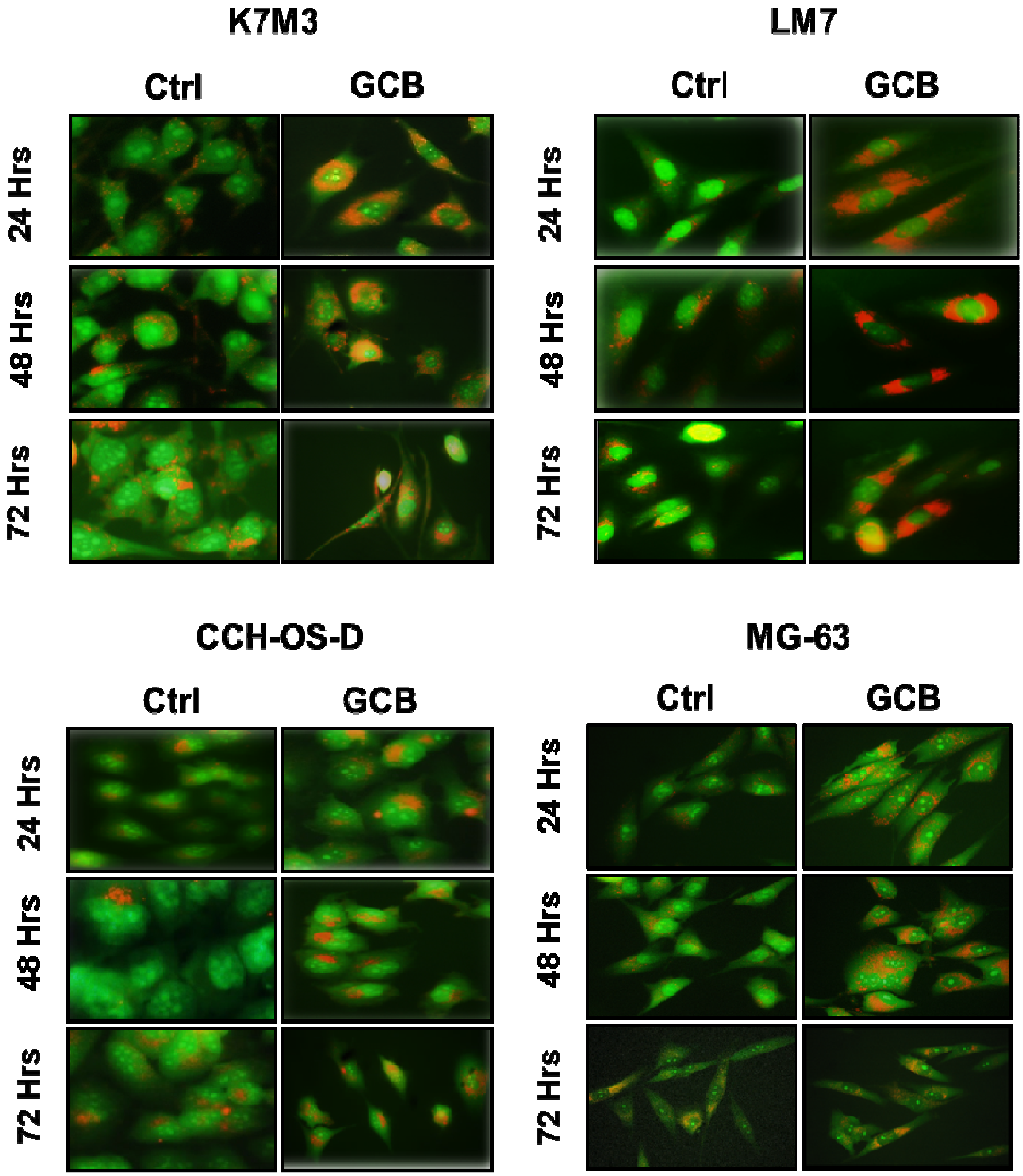


Figure 10. Induction of Acidic vesicular organelles in OS cells after GCB treatment. Cells were treated with 1 µM of GCB during 24, 48 and 72 hrs. Acridine orange (1 µg/mL) was added for 15 minutes. Formation of AVO was visualized using a fluorescence microscope.

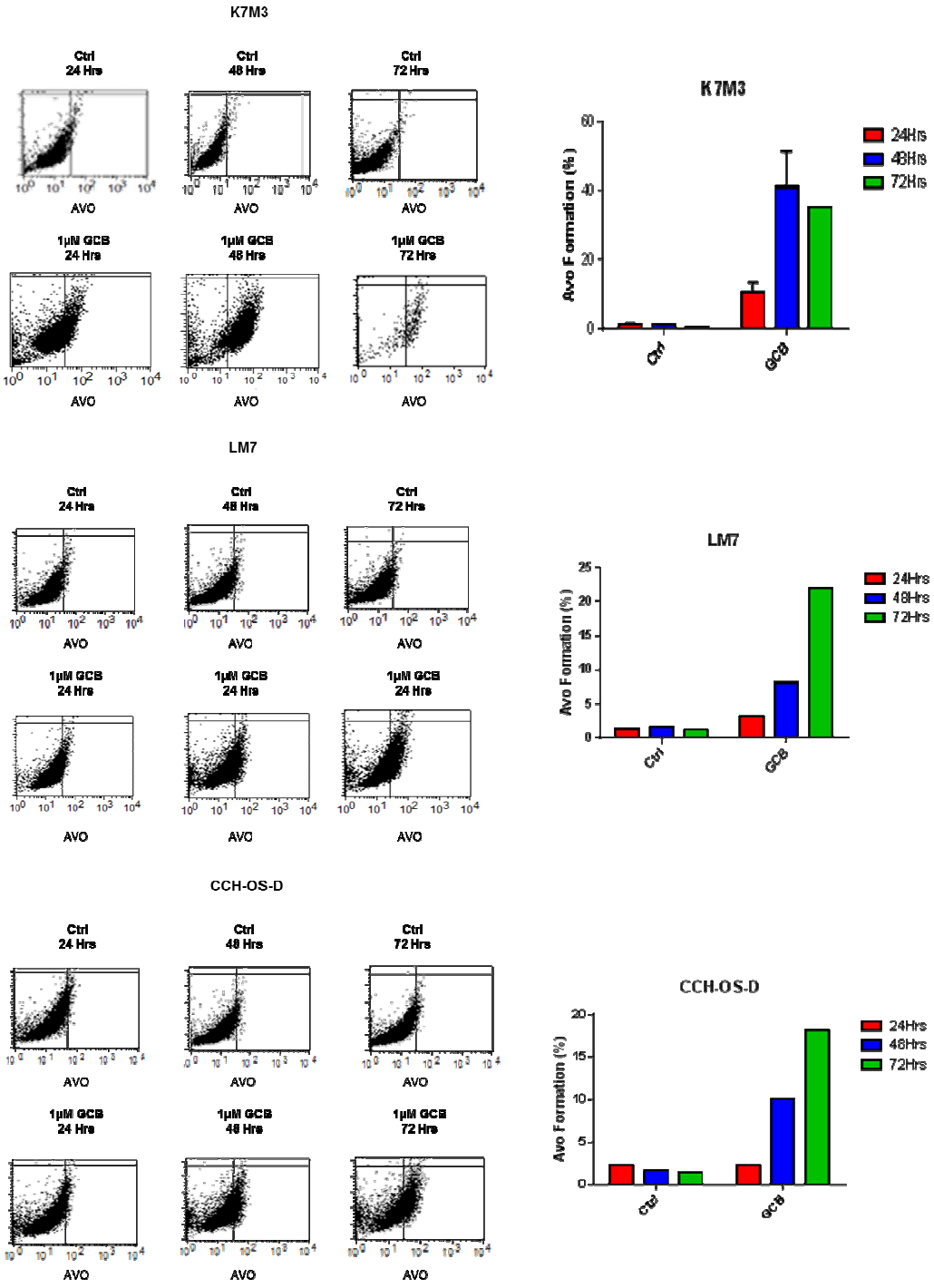


Figure 11. Quantification of Acidic vesicular organelles in OS cells after GCB treatment. Cells were treated with 1 µM of GCB during 24, 48 and 72 hrs. Acridine orange (1 µg/mL) was added for 15 minutes. Quantification of AVO was performed by flow cytometry. Untreated cells were used as a control. Graphs indicate the percentage of AVO-positive cells.

GCB increases Beclin expression, conversion of LC3I to LC3II and decrease of p62.

To confirm autophagy induction after GCB treatment, changes in protein expression of different autophagic markers: Beclin, LC3 and p62 were assessed using Western Blot and quantified by densitometry using ImageJ software. After GCB treatment, an increase in the expression of Beclin, an essential protein for the formation of the isolation membrane, was observed in all OS cells when compared to control group [69].

LC3 is a common autophagic marker. Upon induction of autophagy, LC3I is phospholipidated and converted to LC3II. Therefore the conversion of LC3I to LC3II is a reliable marker of autophagy and an indicative of autophagic activity [55, 69]. As shown in Figures 12-14, there was an increase in the conversion of LC3I/LC3II in GCB-treated OS cells as compared to untreated control.

The adaptor protein p62 is a marker of autophagic flux and is required for the formation of protein aggregates [55]. It is incorporated into the autophagosomes and degraded. Therefore, the expression of p62 inversely correlates with the autophagic activity. As an alternate approach to evaluate autophagic flux, we also assessed the expression of p62 in GCB-treated OS cells. As expected, there was a marked reduction in p62 protein expression in K7M3, CCH-OS-D and LM7 OS cells, further indicating that GCB-induced autophagy in OS cells (Figures 12-14).

Western blots from DLM8 and MG63 cells shows a similar pattern: increased Beclin expression and increased conversion of LC3I to LC3II in figures 15 and 16.

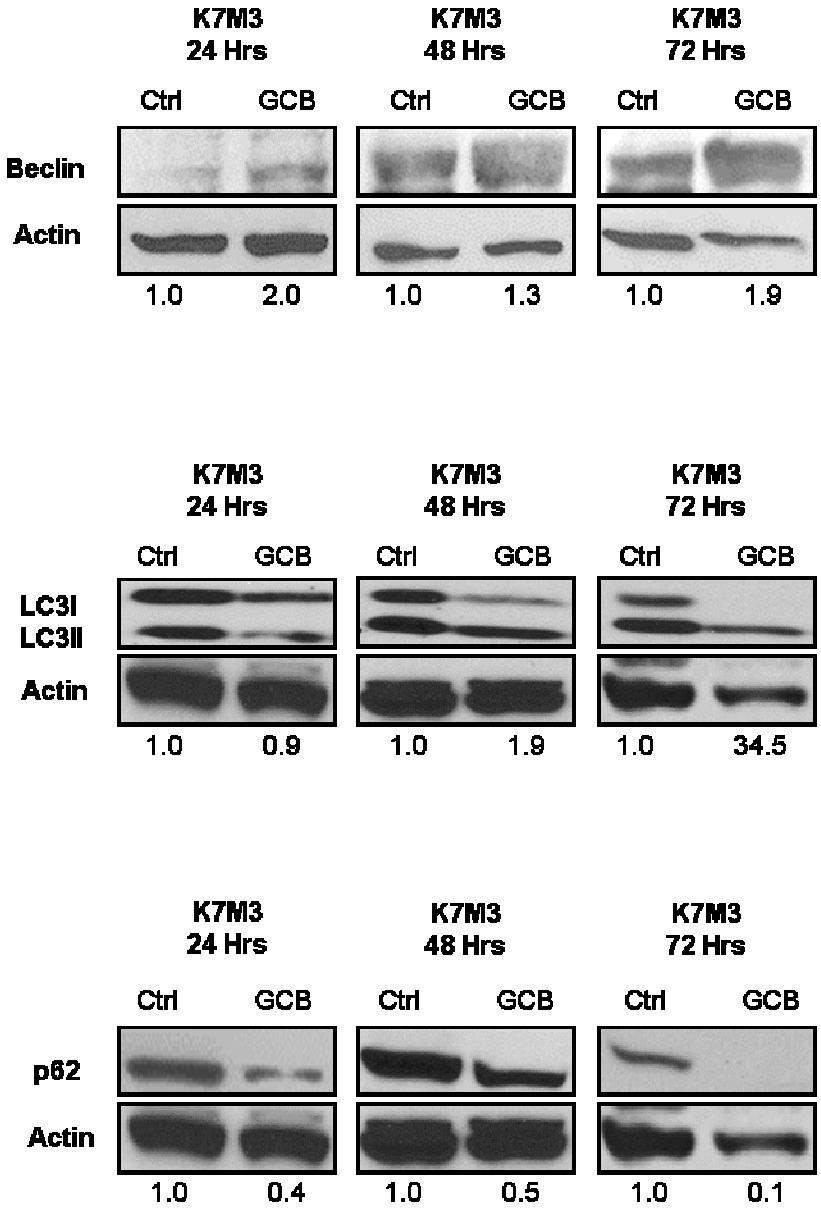


Figure 12. GCB induces autophagy in K7M3 OS cells. OS cells were grown in corresponding media until confluence, treated with GCB during 24, 48 and 72 hrs. Then cells were collected and lysed with RIPA buffer. Equal amount of total protein was resolved in SDS-PAGE, transfer to a nitrocellulose membrane and blotted using antibodies specific for each protein, using actin as a loading control. Western blot analysis and densitometry illustrate changes in Beclin, LC3 and p62 protein expression in response to GCB treatment.

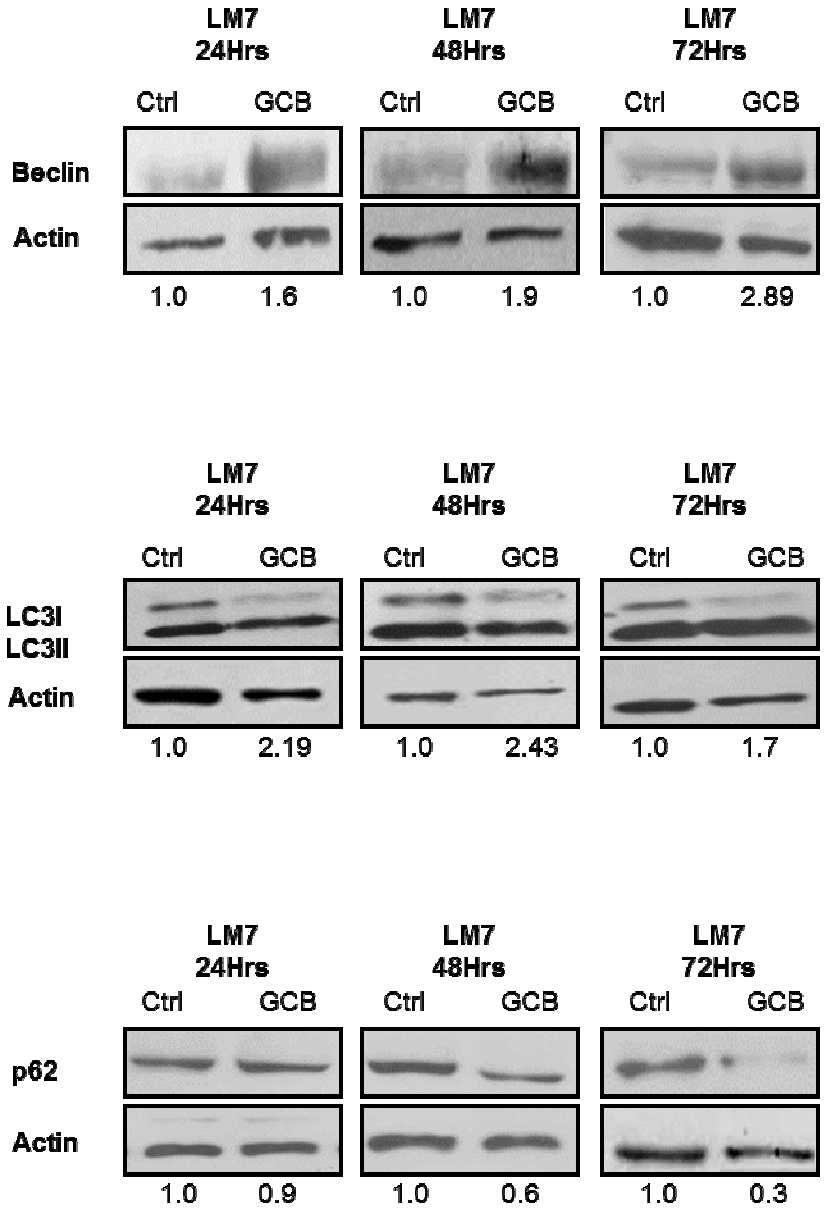


Figure 13. GCB induces autophagy in LM7 OS cells. OS cells were grown in corresponding media until confluence, treated with GCB during 24, 48 and 72 hrs. Then cells were collected and lysed with RIPA buffer. Equal amount of total protein was resolved in SDS-PAGE, transfer to a nitrocellulose membrane and blotted using antibodies specific for each protein, using actin as a loading control. Western blot analysis and densitometry illustrate changes in Beclin, LC3 and p62 protein expression in response to GCB treatment.

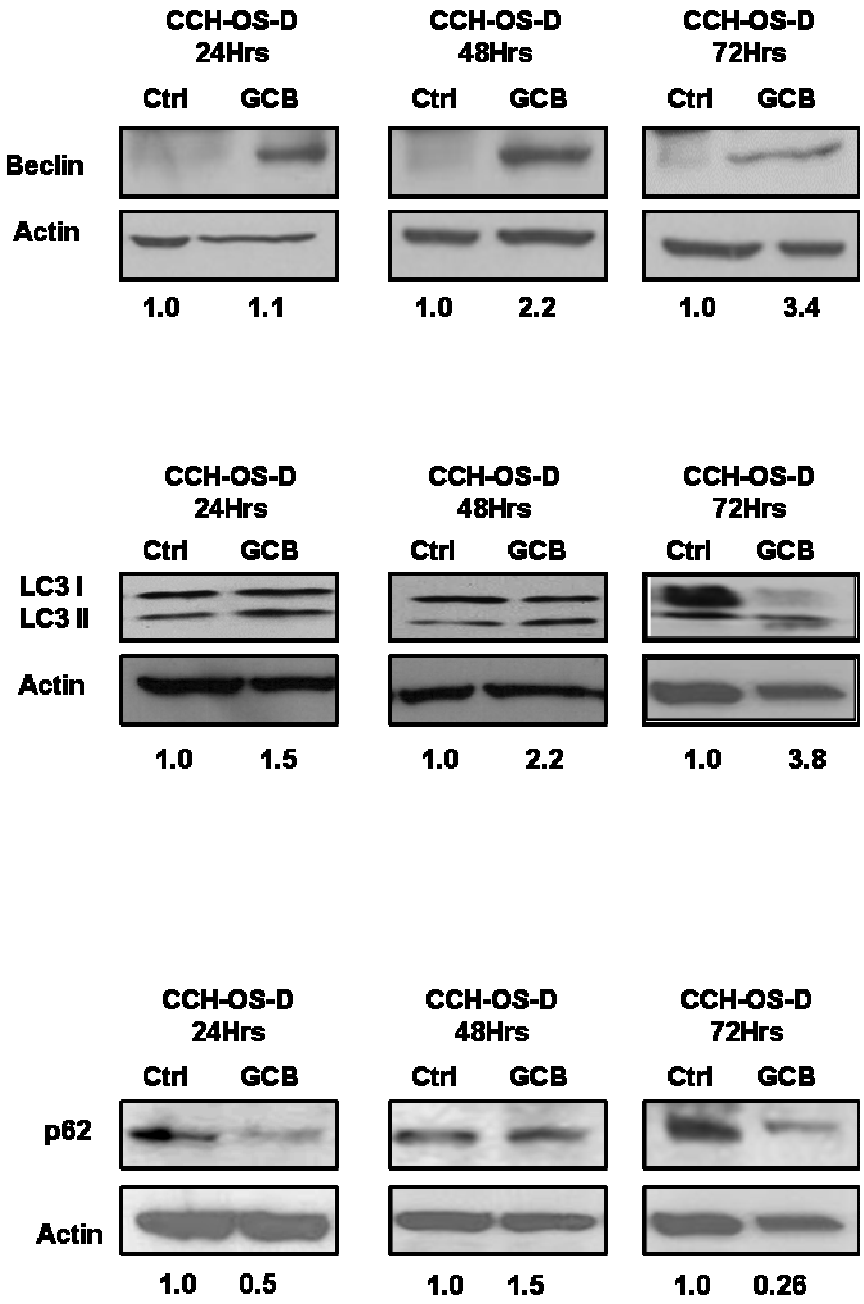


Figure 14. GCB induces autophagy in CCH-OS-D OS cells. OS cells were grown in corresponding media until confluence, treated with GCB during 24, 48 and 72 hrs. Then cells were collected and lysed with RIPA buffer. Equal amount of total protein was resolved in SDS-PAGE, transfer to a nitrocellulose membrane and blotted using antibodies specific for each protein, using actin as a loading control. Western blot analysis and densitometry illustrate changes in Beclin, LC3 and p62 protein expression in response to GCB treatment.

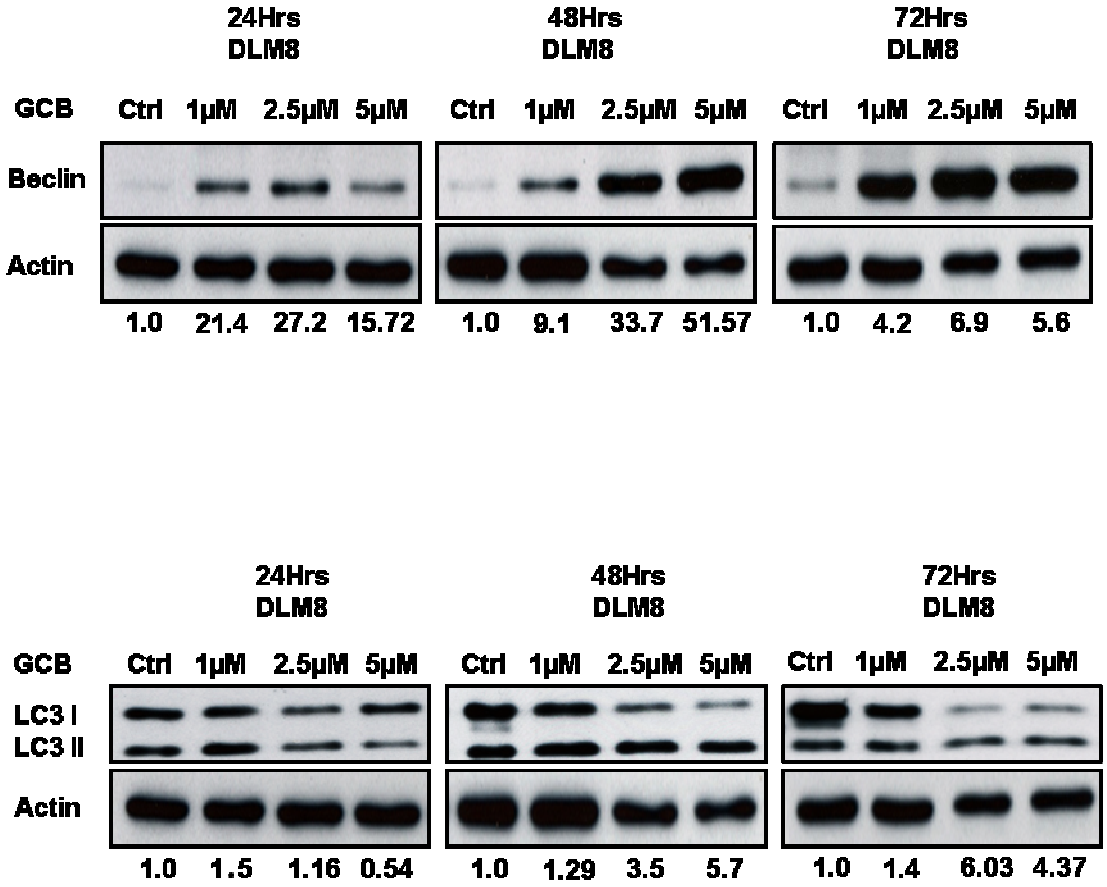


Figure 15. GCB induces autophagy in DLM8 OS cells. OS cells were grown in corresponding media until confluence, treated with GCB during 24, 48 and 72 hrs. Then cells were collected and lysed with RIPA buffer. Equal amount of total protein was resolved in SDS-PAGE, transfer to a nitrocellulose membrane and blotted using antibodies specific for each protein, using actin as a loading control. Western blot analysis and densitometry illustrate changes in Beclin and LC3 protein expression in response to GCB treatment.

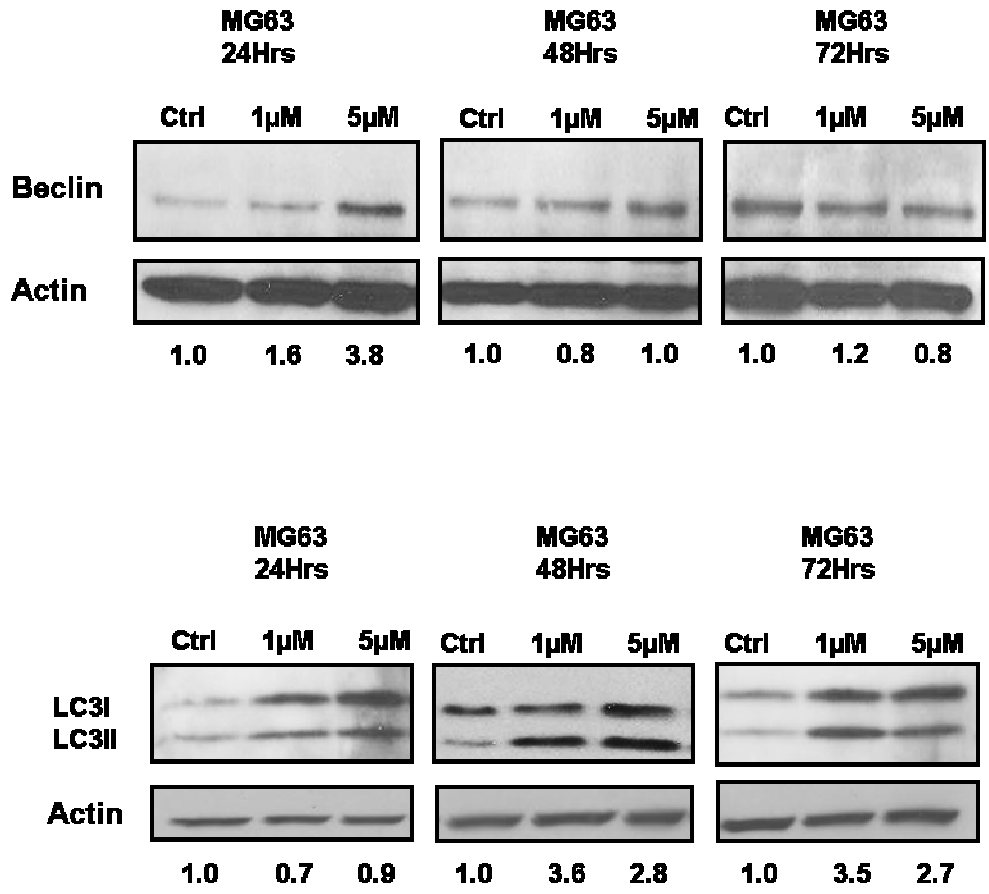


Figure 16. GCB induces autophagy in MG63 OS cells. OS cells were grown in corresponding media until confluence, treated with GCB during 24, 48 and 72 hrs. Then cells were collected and lysed with RIPA buffer. Equal amount of total protein was resolved in SDS-PAGE, transfer to a nitrocellulose membrane and blotted using antibodies specific for each protein, using actin as a loading control. Western blot analysis and densitometry illustrate changes in Beclin and LC3 protein expression in response to GCB treatment.

GCB Increases Immunofluorescent punctate staining of LC3 in OS cells.

Another approach for monitoring autophagy is fluorescence microscopy. In particular, accumulation of LC3 punctate is commonly assessed, as a hallmark of autophagy. Therefore, following exposure to GCB, cells were fixed in coverslips and immunostained with LC3 antibody. After incubation, cells were visualized under a fluorescence microscope. As shown in figure 17, accumulation of LC3 green fluorescent dots was higher in K7M3, CCH-OS-D and LM7 GCB- treated cells, compared to the control that exhibits diffuse and weak LC3 punctate dots.

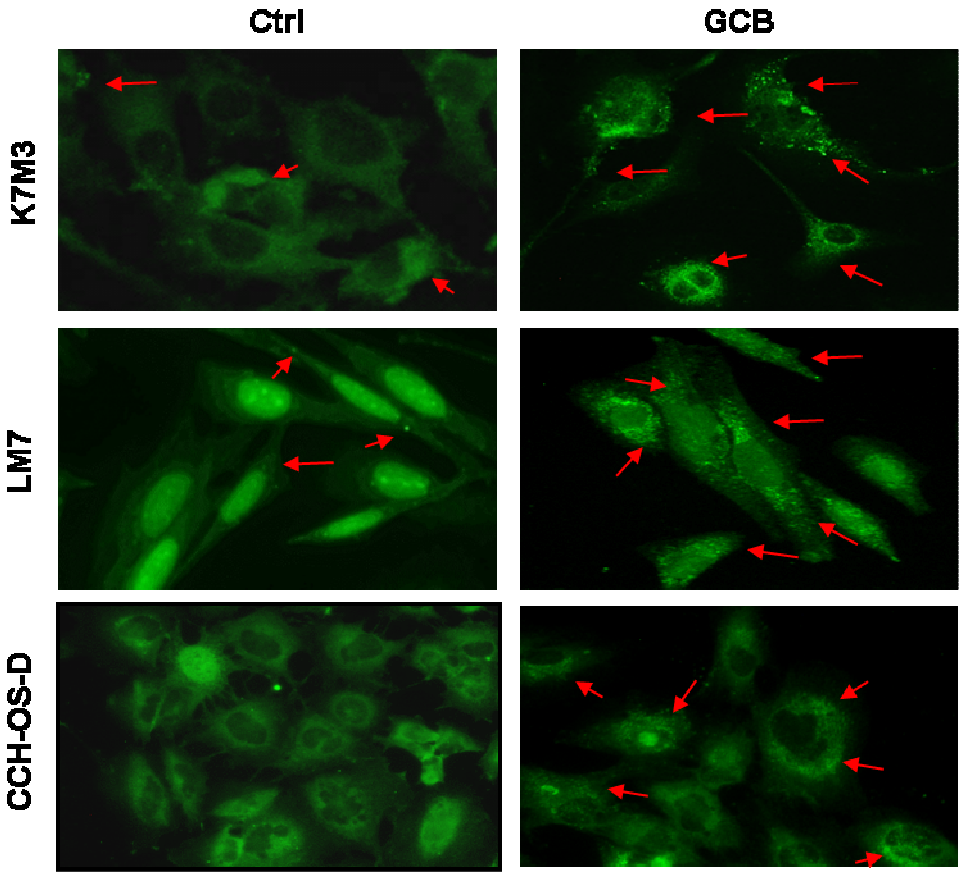


Figure 17: LC3 punctate increased after GCB treatment in OS cells. Cells were treated with GCB during 48 hrs, fixed and immunostained with LC3 antibody. Following treatment, fluorescence was observed under a fluorescence microscope. Arrows indicate LC3 punctate.

Electron microscopy revealed increased formation of autophagosomes in OS cells after GCB treatment.

In 1963, Christian de Duve described autophagy through the use of Electron Microscopy (EM) [70]. Since then, electron microscopy is commonly used for the detection of autophagic vacuoles.

Therefore, we used EM to confirm our previous results. We treated the K7M3, LM7, CCH-OS-D and MG63 OS cells with GCB and examined the formation of autophagosomes via electron microscopy. As shown in figures 18 and 19, GCB-treated OS cells exhibited a high number of double membrane autophagic vacuoles which indicates autophagosome and/or autolysosome formation. On the contrary, untreated cells exhibited low or no number of autophagosomes.

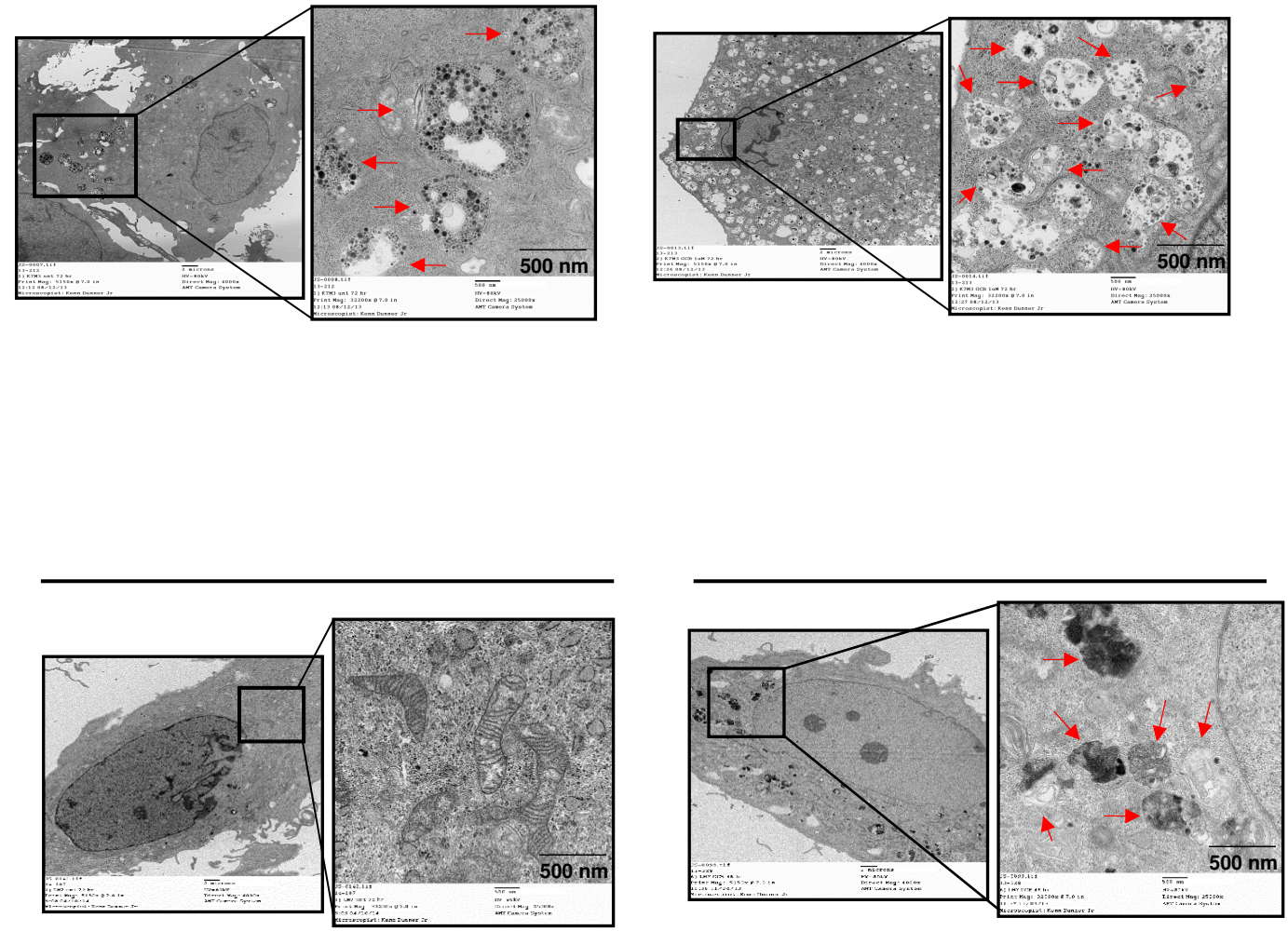


Figure 18: GCB treatment increases autophagosome formation in K7M3 and LM7 cells. A) K7M3, B) LM7 cells were treated with GCB. Electron microscopy was used to visualize the double membrane vacuoles that form during GCB-induced autophagy. Images of whole cells (2 microns) and corresponding higher magnification (500 nm) for each cell line were collected to detect autophagic vacuoles.

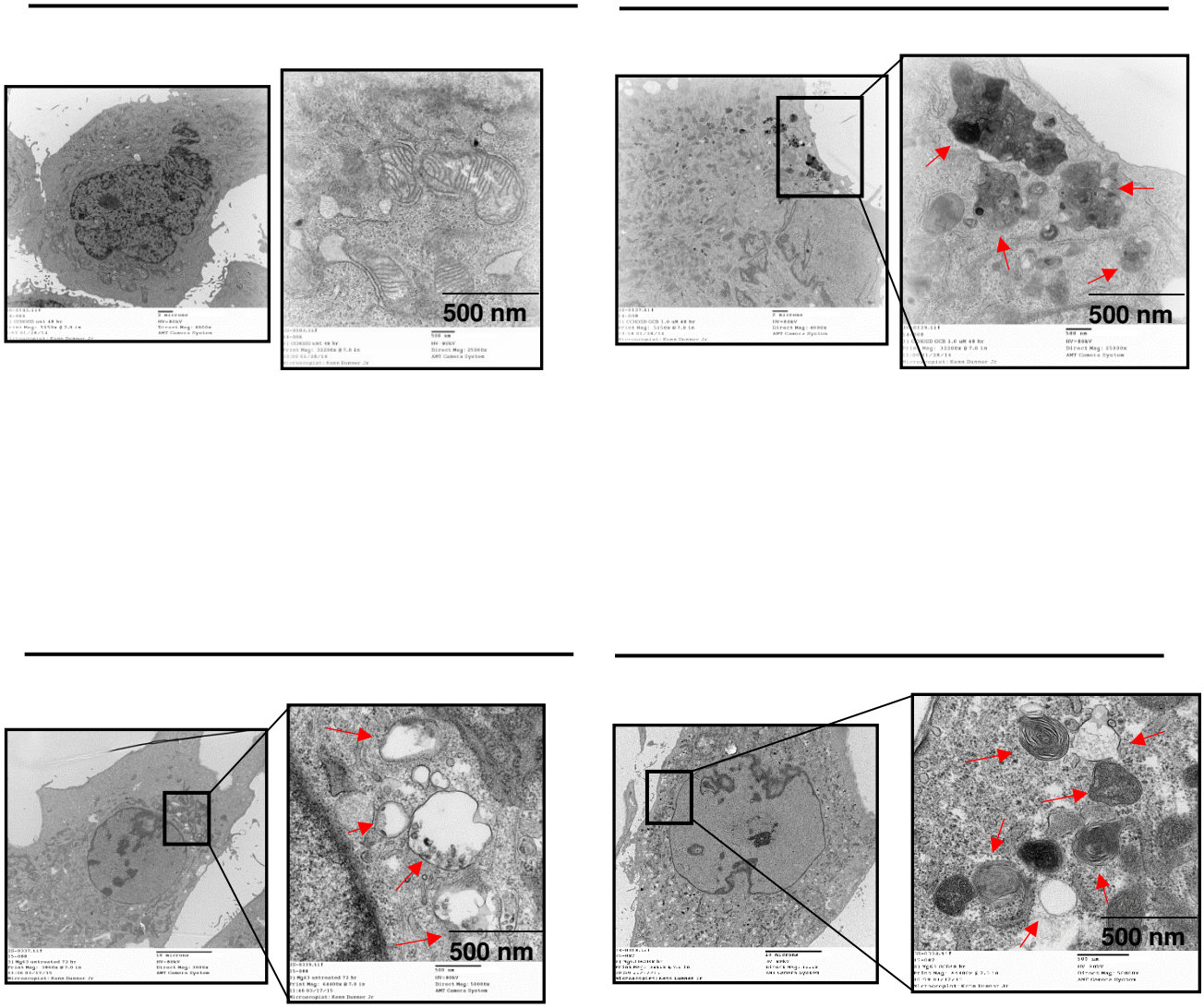


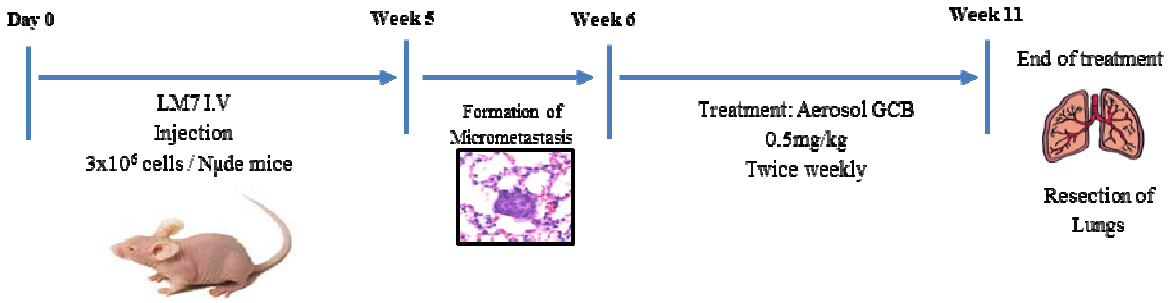
Figure 19: GCB treatment increases autophagosome formation in CCH-OS-D and LM7 cells. A) CCH-OS-D and B) MG63 cells were treated with GCB. Electron microscopy was used to visualize the double membrane vacuoles that form during GCB-induced autophagy. Images of whole cells (2 microns) and corresponding higher magnification (500 nm) for each cell line were collected to detect autophagic vacuoles.

Aerosol GCB induces autophagy in OS lung metastasis

Since our results indicated that GCB induces autohagy in OS cells *in vitro,* we investigated the effect of aerosol GCB on autophagy induction *in vivo* in two different OS mouse models. We anticipated that aerosol GCB would also induce autophagy in OS lung metastasis *in vivo*. Electron microscopy of fixed tissues and detection of some biochemical markers of autophagy such as LC3 and Beclin has been used as tools to monitor autophagy in different mouse models.

We utilized two of our OS pulmonary metastasis models; LM7 and K7M3. Even though these lung metastasis models are well established in our laboratory, we wanted to confirm the ability of these cells to form metastasis before beginning treatment with aeroosol GCB. For that reason, we injected the human LM7 cells intravenously in nude mice and confirmed formation of pulmonary metastasis by H&E staining at 5 weeks post-injection. The mouse K7M3 OS cells were injected intravenously into BALB/C mice. Lungs were extracted and presence of metastasis was demonstrated by H&E at 6 days post-injection (Figure 20 and 21). After formation of metastastasis was confirmed, nude mice were treated with PBS or 0.5 mg/kg of aerosol GCB twice a week for a total of five weeks. Treatment in BALB/C mice was started on week two for up to four weeks. Mice were treated with 0.5 mg/kg of aerosol GCB twice a week. Mice were sacrificed at the end of the treatment and lungs were resected for further studies.

A



B

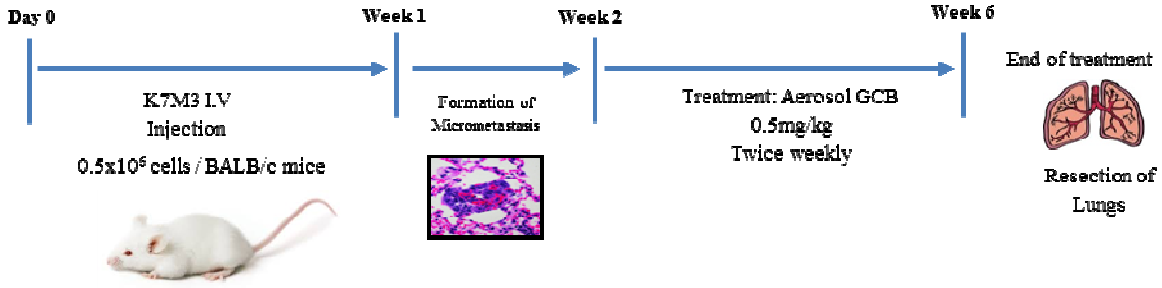


Figure 20 . Schematic diagram of *In vivo* model experimental design. (A) Nude mice were injected intravenously with $3x10^6$ LM7 cells. Formation of micrometastasis was evaluated at week 5 and confirmed by H&E staining. Treatment was started on week six. Mice were treated with PBS or 0.5 mg/kg of aerosol GCB twice a week for a total of five weeks. (B) BALB/C mice were injected with $0.5x10^6$ of K7M3 cells. Micrometastasis were confirmed after one week. Treatment was started on week two for up to four weeks. Mice were treated with 0.5 mg/kg of aerosol GCB twice a week. Mice were sacrificed at the end of the treatment and lungs were resected for further studies.

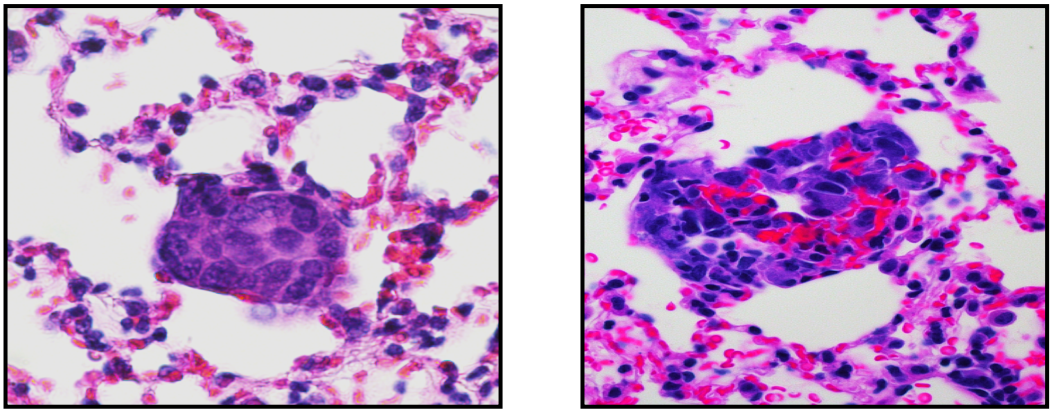


Figure 21. LM7 and K7M3 cells form pulmonary metastasis in mouse models. Mice were sacrified and lungs were extracted. H&E staining was perfomed to confirm the presecence of metastasis.

Aerosol GCB increases the expression of LC3 and Beclin in K7M3 OS lung metastases.

Immunohistochemical staining for LC3 and Beclin was utilized to detected autophagy induction in metastatic OS lung tissues after GCB treatment. Immunohistochemical analysis of metastastic tumors resected from mice of PBS (control) and GCB treated groups demonstrated that LC3 and Beclin expression was significantly increased in pulmonary metastatic tumors from mice of the treated group. Similar results were obtained in LM7 OS lung metastasis from the treated group.

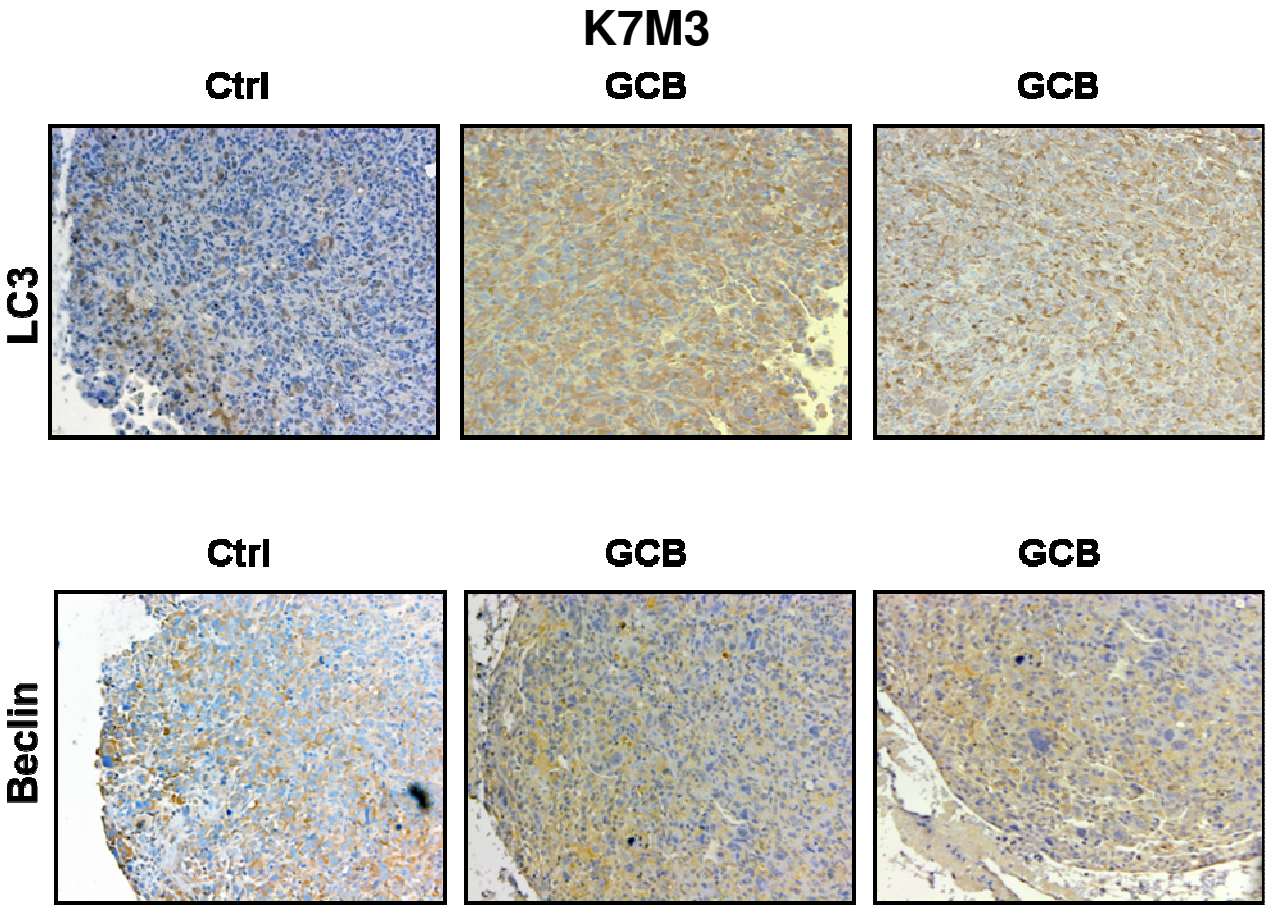


Figure 22. Increased expression of LC3 and Beclin in K7M3 OS lung metastasis after GCB treatment. At the end of the treatment BALB/C mice injected with K7M3 cells were sacrificed, and their lungs were extracted. Immunohistochemistry staining was performed using specific antibodies for LC3 and Beclin.

Aerosol GCB increases the expression of LC3 and Beclin in LM7 OS lung metastases

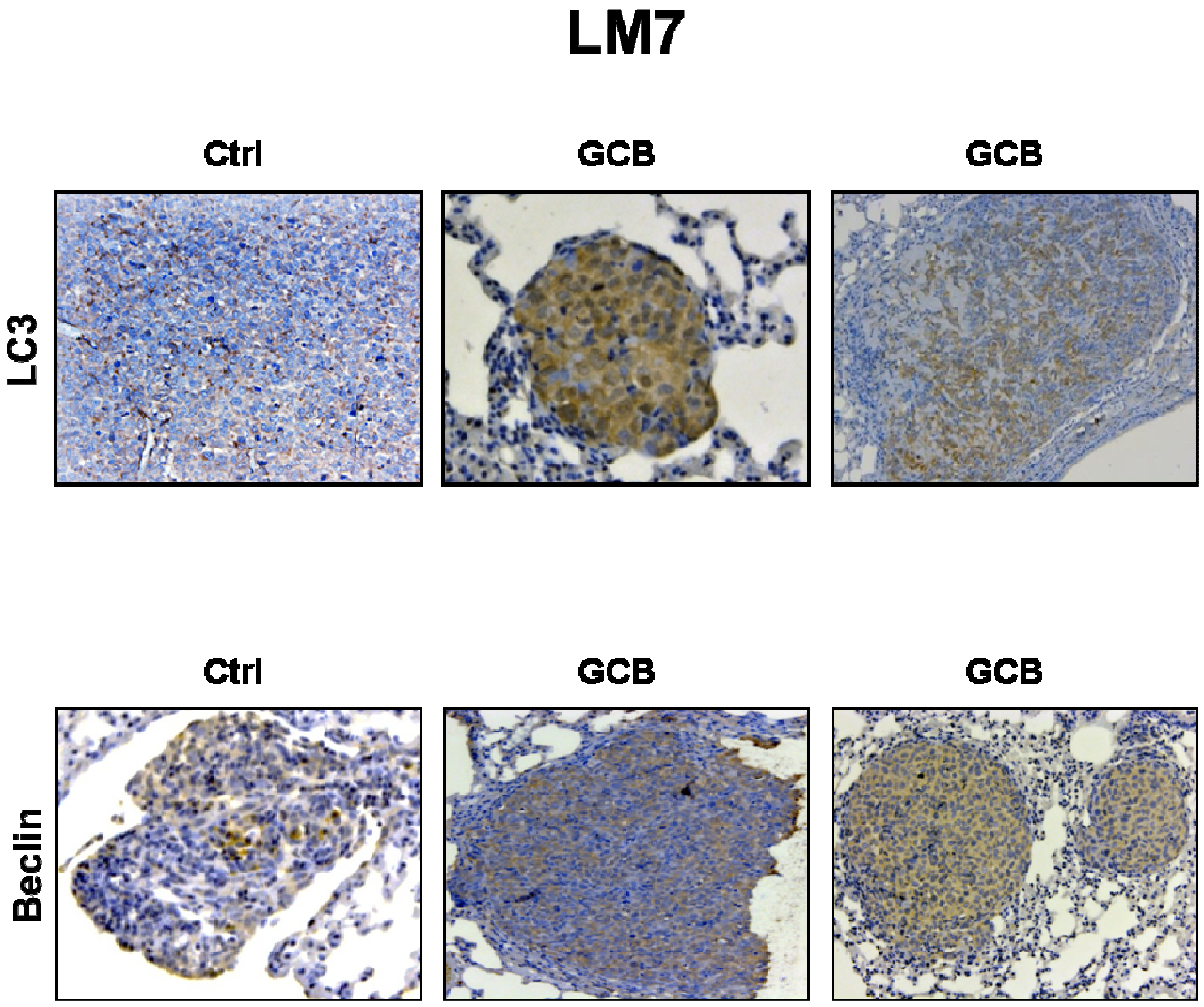


Figure 23. Increased expression of LC3 and Beclin in LM7 OS lung metastasis. At the end of the treatment Nude mice injected with LM7 cells were sacrificed, and their lungs were extracted. Immunohistochemistry staining was performed using specific antibodies for LC3 and Beclin.

Aerosol GCB increases the formation of autophagic vacuoles in K7M3 and LM7 OS lung metastasis.

In order to validate aerosol GCB-induced autophagy in lung metastasis, we resected the lungs and assessed the formation of autophagosomes by electron microscopy. Treated cells exhibited increased formation of double membrane autophagic vacuoles when compared to control cells.

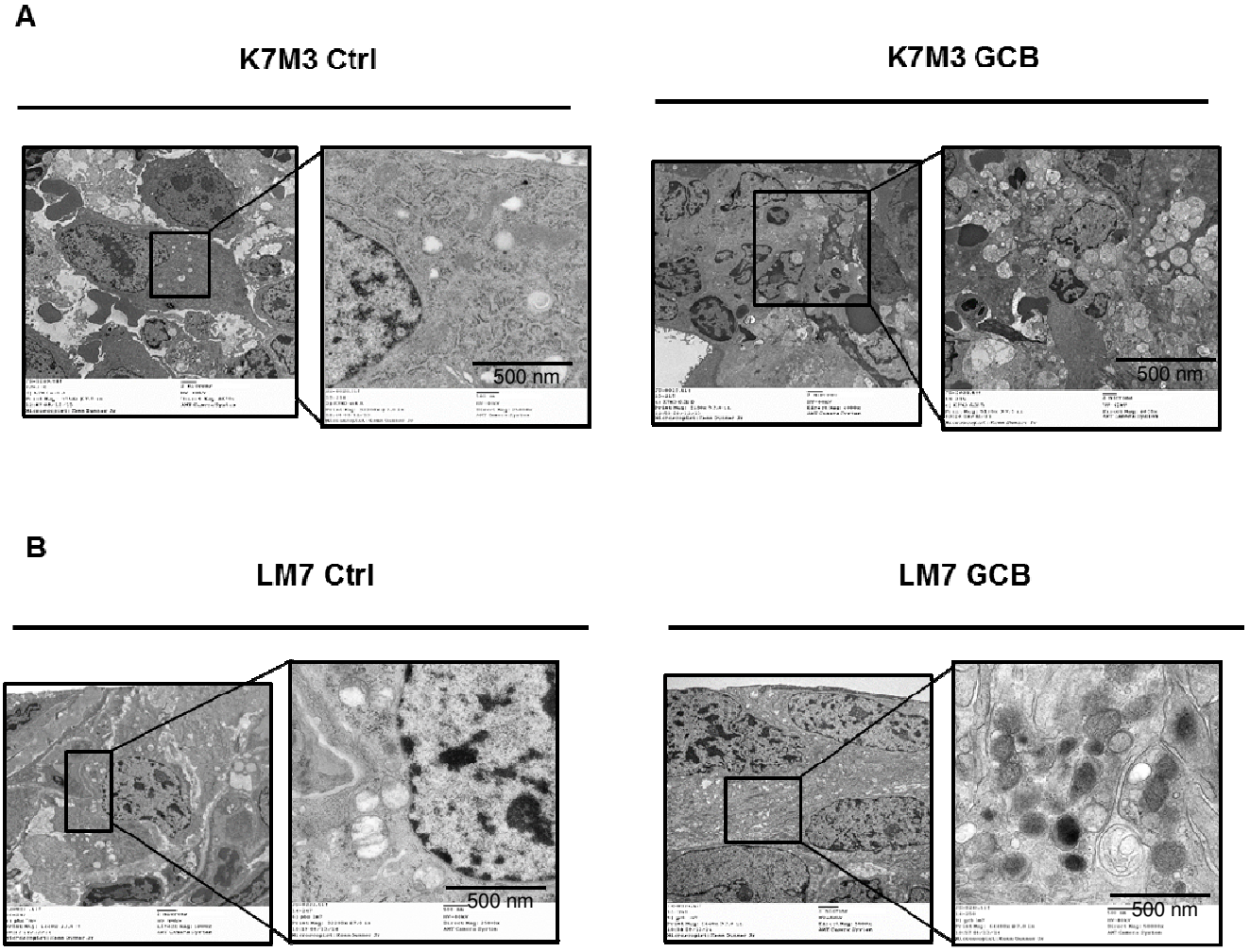


Figure 24. GCB induces formation of double membrane autophagic vacuoles in OS lung metastasis. Following aerosol GCB treatment, lungs were resected and examined by electron microscopy. Representative pictures of electron microscopy in mouse OS lung metastasis are shown as whole cells (2 microns) and corresponding higher magnification (500 nm).

4.2.5 Summary

In the previous chapter, it was observed that after GCB treatment some cells persist as viable cells. We have previously demonstrated that few isolated lung metastasis remained behind after GCB treatment. It was then hypothesized that a mechanism of resistance was acquired by OS cells that decreases cell sensitivity and GCB efficacy. Since autophagy has been described to be induced by chemotherapeutic agents; in this chapter we aimed to determine whether GCB induces autophagy in OS cells. There are different methods for the monitoring and detection of autophagy, including assessment of AVO, biochemical markers such as LC3, p62 and Beclin and ultrastructure visualization of autophagosome formation.

From our initial results with Acridine Orange, GCB-treated cells have higher AVO formation when compared to the untreated cells, suggesting induction of autophagy. Although Acridine Orange is a well-known and used approach to detect autophagic vesicles, it is not specific to autophagic vesicles. It can also be retained in other acidic compartments such as endocytic vesicles and secretory vesicles [71]. Therefore, we decided to support these results with the use of multiple techniques of autophagy detection.

Induction of autophagy was also evidenced by western blots analysis of different biochemical markers responsible for autophagy induction. Beclin is part of an upstream complex responsible for the initiation step and formation of the isolation membrane [69]. Significant increase in Beclin protein expression was observed in all OS cell lines after GCB treatment.

Upon induction of autophagy, LC3I is conjugated to PE and converted to LC3II. LC3II then binds to the autophagosome membrane [71]. Therefore, LC3 is one the most common autophagic marker. Significant conversion of LC3I to LC3II was

observed in all cell lines, indicating autophagy induction by GCB treatment. This increase in LC3 was confirmed by the fluorescent microscope data, which demonstrates accumulation of LC3 punctate after treatment (Figure 17). During late autophagy, LC3II is degraded and recycled, therefore in some cases the lack of increase in LC3II may be due to continued recycling. For this reason even though LC3 is one of the most common autophagy markers, it is necessary to confirm using other markers.

Since the induction of autophagy goes along with the degradation of p62, determining the status of p62 is commonly used to assess the autophagic flux [55]. p62 is implicated in targeting and recruiting proteins and organelles for degradation [72]. It has also been proposed that it binds to LC3 and localizes to the autophagosome where it becomes degraded. Therefore, during autophagy induction, total levels of p62 are inversely correlated with autophagic activity. Following GCB treatment, protein levels of p62 were evaluated by western blot. There was a significant decrease in p62 levels in the treated cells, which is consistent with induction of autophagic flux.

After assessment of lysosomal formation, as well as proteins involved in autophagy induction, we decided to validate our data by the use of electron microscopy. To date, this is the standard and most reliable method for monitoring autophagy. It was using electron microscopy that the phenomena of autophagy was discovered in rat parenchymal cells [73].

Ultrastructural analysis by electron microscopy showed that GCB-treated OS cells exhibited formation of abundant autophagic vacuoles. A higher magnification of these vacuoles confirmed that they were in fact double membrane vacuoles. On the contrary, untreated cells contain only few or no autophagic vacuoles.

We concluded that GCB treatment induces autophagy in OS cells, suggested by increase in AVO, increase in Beclin expression, conversion of LC3I to LC3II, decrease in p62 and accumulation of double membrane autophagic vacuoles.

Based on the *in vitro* results, that GCB induces autophagy in OS cells, we hypothesized that aerosol GCB would also induce autophagy *in vivo*. To test this hypothesis, we used immunohistochemistry staining to evaluate induction of autophagy following GCB treatment in two OS mouse models.

By comparing the expression of LC3 and Beclin in resected lung tissue between mice treated with aerosol GCB and control mice, we demonstrated that mice in the treated group exhibited an increase in the accumulation of autophagy markers confirming that GCB can also induce autophagy in OS lung metastases.

In support of our hypothesis that aerosol GCB would also induce autophagy in OS lung metastases in mice, we sought to determine if treatment of mice with aerosol GCB resulted in formation of double membrane autophagic vacuoles, a feature of autophagosomes. Following aerosol GCB treatment, mice were sacrificed and lungs were submitted and analyzed using electron microscopy. A significant increase in the formation of autophagic vacuoles was reported associated with the treatment, compared to the control group. All these results confirmed autophagy induction after treatment with GCB in OS cells both *in vitro* and *in vivo*.

Chapter 5: AKT/mTOR signaling pathway as the molecular mechanism involved in Gemcitabine-Induced autophagy in osteosarcoma

Rationale and Aims:

Having demonstrated that GCB induces autophagy in OS, we investigated the potential molecular mechanism underlying the induction of autophagy after GCB treatment. A variety of signaling events that regulate autophagy have been investigated. However, the AKT/mTOR pathway is the most common regulatory signal of autophagy. mTOR is a well-established negative regulator of autophagy [2, 27, 74]**.** Activation of AKT through growth factors and nutrients, serve as signal to the cells to know that recycling of cellular components is not necessary and energy should be spend in other cellular processes such as cell proliferation and protein synthesis through mTOR activation [75].

Therefore, we examined whether GCB inactivates this pathway thereby inducing autophagy. In order to answer this question, we investigated the phosphorylation status of key components of the AKT/mTOR signaling pathway. We expected to see a decreasing trend in the phosphorylation of the key components of this signaling pathway, leading to activation of autophagy.

Results

Phosphorylation of AKT and mTOR is decreased after GCB treatment.

Our studies began by utilizing western blot analysis to determine the phosphorylation status of AKT and mTOR. We treated the K7M3, LM7 and CCH-OS-D OS cells with GCB and the cell lysates were analyzed by western blot with specific antibodies for each protein and their phospho-counterparts. As shown in figure 25, western blot analysis revealed that phosphorylation of AKT (S473) and mTOR (S2448) was significantly decreased in K7M3, LM7 and CCH-OS-D cells by GCB as compared to the untreated cells, suggesting the implication of this pathway in GCB-induced autophagy. The non-phosphorylated form was not affected.

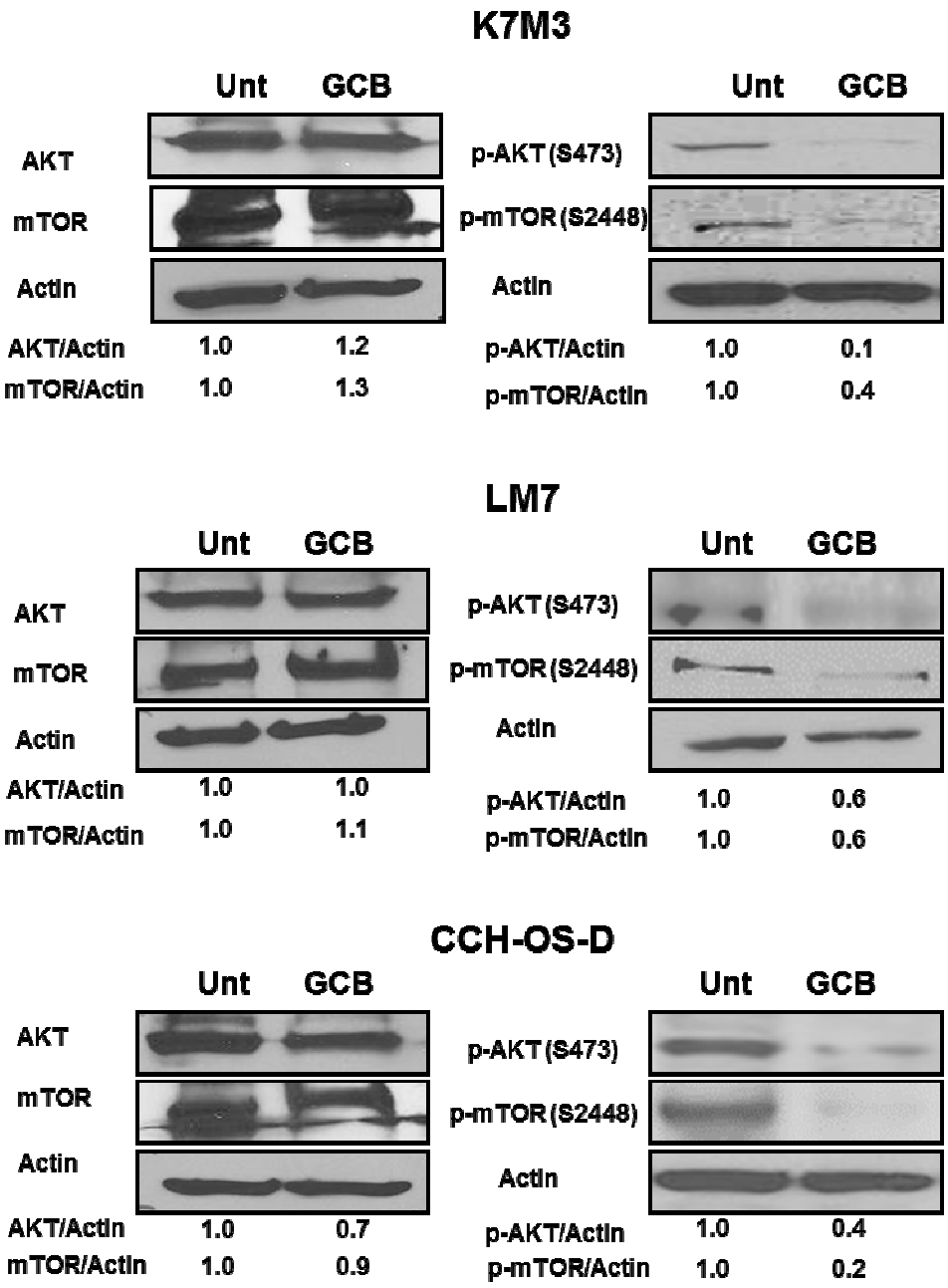


Figure 25. GCB treatment decreases AKT and mTOR phosphorylation in OS cells. K7M3, LM7 and CCH-OS-D OS cells were treated with 1μM of GCB for 48 or 72 hrs. AKT and mTOR phosphorylation was examined with anti-phospho-AKT and anti-phospho mTOR antibody. Total levels were examined with an anti-AKT and anti-mTOR antibody. Actin was used as a loading control.

Phosphorylation of AKT, mTOR, PRAS40 and P70S6K decreased after GCB treatment

To confirm that the AKT/mTOR pathway is involved in GCB-induced autophagy in OS cells, we utilized a high-throughput screening antibody array to evaluate the phosphorylation status of AKT and mTOR and determine the involvement of any other kinase. Following GCB treatment we analyzed the cellular content by using a Human Phospho-Kinase Antibody Array (R&D systems). The results demonstrated that a great number of kinases exhibited changes in phosphorylation after treatment (Figure 26 and 27, Table 5). As expected, we observed a decrease in AKT (S473) and mTOR (2448) phosphorylation after 48 hrs of GCB treatment in CCH-OS-D cells and after 72 hrs in the LM7 cells.

As the proline-rich AKT substrate of 40kDa (PRAS40) has been reported as a substrate of AKT, we investigate the effect of GCB on the phosphorylation status of PRAS40 [76]. In accordance with this idea, as shown in figures 28 and 29 the phosphorylation of PRAS40 decreased after GCB treatment in both OS cell lines. p70S6K is a downstream target of mTOR [77]. To more deeply investigate, we also evaluate the effect of GCB on the phosphorylation status of p70S6K. As shown in figures 28 and 29 the phosphorylation p70S6K (T421/S424) was also decreased after GCB treatment in both OS cell lines.

The Human Phospho-Kinase array was unable to properly detect p70S6k to acceptable levels. In order to assess protein levels, it was decided to perform a western blot analysis and quantification by densitometry. After 48 hrs of GCB treatment, p70S6K expression levels had decreased.

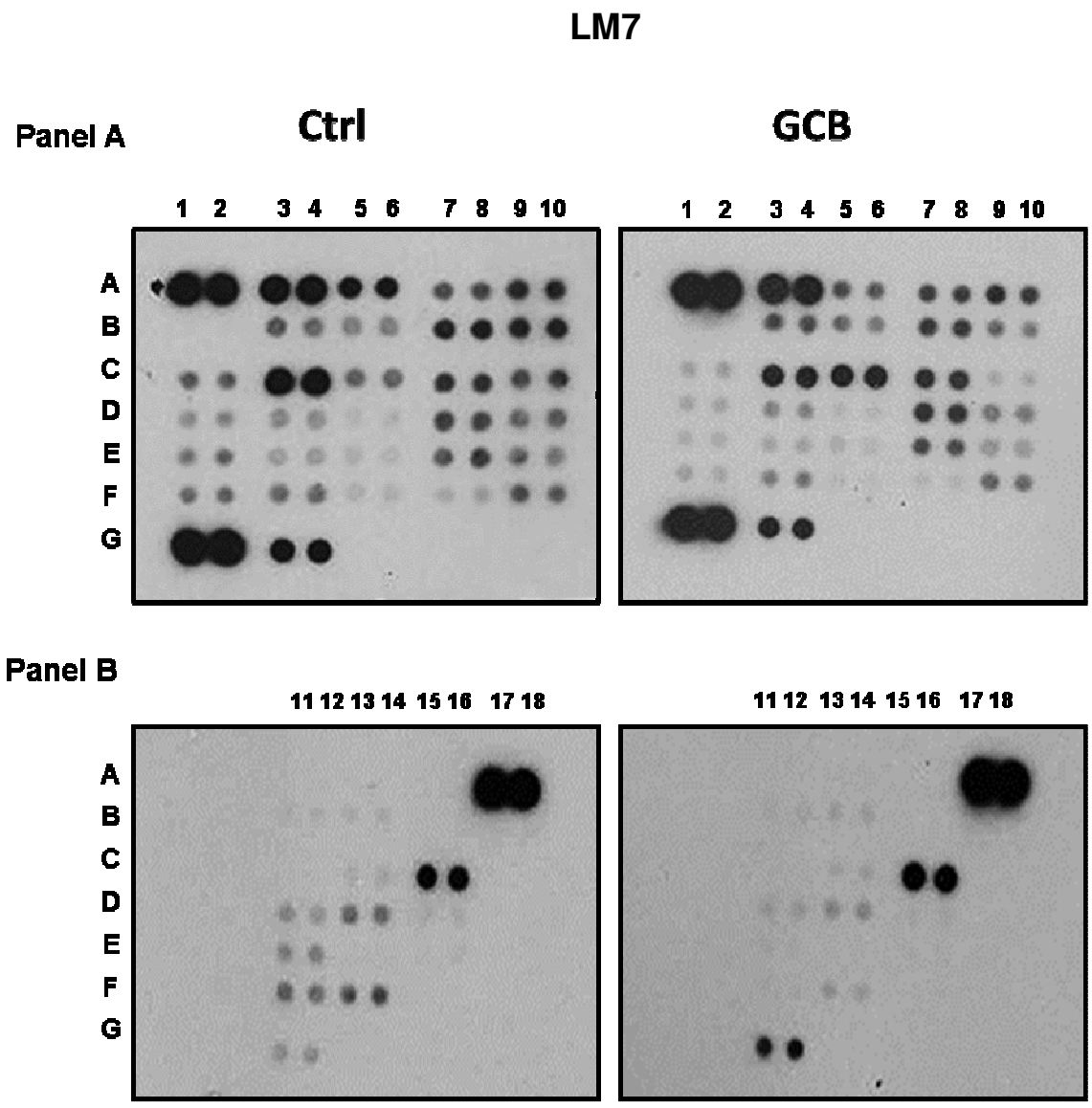


Figure 26. Human phospho-kinase array reveals numerous phosphorylated kinases in LM7 OS cells after GCB treatment. Human phospho-kinase array was utilized to evaluate the phosphorylation status of several kinases during GCB treatment in human OS cells. LM7 cells were treated during 72 hrs and whole cell lysates were analyzed by the array. Each spot indicates phospho-protein levels and controls in duplicate. See Table 5 for the complete list of kinases.

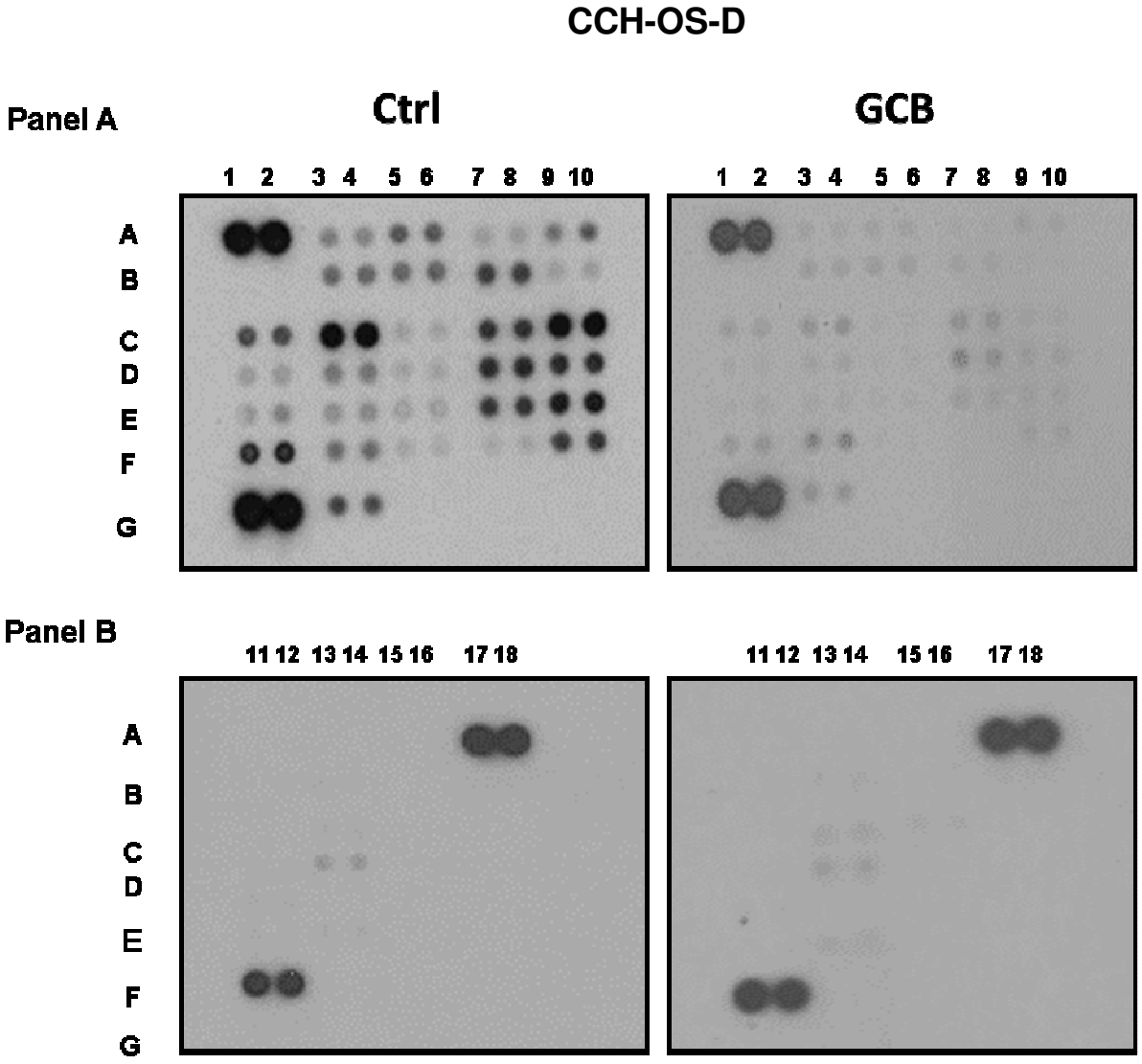


Figure 27. Human phospho-kinase array reveals numerous phosphorylated kinases in CCH-OS-D OS cells after GCB treatment. Human phospho-kinase array was utilized to evaluate the phosphorylation status of several kinases during GCB treatment in human OS cells. CCH-OS-D cells were treated during 48 hrs and whole cell lysates were analyzed by the array. Each spot indicates phospho-protein levels and controls in duplicate. See Table 5 for the complete list of kinases.

Kinase\Control	Phosphorylation Site	Membrane Coordinates
Reference Spot		A-A1,A2
p38α	T180/Y182	A-A3,A4
ERK1/2	T202/Y204/T185/Y187	A-A5,A6
JNK	T183/Y185,T221/Y223	A-A7,A8
GSK-3α/β	S21/S9	A-A9,A10
p53	S392	B-A13,A14
Reference Spot		B-A17,A18
EGFR	Y1086	A-B3,B4
MSK1/2	S376/S360	A-B5,B6
AMPKα1	T183/Y185,T221/Y223	A-B7,B8
AKT	**S473**	**A-B9,B10**
AKT	**T308**	**B-B11,B12**
p53	S46	B-B13,B14
mTOR	**S2448**	**A-C1,C2**
CREB	S133	A-C3,C4
HSP27	S78/S82	A-C5,C6
AMPKα2	T172	A-C7,C8
β-catenin		A-C9,C10
p70S6 kinase	T389	B-C11,C12
p53	S15	B-C13,C14
c-jun	S63	B-C15,C16
Src	Y419	A-D1,D2
Lyn	Y397	A-D3,D4
Lck	Y349	A-D5,D6
STAT2	Y698	A-D7,D8
STAT5a	Y694	A-D9,D10
p70S6 kinase	**T421/S424**	**B-D11-D12**
RSK1/2/3	S380/S386/S377	B-D13,D14
Enos	S1177	B-D15,D16
Fyn	Y420	A-E1,E2
Yes	Y426	A-E3,E4
Fgr	Y412	A-E5,E6
STAT6	Y641	A-E7,E8
STAT5b	Y699	A-E9,E10
STAT3	Y705	B-E11,E12
p27	T198	B-E13,E14
PLC-y1	Y783	B-E15,E16
Hck	Y411	A-F1,F2
Chk-2	T68	A-F3,F4
FAK	Y397	A-F5,F6
PDGF Rβ	Y751	A-F7,F8
STAT5a/b	Y694/Y699	A-F9,F10
STAT3	S727	B-F11,F12
WNK1	T60	B-F13,F14
PYK2	Y402	B-F15,F16
Reference Spot		A-G1,G2
PRAS40	T246	A-G3,G4
PBS(Negative Ctrl)		A-G9,G10
HSP60		B-G11,G12
PBS(Negative Ctrl)		B-G17,G18

Table 5. List of kinases detected by Human Phospho-kinase Array. The table includes the phosphorylation site of each protein as well as the coordinates where each kinase is located within the array. Kinases highlighted in yellow are components of the AKT/mTOR pathway. Kinases in green can also regulate mTOR inactivation.

Human Phospho –Kinase Array

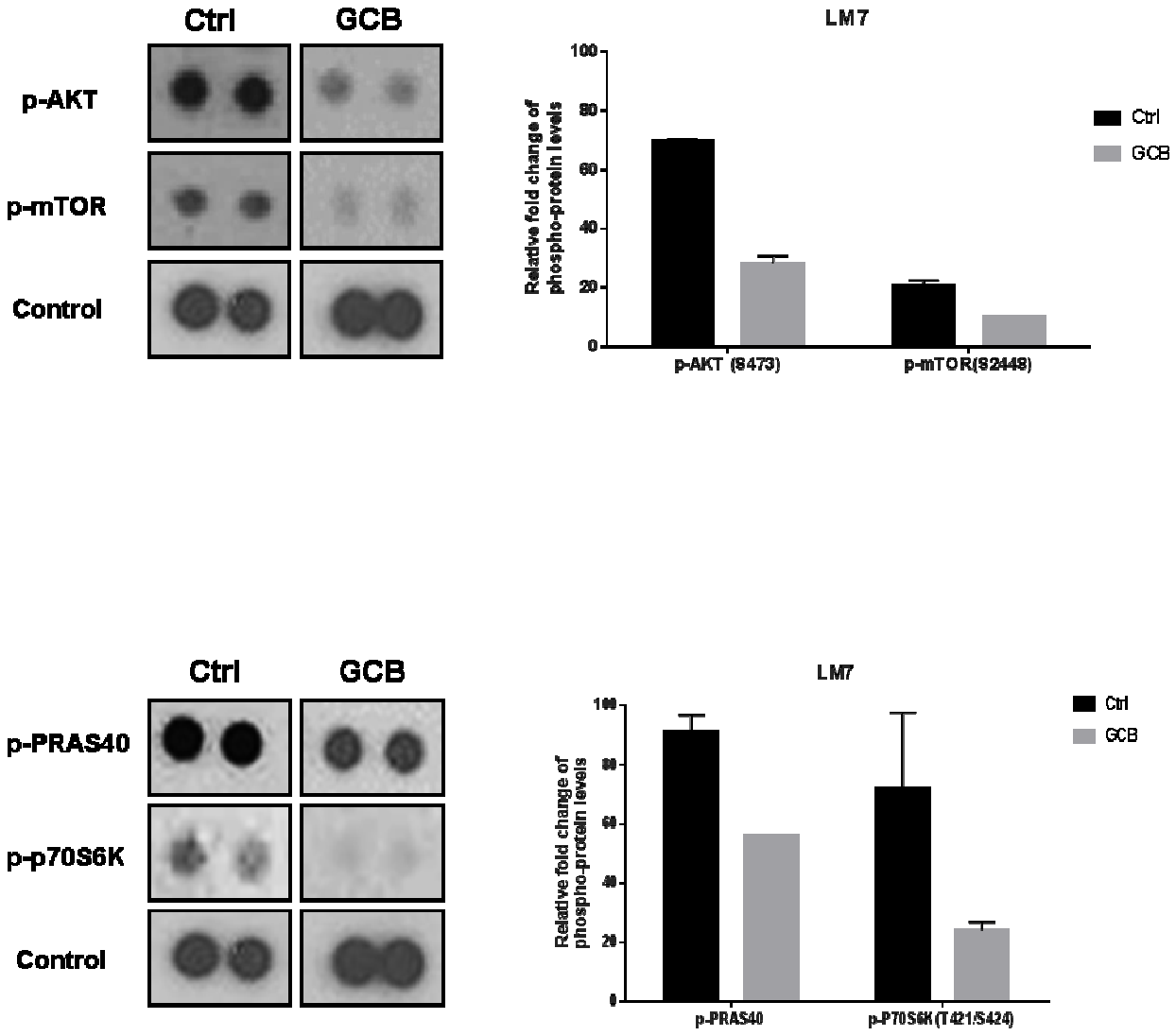


Figure 28. Phosphorylation levels of the components of AKT/mTOR pathway decreases after GCB treatment in LM7 cells. Cells were treated with GCB for 72 hrs and cell lysates were analyzed using the Human phospho-kinase array. The phosphorylation levels of AKT, mTOR, PRAS40 and p70S6K were evaluated. Densitometry was performed and the values were graphed as relative fold change of phospho-protein levels.

Human Phospho –Kinase Array

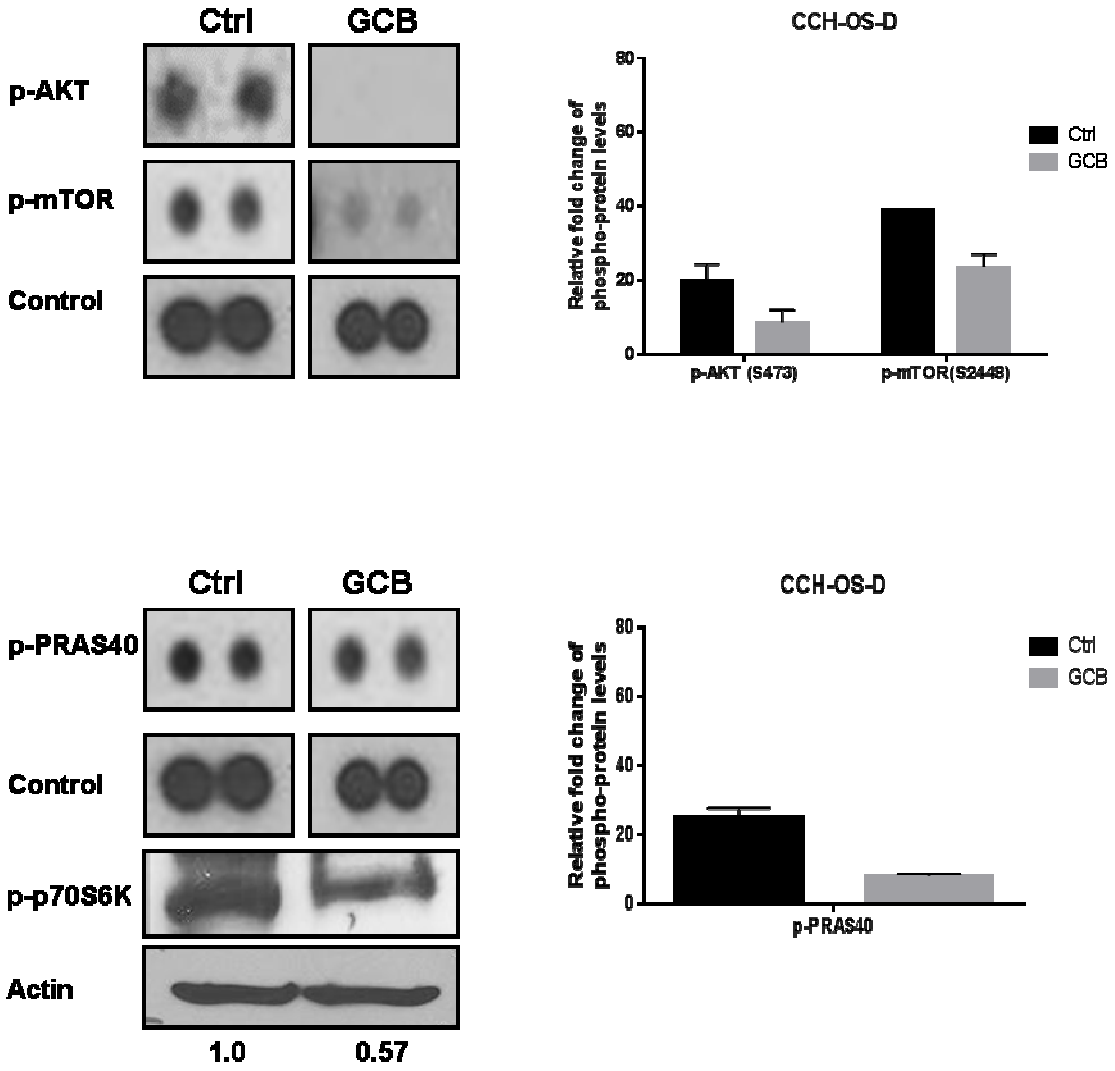


Figure 29. Phosphorylation levels of the components of AKT/mTOR pathway decreases after GCB treatment in CCH-OS-D cells. Cells were treated with GCB for 48 hrs and cell lysates were analyzed using the Human phospho-kinase array. The phosphorylation levels of AKT, mTOR, PRAS40 and p70S6K were evaluated. Densitometry was performed and the values were graphed as relative fold change of phospho-protein levels.

Effect of GCB treatment on the phosphorylation of JNK, AMPK and ERK

As previously discussed, it has been reported that induction of autophagy can also be regulated by other pathways. Activation of other kinases such as JNK, ERK and AMPK, result in mTOR inactivation and autophagy induction [26].

From the Human Phospho-Kinase it was observed that p-ERK levels decreased in GCB-treated cells when compared to untreated cells. Expression levels of both p-AMPK isoforms (AMPKα1 and AMPKα2) did not change when comparing GCB treated cells to control cells. Similar results were obtained for p-JNK expression levels. Together, modification in the phosphorylation of JNK, AMPK and ERK was not observed after GCB treatment, thereby suggesting specificity of the AKT/mTOR pathway as a potential molecular mechanism involved in GCB-induced autophagy.

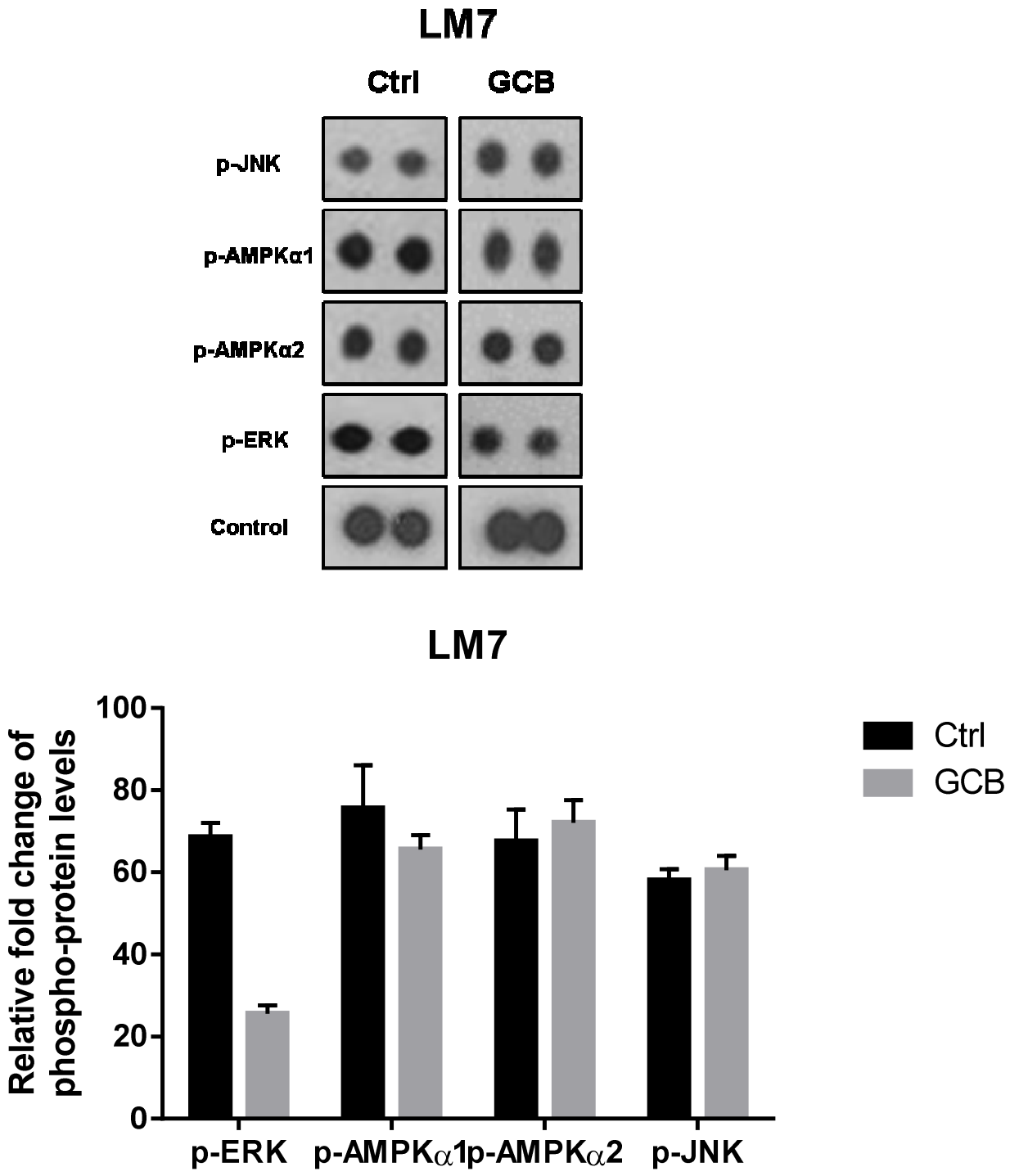


Figure 30. Phosphorylation status of other commonly associated kinases in the regulation of mTOR. LM7 cells were treated with GCB for 72 hrs and cell lysates were analyzed using the Human phospho-kinase array. The phosphorylation levels of JNK, AMPK and ERK were evaluated. Densitometry was performed and the values were graphed as Relative fold change of phospho-protein levels.

Summary:

AKT/mTOR is the major regulatory pathway by which the process of autophagy is inhibited. Previous studies have demonstrated that interruption of this pathway leads to autophagy induction. To investigate if this pathway is involved in GCB-induced autophagy, we measured the phosphorylation of important components of this pathway.

Activation of mTOR mostly depends on the activity of AKT. In accordance with our hypothesis, results demonstrated that GCB treatment decreases phosphorylation of AKT and mTOR, while the non-phosphorylated form of each protein did not change after treatment. To confirm these results, we utilized a Human Phospho-Kinase Antibody Array. Consistent with the results obtained by western blot, the phosphorylation status of both AKT and mTOR was decreased after GCB treatment. We also found that GCB de-phosphorylates PRAS40, an important substrate of AKT and mTOR binding protein of the mTORC1 complex [76]. Furthermore, phosphorylation of p70S6K, a downstream target of mTOR, significantly decreased following GCB treatment. Our results demonstrate that GCB acts on different targets of the AKT/mTOR pathway such as AKT, mTOR, PRAS40 and p70S6K. Taken together, these results strongly suggest that AKT/mTOR pathway is involved in the induction of autophagy.

mTOR activity can also be mediated by JNK, ERK and AMPK. However, in the LM7 cells, the phosphorylation of these kinases, was not affected following treatment. Thus, these results indicate that GCB induces autophagy in OS cells by a mechanism that involves the inhibition of AKT/mTOR signaling pathway.

Chapter 6. The role of autophagy on the sensitivity of OS cells to GCB treatment

Rationale and Aims:

A number of studies have demonstrated that the role of chemotherapy-induced autophagy is associated with cell survival and that inhibition of autophagy can sensitize cancer cells to chemotherapy as well as increase the therapeutic efficacy of certain drugs [42, 74].

Since GCB induces autophagy in OS, as demonstrated in chapter 5, the aim of this chapter is to determine whether autophagy plays a cytoprotective role in OS. For this purpose, we inhibited autophagy using two different approaches. First we used the pharmacologic inhibitor, Hydroxychloroquine (HCQ), which blocks the fusion between the autophagosomes and lysosomes [42]. We examined the effect of HCQ-induced autophagy inhibition on GCB-induced cytotoxicity in the LM7, K7M3, CCH-OS-D and MG63 cells.

Many pharmacologic inhibitors are non-specific [78]. Therefore, we also used shRNA to genetically inhibit the expression of ATG5 and Beclin, two essential autophagy genes. The effect of ATG5 silencing was evaluated in K7M3 and CCH-OS-D cells, while the effect of Beclin silencing was evaluated in K7M3, LM7 and CCH-OS-D cells.

Results

Effect of pharmacologic inhibition of autophagy in combination with Gemcitabine treatment

In order to determine the role of GCB-induced autophagy in OS, as either cell survival or enhanced cell death, we inhibited thea autophagy using two different approaches. First studies were performed with the autophagy inhibitor Hydroxychloroquine (HCQ). HCQ is an FDA approved drug for the treatment of autoimmune disorders, as well as malaria [50]. It prevents the fusion of the autophagosome with the lysosome by changing the pH of the lysosomes. As expected, western blot analysis showed an accumulation of LC3II followed by HCQ treatment, confirming the inhibition of the lysosome- autophagasome fusion which leads to blockade of LC3 degradation (Figure 31).

As shown in figures 32-35, HCQ alone had no significant cytotoxic effect. However, pretreatment of LM7 cells with HCQ resulted in increased cell sensitivity to GCB as demonstrated by a decrease in cell viability (46%) in the combination treatment group, compared to cells treated with GCB alone (64 %). On the contrary, combination treatment of HCQ with GCB in the K7M3 and CCH-OS-D cells significantly decreased OS cells sensitivity to GCB. Viability of K7M3 OS cells treated with the combination therapy was 39% and 24% in the GCB group. In the CCH-OS-D cells the viability in the combination group was 78% vs 54% in the GCB treatment alone. Inhibition of autophagy in the MG63 did not cause any effect in cell viability (71% vs 74%).

We further assessed if decreased or increased in sensitivity of these OS cells to GCB correlated with induction of apoptosis. In the LM7 cells, Annexin/PI staining was used to determine induction of cell death. Combination of HCQ with GCB increased cell death (15.9%) compared to GCB alone (11.0%), confirming the increase in sensitivity. In the K7M3, CCH-OS-D and MG63, we assessed the expression of cleaved caspase 3 as a hallmark of apoptosis. As shown in figures 33 and 34, in K7M3 and CCH-OS-D cells, treated with the combination treatment HCQ-GCB showed decreased cleaved caspase 3 expression compared to GCB alone. In contrast, MG63 had no significant change in the expression of cleaved caspase 3 in the combination group compared to GCB alone.

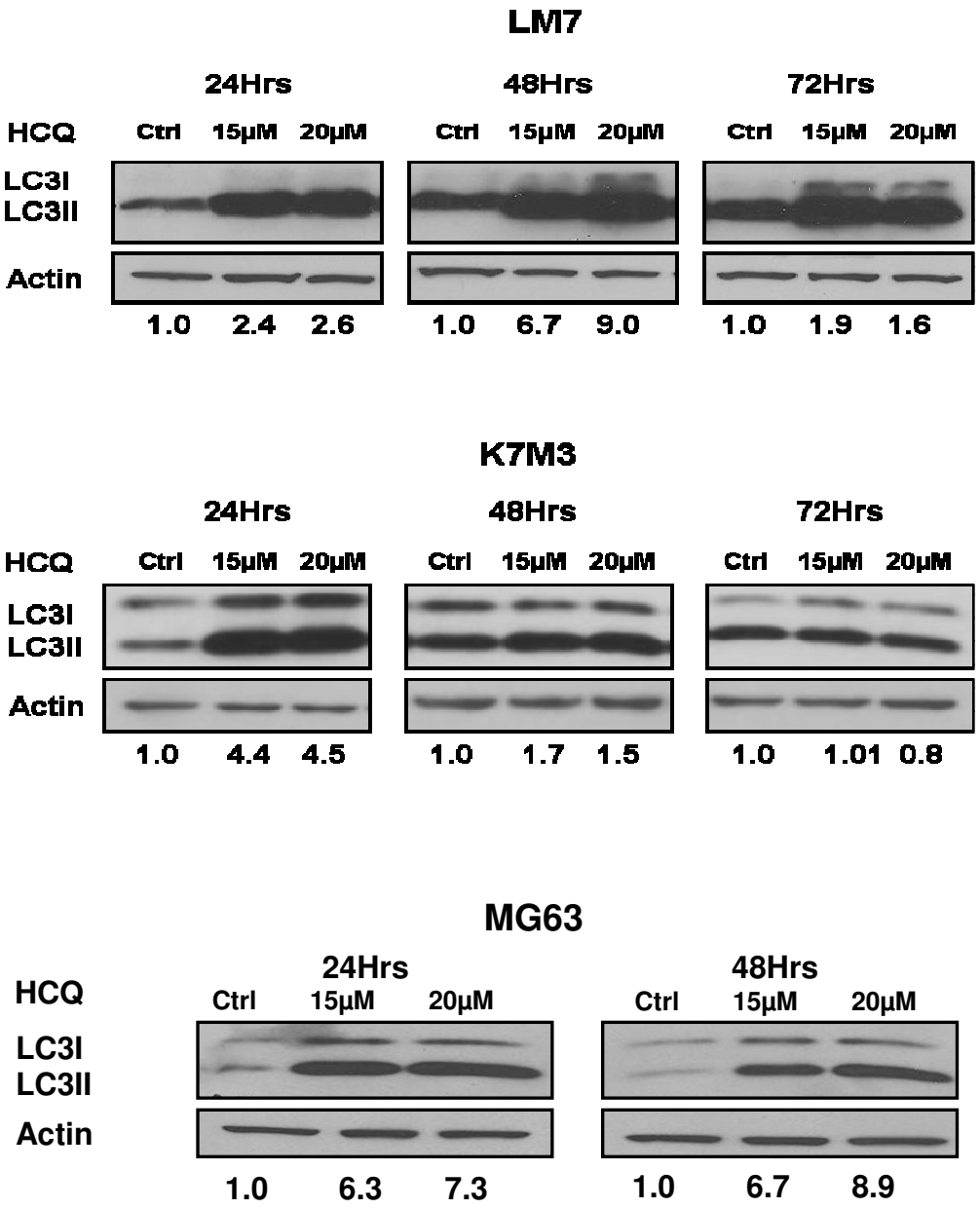


Figure 31. Accumulation of LC3II after HCQ treatment. Cells were treated with 15 or 20 μM of HCQ for up to 72 hrs then lysed using RIPA buffer. Western blot analysis was performed using antibodies for LC3. Actin was used as a loading control.

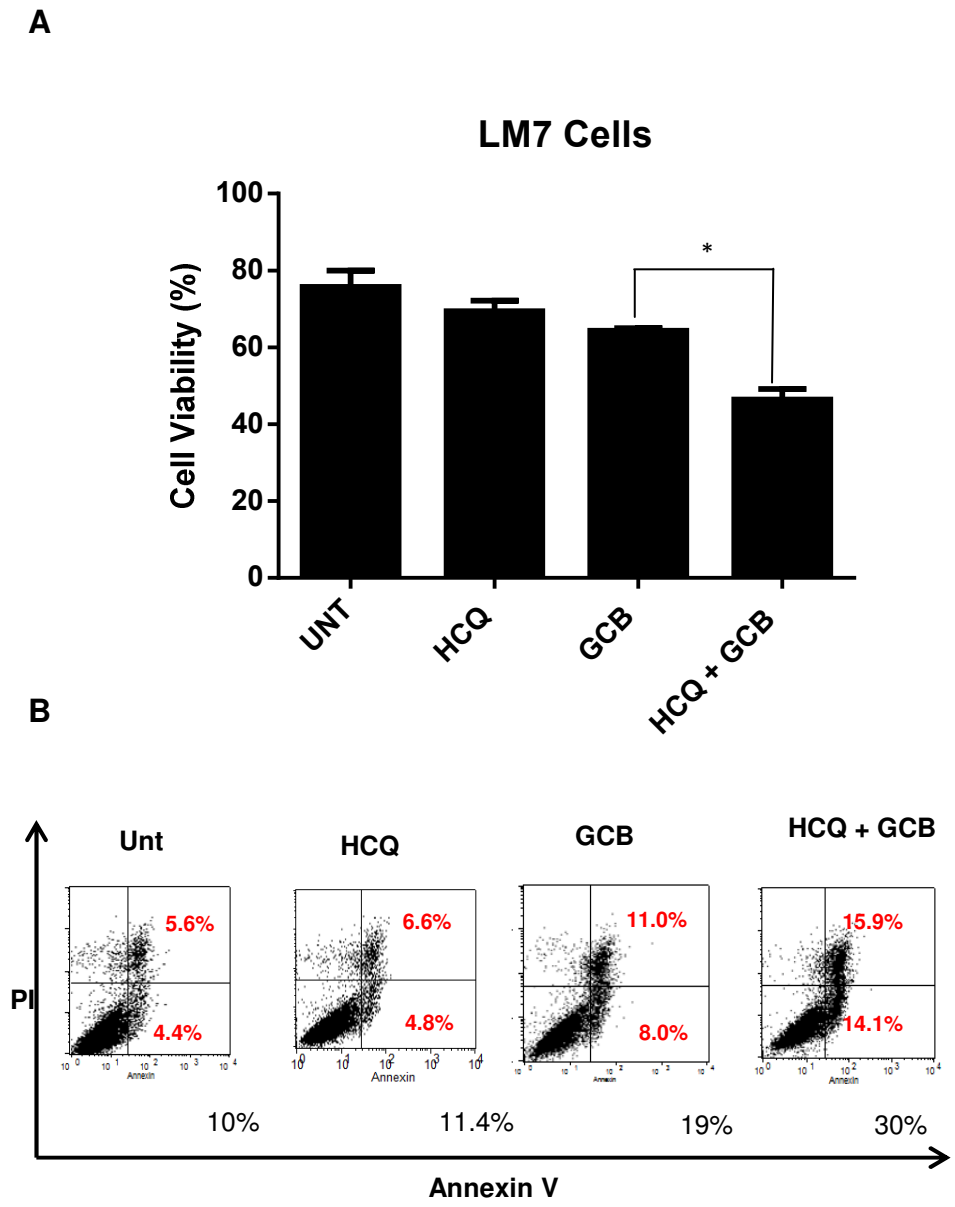


Figure 32. Pharmacologic inhibition of autophagy increased the sensitivity of LM7 OS cells to GCB. (A) LM7 cells were pretreated with HCQ for 30 minutes followed by GCB treatment for 48Hrs**.** Cell viability was measured by trypan blue. (B) Following the corresponding treatment, cell death was determined using double staining with PI and Annexin V.

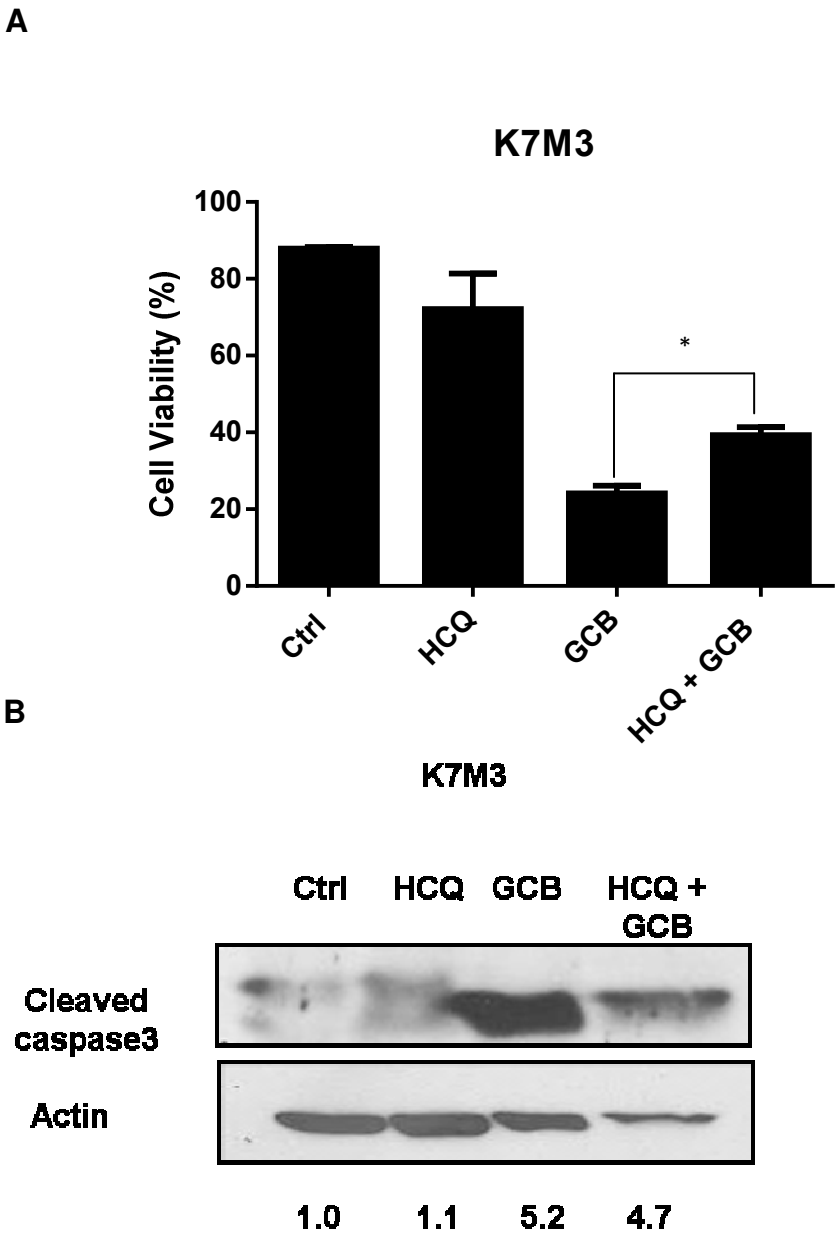
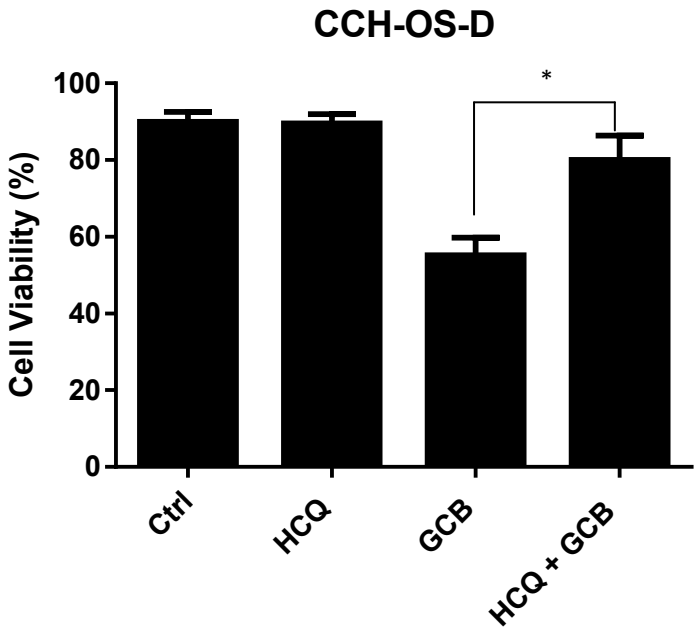


Figure 33. Pharmacologic inhibition of autophagy decreased the sensitivity of K7M3 OS cells to GCB. (A) K7M3 cells were pretreated with HCQ for 30 minutes followed by GCB treatment for 48 hrs. Cell viability was measured by trypan blue. (B) Western Blot analysis was used to detect cleaved caspase 3.

A



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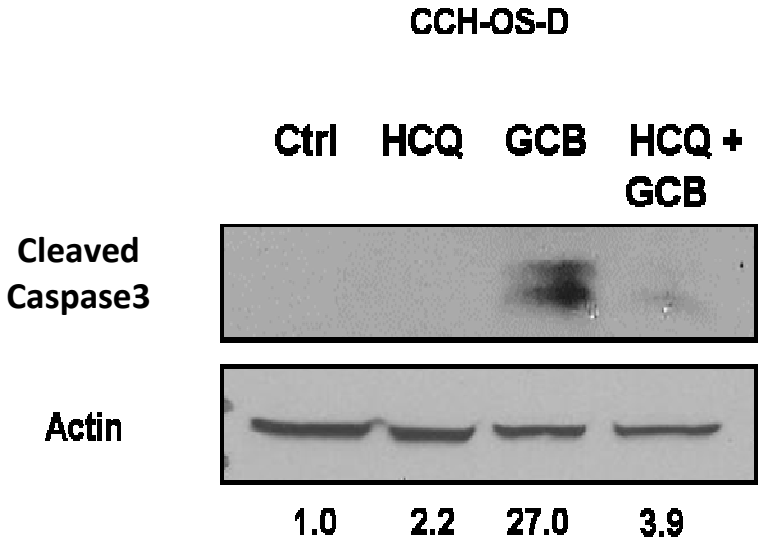


Figure 34. Pharmacologic inhibition of autophagy decreased the sensitivity of CCH-OS-D OS cells to GCB. (A) CCH-OS-D cells were pretreated with HCQ for 30 minutes followed by GCB treatment for 48 hrs. Cell viability was measured by trypan blue. (B) Western Blot analysis was used to detect cleaved caspase 3.

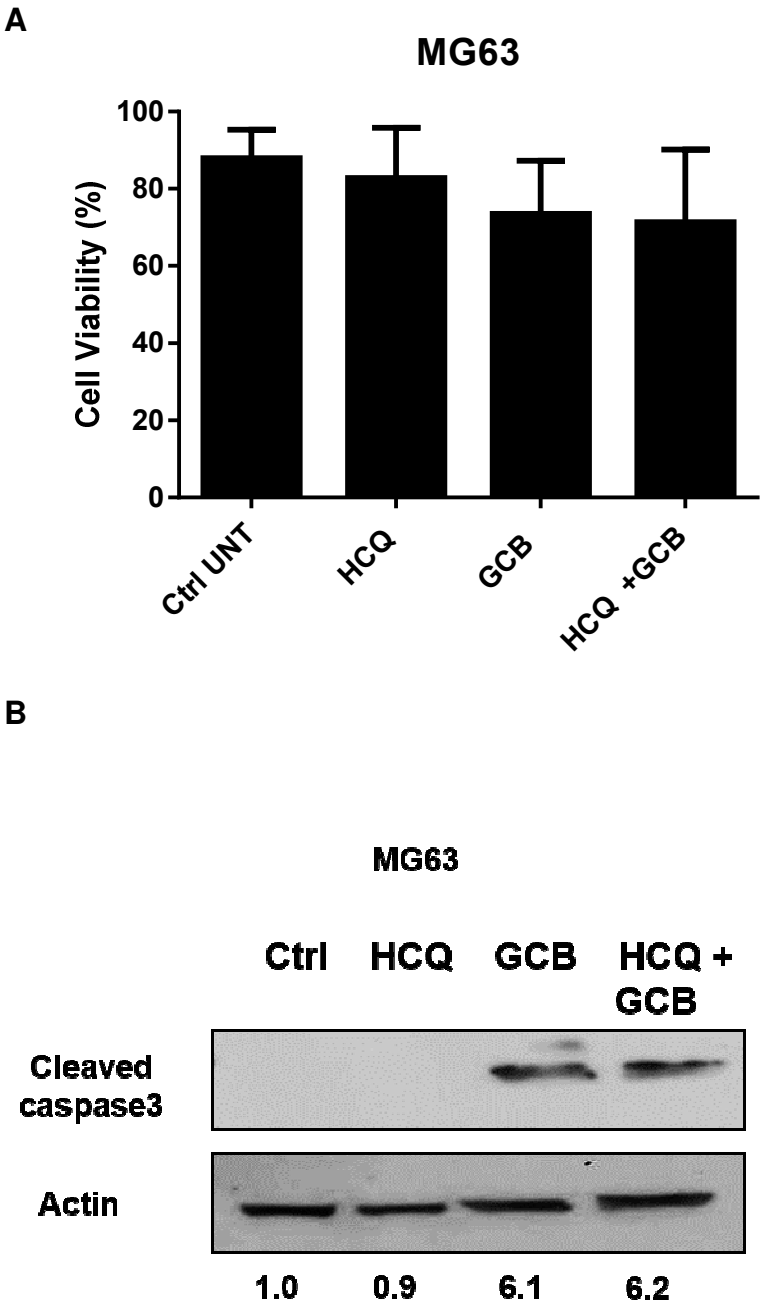


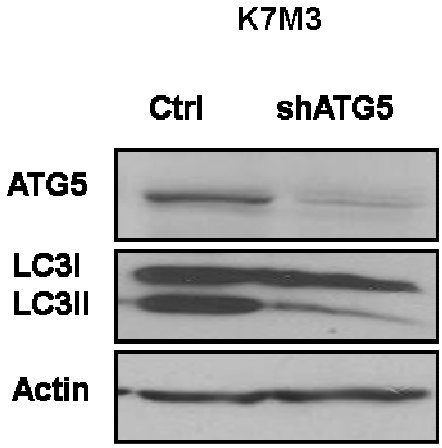
Figure 35. Pharmacologic inhibition of autophagy has no impact in the sensitivity of MG63 OS cells to GCB. (A) MG63 cells were pretreated with HCQ for 30 minutes followed by GCB treatment for 48 hrs. Cell viability was measured by trypan blue. (B) Western Blot analysis was used to detect cleaved caspase 3.

The effect of ATG5 silencing in the viability of OS cells to GCB.

Because HCQ has a wide range of non-specific effects, we decided to confirm the results obtained with HCQ by genetically inhibiting autophagy [78]. ATGs are involved in autophagy regulation and are essential for autophagy activation. Therefore, additional studies were performed in the K7M3 and CCH-OS-D using shRNA against ATG5. ATG5 is part of an important complex with ATG12, essential for the autophagosome formation [20]. As shown in figures 36 and 37, we confirmed ATG5 was stably suppressed. To determine if the knockdown of ATG5 was sufficient to block autophagy, conversion of LC3 was monitored by western blot. Indeed, the cells with ATG5 inhibition had a decreased conversion of LC3 to LC3II compared to the control.

Down-regulation of ATG5 decreased the sensitivity of K7M3 cells to GCB as demonstrated in Figure 36. Viability of shATG5-K7M3 treated with GCB was 57%, which is significantly higher than sh-K7M3 control treated cells at 26%. Similarly, ShATG5-CCH-OS-D GCB treated cells were also less sensitive to the drug with an 80% viability when compared to sh-CCH-OS-D control treated cells at 54%.

A



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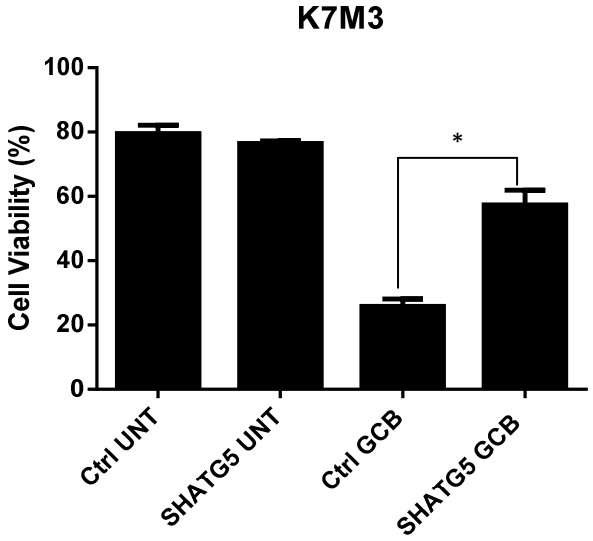

Figure 36. Genetic silencing of ATG5 decreased the sensitivity of K7M3 OS cells to GCB. (A) K7M3 cells were transfected with ATG5 plasmid or with an empty control vector. Down-regulation of ATG5 and LC3 expression was evaluated by western blot analysis. (B) K7M3 control cells and shRNA K7M3 cells were treated with GCB during 48 hrs. Cell viability was detected by trypan blue.

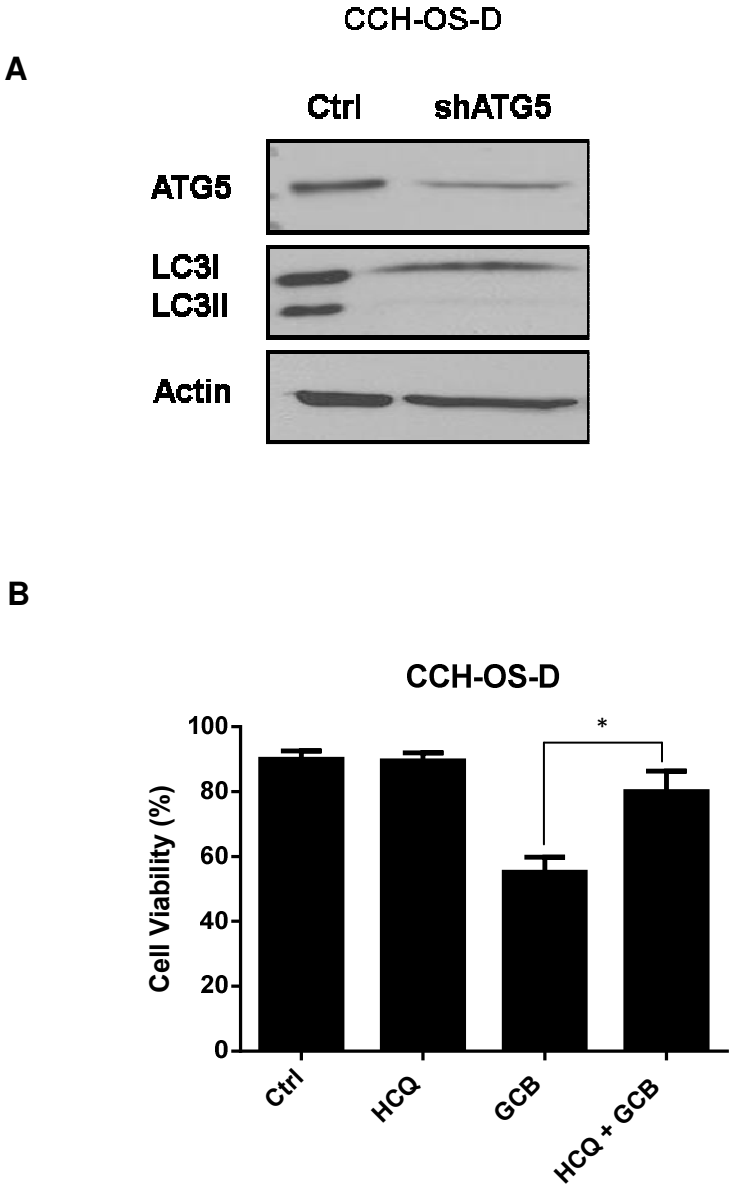

Figure 37. Genetic silencing of ATG5 decreased the sensitivity of CCH-OS-D OS cell to GCB. (A) CCH-OS-D cells were transfected with ATG5 plasmid or with an empty control vector. Downregulation of ATG5 and LC3 expression was evaluated by western blot analysis. (B) CCH-OS-D control cells and shRNA CCH-OS-D cells were treated with GCB for 48 hrs. Cell viability was detected by rrypan Blue.

The effect of Beclin silencing in the viability of OS cells to GCB.

Beclin is important for the induction of autophagy as part of the Vps34 complex [20]. As mentioned before, Beclin expression was increased after GCB treatment. These results led us to investigate if Beclin knockdown would also have an effect in OS cells sensitivity after treatment with GCB. First, we demonstrated that Beclin inhibition resulted in decreased LC3I/LC3II conversion.

Experimental data from the Beclin suppressed cells displayed an identical outcome as with the pharmacologic inhibition by HCQ. That is, a significant decrease in viability was observed in GCB treated shBECN-LM7 cells with a 32% vs 55% control GCB sh-LM7. In contrast, viability of GCB treated shBECN-K7M3 cells was higher (43%) than control GCB sh-K7M3 (28%), while viability of GCB treated shBECN-CCH-OS-D cells was also higher at 57% when compared to control GCB sh-CCH-OS-D at 28%.

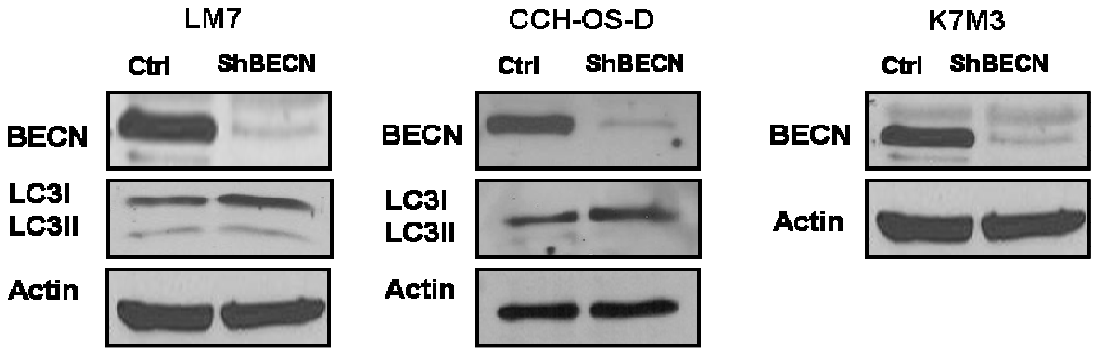

Figure 38. Genetic silencing of Beclin in LM7 CCH-OS-D OS and K7M3 cells. Cells were transfected with Beclin plasmid or with an empty control vector. Downregulation of Beclin and LC3 expression was evaluated by western blot analysis.

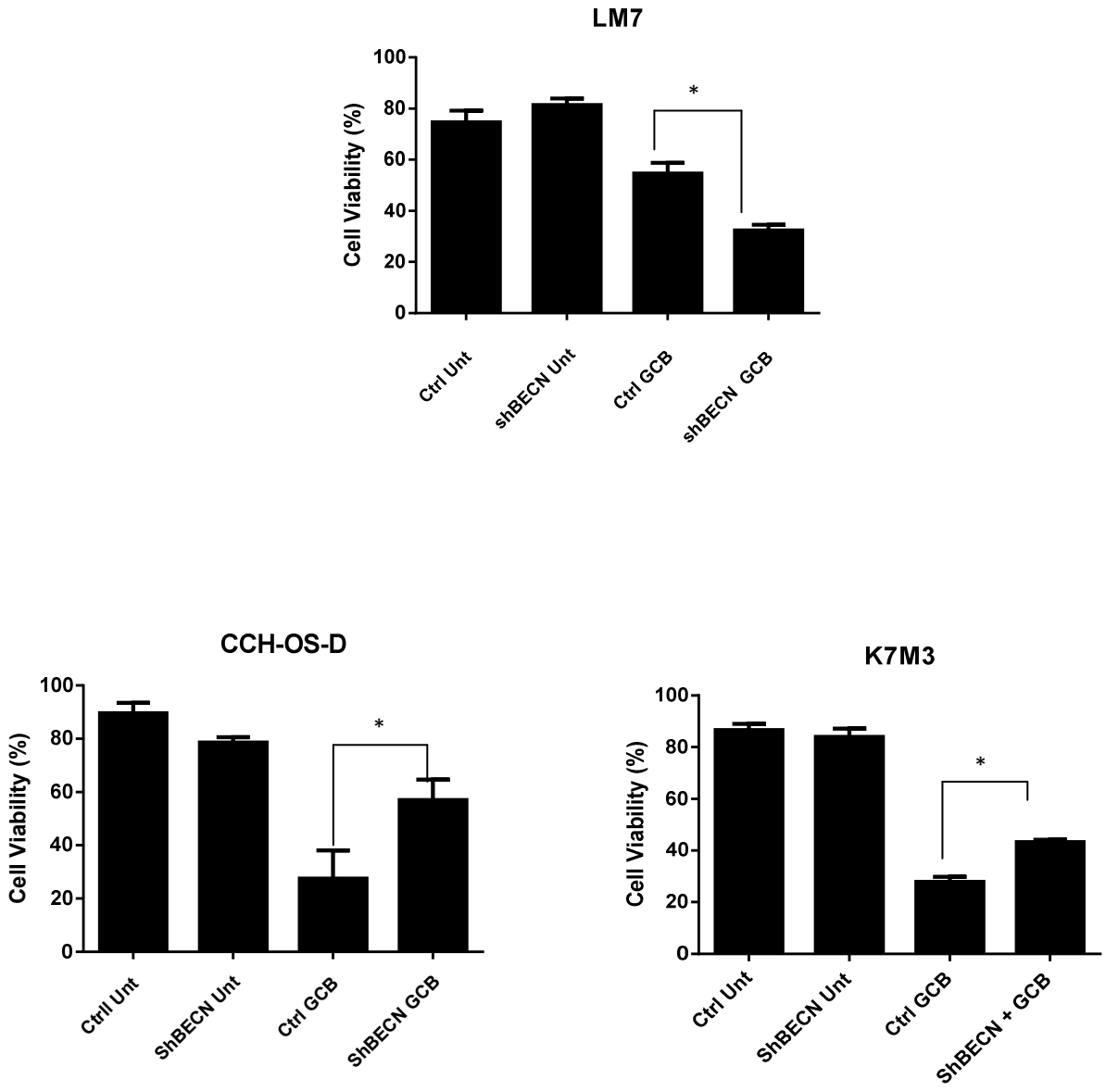

Figure 39. Genetic inhibition of Beclin modulates the sensitivity of LM7, CCH-OS-D and K7M3 to GCB. Cells were transfected with Beclin plasmid or with an empty control vector. Control cells and shRNA cells were treated with GCB for 48 or 72 hrs. Cell viability was detected by trypan blue.

Summary

While several studies have demonstrated both a cytoprotective and cytotoxic role of autophagy in cancer, the role of autophagy in OS is still unknown. The previous observations that GCB induces autophagy in OS cells, led us to examine if inhibition of autophagy can affect the sensitivity of OS cells to GCB treatment. In order to answer this question we used two different methods to inhibit autophagy. Specifically, we used the pharmacologic inhibitor HCQ and genetic inhibition of ATG5 and Beclin.

In the present study, we showed that HCQ did not have any cytotoxic effect in OS cells. Inhibition of autophagy in LM7 cells treated with GCB, resulted in a decrease in cell viability. These results indicate that autophagy regulates the sensitivity of LM7 OS cells and serves as an induced pro-survival cell mechanism. Increase in cell death following autophagy inhibition was confirmed by AnnexinV/PI staining. Conversely, when autophagy was inhibited in K7M3 and CCH-OS-D-treated with GCB, there was a significant increase in cell viability, supporting the concept of autophagy as an induced cell-death mechanism. The analysis of the presence of cleaved caspase 3 in K7M3 and CCH-OS-D revealed a significant decrease in cell death after inhibition of autophagy. Inhibition of autophagy in MG63 cells did not exert any effect on their sensitivity to GCB treatment as confirmed by no change in cleaved caspase 3.

Gewirtz et al, have described a type of autophagy termed “Non-protective autophagy”. This type of autophagy is induced in cancer cells but its inhibition or

induction do not affect the sensitivity of the cells. Therefore, it is possible that in the MG63 cells, autophagy is just a “bystander” [31, 79].

To further confirm the pharmacologic results, we used shRNA to knockdown ATG5 and Beclin. First we demonstrated that inhibition of ATG5 and Beclin, impaired the process of autophagy as indicated by decreased conversion of LC3I/LC3II. Consistent with the HCQ results, we demonstrated by the cell viability assay that ShRNA-transfected cells against ATG5 and Beclin, in combination with GCB treatment have an opposing effect on the sensitivity of OS cells to GCB. These results confirm the existence of the dual role of autophagy in response to chemotherapy in the same type of cancer.

Table 6 Summary of autophagy roles and markers for each cell line.

Type of Cell	Mouse 	Mouse	Human 	Human 	Human
Cell Line	K7M3	DLM8	LM7	CCH-OS-D	MG63
Effect of GCB on Beclin expression					
Effect of GCB on LC3I to LC3II Ratio					
Effect of GCB on p62		No Data			No Data
Effect of GCB on AVOs		No Data			
Effect of GCB on Autophagosome Formation		No Data			
Effect of autophagy inhibition in sensitivity of cells		No Data			No effect

Chapter 7. HSP27 as a potential factor that regulates the fate of autophagy in OS cells

Rationale and aim

The determining factors that distinguish between the cytoprotective and cytotoxic roles of autophagy remain a fundamental question in the field of autophagy. The human phospho- kinase array utilized in chapter 6 encompassed many kinases including Heat shock proteins. Heat shock proteins are proteins whose expression is increase when cells are exposed to high temperatures and other types of stress. Heath shock protein B1 (also known as HSP27) is a chaperone molecule involved in different processes such as unfolding of proteins and resistance to chemotherapy [80]. It is highly expressed in different types of cancer including OS [81, 82]. Studies have shown that its expression correlates with OS lung metastasis [83]. Increased phosphorylated HSP27 has also been shown to be involved in the resistance of pancreatic cancer cells to GCB [80, 82]. We demonstrated that autophagy can either increase or decrease the sensitivity of OS cells to GCB. However, the mechanism that regulates this dual role of autophagy remains undefined. Here, we hypothesized that HSP27 may be an important factor that regulates the fate of autophagy in OS cells.

Results

Phosphorylation status of HSP27 in OS cells after GCB treatment

We used the human phospho-kinase array to test the phosphorylation status of HSP27 in CCH-OS-D and LM7 OS cells. Results demonstrated that following GCB treatment there is a clear increase in the levels of p-HSP27 in treated LM7 OS cells, while decreased levels were observed CCH-OS-D cells (Figure 40 A) as compared to their respective untreated control cells. Many of the functions of HSP27 are regulated by change in phosphorylation, including inhibition of apoptosis [81]. Based on this, we decided to evaluate the protein expression levels of p-HSP27 at Ser 78/82 in a panel of OS cells, including CCH-OS-D, K7M3 and LM7 OS cells following treatment with 1 or 5 μM of GCB. As shown in figure 40 B, protein levels of p-HSP27 at Ser78/82 is decreased in CCH-OS-D and K7M3, while there is no change in MG63. Surprisingly, we found that the phosphorylation status of HSP27 correlates with the role of autophagy in the different OS cells (Table 7).

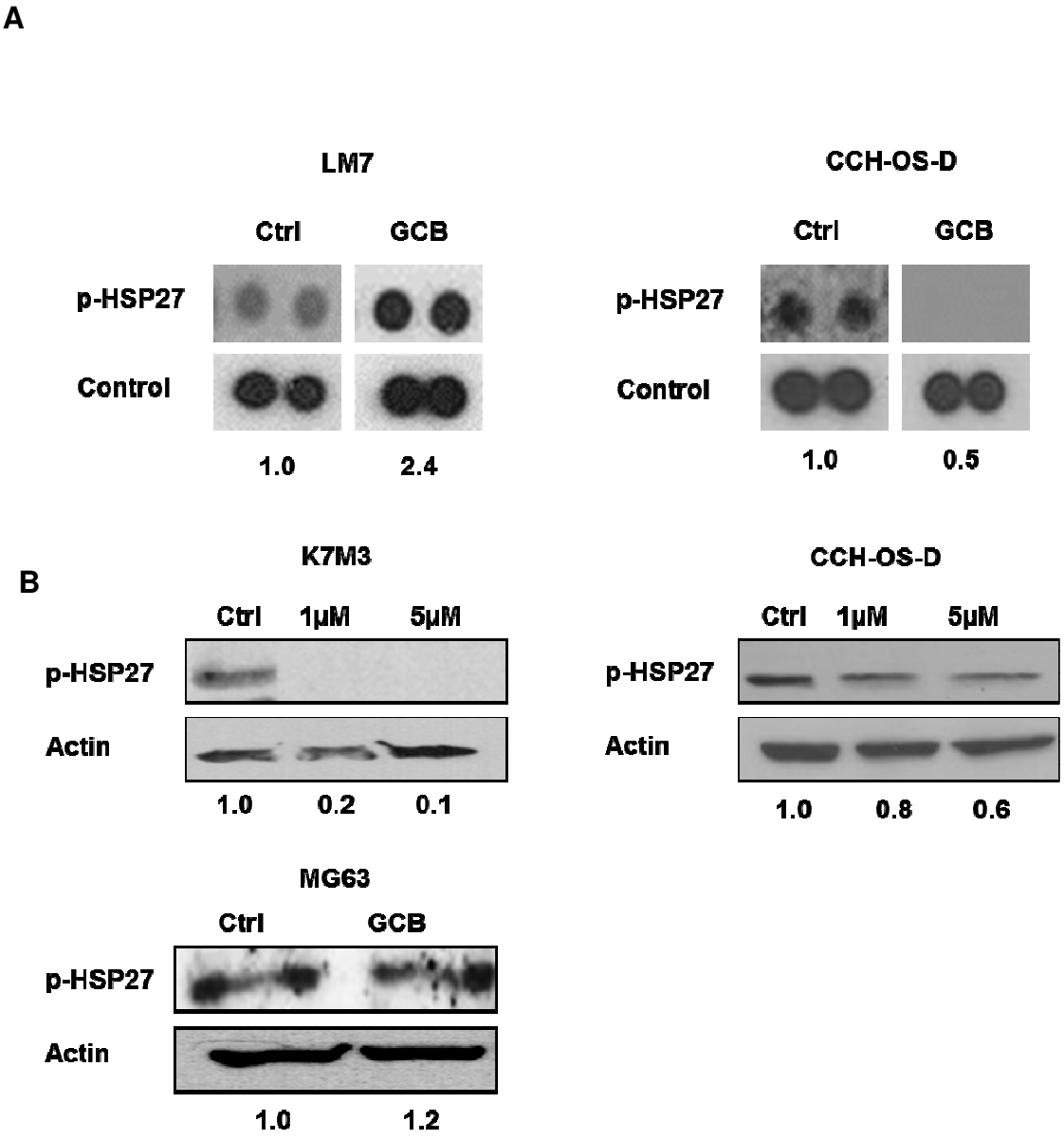

Figure 40. Phosphorylation status of HSP27 in OS cells. (A) CCH-OS-D and LM7 cells were treated during 48 and 72 hrs, respectively. Cells were then analyzed by human phospho-kinase array. (B)K7M3, CCH-OS-D and MG63 cells were treated with indicated doses of GCB. Cells were collected and lysate with RIPA buffer. Western blot analysis was performed using specific antibody for p-HSP27 (Ser78/82). Actin was used as a loading control.

Phosphorylation status of HSP27 correlates with the role of autophagy in K7M3 OS cells treated with 9-NC or GCB

In order to determine if this correlation of HSP27 and the protective effect of autophagy in response to GCB is relevant to other chemotherapeutic treatment agents, we decided to compare the levels of p-HSP27 in the K7M3 cells treated with 9- nitro- camptothecin (9-NC) or GCB. Our laboratory has previously demonstrated that inhibition of autophagy through the genetic inhibition of ATG5 in 9-NC K7M3-treated cells resulted in a significant decrease in cell viability, suggesting autophagy as a cell survival inducing mechanism [84]. As shown in figure 41, HSP27 is dephosphorylated in sh-ATG5 K7M3 cells treated with GCB, while in the sh-ATG5 K7M3 cells treated with 9NC, p-HSP27 levels increased. These results suggest that the phosphorylation status of HSP27 correlates with the role of autophagy.

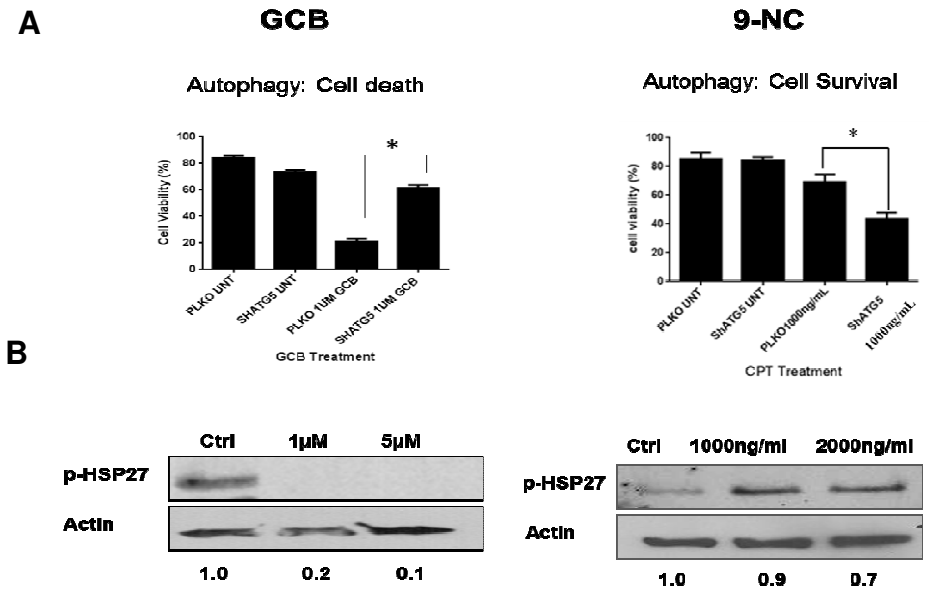

Figure 41. Status of HSP27 phosphorylation after GCB and 9NC treatment in K7M3 cells. (A) ShATG5-K7M3 cells were treated with different doses of GCB or 9NC and cell viability was performed. (B) K7M3 cells were lysed with RIPA buffer and subjected to western blot analysis by using a specific antibody for p-HSP27.

Phosphorylation of p38 MAPK after GCB treatment

We decided to evaluate the signaling pathway involved in HSP27 phosphorylation. HSP27 has been identified as a downstream of the serine/threonine kinase, p38 MAPK [80]. Therefore, we investigated whether p38 MAPK was involved in the phosphorylation of HSP27 in GCB treated CCH-OS-D and LM7 cells. Our preliminary results displayed that compared to control cells, no change in p38 MAPK protein levels was detected in CCH-OS-D or LM7 cells after GCB treatment, suggesting that p38 MAPK may not be involved in the phosphorylation of HSP27 in OS.

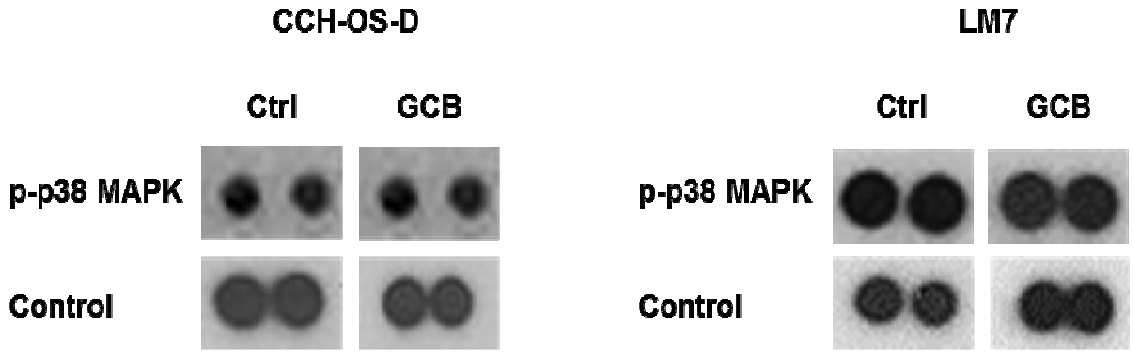

Figure 42. Phosphorylation of p38 after GCB treatment. CCH-OS-D and LM7 cells were treated with GCB during 48 and 72 hrs, respectively. Cell lysates were analyzed using the Human phospho kinase array. The phosphorylation levels of p38-MAPK were evaluated.

Expression of HSP27 in LM7 knockdown cells

In order to explore if HSP27 has any effect in the context of GCB-induced autophagy in OS cells, we used shRNA to create LM7 OS cells with deplete expression of HSP27. Efficacy of HSP27 depletion was confirmed by western blot analysis. Further studies are ongoing to confirm the potential role of HSP27 in GCB-induced autophagy in OS cells.

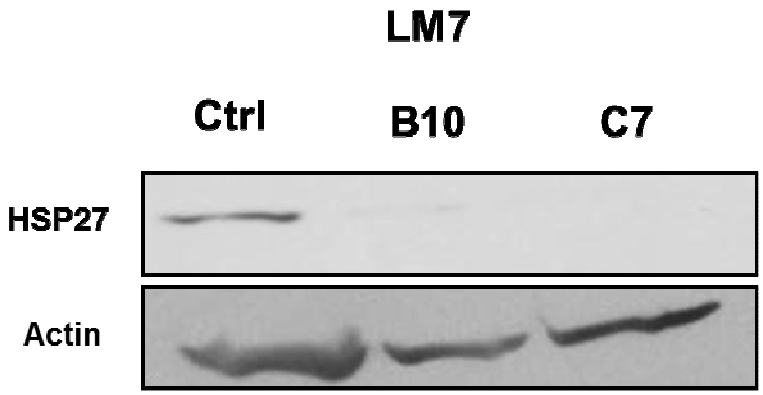

Figure 43. HSP27 knockdown decreases autophagy in LM7 OS cells. LM7 cells were transfected with HSP27 shRNA. Cells were lysed using RIPA buffer and subjected to Western blot analysis using antibodies specific for HSP27. Actin was used as a loading control.

8.3 Summary

On this chapter we demonstrated that there is a visible decrease of HSP27 phosphorylation levels in CCH-OS-D and K7M3 after treatment with GCB. The contrary was found for LM7 cells; HSP27 phosphorylation levels increased after treatment with GCB.

Analysis of the phosphorylation of HSP27 and the role of autophagy in each OS cell line demonstrated that there is a correlation between the phosphorylation status of HSP27 and the role of autophagy (See Table 7). Specifically, phosphorylation of HSP27 correlates with the cytoprotective role of autophagy while in the cells where autophagy have a cytotoxic role, p-HSP27 levels are low.

This data provides information to understand that HSP27 may be a potential regulator in defining the outcome of GCB-induced autophagy in OS. Regulation of HSP27 phosphorylation and the autophagic pathway may help to develop novel therapeutic strategies for OS treatment in the future.

Table 7. Summary of Results. The phosphorylation status of HSP27 correlates with the role of autophagy in OS cells.

Cell Line	P-HSP27 Expression After GCB treatment	Role of Autophagy
LM7	Increase	Cell survival
CCH-OS-D	Decrease	Cell death
K7M3	Decrease	Cell death
MG63	No change	No effect

Chapter 8. Discussion and Future directions

Discussion

OS remains a difficult disease to treat with 30-35% of patients dying of lung metastasis. New better targeted therapies are needed. Studies from our group has previously demonstrated that the nucleoside analogue, GCB, has a significant therapeutic effect against OS lung metastasis. However, a population of cells fails to respond, due to acquired resistance. Better understanding of the molecular mechanisms underlying OS resistance to GCB is necessary.

Autophagy has been described as a potential mechanism that may help cancer cells to survive under stressful conditions, such as in the presence of chemotherapy. The role of GCB-induced cytoprotective autophagy has been studied in various cancer models [68, 85]. However, there is no report on the relevance of autophagy in the sensitivity of OS cells to GCB treatment.

To extend our understanding of the potential role of autophagy in OS, we established three objectives: investigate the induction of autophagy after GCB treatment in OS cells; 2) elucidate the potential mechanism by which GCB induces autophagy in OS; 3) determine the role of autophagy in OS cell sensitivity to GCB. To accomplish these objectives, we conducted a series of studies using different types of OS cells and mouse models. In the current study, for the first time, we demonstrated that GCB can induce both cytoprotective and cytotoxic autophagy in OS cells through a mechanism that involve inactivation of the AKT/mTOR signaling pathway and when HSP27 is induced inhibition of autophagy will increase sensitivity to GCB.

We began by evaluating the sensitivity of a panel of OS cells to GCB. In accordance with previous studies from our lab, we found that GCB exerts a cytotoxic effect in OS cells, suggested by a significant decrease in cell viability and induction of cleaved caspase 3 [18, 19]. K7M3 cells were the most sensitive cell line followed by the CCH-OS-D and LM7, respectively. However, it was observed that after 72 hrs treatment with GCB some cells remain with a viable, suggesting that a population of cells were less sensitive to GCB treatment. We proposed that autophagy was induced as a mechanism of resistance.

GCB-induced autophagy was demonstrated in a panel of 5 OS cell lines Through western blot analysis, we observed an increase in Beclin expression and conversion of LC3I to LC3II. We also observed expression of p62 decreased in treated cells when compared to control cells. Immunofluorescence staining revealed increased formation of acidic vesicles in all treated cell lines. Electron microscopy was used to visualize and verify the nature of the autophagic vacuoles, to verify if in fact they had the characteristic double membrane of autophagic vesicular organelles. All these consistent results allows us to conclude that GCB induces autophagy is OS cells after treatment.

Two mouse OS mouse models were used to verify autophagy induction *in vivo*. Using IHC staining of LC3 and Beclin, the expression of these two autophagy markers was evaluated after aerosol GCB treatment. We found that indeed both markers were upregulated in treated mice when compared to control. Electron microscopy revealed higher number of double membrane autophagic vacuoles in the treated group. These results are consistent with the *in vitro* data allowing us to

conclude autophagy induction in OS after GCB treatment. These results are also in accordance with published research concluding GCB induces autophagy in pancreatic and breast cancer cells [58, 59]. This is therefore the first study to prove that GCB treatment induces autophagy in OS both in vitro and in vivo.

Autophagy activation is associated with the downregulation of the AKT/mTOR pathway [75]. Western blot analysis revealed down-regulation of phosphorylated AKT (S473) and mTOR (S2448) by GCB treatment; total AKT and mTOR levels remained unchanged. Additionally, a human phospho-kinase array was used to verify western blot results and to check for other kinases that may have some involvement in the autophagy induction pathway after treatment with GCB. Results revealed GCB induced de-phosphorylation of AKT at S473 and mTOR at Ser2448, which is one of the most important residues for mTOR activity [77]. Additionally, as revealed by the human phospho-kinase array; GCB decreased phosphorylation of two mTOR related targets: PRAS40 and P70S6K. No changes were observed in phosphorylation of AMPK, JNK or ERK; common mTOR inhibitors. Taken together, these results suggest that AKT/mTOR pathway is involved in the induction of autophagy by GCB in OS cells. These results are consistent with Le et al., who demonstrated that Dasatinib-induced autophagy in ovarian cancer cells depends on the inhibition of the AKT and mTOR pathway [72]. In addition, Wang et al., reported that Quercetin induces cytoprotective autophagy in gastric cancer cells and underscored the involvement of the AKT/mTOR pathway [69].

Regarding the role of autophagy in OS; several studies have proposed that autophagy can either promote cell death or cell survival. We initially hypothesized that GCB-induced autophagy decreased the sensitivity of OS cells to GCB.

Utilizing pharmacologic and genetic inhibition of autophagy, we aimed to study the role of autophagy in OS cells after treatment with GCB. Interestingly, significant decrease in cell viability was observed in LM7 cells treated with GCB after autophagy had been inhibited using HCQ, ATG5 or Beclin shRNA. These results suggest a cytoprotective role in LM7 cells which is consistent with published studies such as: paclitaxel treatment of Saos-2 osteosarcoma cells [2] and Everolimus treatment in Mantle cell lymphoma [86].

However, either pharmacologic or genetic inhibition of autophagy in the K7M3 and CCH-OS-D significantly decreased the sensitivity of these cells to GCB as demonstrated by an increase in cell viability and decrease in expression of cleaved caspase 3. These results confirm that autophagy induction can have dual roles, both cytoprotective and cytotoxic in OS. These results suggest that GCB-induced autophagy has a cytoprotective role in the LM7 cells whereas GCB-induced autophagy in CCH-OS-D and K7M3 enhances tumor cell death.

Even though the dual role of autophagy in cancer has been reported, to our knowledge this is the first study demonstrating that GCB can induces both cytoprotective and cytotoxic autophagy in OS. These results are consistent with the recently published work by Hollomon and coworkers where they showed that Camptothecin (CPT) induces autophagy in DLM8 and K7M3 mouse OS cells and that inhibition of autophagy has an opposite effect on CPT-induced cytotoxicity

within OS cells [84]. In addition, Gewirtz et al., have also demonstrated the induction of both cytoprotective and cytotoxic autophagy in breast cancer cells after radiation treatment [66].

Although the factor that distinguishes between the cytoprotective or cytotoxic role of autophagy is unknown, we proposed HSP27 as a potential factor for the regulation of GCB-induced autophagy in OS cells. We demonstrated that following GCB treatment there is a clear increase in the levels of p-HSP27 in the treated LM7 OS cells, while decreased levels were observed in the CCH-OS-D and K7M3 treated OS cells. However, in MG63 cells, there was no significant difference in p-HSP27 between treated and un-treated cells. Interestingly, on K7M3 and CCH-OS-D cells, the role of autophagy was cytotoxic, whereas in the LM7 cells the role was found to be cytoprotective. These results suggest that phosphorylation of HSP27 correlates with autophagy as a mechanism of cellular resistance.

In contrast to Nakashima et al., who previously reported that p38 MAPK is involved in phosphorylation of HSP27 in GCB-treated pancreatic cancer cells, our results indicate that after GCB treatment there was no change in the phosphorylation of p38, suggesting that phosphorylation of HSP27 is regulated by other kinase. More detailed studies are necessary to underscore the potential role of HSP27 in the regulation of autophagy.

In conclusion, we have demonstrated that GCB can induce both cytoprotective and cytotoxic autophagy in OS cells. In addition, we also demonstrated the involvement of AKT/mTOR pathway in GCB-induced autophagy and HSP27 as the potential factor that regulates the fate of autophagy. Recently,

there has been significant enthusiasm around the modulation of autophagy as a novel strategy for cancer treatment in multiple tumor types. Current clinical trials involving combination of chemotherapeutic agents with autophagy inhibitors such as HCQ are ongoing. However, the autophagy paradox remains. The role of autophagy, cytoprotective or cytotoxic, should be established on the specific intended target/ treatment combination. There is still a need for better understanding of the molecular pathways that govern the process of autophagy and the possible outcome of the cells. While autophagy can be induced in OS, the role of autophagy in treatment response and its potential as therapeutic target through either upregulation or inhibition of the process requires further investigation.

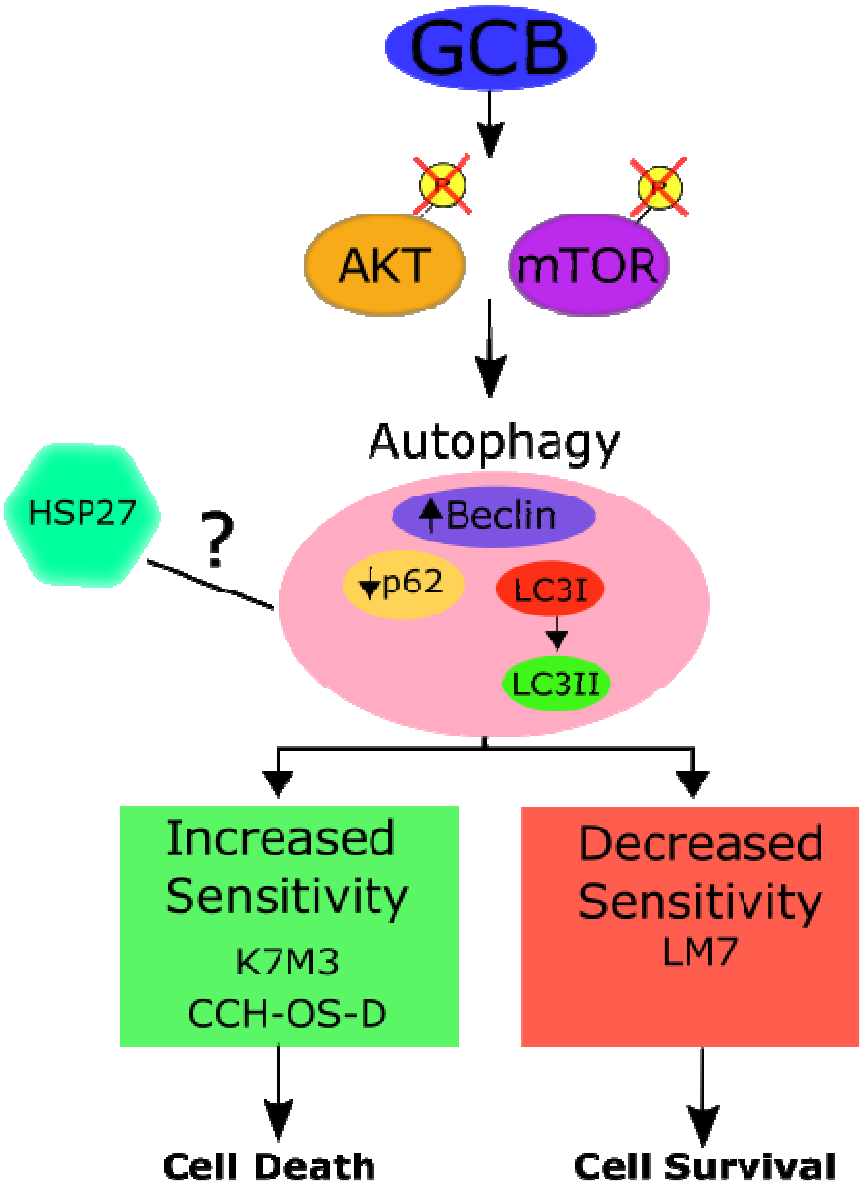

Figure 44. Schematic of proposed model. After GCB treatment there is a decrease in the phosphorylation of AKT and mTOR which leads to the induction of autophagy. The process of autophagy is suggested by activation of Beclin, degradation of p62 and conversion of LC3I/LC3II. The role of autophagy is cell dependent leading to a decreased in sensitivity of LM7 cells OS to GCB and an increase sensitivity of CCH-OS-D and K7M3. HSP27 is proposed as a potential modulator of the fate of autophagy.

Future Directions

Determination of the role of autophagy in vivo

We have demonstrated induction of autophagy in K7M3 and LM7 OS lung metastasis followed aerosol GCB treatment. However the role of GCB-induced autophagy in OS lung metastasis is unknown. Therefore, a critical aspect for further investigation would be to evaluate the role of autophagy in the sensitivity of OS cells *in vivo.*

In chapter 6, we demonstrated that inhibition of autophagy using the pharmacologic inhibitor HCQ leads to either increased or decreased OS cell sensitivity to GCB *in vitro*. Therefore, using OS mouse models, the therapeutic effect of GCB can be evaluated when used alone and in combination with HCQ. The therapeutic effect can be measured by wet-lung weight and number of macro and micro-metastasis in the different mice populations, while evidence of autophagy will be determined by the analysis of tissue samples using immunohistochemistry and electron microscopy.

To inhibit autophagy more specifically at the molecular level, we can target specific proteins that are required for the process of autophagy. Some proteins to be studied include: Beclin and ATG5. OS cells will be transfected by means of shRNA to suppress the expression of the intended target proteins. shRNA transfected OS cells will be injected I.V. into the tail vein. Evidence of lung metastases will be determined. Therapy with aerosol GCB will be given and therapeutic effect and autophagy induction will be measured as previously described.

Mechanistic studies

As shown in chapter 5, we have demonstrated a decrease in the phosphorylation of key components of the AKT/mTOR pathway suggesting a correlation between the inactivation of this pathway and induction of autophagy. However, studies to confirm that autophagy induction is due to mTOR inhibition are needed. To further confirm this, Rapamycin, an mTOR inhibitor, will be used to inactivate mTOR. We will expect to see that the combination treatment of Rapamycin and GCB will suppress the activation of mTOR and lead to an accumulation of LC3, indicating induction of autophagy. In addition, the effect of this combination on the sensitivity of OS cells can be measured by cell viability and induction of cell death. We will expect to see that inhibition of mTOR by Rapamycin will increase the sensitivity of LM7 cells and decrease the sensitivity of CCH-OS-D and K7M3, confirming that GCB induces autophagy through inhibition of the AKT/mTOR pathway.

Another important question that remains is to determine how GCB treatment causes a decrease in AKT/mTOR pathway. Since one of the mechanisms of GCB is to inhibit the function of Ribonucleotide Reductase, the enzyme involved in the formation of deoxyribonucleotides, we hypothesized that the AKT/mTOR pathway will be inactivated as a signal to the cells for recycling cellular components necessary for energy and sustain survival, thus activating autophagy.

HSP27 as potential factor that regulates the fate of Gemcitabine- induced autophagy in osteosarcoma

We have demonstrated that following treatment with GCB the phosphorylation status of HSP27 correlates with the role of autophagy. However, whether HSP27 is involved in the regulation of the role of autophagy is unknown. It would be beneficial to continue exploring the potential of HSP27 as a candidate factor that might regulate the observed dual role of autophagy in OS, both on a cellular and systemic level. Currently, there are no specific HSP27 inhibitors. We propose to use specific genetic inhibition. We would also determine the sensitivity of OS cells to GCB after inhibition of HSP27. The information obtained from these experiments would provide knowledge to design future experiments.

Translational Implications

A fundamental piece of information that needs to be assessed is whether autophagy is induced in OS tumor specimens following chemotherapy treatment. A potential approach to address this aim would be to analyze patient samples (pre-treatment and primary resection) for autophagy markers, such as LC3, and expression of HSP27 and to correlate the levels of expression with clinical outcomes. Based on the premise that autophagy is a mechanism of chemoresistance and that HSP27 may be a potential factor that regulates the fate of autophagy in osteosarcoma, we hypothesize that patients with significant induction of autophagy and high levels of HSP27 will have a poorer response to chemotherapy, thus worse clinical outcomes. The results of this study will provide knowledge for using autophagy and HSP27 as a prognostic biomarkers. Further,

this could potentially serve as the rationale for the use of autophagy inhibitors or inducers in combination with GCB for future clinical trials.

Another potential future direction will be to assess what are the transcriptional regulators of autophagy through gene expression profiling. We can test OS cell lines that have shown the dual role of autophagy (LM7 and CCH-OS-D), to better understand which genes are associated with the induction of cytoprotective or cytotoxic autophagy.

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Vita

Janice M. Santiago O'Farrill was born in Puerto Rico on August 26, 1985 and raised by her parents Jesusa O'Farrill and Ramón Santiago. She graduated with a bachelor's degree in General Sciences from the University of Puerto Rico, Rio Piedras Campus in May 2008. In August 2008, she enrolled at the School of Pharmacy, University of Puerto Rico-Medical Sciences Campus and graduated with a Master's Degree in Pharmaceutical Sciences. During this time she was focused in trying to identify potential compounds with inhibitory action on the growth of breast cancer cells. The experiences she gained during her graduate career, have confirmed her devotion to continue working in the cancer research field. For this reason, in August 2010 Janice joined The University of Texas Graduate School of Biomedical Sciences at Houston and M.D Anderson Cancer Center to pursue a doctoral degree in cancer biology. Janice then joined the Department of Pediatrics in Dr. Eugenie Kleinerman's laboratory to carry out her dissertation project, determining how autophagy contributes to the resistance of osteosarcoma cells to chemotherapy. Thus far, her work on autophagy and osteosarcoma has resulted in the co-author publication in *BMC Cancer 2013; 13(500)* and two book chapters in *Advances in Experimental Medicine and Biology (*2014) and *IBMS BoneKEy-Nature* (2014).