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Dissertação de Mestrado
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Trabalho efetuado sob a orientação do
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e co-orientação da
Professora Doutora Cristina Pereira-Wilson

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DECLARAÇÃO

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"...All our science, measured against reality, is primitive and childlike - and yet, it is the most precious thing we have."

Albert Einstein

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Oleanolic acid but not ursolic acid induces cell death in HepG2 cells under starvation-induced autophagy

Abstract

Cancer incidence is increasing worldwide mainly due to changes in diet, life style and increased lifespan. In particular, liver cancer is the fifth most common cancer in the world and the third most common cause of cancer mortality. Plant phytochemicals are a good and promising source of anticancer compounds. In a previous study, we reported the potential of ursolic acid (UA) to induce cell death and to inhibit proliferation in colorectal cancer cells. This natural triterpenoid, UA, was also shown to activate JNK and to modulate molecular markers of autophagy. In the present study, the ability of two isomer triterpenoids, UA and oleanolic acid (OA), to induce cell death and modulate autophagy in the human hepatocellular carcinoma cell line (HepG2 cells) was tested. For that, the effect of these phytochemicals on cell death was evaluated by MTT assay and propidium iodide staining, in complete and starvation medium. Autophagy markers were evaluated by western blot and fluorescence microscopy. Contrary to our previous data with other cell lines, HepG2 cells were less susceptible to UA and, unexpectedly, OA was a more potent inducer of cell death than UA. Interestingly, starvation-induced autophagy sensitized HepG2 cells to cell death caused by OA, but not by UA. The IC_{50} of OA decreased from about 50 μM in complete medium to 3.5 μM in starvation medium. Although UA and OA increased the levels of autophagy markers LC3 and p62, as well as the number of acidic vacuoles (as assessed by MDC staining), the cell death induced by OA was not prevented by inhibitors of autophagy and of lysosome proteases. Overall, the results seem to indicate that autophagy is not directly involved in cell death induced by OA. Interestingly, methyl-β-cyclodextrin (a polymer able to decrease membrane cholesterol content) prevented OA-induced cell death, which indicates that disruption of cholesterol homeostasis, and in particular in lipid rafts, may be involved in OA effects under starvation conditions. The present results suggest the application of OA as a specific drug for cancer treatment in particular cell physiological conditions, such as under metabolic stress.

Potencial efeito anticancerígeno do ácido oleanólico e do ácido ursólico em células HepG2 sob indução de autofagia por privação de nutrientes

Resumo

A incidência do cancro está a aumentar em todo o mundo principalmente devido a alterações da alimentação, do estilo de vida e do aumento da esperança média de vida. Em particular, o cancro do fígado é o quinto cancro mais comum no mundo e a terceira maior causa de morte por cancro. Os fitoquímicos são uma excelente e promissora fonte de compostos anticancerígenos. Num estudo anterior o nosso grupo descreveu o potencial do ácido ursólico (AU) na indução de morte e na inibição da proliferação de células do cancro coloretal. Este triterpenóide de origem natural foi também descrito como ativador da sinalização JNK e modulador de marcadores moleculares de autofagia. No presente trabalho foi testada a capacidade de dois isómeros triterpenóides, o AU e o ácido oleanólico (AO), em induzir morte celular e modular a autofagia numa linha celular do carcinoma hepatocelular humano (células HepG2). Para tal, o efeito destes fitoquímicos na morte celular foi avaliado pelo ensaio de MTT e pela marcação com o iodeto de propídio, tanto em meio completo como em meio com privação de nutrientes. Os marcadores de autofagia foram avaliados por western blot e também por microscopia de fluorescência. Contrariamente a resultados anteriores com outras linhas celulares, as células HepG2 foram menos suscetíveis ao AU, bem como o AO mostrou ser mais potente na indução de morte celular do que o AU. Além disso, a autofagia induzida pela privação de nutrientes suscetibilizou marcadamente as células HepG2 para a morte celular causada pelo AO, e tal já não se verificou com o AU. Nestas condições o IC_{50} do AO foi de 3,5 μM, enquanto em meio completo era de cerca de 50 μM. Embora, o AU e o AO aumentem os níveis dos marcadores autofágicos LC3 e p62, bem como o número de vacúolos acídicos (avaliado pela marcação com MDC), a morte celular induzida pelo AO não foi prevenida por inibidores de autofagia e nem por inibidores de proteases lisossomais. Em geral, os resultados parecerem indicar que a autofagia não está diretamente envolvida na morte celular induzida pelo AO. No entanto, um polímero capaz de diminuir o conteúdo de colesterol nas membranas celulares, a metil-β-ciclodextrina, preveniu a morte celular induzida pelo AO. Este resultado indica-nos que o AO pode induzir uma alteração na homeostasia do colesterol, em particular em domínios lipídicos ricos em colesterol, com efeitos drásticos sob a viabilidade das células quando estas estão sob privação de nutrientes. Estes resultados sugerem-nos que o AO pode ser utilizado no tratamento do cancro em condições fisiológicas específicas, tal como sob stress metabólico.

Index

Abbreviations

AKT	Protein kinase B (PKB)
AMP	Adenosine monophosphate
AMPK	AMP-activated kinase
ATF-2	Activating transcription factor 2
ATP	Adenosine triphosphate
BCL-2	B-cell lymphoma protein-2
BECLIN 1	The human ortholog of murine Atg6
Bnip3	BCL-2/adenovirus E1B 19 kDa protein – interacting protein 3
BRAC2	Breast cancer 2
CMA	Chaperone-mediated autophagy
CREB-1	cAMP-response element-binding
DLC-1	Deleted in liver cancer 1
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
eIF4E	Eukaryotic initiation factor 4E
ERK	Extracellular regulated kinase
FIP200	FAK family – interacting protein of 200 kDa
FOXO3	Forkhead box O3
GPCRS	G-protein-coupled receptors
GSK-3	Glycogen synthase kinase 3
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
hVps34	Human vacuolar protein sorting
ICAM-1	Intercellular adhesion molecule 1
IGF	Insulin – like growth factor
IGF-IR	Insulin-like growth factor-1 receptor
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IR	Insulin receptor
IRS	Insulin receptor substrate
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
LAMP-2	Lysosomal-associated membrane proteins 2
LC3	Microtubule-associated protein light chain 3
LD	Lipid droplets
MDC	Monodansylcadaverine
MβCD	Methyl-β-cyclodextrin
MEK	Mitogen-activated protein extracellular kinase

mLST8	Mammalian LST8
MMP	matrix metalloproteinase
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NF-KB	Nuclear transcription factor kappa B
OA	Oleanolic acid
PDK1	3' phosphoinositide-depedent kinase 1
PepA	Pepstatin A
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI3K	Phosphatidylinositide 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PIK3CA	Phosphoinositide-3-kinase catalytic alpha polypeptid
PIP_3	Phosphatidylinositol (3,-4,-5)-triphosphate
PKC	Protein kinase C
PPAR-α	Peroxisome proliferator-activated receptor
PRAS40	Proline-rich Akt substrate of 40 kDa
PTEN	Phosphatase and tensin homolog
RAF	Rat sarcoma-activated factor
RAPTOR	Regulatory associated protein of mTOR
RAS	Rat sarcoma
Rb	Retinoblastoma protein
Rheb	Ras homolog enriched in Brain
RICTOR	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinases
S6K1	Ribosomal protein S6 kinase 1
Sin1	SAPK-interacting protein 1
SLC1A5	Solute carrier family 1 member 5
SLC7A5	Solute carrier family 7 member 5
SMAD2	SMAD family member 2
SMAD4	SMAD family member 2
SNARE	Soluble NSF attachment receptor
SOCS-1	Suppressor of cytokine signaling 1
Stat	Signal transducers and activators of transcription
TG	Triglycerides
TMRM	Tetramethylrhodamine methyl ester
TNF-α	Tumor necrosis factor-α
TP53	Tumor protein p53
TP73	Tumor protein p73
TSC2	Tuberous sclerosis complex 2

UA	Ursolic acid
ULK	UNC51-like kinase
UVAR	Ultraviolet A radiation
XIAP	X-linked inhibitor of apoptosis protein
3-MA	3-methyladenine
4E-BP1	4E binding protein-1
5' Top	5' Terminal oligopyrimidine tract

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Introduction

1. Cancer

Cancer is currently a major health issue due to its high incidence being responsible for 13% of all deaths worldwide annually. Overall, cancer is characterized by alterations in cell proliferation, differentiation and development due to accumulation of genetic mutations [Knowles and Selby, 2005]. Carcinogenesis is the process by which these changes occur and can be divided into three distinct phases: initiation, promotion and progression [Hennings *et al.*, 1993]. During the initiation phase, irreversible mutations occur in DNA that confer cells the ability to grow faster and to avoid normal cellular growth control mechanisms. During the promotion phase, there is an increase in the proliferation ability of initiated cells. This stage is associated with accumulation of further mutations and the consequent formation of a mass of abnormal cells. The capacity of cancer cells to invade surrounding tissues and to metastasize is acquired through the progression phase [Abraham, 2003; Thyparambil *et al.*, 2010]. Genes that are usually expressed inappropriately or mutated, such as oncogenes and tumor suppressor genes contribute to the development of cancer. Oncogenes act in a dominant way at the cellular level to drive proliferation or to prevent normal differentiation; in fact, impaired regulation of cell cycle is a basis for tumor formation [Knowles and Selby, 2005; Kopnin, 2000]. For example, mutational activation of *RAS* oncogene can decrease functions of checkpoints at G1 and G2 leading to genetic instability and consequently tumor progression [Kopnin, 2000]. Cancer development also occur due to inactivation/dysfunction of tumor suppressors genes, such as *TP53* (*tumor protein p53)* gene that is typically mutated in most human tumors, resulting in dysfunctions of the cell cycle checkpoints and simultaneously inhibition of apoptosis [Levine, 1997; Orr-Weaver and Weinberg, 1998]. In addition to inactivating mutations in genes that provide negative regulation signals, such as *TP53*, mutation in genes of DNA repair systems can also lead to the development of certain tumors, since they are important to avoid mutations in other genes [Knowles and Selby, 2005].

1.1. Liver cancer

Cancer of the liver is the sixth most common cancer in the world and can have different etiologies. Risk factors that are associated directly or indirectly with different diseases of the liver, include infection with hepatitis B and C virus (HBV and HCV, respectively), contamination of food with aflatoxins, heavy alcohol consumption, tobacco smoking, obesity, diabetes, iron overload, infestation with the liver flukes, exposure to vinyl chloride, as also children affected with Beckwith-Wiedemann syndrome [Boffetta and Hashibe, 2006; Chuang *et al.*, 2009; El-Serag and Rudolph, 2007]. Less common types of hepatic cancer include: (i) hepatoblastoma that represents 1% of malignances of children younger than 20 years old; (ii) cholangiocarcinoma that is relative to the cancer of the intrahepatic biliary ducts; and, (iii) angiosarcoma - a liver mesenchymal tumor that usually occurs in elderly men [Chuang *et al.*, 2009]. The most common form of liver cancer is the hepatocellular carcinoma (HCC), which has a high incidence worldwide and cause a high number of deaths.

1.1.1. Hepatocellular carcinoma

Hepatocellular carcinoma is the most common cause of cancer mortality. The incidence of HCC varies widely, with high rates in sub-Saharan Africa, eastern and southeastern Asia, and Melanesia, and with a low incidence in Northern and Western Europe and the Americas [Parkin *et al.*, 2005]. Approximately 90-95% of HCC are caused by persistent HBV and HCV infections (chronic hepatitis) and cirrhosis, particularly with dysplastic hepatocytes [Grisham, 2001; Motola-Kuba *et al.*, 2006; Seki *et al.*, 2000; Takayama *et al.*, 1990]. These tissue lesions are precancerous histological changes that normally precede HCC and allow identifying the temporal order with which genomic alterations develop during hepatocarcinogenesis (Figure 1) [Thorgeirsson and Grisham, 2002]. In western countries the HCC is caused mainly due to alcoholic cirrhosis [Chiesa *et al.*, 2000].

The hepatocarcinogenesis is the result of a multistep process characterized by the accumulation of genetic alterations in hepatocytes, the major cell type in the liver [Motola-Kuba *et al.*, 2006]. When compared with other cancers, such as colon and breast cancers, HCC has not

so predominant genetic mutations [Wong and Ng, 2008]. Mutations in *TP53* gene, have an important role in hepatocarcinogenesis, and are associated with about 30% of HCC cases [Ozturk, 1999]. This gene mutation leads to a reduction of apoptosis and coincides with overexpression of oncogenes and underexpression of other tumor suppressor genes [Wong and Ng, 2008]. Genetic alterations in *β-catenin* gene, which is involved in Wnt signaling, protects the protein from degradation and, therefore, increase its nuclear accumulation. This leads to the upregulation of target genes, including the proto-oncogene c-*myc* and *cyclin D1* [Shtutman *et al.*, 1999; Thyparambil *et al.*, 2010]. Other genetic alterations associated with the development of HCC include mutations in *TP73 (tumor protein p73)*, *Rb (retinoblastoma protein)*, *DLC-1 (deleted in liver cancer 1)*, *PTEN (phosphatase and tensin homolog)*, *IGF-2 (insulin-like growth factor 2)*, *SMAD2 (SMAD family member 2)* and *SMAD4* (*SMAD family member 4)*, *BRAC2 (breast cancer 2)* and *SOCS-1* (*suppressor of cytokine signaling 1)* genes [Fujimori *et al.*, 1991; Motola-Kuba *et al.*, 2006].

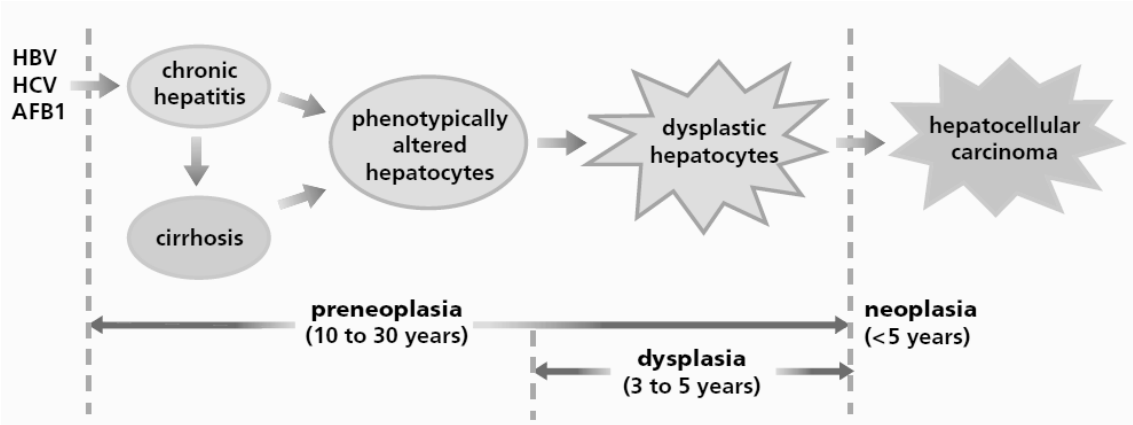


Figure 1. Representation of chronological sequence of cellular lesions promoted by hepatitis B virus (HBV), hepatitis C virus (HCV) and aflatoxin B1 (AFB1), culminating in the development of hepatocellular carcinoma in humans (from [Thorgeirsson and Grisham, 2002]).

1.1.2. Signaling pathways associated with hepatocellular carcinoma

Many signaling pathways are deregulated in HCC and associated with the carcinogenesis process, being the most common activated through receptor tyrosine kinases, RTKs, which will result in increased proliferation, invasion, metastasis and survival of tumor cells (Figure 2)

[Huynh, 2010]. These include the rat sarcoma/rat sarcoma-activated factor/mitogen-activated protein extracellular kinase/extracellular regulated kinase (RAS/RAF/MEK/ERK) pathway, the phosphatidylinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway, as well as the janus kinase/signal transducers and activators of transcription (Jak/Stat) and the Wnt/β-catenin signaling pathways (Figure 2) [Avila *et al.*, 2006; Huynh, 2010]. In this thesis, only the PI3K/AKT/mTOR pathway was object of study.

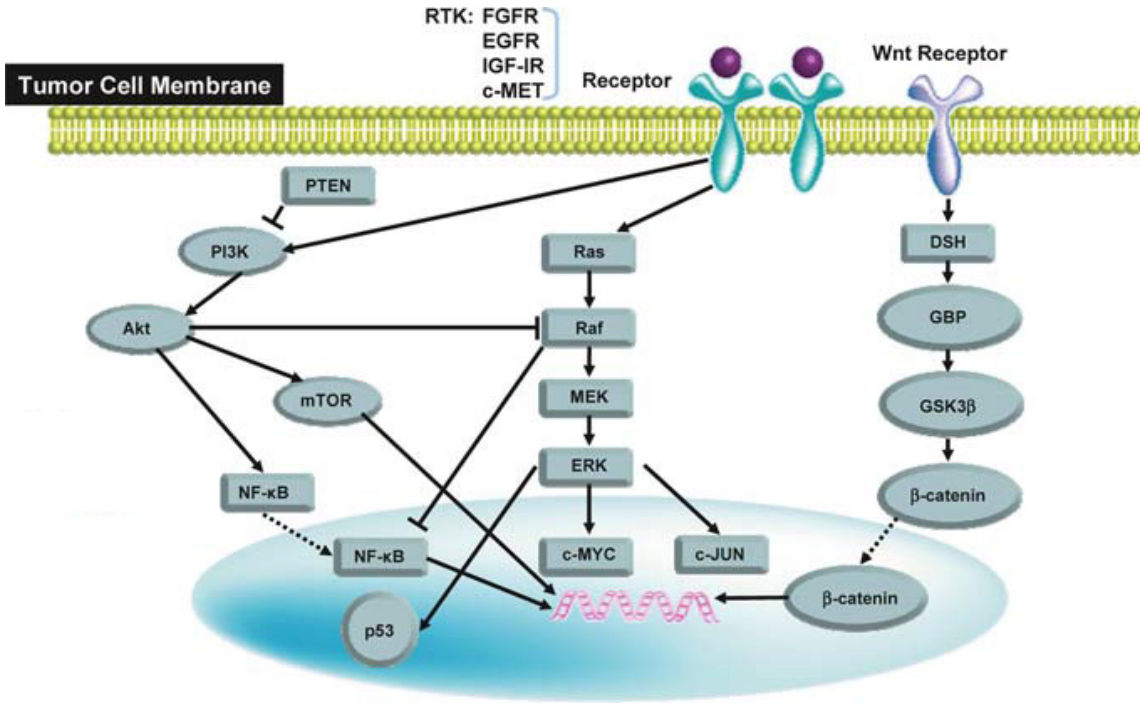


Figure 2. Schematic representation of pathways involved in development of HCC that modulate apoptosis, cell division, cell survival, and angiogenesis. Included are the rat sarcoma/rat sarcoma-activated factor/mitogen activated protein kinase/extracellular regulated kinase (Ras/Raf/MAP/ERK) pathway, the phosphatidylinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway and Wnt/β-catenin pathway (modified from [Carr and Kralian, 2010]).

2. The PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR is one of major signaling pathways associated with and constitutively activated in many types of cancer including HCC [Llovet and Bruix, 2008]. PI3K is activated by RTKs, such as insulin-like growth factor-1 receptor (IGF-IR) and epidermal growth

factor receptor (EGFR), the oncogene RAS, integrins that are molecules of cell adhesion, and G-protein-coupled receptors (GPCRS); and is negatively regulated by the *PTEN* tumor suppressor gene [LoPiccolo *et al.*, 2008; Wang *et al.*, 2011b]. In tumor cells, the mechanisms for this pathway activation include mutations in the p110 catalytic domain of phosphoinositide-3 kinase *(PIK3CA)* gene, amplification or mutation of AKT, and loss of function of PTEN by underexpression or epigenetic silencing [Hu *et al.*, 2003; LoPiccolo *et al.*, 2008; Wang *et al.*, 2007; Wang *et al.*, 2011b]. The serine/threonine kinase AKT occurs in three isoforms, AKT1, AKT2, AKT3, being expressed distinctively depending of the tissues, and in particular to HCC cell mass there is the expression of AKT2 but not of AKT1 [Xu *et al.*, 2004; Zinda *et al.*, 2001]. mTOR is a major mediator of this signaling pathway and is a downstream substrate of AKT, which can phosphorylate directly mTOR or indirectly by phosphorilation and inactivation of tuberous sclerosis complex 2 (TSC2) [LoPiccolo *et al.*, 2008; Wang *et al.*, 2011b]. mTOR is presented in two forms, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2): the first is implicated on control protein translation and autophagy, and promotes the expression of *c-myc*, *cyclin D* and other genes involved in cell proliferation, growth and angiogenesis; in turn mTORC2 is responsible for the activation of AKT by phosphorylation at serine 473, promoting cell survival (Figure 4) [Carr and Kralian, 2010; Sahin *et al.*, 2004; Sarbassov *et al.*, 2005b]. The involvement of PI3K/AKT/mTOR pathway in the autophagy process is of special interest in this work and, therefore, it will be detailed as follow.

3. Autophagy

Autophagy is a cellular dynamic process of "self-eating" that was first described by de Duve and Wattiaux [De Duve and Wattiaux, 1966]. There are three primary forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), differing from each other on their physiological functions [Rautou *et al.*, 2010]. Macroautophagy is the most prevalent and commonly referred to as autophagy (term hereafter used to refer to macroautophagy), and is characterized as a non-selective vacuolar degradative cellular process by which intracellular membrane structures sequester proteins and organelles to degrade and turn over these materials [Meijer and Codogno, 2004]. When cells are exposed to different

situations of stress, such as nutrient starvation, oxidative stress, and hormonal signaling, autophagy takes place and can result in adaptation and survival, or cell death [Kondo *et al.*, 2005; Meijer and Codogno, 2004]. Extracellular pathogens are also eliminated in this pathway as part of a cellular defense mechanism [Eskelinen and Saftig, 2009; Todde *et al.*, 2009]. The capacity for degradation is an important autophagic function but if unregulated it can be lethal. Basal levels of autophagy are important for maintaining normal cellular homeostasis and to generate energy and building blocks for reuse in order to cells survive under nutrient starvation [Kroemer *et al.*, 2010]. However, excessive autophagy may lead to autophagic cellular death, the also called type II programmed cell death [Galluzzi *et al.*, 2009].

In cancer cells, autophagy is suppressed during the early stages of tumorigenesis, because there is a need of higher level of protein synthesis than protein degradation to promote tumor growth [Cuervo, 2004]. In addition, with inhibition of autophagy there is less removal of damaged organelles, which allows accumulation of genotoxic free radicals contributing for cancer promoting stages [Edinger and Thompson, 2003]. Although autophagy is generally decreased in cancer cells compared with normal ones, in later stages of tumorigenesis stimulation of autophagy is observed in cancer cells that are located in the central areas of the tumor, where there is poor vascularization allowing them to survive under conditions of nutrient starvation and low oxygen conditions [Cuervo, 2004]. In addition, induction of autophagy was also observed in response to anticancer drugs, and therefore, autophagy is currently viewed as a good target for cancer therapy [Bursch *et al.*, 2000; Inbal *et al.*, 2002; Paglin *et al.*, 2001].

3.1. The process of autophagy

Autophagy is an evolutionarily conserved lysosomal degradation pathway that occurs in all eukaryotic cells, from yeast to mammals [Klionsky and Emr, 2000; Meijer and Codogno, 2004]. The autophagic process begins with the sequestration of cytoplasmatic constituents, including organelles, by a double-membrane-bound structure known as phagophore or isolation membrane [Mizushima, 2007; Stromhaug *et al.*, 1998]. The elongation and complete closure of the phagophore, results in the formation of the autophagosome, which after maturation fuse with lysosome to form the autolysosome. Then, the sequestered content together with the inner

autophagosomal membrane are degraded by acidic lysosomal hydrolases for recycling (Figure 3) [Mizushima, 2007; Shintani and Klionsky, 2004].

Several Atg (autophagy-related) proteins are necessary to the execution of autophagy and have been first characterized in yeast, and many of these have mammalian orthologs [Yang *et al.*, 2005]. Together with other autophagy proteins, the class III PI3K, also named human vacuolar protein sorting (hVps34) is involved in initial formation of the phagophore membrane, as well as in the sequestration of all contents that will be degraded [Liang *et al.*, 1999; Petiot *et al.*, 2000; Sun *et al.*, 2008]. The first evidence of implication of this enzyme in the autophagic process was due to discovery of 3-methyladenine (3-MA) that is able to block the formation of autophagosomes by inhibition of class III PI3K (Figure 3) [Blommaart *et al.*, 1997b; Furuya *et al.*, 2005; Petiot *et al.*, 2000; Zeng *et al.*, 2006]. *BECLIN 1* was the first tumor suppressor gene related with autophagy to be reported, and its interaction with class III PI3K is necessary for autophagy. BECLIN 1, can be inhibited by the interaction with the antiapoptotic protein BCL-2 [Sinha and Levine, 2008; Yang *et al.*, 2005]. In other words, dissociation of BECLIN 1 from BCL-2 is necessary to occur induction of autophagy and, consequently, there is a relationship between the induction of autophagy and increased expression of BECLIN 1 [Liang *et al.*, 1999; Nice *et al.*, 2002]. Microtubule-associated protein light chain 3 (LC3), a mammalian homolog of yeast Atg8, is a major constituent of the autophagosomes and also important for their formation [Mizushima *et al.*, 2004]. LC3 is synthesized as a pro-protein that is cleaved at the glycine residue, forming the cytosolic LC3-I [Farkas *et al.*, 2009]. The C-terminal glycine of LC3-I when coupled to phosphatidylethanolamine through an ubiquitin-like conjugation reaction result in the formation of LC3-II [Ichimura *et al.*, 2000; Kabeya *et al.*, 2004]. LC3-II is important for the elongation and closure of the phagophore and remains associated with the inner and outer membrane of the autophagosome (Figure 3) [Nakatogawa *et al.*, 2007]. After fusion with the lysosome, the LC3-II content in the autolysosome decreases either by cleavage (outer) or by degradation by lysosomal enzymes (inner membrane-associated) [Kabeya *et al.*, 2000; Mizushima *et al.*, 2010]. Consequently, LC3-II is considered as a good autophagy marker [Kouri *et al.*, 2002]. p62 protein/sequestome 1 (p62/SQSTM1) is a ubiquitin-binding protein that binds directly to LC3-II and is transported into the autophagosome, where it will be degraded on the final phase of the autophagic process [Ichimura and Komatsu, 2010]. p62 is also used as marker of autophagic flux, because when occurs a inhibition or induction of autophagy, there is an accumulation or decline of p62 levels, respectively [Bjorkoy *et al.*, 2009].

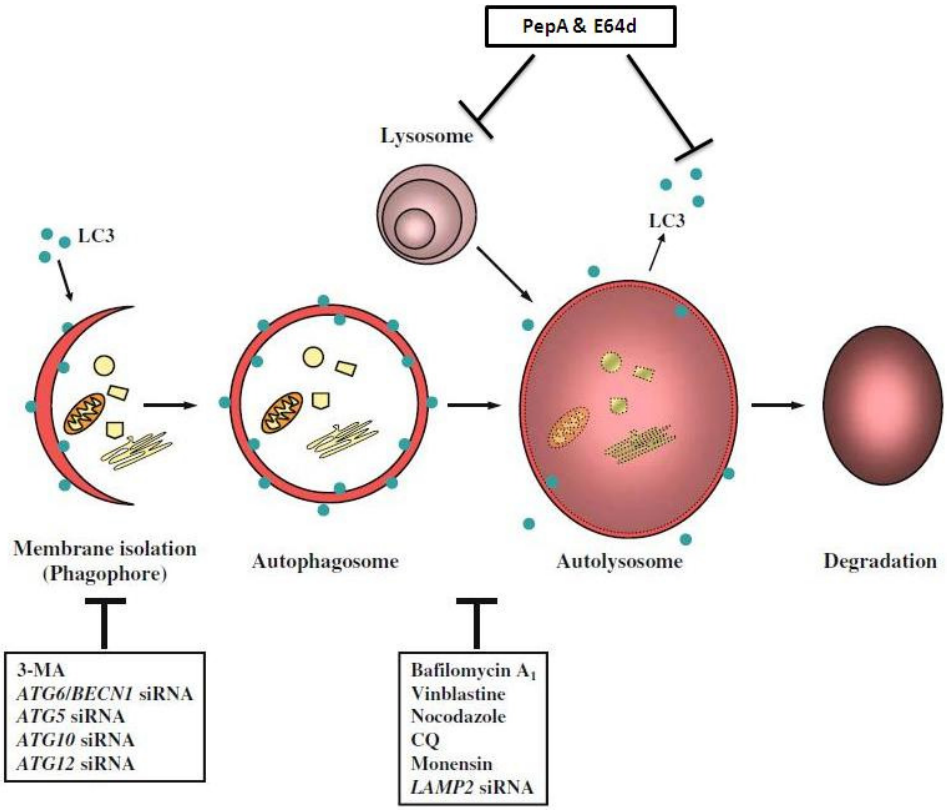


Figure 3. Schematic illustration of the autophagic process. Autophagy begins with the isolation of a double membrane that sequesters and engulfs cellular proteins, organelles and cytoplasm to form a double membrane vesicle named autophagosome. The isolation membranes elongate and mature, with the necessary recruitment of the microtubule-associated protein 1 light chain 3 (LC3). The formation of the pre-autophagosomal structure can be inhibited by the class III PI3K inhibitor 3-methyladenine (3-MA). Lysosomes fuse with autophagosomes and intraautophagosomal contents are lysed by lysosomal hydrolases for recycling. This process can be inhibited by lysosomotropic agents such as chloroquine [Andjelkovic *et al.*, 1997]. Pepstatin A (PepA) and E64d, inhibitors of cathepsins, also inhibit the final step of autophagy that is the degradation of intraautophagosomal contents. Represented are also other forms of inhibition of autophagy, either pharmacologically or by molecular tools (modified from [Mehta and Siddik, 2009]).

3.2. Regulation of autophagy

Autophagy has an important role in cancer and the initial signals that induce autophagy are mainly due to stress conditions, such as anticancer treatments, and low nutrient availability [Kondo *et al.*, 2005]. There is thus a growing interest in pathways and molecules that regulate autophagy that can be used as targets in cancer treatment [Sridharan *et al.*, 2011]. The autophagic process and the molecular machinery associated suggest that its regulation can be complex and may involve multiple signaling inputs [Periyasamy-Thandavan *et al.*, 2009]. Kinases

such as mTOR, PI3K and AKT play an essential role in induction or inhibition of autophagy depending on different stimuli, where mTOR occupies an important position because it is the principal regulator of autophagy [Periyasamy-Thandavan *et al.*, 2009]. Bellow will be described the most important pathways that regulate autophagy.

3.2.1. Amino acid signaling

The amino acids are the major end products of protein degradation process in autophagy and promote its regulation by inhibition of this process, maintaining cellular homeostasis [Yang *et al.*, 2005]. However, not all amino acids are involved in autophagy regulation [Mortimore and Poso, 1987; Periyasamy-Thandavan *et al.*, 2009; Seglen *et al.*, 1980]. In particular, alanine, leucine, glutamine and phenylalanine seem to be effective inhibiting autophagy [Bergamini *et al.*, 1995; Mortimore *et al.*, 1991; Seglen *et al.*, 1980]. These amino acids have their own recognition sites at the cell surface and promote signal transduction that will act by phosphorylation of mTORC1 through the Ras-related small GTPases (Rag proteins), suppressing autophagy [Kadowaki *et al.*, 2006; Kim *et al.*, 2008a; Long *et al.*, 2005]. The stimulation of autophagy through nutrient starvation (nitrogen starvation) was first demonstrated by Schworer and Mortimore in 1977 [Chan *et al.*, 2006a]. Recently, it was demonstrated that it is L-glutamine that is the rate-limiting factor that enables amino acids shortage to inhibit mTORC1 signaling [Mortimore and Schworer, 1977; Nicklin *et al.*, 2009]. Under conditions of availability of extracellular amino acids the influx of L-glutamine by its high-affinity transporter SLC1A5 (solute carrier family 1 member 5) occurs increasing its intracellular concentration (Figure 4) [Ravikumar *et al.*, 2010]. The heterodimeric SLC7A5 (solute carrier family 7 member 5)/SLC3A2 is a bidirectional antiporter that uses L-glutamine as an efflux substrate in exchange for the cellular uptake of essential amino acids that consequently allows activation of mTORC1 through the Rag GTPases [Nicklin *et al.*, 2009]. The stimulation of autophagy by amino acid starvation reproduces what happens in advanced stages of cancer, where autophagy may be required to provide essential nutrients to cells in the inner part of a solid tumor that do not have direct access to the blood supply [Ogier-Denis and Codogno, 2003].

3.2.2. Insulin/Insulin-like Growth Factor signaling

The pathways through which hormones regulate autophagy are distinct from those regulated by nutrients, but both converge in the activation or inhibition of the key regulator of autophagy - mTOR. The hormone insulin that is secreted by the pancreas in response to high blood glucose, binds to its receptor (insulin receptor – IR) on the surface of cells and promotes its autophosphorylation on tyrosine residues. This induces the recruitment and phosphorylation of IRS1 and IRS2 (insulin receptor substrate 1 and 2) [He and Klionsky, 2009; Neely *et al.*, 1977; Pfeifer, 1978]. The insulin-like growth factor is mainly secreted in the liver as a result of stimulation by the growth hormone, and has the same effect on the IR as insulin. High levels of circulating insulin-like growth factor 1 (IGF1) and certain genetic polymorphisms of IGF1 are associated with increased risk of several cancers [Tao *et al.*, 2007]. In association with the phosphorylation of insulin receptors, p85 (a regulatory subunit of class I PI3K) promotes activation of PI3K (Figure 4) [Yang *et al.*, 2005]. Thereby, activation of PI3K generates phosphatidylinositol (3,-4,-5)-triphosphate (PIP_3) that allows membrane recruitment of AKT and 3' phosphoinositide-depedent kinase 1 (PDK1) [Alessi *et al.*, 1997; Stokoe *et al.*, 1997]. This last in turn phosphorylates and activates AKT, leading to activation of mTOR and thus inhibition of autophagy (Figure 4) [Blommaart *et al.*, 1997a; Klionsky, 2004]. This effect can be reversed by the tumor suppressor gene PTEN, which reverse PIP_3 production and decreases the downstream AKT signaling, positively regulating autophagy (Figure 4) [Arico *et al.*, 2001].

3.2.2.1. PI3K

The enzymes PI3K catalyze the phosphorylation of the 3' position hydroxyl group of the inositol ring in phosphatidylinositol, and have been classified into three classes [Cantley, 2002]. Each class has its own structure, function, substrate specificity and lipid products [Engelman *et al.*, 2006; Katso *et al.*, 2001]. The classes I PI3K is often activated in response to growth factors and once activated, the generated PIP_3 binds to the pleckstrin homology (PH) domains of PDK-1 and AKT, leading to the translocation of both proteins to the cell membrane where they are consequently activated (Figure 4) [Adjei and Hidalgo, 2005; LoPiccolo *et al.*, 2008]. About the class II PI3K little is known of their function but it is thought to play a role in processes such as cell migration and vascular smooth muscle contraction [Domin *et al.*, 2005; Wang *et al.*, 2006].

The class III PI3K specifically produces the lipid phosphatidylinositol 3-phosphate (PI3P) and is involved in the formation of autophagosomes and initiation of autophagy [He and Klionsky, 2009; Schu *et al.*, 1993; Volinia *et al.*, 1995; Yang *et al.*, 2005]. The sole member of class III PI3K is hVps34 and was first identified as an important regulator of vesicular trafficking in the endosomal/lysosomal system [Lindmo and Stenmark, 2006; Odorizzi *et al.*, 2000]. It is implicated in the recruitment of proteins that have PI3P binding domains to the intracellular membranes [Backer, 2008; Lindmo and Stenmark, 2006; Odorizzi *et al.*, 2000]. hVps34 interacts with the autophagy-related proteins such as BECLIN 1, UVRAG (UV radiation resistance-associated gene), Bif-1 (Bax-interacting factor 1) and p150 (Vps15 in yeast), forming a complex that produces PI3P by phosphorylation of phosphatidylinositol (PI) important for the execution of autophagy [Furuya *et al.*, 2005; Kihara *et al.*, 2001; Liang *et al.*, 2006; Takahashi *et al.*, 2007].

3.2.2.2. AKT

AKT is a serine/threonine kinase also named protein kinase B (PKB), which is activated by various signals upstream class I PI3K, resulting in the phosphorylation of multiple downstream effectors [Nogueira *et al.*, 2008; Vivanco and Sawyers, 2002]. This kinase is present in three isoforms, AKT1, AKT2 and AKT3, and their activation occur first by phosphorylation at threonine 308 in the catalytic domain by PDK-1 and then requires a subsequent phosphorylation at serine 473, which can be mediated by several kinases such as PDK-1, AKT itself or mTORC2 complex [Andjelkovic *et al.*, 1997; Balendran *et al.*, 1999; Santos *et al.*, 2001; Toker and Newton, 2000]. Once activated, one of the downstream effectors of AKT is the tumor suppressor proteins mutated in tuberous sclerosis which form a complex named the tuberous sclerosis complex (TSC) [Ravikumar *et al.*, 2010]. TSC consists of TSC1 and TSC2, where their phosphorylation is inhibitory by blocking TSC2 interaction with TSC1. This leads to activation of the GTP-binding protein Rheb (Ras homolog enriched in Brain), which directly binds and activates the mTORC1 resulting in protein synthesis, cell growth and suppression of autophagy (Figure 4) [Gao *et al.*, 2002; Mathew *et al.*, 2009; Ravikumar *et al.*, 2010; Teckman and Perlmutter, 2000].

3.2.2.3. mTOR

The serine/threonine kinase mTOR is the master regulator that integrates upstream pathways that include the response to insulin/growth factor signaling through PI3K/AKT and to nutrient and energy conditions (see 3.2.1 and 3.2.3), leading to the regulation of autophagy and

other cellular functions, such as initiation of mRNA translation, cell growth and proliferation, and transcription [Sarbassov *et al.*, 2005a]. mTOR can exist in two distinct protein complexes, of which only one is involved directly with autophagy regulation.

Rapamycin-sensitive mTORC1 consists of the mTOR catalytic subunit, RAPTOR (regulatory associated protein of mTOR) mLST8 (mammalian LST8) and PRAS40 (proline-rich Akt substrate of 40 kDa) [Guertin and Sabatini, 2009; Yang and Guan, 2007]. When activated, mTORC1 phosphorylates the ribosomal protein S6 kinase 1 (S6K1 also named p70S6K) to positively regulate the translation of mRNAs containing 5' terminal oligopyrimidine tract (5' Top). In addition, the eukaryotic initiation factor 4E binding protein-1 (4E-BP1) is also phosphorylated by mTORC1, which induces its dissociation from the eukaryotic initiation factor 4E (eIF4E), liberating this factor to bind the 5' terminal cap structure of RNA promoting the initiation of translation [Yang *et al.*, 2005]. mTORC1 also phosphorylates and regulates proteins involved in autophagy (Figure 4). Active mTORC1 interacts with and phosphorylates the UNC51-like kinase 1/2 (ULK1/2) and Atg13, leading to their inhibition, and together with FAK family – interacting protein of 200 kDa (FIP200) form a ULK1/2-Atg13-FIP200 complex that promote inhibition of autophagy [Ravikumar *et al.*, 2010]. Under starvation conditions or rapamycin treatment, mTORC1 is inhibited due to the dissociation of mTOR from the complex. Inactive mTORC1 dissociates from the ULK1/2-Atg13-FIP200 complex resulting in the dephosphorylation of ULK1/2, activating it, which promotes the ULK1-mediated phosphorylations of Atg13, FIP200, and ULK1 itself, triggering the autophagy cascade (Figure 4) [Ganley *et al.*, 2009; Hosokawa *et al.*, 2009; Jung *et al.*, 2009; Ravikumar *et al.*, 2010]. Therefore, the ULK1/2-Atg13-FIP200 complex signals the autophagic machinery downstream of mTORC1.

In contrast, rapamycin-insensitive mTORC2 is composed by mTOR, RICTOR (rapamycin-insensitive companion of mTOR), mLST8 and Sin1 (SAPK-interacting protein 1), and it is able to phosphorylate members of the AGC kinase family, such as AKT and protein kinase C (PKC), promoting cellular survival and actin cytoskeleton organization, respectively [Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004].

3.2.2.4. Feedback mechanism that regulates autophagy

The role of mTOR in regulation of autophagy can also involve the mTORC2 complex (Figure 4). Chen *et al.* have shown that low concentrations of rapamycin induce AKT phosphorylation by inhibition of mTORC1 but higher concentrations suppress AKT

phosphorylation through inhibition of mTORC2 [Chen *et al.*, 2010]. The mTORC2 have an important role in activation of AKT that is implicated in the phosphorylation and inactivation of the forkhead box O3 (FOXO3) transcriptional factor, which has been reported to stimulate autophagy by increasing the expression of proteins involved in autophagy, such as LC3 and BCL-2/adenovirus E1B 19 kDa protein – interacting protein 3 (Bnip3) [Brunet *et al.*, 1999; Mammucari *et al.*, 2007; Sarbassov *et al.*, 2005b].

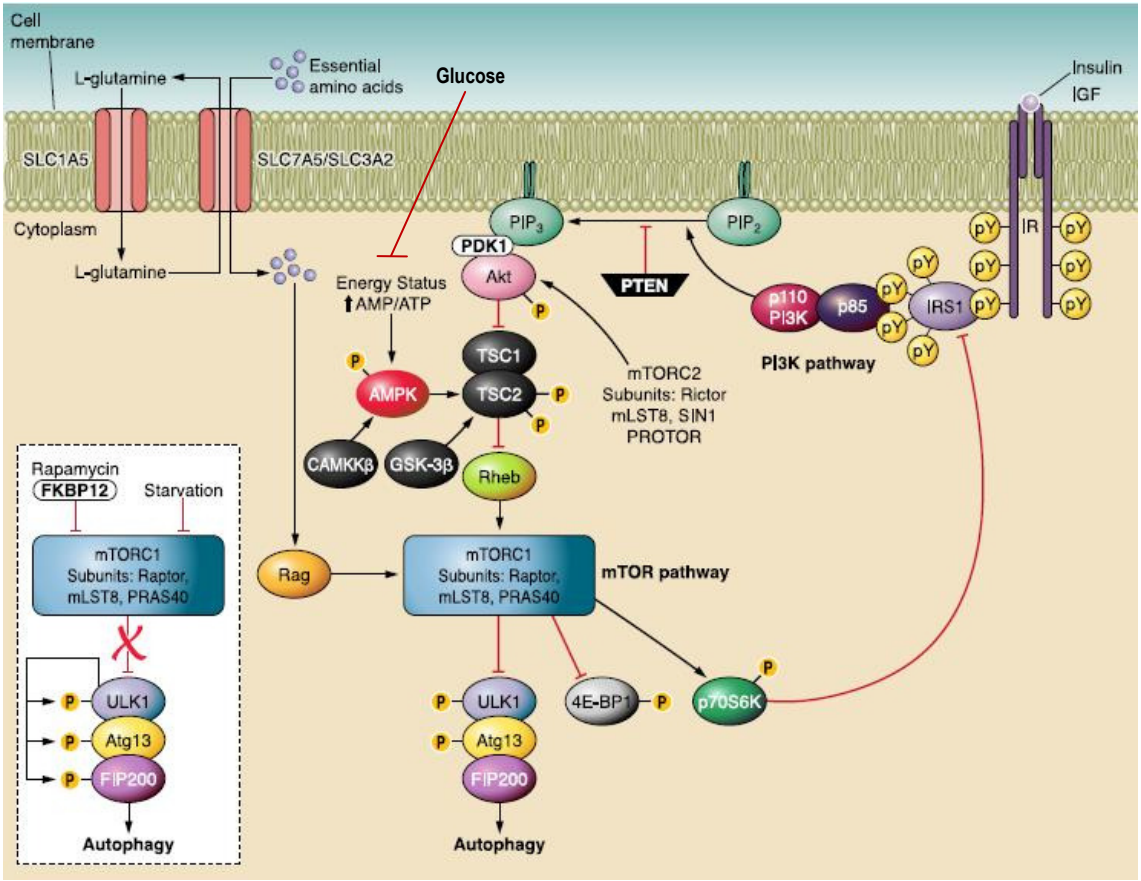


Figure 4. Schematic representation of mTOR-dependent main pathways regulating autophagy. The activation of PI3K pathway trough binding of insulin (or growth factors) to insulin receptor (IR) leads to activation of AKT, and in turn inhibition of TSC-1/2 complex that promotes activation of Rheb and consequently mTORC1. AKT can also be phosphorylated and activated by mTORC2. Downstream of mTORC1, the ULK1-Atg13-FIP200 complex acts as an integrator of the autophagy signals. Under nutrient-rich conditions, mTORC1 suppresses autophagy by interacting with this complex and mediating phosphorylation dependent inhibition of Atg13 and ULK1. Under starvation conditions or rapamycin treatment, mTOR dissociates from the complex, resulting in dephosphorylation-dependent activation of ULK1 and ULK1-mediated phosphorylations of Atg13, FIP200, and ULK1 itself, which triggers autophagy. Amino acids activate mTORC1 via Rag GTPases and suppress autophagy, being L-glutamine the rate limiting factor. The mTORC1 pathway regulates cell growth mainly through 4E-BP1 and p70S6K. Phosphorylation dependent activation of p70S6K can also inhibit IRS1, thereby exerting a negative feedback loop mechanism (modified from [Ravikumar *et al.*, 2010]).

Therefore, mTORC2 can negatively regulate autophagy indirectly by AKT through a negative feedback loop. In addition, autophagy can be also regulated by other feedback loop through p70S6K [Scott *et al.*, 2004]. Once activated by PI3K/AKT signaling, mTOR/p70S6K phosphorylates the IRS-1 on serine residues, resulting in its inhibition by targeting it to degradation. This work as a negative feedback loop that attenuate PI3K/AKT signaling, and therefore mTOR activity (Figure 4) [Manning and Cantley, 2007; O'Reilly *et al.*, 2006; Ravikumar *et al.*, 2010].

3.2.3. Energy-dependent AMPK signaling

The autophagic process is ATP-dependent and lower cellular energy levels (for example, due to glucose starvation or other stress) signals mTORC1 by activating AMPK [Chan *et al.*, 2006b; Farkas *et al.*, 2011]. AMPK, which senses changes in the intracellular ATP/AMP ratio, directly phosphorylates TSC2, thereby providing the priming phosphorylation for subsequent phosphoryalation of TSC2 by glycogen synthase kinase 3 (GSK-3) to inhibit mTOR signaling, thereby inducing autophagy and inhibiting protein synthesis (Figure 4) [Hoyer-Hansen and Jaattela, 2007; Inoki *et al.*, 2006].

4. Autophagy and lipids

Autophagy is an essential cellular process that mediates the degradation of intracellular components since they are dysfunctional or to meet cellular energetic demands. Therefore, during nutrient deprivation not only autophagic degradation of cytosolic proteins and organelles are in place, but also lipid and glycogen stores are mobilized for energy production (Figure 5) [Eskelinen and Saftig, 2009; Singh and Cuervo, 2011; Todde *et al.*, 2009]. Lipids are essential to all organisms as substrates for energy production, as precursors of membrane lipids and as signaling molecules of several cellular processes. The cells store the lipids as triglycerides in the form of dynamic organelles called lipid droplets (LD) that, when necessary, are breakdown into free fatty acids by the process of lipolysis [Martin and Parton, 2006]. Both autophagy and lipolysis are regulated hormonally by insulin and glucagon and are increased during starvation

[Mizushima and Klionsky, 2007; Singh *et al.*, 2009a]. Interestingly, it has been established recently a link between these two catabolic processes, in which part of lipid droplets have been incorporated into autophagic vesicles of double membrane, that subsequently fuse with lysosomes, for degradation of their contents (Figure 5) [Singh *et al.*, 2009a]. Genetic or pharmacology inhibition of autophagy in hepatocytes resulted in increased content of triglycerides (TG) and LD, supporting the idea that lysosomal degradation of intracellular LD by autophagy is a constitutive process and that can also contribute to lipid mobilization *in vivo* [Singh *et al.*, 2009a]. However, other study revealed that autophagy is involved in LD formation, during starvation in hepatocytes and cardiomyocytes. The lipidated LC3 protein required for autophagosome formation was shown to be present in isolated LD. In addition, knockout of the essential autophagy gene Atg7 led to a reduced content of LD during fasting [Shibata *et al.*, 2009]. These contrasting effects between both studies may be due to the model used: in the first adult mice were used whereas in the second young mice were used [Rodriguez-Navarro and Cuervo, 2010]. Interestingly, studies in pre-adipocytes in culture also showed that autophagy can regulate adipogenesis (the differentiation process of pre-adipocytes into mature adipocytes) [Singh *et al.*, 2009b].

Despite the importance of autophagy to regulate lipid metabolism, many evidences also support that lipids and lipid modifications modulate autophagy. For example, it has been reported that exposure of cells to a high lipid load significantly decreases the degradation of proteins mainly due to reduced autophagy [Koga *et al.*, 2010]. This high lipid load affects the cholesterol content of membranes reducing it, which can lead to defects in the membrane fusion between autophagosome and lysosomes, inhibiting therefore autophagy in later steps [Koga *et al.*, 2010]. Conversely, a previous study reports that depletion of cholesterol in fibroblasts induces autophagy [Cheng *et al.*, 2006]. However, these authors studied autophagy only by measuring LC3 levels by western blot and immunofluorescence. They observe a significant increase of LC3 that suggest them to be in the presence of increased of autophagic activity. However, it is known currently that this effect is most probably due to the inhibition of autophagy at later steps [Mizushima *et al.*, 2010]. Also CMA is known to be affected by cholesterol levels. The receptor of this autophagic pathway, LAMP-2A, associates in a dynamic manner with lipid microdomains (enriched in cholesterol and sphingolipids) at the lysosomal membrane. LAMP-2A undergoes regulated degradation in these regions, and therefore lower cholesterol levels will increase CMA activity [Rodriguez-Navarro and Cuervo, 2010]. The PI3P lipid molecules are also required and

essential for autophagy execution, serving as a scaffold for the assembly of autophagosomes, their trafficking in microtubules, lysosomal fusion and possibly in cargo recognition [Singh and Cuervo, 2011]. Therefore, modulation of these lipid molecules by the coordination between kinases and phosphatases are essential for regulating autophagy.

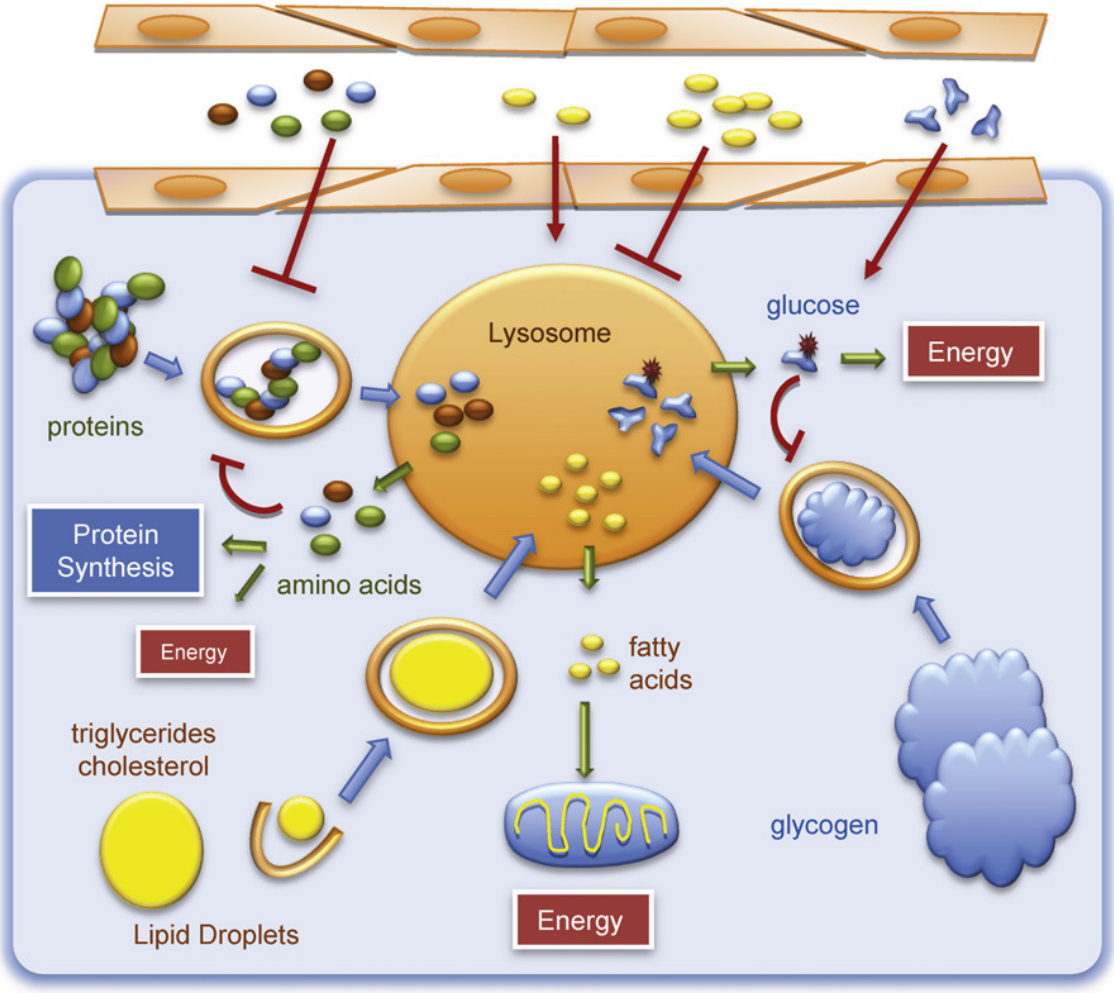


Figure 5. Macroautophagy contributes to the delivery of proteins, lipid stores, and glycogen for breakdown into lysosomes. The constituent components of these macromolecules exit the lysosome and become available for production of energy. In the case of protein breakdown, the resulting amino acids may have less energetic value and be preferentially utilized for the synthesis of new proteins. Levels of amino acids, free fatty acids, and sugars circulating in blood or in the extracellular media have a direct impact on intracellular marcoautophagy (from [Singh and Cuervo, 2011]).

5. Cancer therapy and pharmacological autophagy modulation

Autophagy is a process with an important role in cancer development and therapy. Increased levels of autophagy are commonly observed in tumor cells after cancer therapy, such as by radiotherapy, with chemotherapeutics (e.g., doxorubicin, temozolomide, camptothecin), by histone deacetylase inhibitors and with hormonal therapeutics (e.g., tamoxifen) [Roy and Debnath, 2010]. Considering that increased autophagic activity provide resistance of cancer cells to anticancer treatment, inhibitors of autophagy have been proposed as good adjuvants for cancer therapy [Roy and Debnath, 2010]. In established tumors, increased autophagy has been reported in central parts of the tumor as a strategy for survival in a very stressful and nutrient-deprived environmental. Autophagy-dependent cell death has also being explored in cancer treatment, where extensive degradation of cytoplasmic materials beyond a critical point is believed to drive cell death. Therefore, the signaling pathways that regulate autophagy and also proliferation and apoptosis, which commonly are altered in cancer, are therefore potential therapeutic targets (Figure 6) [Kondo *et al.*, 2005].

A range of chemical inhibitors of autophagy, such as 3-MA, chloroquine (CQ), pepstatin A (PepA) and E64d, LY-294002 and wortmannin, can be used to study the role of autophagy in tumorigenesis and in response to therapy (Figure 6). However, due to lack of specificity, low solubility and/or high toxicity of many of these compounds, their clinical application is compromised of low value. For example, 3-MA inhibits both class I and class III PI3K; the inhibition of class I PI3K is persistent, whereas its effect on class III PI3K is transient, providing a temporal different effect and an inhibition of autophagosome formation [Bursch *et al.*, 1996; Wu *et al.*, 2010]. In addition to 3-MA, other pharmacologic agents such as LY-294002 and wortmannin target the p110 catalytic subunit of class I PI3K, but also lack specificity like 3-MA, which compromise also their use in modulating autophagy [LoPiccolo *et al.*, 2008]. Chloroquine, known as an anti-malarial drug, is an inhibitor of lysosomal acidification, because leads to disruption the lysosomal pH gradient. Thus, it blocks the terminal stages of autophagic proteolysis by preventing the fusion of autophagosomes with lysosomes. Currently, CQ is being tested in clinical trials for cancer treatment through autophagy inhibition [Farkas *et al.*, 2011; Roy and Debnath, 2010; Yamamoto *et al.*, 1998]. The inhibition of lysosomal function by inhibition of cathepsins is another form of promoting the blockage of autophagy. To do this experimentally it

can be used a combination of PepA and E64d, two inhibitors of cathepsins, in which PepA is a membrane-permeable inhibitor of cathepsins D and E, whereas E64d is a membrane-permeable inhibitor of cathepsins B, H and L [Kirschke and Wiederanders, 1987; Tamai *et al.*, 1987; Tanida and Waguri, 2010; Umezawa *et al.*, 1970]. Inhibition of these proteases will promote a blockage of the final step of autophagy, easing the evaluation of autophagic flux [Kim *et al.*, 2008b].

As discussed above, inhibition of mTOR (and, therefore, induction of autophagy) is also a strategy to kill cancer cells since they have a higher demand on protein synthesis [Cuervo, 2004]. Frequently, rapamycin is used to inhibit mTOR and exhibits considerable anticancer activity (Figure 6) [Faivre *et al.*, 2006]. However, therapy mediated by rapamycin shows some problems, such as the need of a wide dose range to inhibit mTOR under different conditions, as also the resistance to therapy through mechanism of negative feedback loop [Chen *et al.*, 2010; Foster and Toschi, 2009].

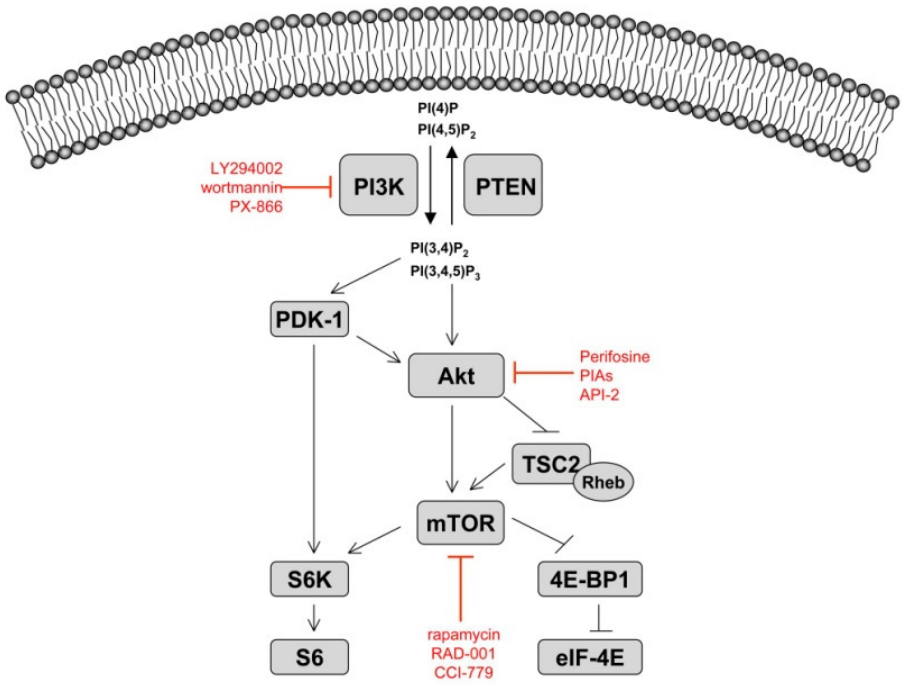


Figure 6. Schematic representation of PI3K/AKT/mTOR pathway with targets of the pharmacological inhibitors with modulatory effects in autophagy (from [LoPiccolo *et al.*, 2008]).

6. Natural compounds and cancer

Throughout of time, natural compounds from plants, marine organisms and microorganisms have been a rich source of agents that are used in many applications and fields, including in medicine, due to their structural diversity and bioactive potential [Nobili *et al.*, 2009]. Today, the natural products play a relevant role in cancer therapy and others are in development with a significant number of compounds in different phases of clinical trial [Cragg *et al.*, 1997; Gordaliza, 2007]. In fact, a high percentage of pharmaceutical drugs in use are of natural origin or develop from research in natural compounds, where most of them are used in anticancer treatment [Cragg *et al.*, 1997; Newman *et al.*, 2000].

Due to the diverse molecular alterations that occur in cancer cells that are associated with their tumorigenesis, the structural diversity of natural compounds make them good source of potential anticancer drugs with a specific target of action or multi-targets in key cancer regulators. In addition, natural compounds are a more attractive option than standard chemotherapy agents, due to their recognized low-toxicity [West *et al.*, 2002]. Therefore, these chemicals of natural origin can contribute to decrease resistance of anticancer treatments or to be used as substitutes of common agents in chemotherapy [Roy and Debnath, 2010].

6.1. Triterpenoids

Plants synthesize a large number of secondary metabolites, including many phytochemicals, such as phenolic compounds, essential oils, alkaloids and terpenes. Terpenes are compounds with a cyclic structure and based on the C_5 isoprene units [Cowan, 1999]. Triterpenoids are composed from six isoprene units ($C_{30}H_{48}$), where additional elements, normally oxygen, are added [Cowan, 1999; Phillips *et al.*, 2006]. Triterpenoids are aglycones, but when linked to one or more sugar chains are in turn named triterpenoid saponins [Price *et al.*, 1987].

Throughout of years, triterpenoids were considered to be biologically inactive. But their low toxicity profile led to their use for medicinal purpose in many countries, primordially in Asia, and evidences of their pharmacologic activities of triterpenoids have been emerging [Bishayee *et*

al., 2011]. The anticancer efficacy of several triterpenoids has been recently reported, where these compounds exhibit cytotoxicity against a variety of cancer cells without major toxicity in normal cells [Laszczyk, 2009; Petronelli *et al.*, 2009; Setzer and Setzer, 2003]. In the present work we used two pentacyclic triterpenoid isomers – ursolic acid (UA) and oleanolic acid (OA).

6.1.1. Ursolic acid

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpene compound that exists in plants, medicinal herbs and fruits (Figure 7). UA has shown interesting biological activities such as anti-inflammatory, anti-hyperlipidemic, antioxidant, anti-angiogenic and anticancer effects [Banno *et al.*, 2004; De Angel *et al.*, 2010; Ramos *et al.*, 2010; Sohn *et al.*, 1995]. Thereby, this triterpenoid has been shown to inhibit the growth of tumor cells through cell cycle arrest and induction of apoptosis in many cancer cell types [Harmand *et al.*, 2003; Tang *et al.*, 2009]. Related to apoptosis induction, it was reported recently that UA activated caspase -3, -8, and -9, as well as downregulated the expression of BCL-2 in gastric cancer cells [Wang *et al.*, 2011a]. It has also been suggested that UA may be involved in modulation of autophagy through activation of JNK signaling, and in turn exerting anticancer effects in apoptosis-resistant HCT15 colorectal cancer cells [Xavier *et al.*, 2012, submitted]. In HCC, it has been reported that UA induces apoptosis through activation of caspase 3 and cell cycle arrest via inhibition of DNA replication and increased p21 expression [Kim *et al.*, 2000]. In other study, it was also observed that UA decreased the activation of nuclear transcription factor NF-kB and its downstream effectors BCL-2 and X-linked inhibitor of apoptosis protein (XIAP), the later with ability to inhibit caspase-3, -7, and -9 [Shyu *et al.*, 2010; Stennicke *et al.*, 2002]. UA was also able decrease the mitochondrial membrane potential of the HCC cells HuH7 with the consequent release of cytochrome c from the mitochondria into the cytosol and with activation of caspases [Shyu *et al.*, 2010; Yan *et al.*, 2010]. In breast cancer cells, UA inhibited migration and invasion of tumor cells through suppression of AKT/mTOR and NF-kB signaling, with the corresponding decrease of matrix metalloproteinase (MMP) levels [Yeh *et al.*, 2010]. Anti-angiogenic effects of UA by decreasing the expression of vascular endothelial growth factor (VEGF), interleukin-8, and transcription factor HIF-1α was also reported in liver cancer [Lin *et al.*, 2011], while in melanoma cancer cells UA suppressed VEGF, MMP-2 and MMP-9 [Kanjoormana and Kuttan, 2010].

6.1.2. Oleanolic acid

Oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid) is a isomer of UA, differing these two compounds in the position of a methyl residue (Figure 7). In OA a methyl group is together with other in position 20, while in UA the methyl group is in position 19 of the cyclic ring system [Liu, 1995]. Like UA, it has been shown that OA possess many biological activities such as anti-inflammatory, anticancer, antiviral, hepatoprotective and anti-hyperlipidemic effects, occurring in more than 120 plants species [Chen *et al.*, 2011; Wang and Jiang, 1992]. The antitumor activity of OA has been reported in colon cancer cells due to its inhibitory effect on cell proliferation through cell cycle arrest [Li *et al.*, 2002]. The apoptotic effects of OA was described in leukemia cells, in which it activated caspase-9 and caspase-3, accompanied by the cleavage-induced inactivation of the DNA repair enzyme Poly (ADP-ribose) polymerase [Zhang *et al.*, 2007]. It has also been shown that OA downregulates the expression of cyclin D1 and upregulates p21 and p27, that inhibits the activation of the nuclear translocation of transcription factors such as NF-kB, c-fos, ATF-2 and CREB-1, and that also downregulates the production and expression of TNF-α, IL-1β and IL-6, in melanoma cells [Pratheeshkumar and Kuttan, 2011]. In HCC cells, OA induced also apoptotic effects via increasing DNA fragmentation, decreasing mitochondrial membrane potential, lowering Na^+-K^+-ATPase activity, and elevating caspase-3 and caspase-8 activities [Yan *et al.*, 2010]. In other HCC cell lines, it was reported the antiproliferative effects of OA through apoptosis associated with alterations in BCL-2 family proteins and downregulation of NF-kB and XIAP [Shyu *et al.*, 2010]. OA was also able to suppress cell adhesion and to reduce the production of VEGF and the intercellular adhesion molecule 1 - ICAM-1 [Yan *et al.*, 2010]. In osteosarcoma cells, OA inhibited both mTORC1 downstream targets S6K, and 4E-BP1, induced cell cycle arrest and inhibited mTORC2 and its target AKT, leading to induction of apoptosis [Zhou *et al.*, 2011].

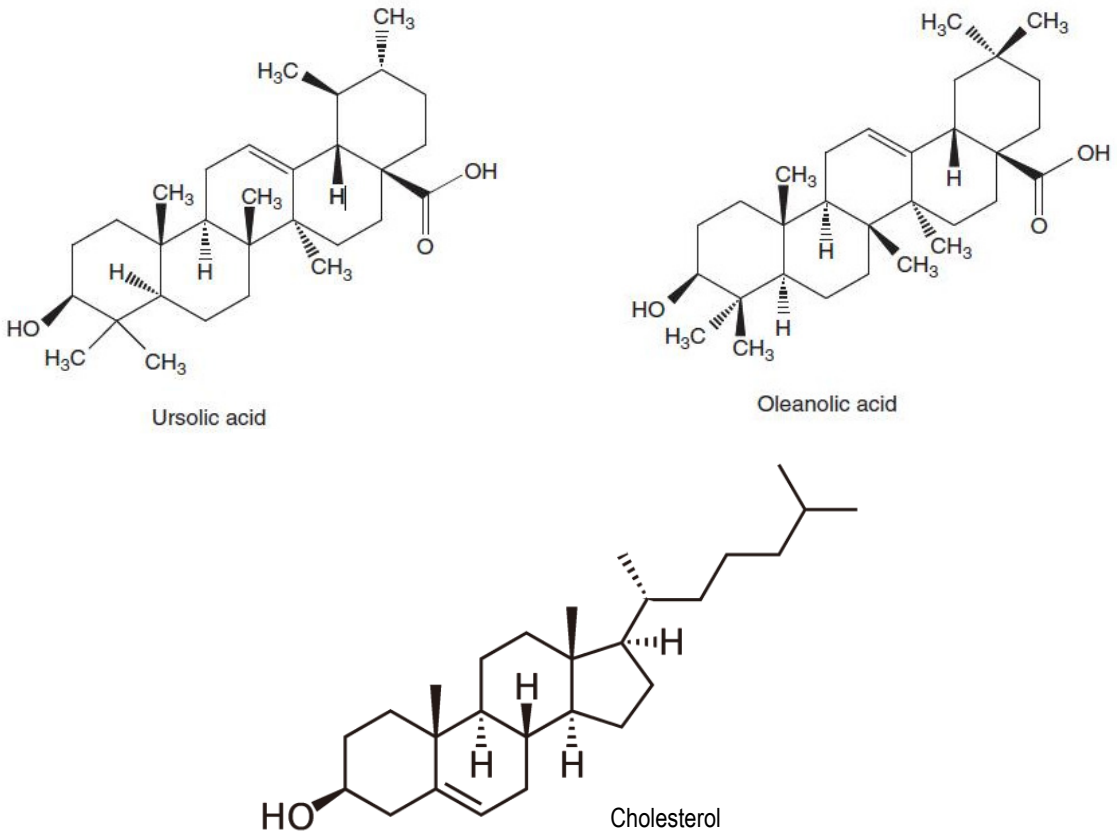


Figure 7. Structure of ursolic acid, oleanolic acid and cholesterol.

7. Triterpenoids and cholesterol

Cholesterol is a molecule formed by squalene cyclization and in humans can be synthesized or absorbed from the diet [Stryer *et al.*, 2002]. It is an essential component of cellular membranes and modulates various of their properties such as fluidity, permeability, stability and contour [Deng *et al.*, 2009]. Lysosome is one of the organelles that contain a high concentration of cholesterol in its membrane [Gallegos *et al.*, 2002]. Cholesterol can interact with the phospholipids of membranes, integrating regions with a characteristic structural composition that appear to act as platforms to colocalize proteins, these microdomains are named lipid rafts [Calder and Yaqoob, 2007]. Such lipid rafts play important roles in several cellular process that include signal transduction, membrane trafficking, cytoskeletal organization and pathogen entry [Ikonen, 2001]. As mentioned before, cellular cholesterol levels can have dramatic effects in autophagy, in particular in the fusion of autophagosomes with lysosomes [Koga *et al.*, 2010]. The

proteins involved in fusion of the two compartments, such as SNAREs- mediated vesicular fusion, are localized in these microdomains and their activity are affected by depletion of cholesterol [Koga *et al.*, 2010]. Another effect of low cholesterol in lysosomal membrane is the increased permeability to K^+ and H^+, destabilizing the lysosomes by affecting pH, leading to loss of the lysosomal function as well as the fusion ability with autophagosomes [Deng *et al.*, 2009]. On the contrary, high levels of cholesterol can increase mitochondrial membrane condensation. This can occur due to deficiency in caveolins, proteins that reside in lipid rafts microdomains and that bind cholesterol, promoting an increase of influx and accumulation of free cholesterol. Consequently, it occurs a decreased efficiency of the respiratory chain and intrinsic antioxidant defenses leading to accumulation of reactive oxygen species (ROS) and eventually cell death [Bosch *et al.*, 2011].

The bioactive triterpenoids have structural similarities to cholesterol (Figure 7) and are good candidates to interact with biomembranes, potentially modulating their structural properties and leading to functional changes of proteins associated to membranes. A previous report described the ability of both UA and OA to perturb membrane domains rich in cholesterol and, therefore, affecting the lipid membrane physical properties [Prades *et al.*, 2011]. Also recently, it was shown the potential of OA to strongly decrease the phosphorylation of AKT and lipid rafts-mediated mTORC1 and mTORC2 signaling, as well as their contents decreased in the lipid raft fractions. Moreover, addition of exogenous cholesterol restored the signaling events disrupted by OA in breast cancer cells [Chu *et al.*, 2010]. All these aspects confer a certain capacity of pentacyclic triterpenoids to interfere with cholesterol homeostasis and associated cellular functions, which may be explored as a novel approach to disrupt the survival of resistance of tumor cells.

The triterpenoids UA and OA have also been considered as potential good drugs for the treatment of hyperlipidemia, which also indicate their potential to control cholesterol levels *in vivo*. Azevedo *et al.* reported the ability of UA to ameliorate the lipid profile in rats [Azevedo *et al.*, 2010]. It has also been reported that both triterpenoids are able to inhibit acylCoA:cholesterol acyltransferase (a enzyme that is require for the storage of cholesterol), promoting decreased levels of cholesterol *in vivo* [Lin *et al.*, 2009]. Effects on cholesterol biosynthesis has also been associated with UA, where it was reported its capacity to increase the mRNA expression of cholesterol 7α-hydroxylase and to accelerate the conversion of cholesterol into bile acid, which may explain the reduction in cholesterol and low density lipoprotein-cholesterol levels in the circulation induced by UA [Xue *et al.*, 2006]. Recently, a study by Jia *et al.* indicates that UA

regulates hepatic lipid metabolism by acting as a peroxisome proliferator-activated receptor (PPAR-α) agonist *in vitro.* UA altered the expression of key genes in lipid metabolism, significantly reducing intracellular triglyceride and cholesterol concentrations in hepatocytes, but without being a direct ligand of PPAR-α [Jia *et al.*, 2011].

Objectives of the work

These works are integrated in the Nutriomics and Pharmacology Group of the Department of Biology, University of Minho, that studies the cellular and molecular mechanisms of action of health improving properties of phytochemicals. In this regard, many natural compounds and plant extracts have been characterized as potential anticancer drugs or sensitizers of chemotherapeutics. A previous work in our group reported the potential of UA to induce cell death and to inhibit proliferation in colorectal cancer cells. This natural triterpenoid UA was also shown to enhance the efficacy of the chemoterapeutic 5-fuorouracil, to activate JNK signaling and to modulate molecular markers of autophagy.

In the present report, we decided to evaluate the applicability of two isomer triterpenoids, UA and OA, to induce cell death and modulate autophagy in the HepG2 human hepatocellular carcinoma cell line. Resistance to chemoterapeutics and survival of cancer cells in poor vascularised central areas of tumors are mediated by autophagy. Therefore, starvation-induced autophagy was used in this work to mimic metabolic stress in cancer cells and to study the potential anticancer effects of UA and OA in HepG2 cells. The mechanisms of cell death induced by the triterpenoids was investigated using pharmacological inhibitors of several molecular targets and signalling pathways, and cell viability measured by MTT assay and PI staining. Considering previous effects of UA in autophagy molecular markers, we also studied whether cell death induced by these triterpenoids is mediated by autophagic flux inhibition. Triterpenoids are structurally similar to cholesterol, which is an important component of cell membranes and involved in central cellular functions. Therefore, the potential of these compounds to disrupt cholesterol homeostasis was also explored in this work. With this study we intend to give one more step ahead in the elucidation of the mechanisms of action of triterpenoids as potential anticancer compounds.

Material and Methods

1. Chemicals and antibodies

Oleanolic acid (OA), ursolic acid (UA), 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), methyl-β-cyclodextrin (MβCD), Minimum Essential Medium Eagle (MEM), MEM without L-glutamine, RPMI-1640, antibiotic/antimycotic solution, bovine serum albumin (BSA), N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), Monodansyl-cadaverine (MDC) and chloroquine (CQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin (Rap) was purchase from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protease inhibitors PepA and E64D were from PeptaNova GmbH (Germany). 2',7'-Dichlorodihydrofluorescein diacetate (DCF) was purchased from Molecular Probes (Eugene, OR, USA). Fetal bovine serum was bought from Lonza (Verviers, Belgium). The source of primary antibodies were the following: anti-phospho-mTOR from Cell Signaling (Danvers, MA, USA); ant-rabbit p62 (SQTM1) from Enzo Life Sciences (Lorrach, Germany); anti-MAPLC3 (clone 5F10) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and anti-β-actin from Sigma-Aldrich. Secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were purchased from Santa Cruz Biotechnology.

2. Cell culture

HepG2 cells (human hepatocellular liver carcinoma cell line), obtained from the American Type Culture Collection (ATCC), were maintained in culture in 75 cm^2 polystyrene flasks (TPP, Switzerland) with MEM medium, containing 10% FBS, 1% antibiotic–antimycotic solution, 1 mM sodium pyruvate, 10 mM HEPES and 2.2 g/l sodium bicarbonate under an atmosphere of 5% CO_2 and 95% air at 37°C. HCT116 cells (human colorectal carcinoma cell line) were kindly provided by Prof. Raquel Seruca from IPATIMUP, Porto. The cell line was maintained in culture in 25 cm^2 polystyrene flasks (TPP, Switzerland) with RPMI 1640 medium, containing 6% FBS, 1% antibiotic–antimycotic solution, 0,1 mM sodium pyruvate, 10 mM HEPES and 2 g/l sodium bicarbonate under an atmosphere of 5% CO_2 and 95% air at 37°C.

3. MTT reduction assay

A MTT reduction assay was performed to study the potential of test compounds to decrease the number viable cells, as previously described [Lima *et al.*, 2011]. Briefly, HepG2 cells were plated in 24-multiwell culture plates at $0.1x10^6$ cells per well one or two days before incubation with test compounds (for pre-incubation and co-incubation procedures, respectively). In the pre-incubation procedure, HepG2 were incubated with test compounds for 24h followed by a period of 48h with fresh complete medium or fresh starvation medium (MEM medium without glutamine and FBS). In the co-incubation procedure, HepG2 were incubated with test compounds dissolved in complete medium or starvation medium for 48h. One hour before the end of the incubation period MTT (final concentration 0.5 mg/ml) was added to each well. Then, the medium was removed, and the formazan crystals formed by the cell's capacity to reduce MTT were dissolved with a 50:50 (v/v) DMSO:ethanol solution, and absorbance measured at 570 nm (with background subtraction at 690 nm). The results were expressed as percentage relative to the control (cells without any test compound, vehicle only – DMSO 0.5%). The concentration of test compound that decreases the number of viable cells to 50% (IC_{50}) was calculated using mathematical modeling with the program GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

4. Cell death analysis by PI staining

HepG2 cells were plated in 24-multiwell culture plates at $0.1x10^6$ cells per well. After treatment with different incubation times and/or different concentrations of OA, cells were collected (both floating and attached cells) and washed in ice cold PBS containing 6% (v/v) FBS. Cells were then resuspended in ice cold PBS with propidium iodide (PI) and Hoechst, added to a final concentration of 25 μg/ml and 5 μg/ml, respectively. Twenty microliters of the stained cell suspension were placed on microscope slides and overlaid carefully with a coverslip. Immediately, cells were visualized on a fluorescent microscope and photos taken from different fields. The percentage of dead cells (PI positive) was calculated from the ratio between PI positive

cells and total number of cells (visualized with Hoechst staining), from a count higher than 500 cells per slide.

5. Western blotting

HepG2 cells were plated in 6-multiwell culture plates at $0.5x10^6$ cells per well. After treatment with different incubation times or different concentrations of compounds, cells were washed with PBS and lysed for 15 min at 4°C with ice cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris-HCl (pH 8), 2 mM EDTA) containing 1 mM PMSF, phosphatase inhibitors (20 mM NaF, 20 mM $Na_2V_3O_4$) and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and BSA used as protein standard. For Western blot, 20 μg of protein was resolved in SDS-polyacrylamide gel and then electroblotted to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) nonfat dry milk, washed in TPBS and incubated with primary antibody overnight. Then, after washing, membranes were incubated with secondary antibody conjugated with horseradish peroxidase and immunoreactive bands were detected using the Immobilon solutions (Millipore) under a chemiluminescence detection system, the ChemiDoc XRS (Bio-Rad Laboratories). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-Actin was used as loading control.

6. Immunofluorescence analysis

HepG2 cells were plated in chamber slides at $0.1x10^6$ cells per well. After treatment cells were fixed for 30 min with 4% PFA in PBS, washed twice with PBS, followed by permeabilization with 0.2% Triton-X-100 for 2 min and by blocking with 5% normal goat serum diluted in PBS containing 1% BSA and 0.3% Triton-X-100 for 20 min. Chamber slides were incubated with primary antibodies overnight at 4°C in humidity chambers with anti-mouse LAMP-2 (1:200) from the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA) and anti-mouse LC3 (1:50) from Santa cruz. The monoclonal antibody LAMP-2 (clone H4B4) developed by August JT

and Hildreth JEK was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. After incubation with primary antibodies, slides were washed in PBS containing 0.25% BSA and 0.1% Triton X-100, and incubated with secondary antibodies (goat anti-mouse IgG Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 488, Invitrogen) for 1h at room temperature. Slides were washed with TPBS and mounted with Vectashield antifading solution (Vector Laboratories, Inc., Peterborough, UK). Immunofluorescence signal were visualized under a fluorescent microscope (Olympus IX71) and photos taken from different fields.

7. Measurement of reactive oxygen species

To determinate the levels of reactive oxygen species (ROS) by flow cytometry, cells were seeded in petri dishes (60 mm) with $1x10^6$ cells and treated with OA overnight. After that, cells were washed with PBS and incubated with 2',7'-Dichlorodihydrofluorescein diacetate (DCF) at a final concentration of 10 μM for 30 min, 37°C. After incubation, cells were washed with PBS, collected and washed with ice cold PBS, centrifuged at 500 xg for 5 min and resuspended in PBS. Samples were then run on a flow cytometer (Beckman-FC500, Beckman Coulter) and ROS levels expressed as the ratio between mean fluorescence intensity of each sample and autofluorescence.

8. Statistical analysis

Data are expressed as the mean ± SEM of at least 3 independent experiments. Statistical significances among data groups were analyzed by one-way ANOVA followed by the Newman–Keuls multiple comparison test, or analyzed by the Student's *t*-test, as appropriate, using GraphPad Prism 5.0 software (San Diego, CA, USA). Differences between groups were considered statistically significant when $P \leq 0.05$.

Results and Discussion

1. Effects of UA and OA in molecular markers of autophagy

In a previous work, in our laboratory, UA was tested in colorectal cancer cells and shown to have anti-proliferative effects and to induce cell death by apoptosis [Xavier *et al.*, 2009]. In the apoptosis-resistant HCT15 cell line, however, apoptosis did not account for all cell death induced by UA [Xavier *et al.*, 2012, submitted]. In these cells, UA activated JNK and modulated molecular markers of autophagy, which suggested that these pathways might be involved in cell death [Xavier *et al.*, 2012, submitted]. Both in HCT15 and in MCF7 breast cancer cell line, UA induced the levels of LC3 and p62 that, in addition with other molecular assays, suggested that UA is inhibiting autophagy at later steps, in particular in the fusion of autophagosomes with lysosomes [Xavier, 2010]. In the present study, we tested the ability of the two isomeric triterpenoids UA and OA to induce cell death and to modulate autophagy in the HCC HepG2 cell line. First, we evaluated by western blot the effects of these triterpenoids in the levels of p62 and LC3 that are considered good molecular markers of autophagy [Bjorkoy *et al.*, 2009; Kouri *et al.*, 2002]. As shown in Figure 8A, both UA and OA induced the accumulation of p62 and LC3-II along the time. Therefore, like the previous results in colon cancer cells, UA also induced the increase of autophagy markers in HepG2 cells. OA also present similar results, which is not surprising having in count the very similar structure with UA.

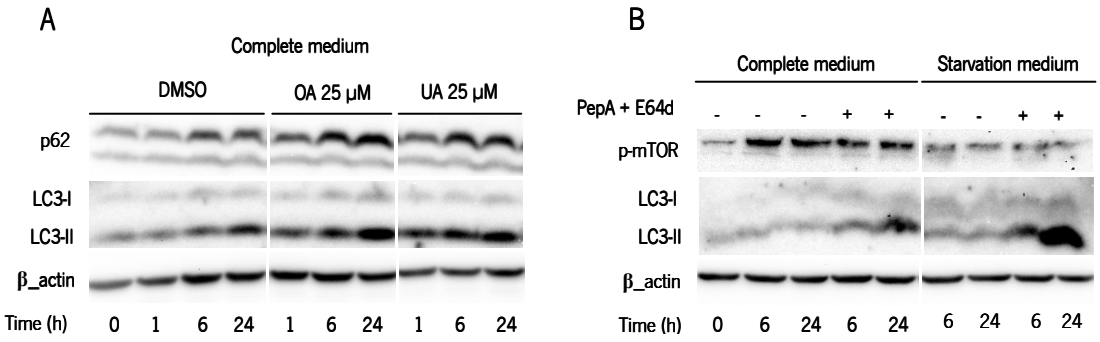


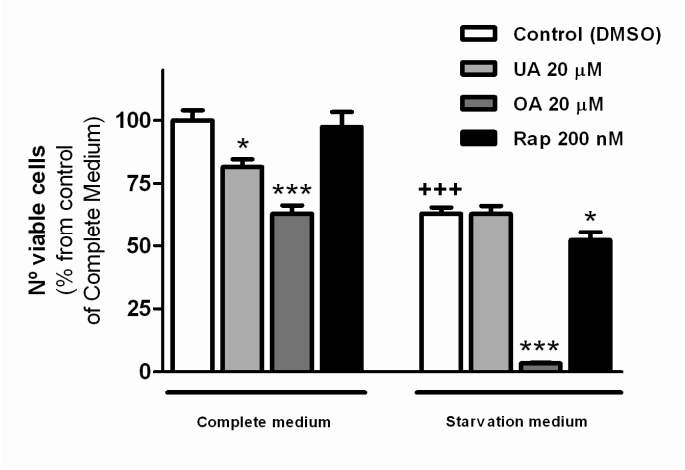
Figure 8. Modulation of autophagic markers by triterpenoids and starvation in HepG2 cells. A) Effect of 25 µM of UA and OA in the levels of LC3 and p62 in complete medium along the time. B) Effect of starvation medium (complete medium without FBS and glutamine) in the levels of p-mTOR and LC3 along the time, in the presence or absence of the protease inhibitors PepA (pepstatin A) and E64d.

To compare with the effects of both UA and OA, we also induced autophagy by starvation, in particular with serum and glutamine deprivation as reported before [Yu *et al.*, 2010]. As shown in Figure 8B, starvation decreased p-mTOR levels but did not increase considerably the levels of LC3-II after 6h and 24h of incubation. This is in agreement with the recent literature, because when the autophagic flux is induced there is only a transient increase in LC3-II levels that then return to normal levels due to its degradation in the autolysosome [Kabeya *et al.*, 2000; Mizushima *et al.*, 2010]. Therefore, in these conditions increased autophagic flux can be studied using inhibitors of autophagy in later steps, such as by using the lysosome protease inhibitors PepA and E64d [Mizushima *et al.*, 2010; Tanida and Waguri, 2010]. Blocking the autophagic flux we observed, in fact, that starvation medium induced a higher accumulation of LC3-II as compared with complete medium, indicating therefore induction of autophagy. Thus, in opposition to the starvation-induced autophagic flux, increased levels of both LC3 and p62 by UA and OA may indicate an inhibition of autophagy at later steps (Figure 8).

2. Effect of UA and OA in HepG2 cell viability

Considering that both UA and OA also remarkably increased autophagy markers in HepG2 cells, we then evaluated their effects on the viability of HepG2 cells by MTT assay in both complete and starvation medium. As shown in Figure 9A, OA induced a higher decrease of the number of viable cells as compared with UA at 20 μM, in both complete and starvation medium. On the contrary, in previous results in colon and breast cancer cells UA was much more effective than OA in decreasing cell viability [Xavier, 2010]. Interestingly, OA remarkably decreased the number of viable cells in starvation medium as compared with UA, suggesting therefore different modes of action of these isomer triterpenoids. Under the microscope, UA induced remarkable cell morphological changes with the appearance of floating cellular debris but without floating cells in the medium (Figure 9B). These debris increased in starvation medium and cells present bigger and higher number of shining vacuolar structures, but without cell death (floating cells). On the contrary, although OA also induced some cellular debris in complete medium, when cells

A



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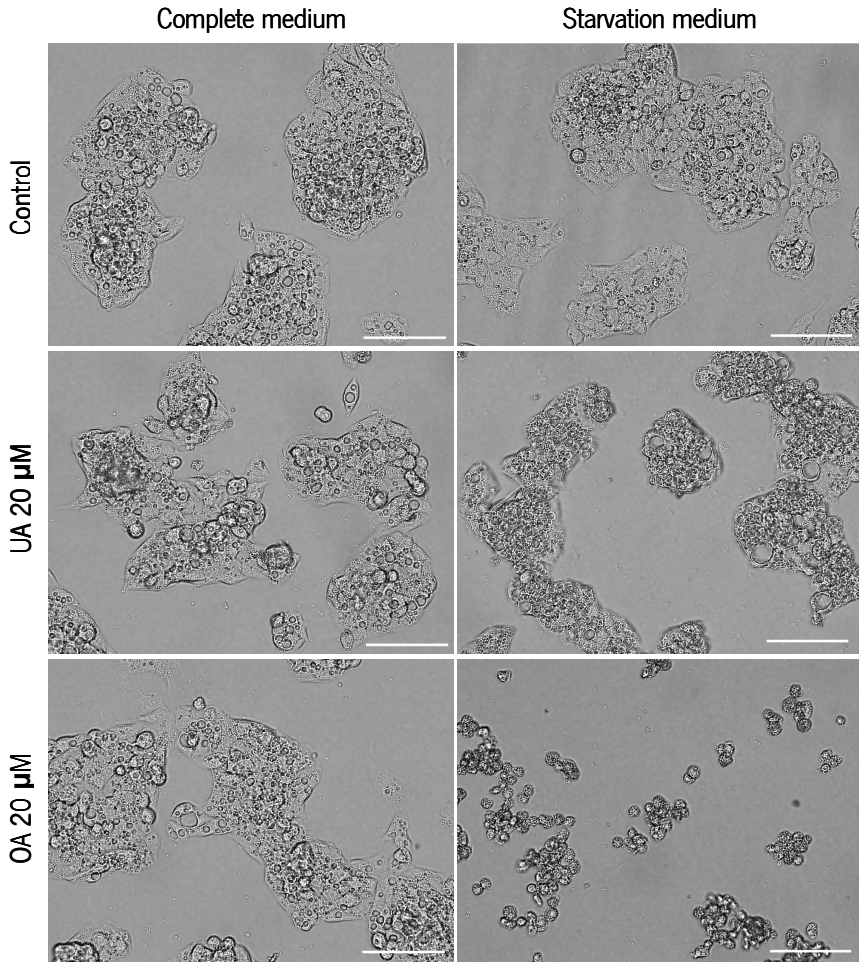

Figure 9. Effect of ursolic acid (UA), oleanolic acid (OA) and rapamycin (Rap) in the viability of HepG2 cells. A) Cells were incubated with test compounds (controls received vehicle only – DMSO) for 48h either in complete medium or starvation medium, and cell viability assessed by MTT reduction assay. Values are mean ± SEM of at least three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, when compared with respective control, analyzed by the Student's *t*-test. $^{+++}$ $P \leq 0.001$, when compared with same condition between different media, analyzed by the Student's *t*-test. B) Representative images of the effect of test compounds in complete and starvation medium for 48h (bar=100μm).

were incubated in starvation medium for 48h, most of cells were floating with morphological features of cell death, confirming therefore the MTT results (Figure 9). The effect of Rap in cell viability was also studied to test the effects of a compound known to induce autophagy and to have anticancer effects [Faivre *et al.*, 2006; Oshiro *et al.*, 2004]. As shown in Figure 9A, HepG2 cells were not susceptible to cell death induced by Rap, and only in starvation medium there was a significant but slight decrease in the number of viable cells. Also comparing the control situation in both media, we observe a decrease in the number of viable cells in starvation medium (Figure 9). This decrease is probably due to inhibition of cell proliferation during the 48h of incubation due to serum and glutamine starvation, since no cell death was observed under the microscope as shown by the absence of floating cells and cells with blebs.

It is not surprising that starvation-induced autophagy increases the susceptibility to cell death induced by other drugs, such as what happened here with OA. It is known that starvation-induced autophagy mediates protection from apoptosis [Boya *et al.*, 2005], and that inhibitors of autophagy inhibits the survival of cells under starvation [Farkas *et al.*, 2011]. Here, although both UA and OA increase the levels of LC3 and p62 probably due to autophagy inhibition, only OA sensitized HepG2 cells to death under starvation (Figure 9). To test if OA also sensitize other cells to cell death under starvation, we did a similar experiment in the colorectal carcinoma cell line HCT116. Confirming previous results by Xavier (2010), UA was more toxic than OA in the HCT116 cells line (see Supplementary Fig. 1A). When the compounds were tested in starvation medium the number of viable cells decrease (see Supplementary Fig. 1B). However, the decrease was much more significant in cells treated with OA (see Supplementary Fig. 1). For UA the IC_{50} decrease from 8 μM in complete medium to 4 μM in starvation medium, after 48h of incubation, whereas for OA, the IC_{50} decrease from >>50 μM (viability was 93% at 50 μM – higher concentration tested) to 12 μM. These results show that OA also remarkably sensitize cells to death in other cell lines under starvation as compared with UA.

To further test that UA and OA have different effects as compared with Rap, we next evaluated the effects of pre-incubation with test compounds in complete medium followed by a period of starvation of 48h without test compounds in cell viability by the MTT assay. Induction of autophagy by Rap 24h prior to starvation remarkably decreased cell viability induced by 48h of starvation (Figure 10A). This effect was in part due to cell death since floating cells were observed (data not shown), contrarily to the effect of Rap incubated in starvation medium (co-incubation procedure – Figure 10A). Here, the observed decrease in cell viability was probably

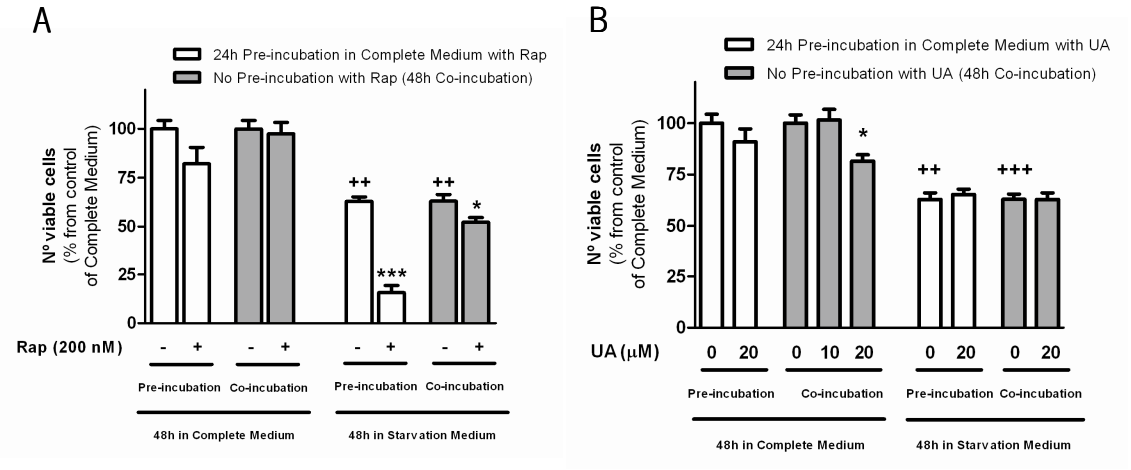

Figure 10. Effect of Rap (A) and UA (B) in the viability of HepG2 cells, as assessed by MTT reduction assay. In the pre-incubation regime, cells were incubated with Rap (A) or UA (B) at indicated concentrations for 24h in complete medium followed by a period of 48h with the indicated fresh medium without Rap or UA. In the co-incubation regime, cells were incubated with Rap (A) or UA (B) at indicated concentrations for 48h either in complete medium or starvation medium. Values are mean ± SEM of at least three independent experiments. $^{*}P \leq 0.05$, $^{***}P \leq 0.001$, when compared with respective control, analyzed by the Student's *t*-test. $^{++}P \leq 0.01$, when compared with same condition in complete medium, analyzed by the Student's *t*-test.

due to the presence of a less number of viable cells due to inhibition of proliferation or due to a decrease in cell metabolic activity induced by Rap together with starvation as compared with starvation alone. Therefore, autophagy induction by Rap was detrimental to cells in a following period of starvation-induced autophagy. This might happen because cells possess less cellular contents for degradation by autophagy for generation of energy and building blocks in a period of starvation that immediately follows other. In the case of UA, this pre-incubation procedure did not result in a decrease of the number of viable cells (Figure 10B). Regarding the effects of OA, likewise Rap, the pre-incubation procedure resulted in a decrease of cell viability (Figure 11A&B). However, contrarily to Rap, the effect of OA in cell viability was stronger when incubated along with starvation than that in the pre-incubation regime (Figure 11B). Therefore, these results corroborate that the effect of OA in autophagy and cell death is different than that of Rap and UA.

Considering all the results we decided then to focus the following work in the effect of OA in the induction of cell death under starvation conditions. Thus, we next tested the effect of different concentrations of OA in cell viability by the MTT test. As shown in Figure 11B, OA induced a concentration-dependent decrease in the number of viable cells after 48h under starvation conditions, from 1 μM to 20 μM. Under starvation OA present an IC_{50} of 3.5 μM

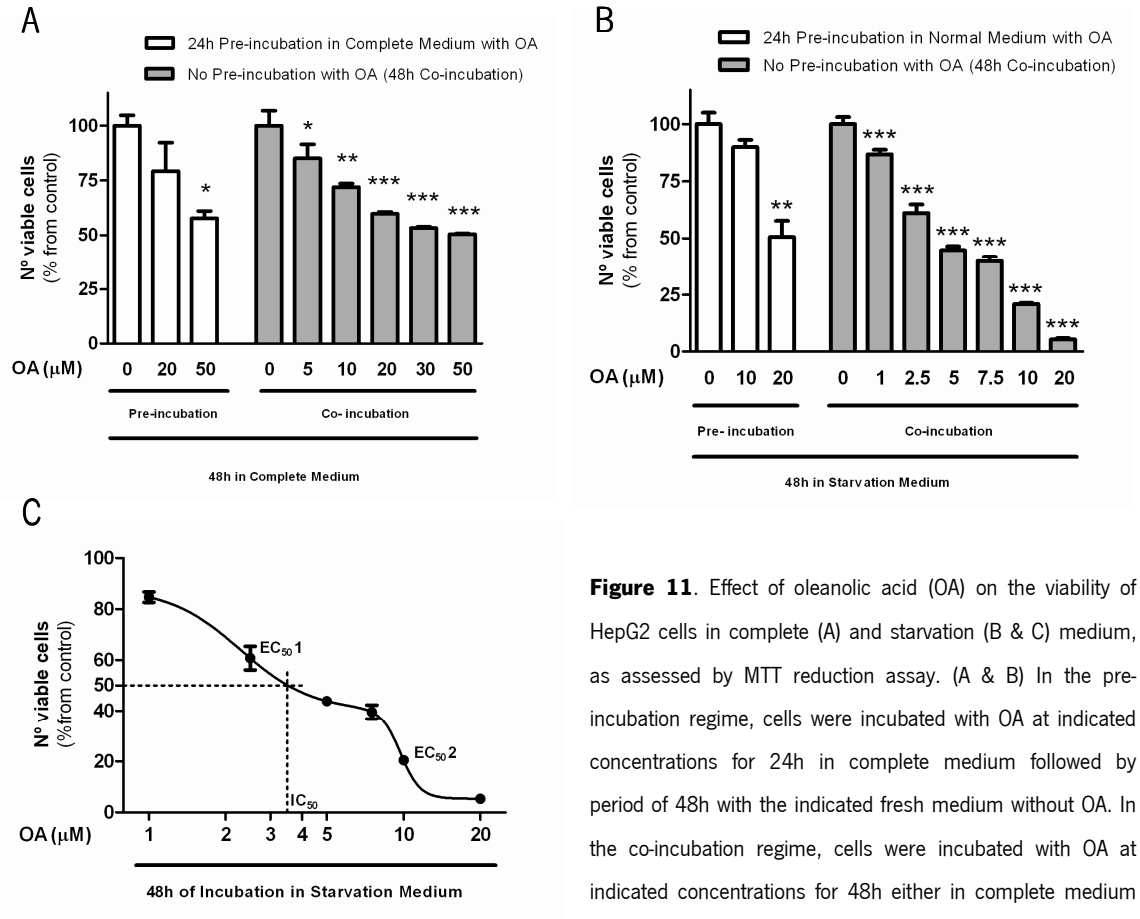

Figure 11. Effect of oleanolic acid (OA) on the viability of HepG2 cells in complete (A) and starvation (B & C) medium, as assessed by MTT reduction assay. (A & B) In the pre-incubation regime, cells were incubated with OA at indicated concentrations for 24h in complete medium followed by period of 48h with the indicated fresh medium without OA. In the co-incubation regime, cells were incubated with OA at indicated concentrations for 48h either in complete medium (A) or starvation medium (B). Values are mean ± SEM of at least three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ when compared with respective control, analyzed by one-way ANOVA (Newman-Keuls Multiple Comparison Test). (C) Graphic of the mathematical modelling of data present in Figure 11B in the co-incubation regime following a bell-shaped dose-response curve, using the GraphPad Prism software. Shown in the graphic are the IC_{50} of 3.5 μM as well as the $EC_{50}1$ of 2.3 μM and $EC_{50}2$ of 9.8 μM for cell viability.

(Figure 11C) whereas in complete medium is about 50 μM (Figure 11A), representing about a 15x increase of susceptibility.

Usually, the effect of a drug on cell viability follows a sigmoidal curve. However, in this case we find that the effect of OA followed a bell-shaped dose-response curve (composed of two sigmoidal curves) showing two EC_{50} values (Figure 11C). Based on the morphological observations after OA treatment under starvation and on the PI staining results (Figure 12), we believed that this effect is due to different effects of the drug in two concentration ranges. In particular, in the first sigmoidal curve that presents an EC_{50} (half maximal effective concentration) of 2.3 μM OA might induces a decrease of the number of viable cells due to the inhibition of cell proliferation (under starvation this factor will not contribute much) and/or due to a decrease in cells' metabolic activity. On the other hand, in the second sigmoidal curve that

presents an EC_{50} of 9.8 μM, the effect of OA in the decrease of the number of viable cells is most likely due to induction of cell death in a concentration-dependent manner. This latter assumption is corroborated by the results of PI staining shown below.

As shown in Figure 12A by the PI staining assay, OA induced cell death in a concentration-dependent manner from 5 μM to 15 μM after 48h of incubation in starvation medium. Cell death significantly increased from 10% in control condition to 60% and 95% with 10 μM and 15 μM of OA, respectively. Studying cell death along the time we found that OA 10 μM increased significantly the number of PI positive cells only after 24 h of incubation (Figure 12B). These results indicate that cell death induced by OA is not abrupt and may be programmed.

Considering the effect of OA on cell death, we then tested the ability of OA to induce apoptosis. Incubation of HepG2 cells with OA 15 μM in starvation medium for 48h did not result in nuclear condensation and cleavage of caspases and PARP1 (data not show). These results are not consistent with previous reports where it was shown that OA and UA induced apoptosis in hepatocellular carcinoma cells [Shyu *et al.*, 2010; Yan *et al.*, 2010]. However, those results were done in complete medium and with higher concentrations of drug and/or fewer cells.

It is considered that starvation-induced autophagy protects cells from cell death by apoptosis, process that is used by cancer cells for survival [Boya *et al.*, 2005; Roy and Debnath, 2010]. This process can be reverted by small inhibitors of autophagy [Farkas *et al.*, 2011]. Thus, suppression of autophagy with pharmacological agents under conditions of starvation or chemotherapy may significantly increase cell apoptosis and retard proliferation of

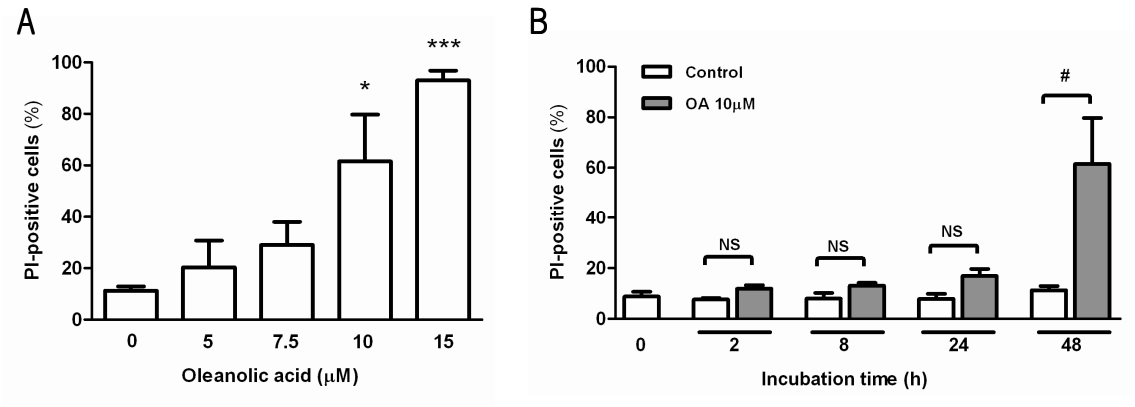

Figure 12. Effect of oleanolic acid (OA) on cell death in HepG2 cells under starvation, as assessed by the PI staining assay. (A) Effect of different concentrations of OA for 48h. (B) Effect of OA 10 μM along time. Values are mean ± SEM of at least three independent experiments. *$P \leq 0.05$, *** $P \leq 0.001$ when compared with control by the one-way ANOVA (Newman-Keuls Multiple Comparison Test), # $P \leq 0.05$ when compared with each other by the Student's *t*-test. NS, not significant ($P > 0.05$) when compared with each other by the Student's *t*-test.

cancer cells [Chang *et al.*, 2011]. However, in our study, although OA seem to be modulating autophagy by inhibiting it, the induction of cell death by OA under starvation conditions seems to not involve apoptosis.

3. OA and UA induce accumulation of acidic vacuoles

Considering the effects of UA and OA in the modulation of autophagy molecular markers and the differential effects that they present on induction of cell death, we decide to further study their kinetics in the formation of acidic degradative compartments. Monodansylcadaverine (MDC) has been reported as a specific marker for autophagic-related vacuoles [Biederbick *et al.*, 1995]. In addition, the accumulation of MDC-labelled vesicles correlates with the induction of autophagy by starvation conditions for 2h [Munafo and Colombo, 2001]. However, it is currently accepted that MDC does not stain nascent autophagosomes until prior to their acquisition of acidic properties, and, therefore, it only stains acidic compartments that include late endosomes, lysosomes and autolysosomes [Bampton *et al.*, 2005]. As shown Figure 13, cells treated with UA for 16h showed a remarkable increase of MDC-labelled large acidic vesicles both in complete and starvation medium. In the case of OA, there was the appearance of few and small acidic vacuoles in complete medium, which remarkably increased upon treatment of cells under starvation conditions (Figure 13). In the case of Rap-induced autophagy conditions, accumulation of acidic vacuoles was only observable after inhibition of autophagy at later steps with chloroquine (Figure 13A and Supplementary Fig. 2). However, the increase of acidic valuoles by Rap was not significant as compared with control condition (Figure 13A). Therefore, also here both triterpenoids behaved differentially than rapamycin showing an effect similar of an autophagy inhibitor at later steps. In addition, comparing the results between the two isomer triterpenoids also agrees that they act in a different way in HepG2 cells, since UA induced accumulation of acidic vacuoles in both normal and starvation conditions whereas OA only did that remarkably under starvation conditions. Importantly, this accumulation of acidic vesicles induced by OA under starvation might not be involved in cell death, since UA have more capacity to induce that in HepG2 but without significant cell death.

The kinetics of accumulation of acidic vacuoles induced by OA were also followed in

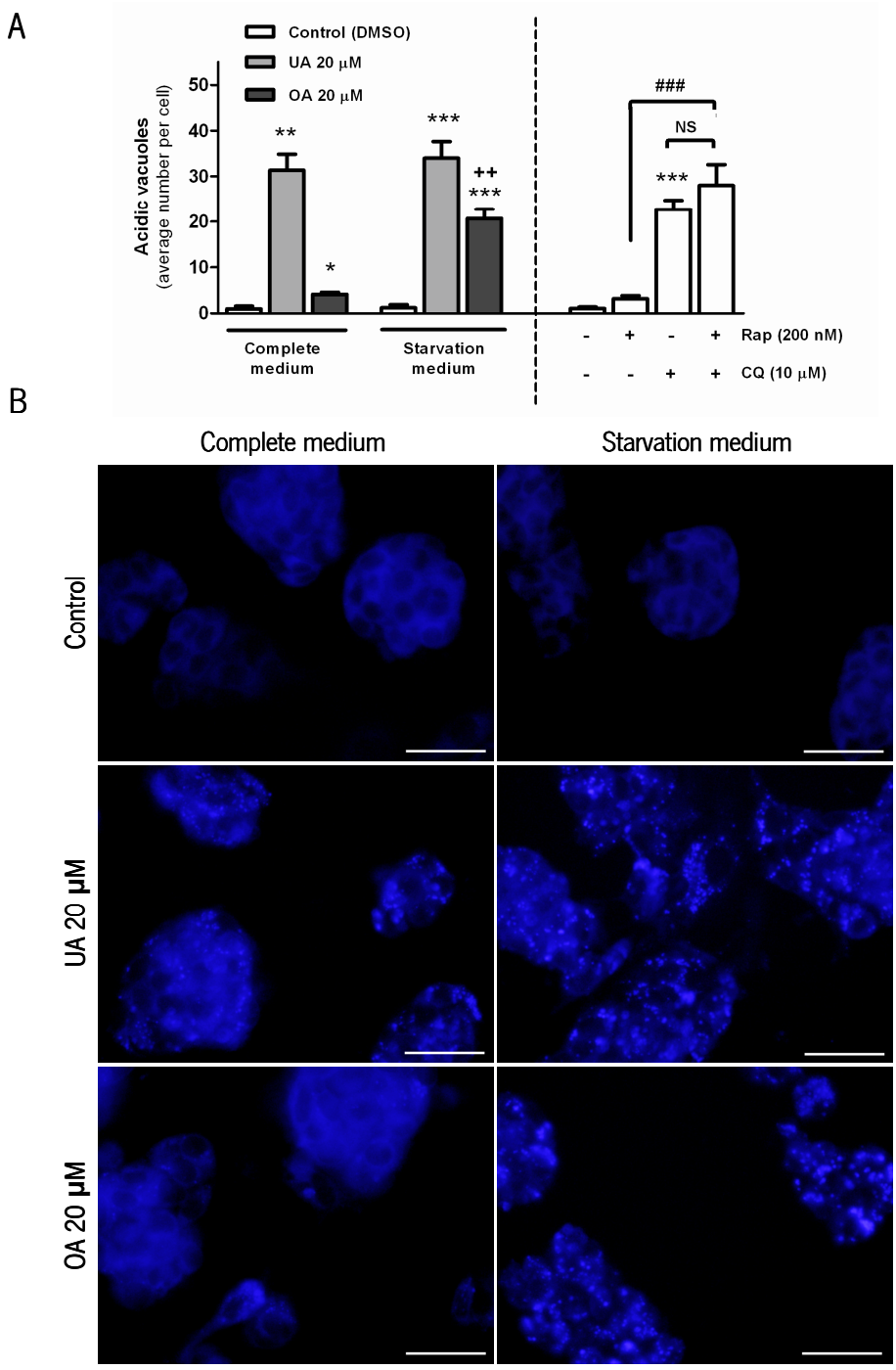

Figure 13. Effect of oleanolic acid (OA) and ursolic acid (UA) in the appearance of cellular acidic vacuoles, as assessed by MDC-staining. HepG2 cells were treated with the indicated compounds for 16h in complete or starvation medium before being stained with 50 μM monodansylcadaverine (MDC) for 10 minutes. Chloroquine (CQ) at 10 μM was used to observe the effects of rapamycin (Rap) in complete medium. (A) Quantification of the number of MDC-labelled acidic vacuoles per cell by morfometric analysis performed in photos taken in different areas of the samples. Values are mean ± SEM of at least three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ when compared with control by the Student's *t*-test. $^{++}$ $P \leq 0.01$ when compared with same treatment but in different medium by the Student's *t*-test. $^{###}$ $P \leq 0.001$ when compared with each other by the Student's *t*-test. NS, not significant ($P > 0.05$) when compared with each other by the Student's *t*-test. (B) Representative images of the effect of tested compounds for 16h in the appearance of cellular acidic vacuoles in HepG2 cells cultured in complete and starvation medium (bar=50μm).

HepG2 cells under starvation conditions. As shown in Figure 14, OA induced a concentration- and time-dependent accumulation of MDC-labelled vesicles. The effect is significant after the concentration of 10 μM (Figure 14A) and begins to occur after 4h of incubation, increasing the number of acidic vacuoles along the time (Figure 14B). These results, in addition to the levels of autophagy markers shown in Figure 8, indicate that OA (as well as UA) modulates autophagic flux very early after compound incubation.

To complement these results we also studied the expression of LC3 and LAMP-2-associated lysosomal marker by immunofluorescence. Under autophagy induction and inhibition at later steps LC3 positive punctae accumulate in cells due to the increase of autophagosomes. A portion of these LC3 punctae may or may not be colocalized with LAMP-2 staining, depending on the interference that we may be inducing to the autophagic flux [Bampton *et al.*, 2005]. Unfortunately, we were not able to get a positive immunostaining of LC3 with the antibody and conditions that we used. However, as shown before, we observed a remarkably increase of LC3 levels by western blot (Figure 8). Regarding the LAMP-2 we get a positive signal and we observe that stained vesicles seem to increase in cells treated with UA or OA as compared to the control condition under starvation (see Supplementary Fig. 3). But interestingly, vesicles stained by

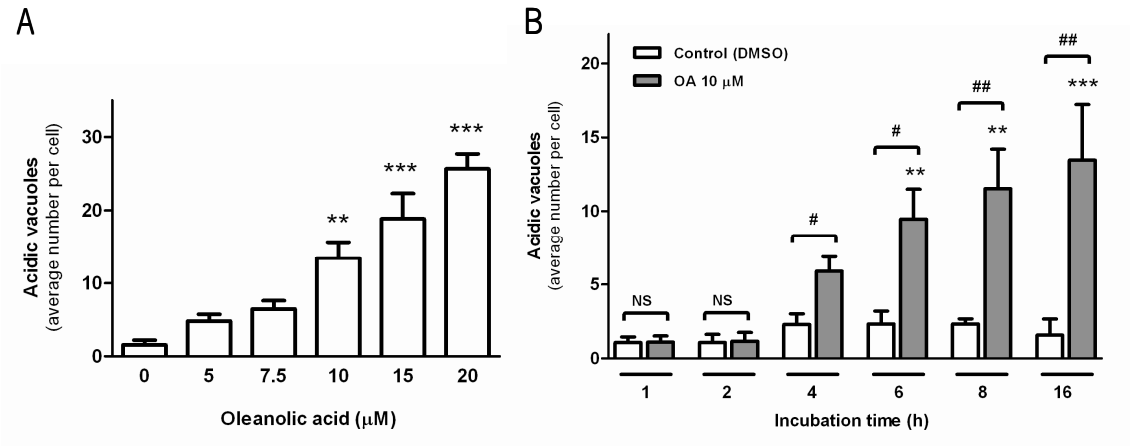

Figure 14. Effect of oleanolic acid (OA) in the accumulation of acidic vacuoles in HepG2 cells under starvation medium, as assessed by MDC-staining. Cells were incubated with different concentrations of OA for 16h (A) or along the time with 10 μM OA (B) before being stained with 50 μM of monodansylcadaverine (MDC) for 10 minutes. Represented are the quantification of the number of MDC-labelled acidic vacuoles per cell by morfometric analysis performed in photos taken in different areas of the samples. Values are mean ± SEM of at least three independent experiments. ** $P \leq 0.01$ and *** $P \leq 0.001$ when compared with control (A) or with 1h-control (B) by the one-way ANOVA (Newman-Keuls Multiple Comparison Test). $^{\#}$ $P \leq 0.05$ and $^{\#\#}$ $P \leq 0.01$ when compared with each other by the Student's *t*-test. NS, not significant ($P > 0.05$) when compared with each other by the Student's *t*-test.

LAMP-2 antibody were larger in cells treated with OA being possible to observe the contour of the structures in the amplification used, whereas in cells treated with UA we only observed bright LAMP-2-labbeled punctae (see Supplementary Fig. 3). Although these results need to be confirmed in following experiments, these larger lysosomes induced by OA but not by UA may reflect or be involved in the cell death induced specifically by OA in starvation conditions.

4. Autophagy inhibitors do not protect cells from death induced by OA

In the present work we have shown that OA induces significant cell death independent of apoptosis in HepG2 cells under starvation conditions. Concomitant with this, we also found accumulation of several markers of autophagy, such as LC3, p62 and acidic vesicles. Therefore, we speculate whether autophagy would be involved in OA-induced cell death under starvation conditions. If autophagy is induced above a critical threshold it can induce cell death due to extensive degradation of cytoplasmatic material [Galluzzi *et al.*, 2009]. As a control of this condition, we used the pre-incubation regime with Rap described above that leads to cell death in a subsequent period of starvation-induced autophagy. As shown in Figure 15A, Rap pre-treatment increased the susceptibility of cells to die under a subsequent starvation period, which was prevented by CQ but not by the PepA + E64d. Both of these autophagy inhibitors, that act at later steps, were used in non-toxic concentrations (Figure 15A) and in effective concentrations as can be observed in Figure 8B and Figure 13A for PepA + E64d and CQ, respectively. The fact that PepA + E64d did not work as CQ protecting cell death induced by Rap could be because they are degraded faster than CQ and, therefore, they are inappropriate to be used for 48h in the concentrations tested. On the other hand, the cell death induced by OA under starvation conditions was not prevented by CQ and PepA + E64d (Figure 15B). On the contrary, co-incubation of these inhibitors with OA even promoted slightly the decrease of cell viability. Therefore, this result does not support the idea that OA is inducing cell death by increasing chronically autophagy, which is in agreement with our previous results.

In turn, as discussed above, the accumulation of LC3, p62 and acidic vesicles by OA is most likely due to inhibition of autophagy at later steps. To check if inhibition of autophagy was

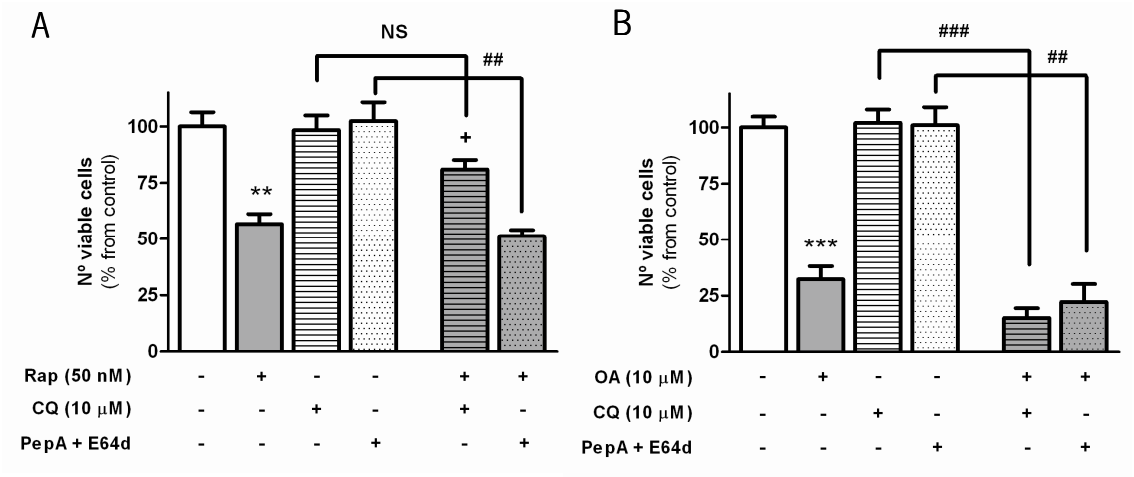

Figure 15. Effect of autophagy inhibitors in the cell death induced by rapamycin (Rap) and oleanolic acid (OA) in HepG2 cells, as assessed by MTT assay. A) Cells were pre-incubated with 50 nM Rap (controls received vehicle only – DMSO) for 24h in complete medium followed by period of 48h with fresh starvation medium containing 10 μM CQ or PepA/E64d (10μg/ml, each). B) Cells were incubated with 10 μM OA for 48h in starvation medium in the presence of 10 μM CQ or PepA/E64d (10μg/ml, each). Values are mean ± SEM of at least three independent experiments. ** $P \leq 0.01$, *** $P \leq 0.001$, when compared with respective control, analyzed by the Student's *t*-test. $^{+}P \leq 0.05$ when compared with the respective test compound alone, analyzed by the Student's *t*-test. $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$, when compared with each other by the Student's *t*-test. NS, not significant ($P > 0.05$) when compared with each other by the Student's *t*-test.

involved in OA-induced cell death we decreased autophagic flux by using pharmacological inhibitors of autophagosome formation. For that we used the inhibitors 3-MA, LY-294002 and wortmannin; but when incubated alone, all of them already decreased cell viability of HepG2 cells under starvation as measured by the MTT assay (data not show). That is not surprising since it is known that these inhibitors lack of specificity to class III PI3K, inhibiting also class I PI3K, which affect cell growth and survival [Farkas et al, 2011]. A possible solution would be to inhibit autophagosome formation by genetic tools, such as with the use of small interfering RNA targeting *Atg5* or *Beclin 1*. However, under starvation conditions, inhibition of autophagy by any mean result in cell death [Boya *et al.*, 2005; Farkas *et al.*, 2011], being therefore difficult to speculate whether OA is inducing cell death due to inhibition of autophagy at later steps. Nevertheless, here we have used two isomer triterpenoids, and because UA is more effective inhibiting autophagy than OA, but only OA is inducing cell death under starvation conditions, it indicates that the toxic effect of OA is probably independent of inhibition of autophagy at later steps. Altogether, these data point out that OA is modulating autophagy and doing something

else to the cells that promotes cell death under starvation conditions.

To explore other possible mechanisms of induction of cell death by OA in HepG2 cells under starvation conditions, we have further tested other inhibitors of molecular targets and/or signalling pathways using the MTT assay. In a previous work, it was shown that UA induced apoptosis and modulation of autophagy proteins through JNK pathway in the colorectal carcinoma HCT15 cells [Xavier *et al.*, 2012, submitted]. The stress-activated protein kinase JNK has been implicated in many cellular events including apoptosis signalling and more recently autophagic cell death [Cheng *et al.*, 2008; Wong *et al.*, 2010]. In line with this, we also tested the use of the JNK inhibitor SP600125 (SP) in co-incubation with OA. The results showed that SP does not prevent cell death induced by OA (data not shown), suggesting that it is not dependent on activation of JNK.

Mitochondrial membrane permeabilization may result from bioenergetic failure, which may occur due to nutrient depletion combined with the impossibility of recruiting endogenous nutrients by autophagy-dependent catabolic reactions [Boya *et al.*, 2005; Plas and Thompson, 2002]. On the other hand, autophagy is the process that leads to the removal of damaged mitochondria and its inhibition has been shown to increase the production of ROS [Tal *et al.*, 2009; Zhang *et al.*, 2007]. Also in the study of Xavier *et al.* (2012, submitted), the antioxidant N-acetylcysteine (NAC) was found to reduce partially the apoptosis-like cell death induced by UA in HCT15 cells, suggesting an implication of ROS. Therefore, we also test here the possible involvement of ROS in the OA-induced cell death under starvation conditions by using known antioxidants such as NAC, α-tocoferol and glutathione ethyl ester (GSH-EE – a cell permeable form of glutathione). However, none of these antioxidants protected HepG2 cells against death induced by OA (data not shown). Corroborating these results we have measured the cellular content of ROS and instead of an increase we observed a decrease of ROS induced by OA. As shown in Supplementary Fig. 4, ROS levels measured by flow cytometry using the DCF probe decreased about 35% in the presence of OA as compared with control. This decrease in ROS levels may indicate a decrease of oxidative phosphorylation activity in the mitochondrial electron transport chain induced by OA, which can be detrimental in a condition of starvation. To test if there was also some effect of OA on the mitochondrial membrane potential we also measured it by flow cytometry with the fluorescent probe tetramethylrhodamine methyl ester (TMRM), but we were not able to get good stain in preliminary experiments (data not shown). Therefore, we

should establish better conditions of HepG2 cells' staining with TMRM and/or JC-1 probes in future experiments to investigate whether OA affect mitochondria number and activity.

5. Methyl-β-cyclodextrin protects against OA-induced cell death

Cholesterol is an essential component of cellular membranes and controls its physical properties and important biological functions [Deng *et al.*, 2009]. Considering the structural similarity of triterpenoids with cholesterol we hypothesized that OA could be promoting cell death by changing cholesterol homeostasis. To test that we have used methyl-β-cyclodextrin (MβCD) - a polymer able to decrease membrane cholesterol content [Ohtani *et al.*, 1989]. As shown in Figure 16, when MβCD was added 3 hours after addition of the triterpenoid, the cell death induced by OA in HepG2 cells under starvation conditions were significantly prevented. The protection of cell death was in a concentration-dependent manner, except for the highest concentration tested in which there was some toxicity of MβCD alone. Since removal of membrane-associated cholesterol by MβCD prevented OA-induced cell death, it indicates that this triterpenoid is interfering with cholesterol levels and cellular functions that result in toxic effects under starvation conditions. However, due to the lipophilic nature of OA and structural similarity with cholesterol, we cannot exclude that MβCD is protecting cells against death by removing also OA. In an attempt to avoid this possibility MβCD was added to the culture medium 3 hours after OA to allow its internalization and induction of cellular effects. In preliminary experiments, MβCD was also added 6 and 24 hours after OA incubation and protection of cell death was also observed, but of less extent and decreasing with time (data not shown). This indicates that MβCD is preventing OA-induced cell death most likely by removing cholesterol from cellular membranes.

We have also tested an inhibitor of cholesterol synthesis to check whether it has the same effect of OA under starvation conditions or if in combination with OA it would prevent cell death. For that we used pravastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A [Tsujita *et al.*, 1986], at 1 µM and nor cell death was induced by it alone, nor it prevented cell death induced by OA (data not shown). The lack of any effect of pravastatin may indicate that the dose used was not effective decreasing cholesterol levels. That should be confirmed in future experiments by quantifying cellular cholesterol levels.

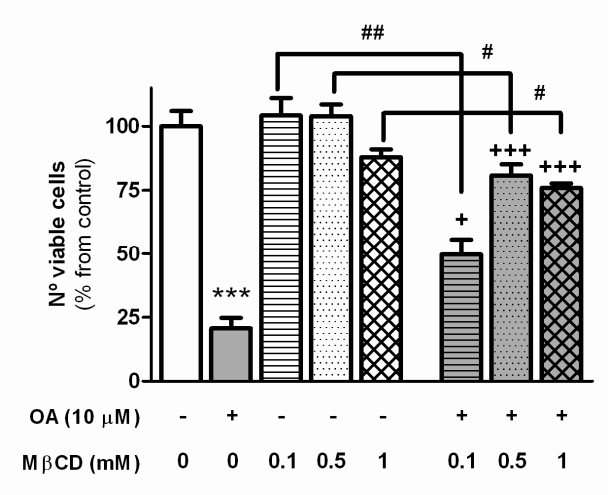

Figure 16. Effect of methyl-β-cyclodextrin (MβCD) on oleanolic acid (OA)-induced cell death of HepG2 cells under starvation conditions. Cells were incubated with OA 10 μM and, 3h after, MβCD was added at the indicated concentrations. Cell viability was measured 48h after OA incubation by the MTT assay. Values are mean ± SEM of at least three independent experiments. *** $P \leq 0.001$, when compared with respective control, analyzed by the Student's *t*-test. # $P \leq 0.05$, ## $P \leq 0.01$, when compared with each other by the Student's *t*-test. + $P \leq 0.05$, +++ $P \leq 0.001$, when compared with the respective compound alone, analyzed by the Student's *t*-test.

Considering that the removal of cholesterol with MβCD protected OA-induced cell death, that indicates that OA may be increasing cholesterol content of cellular membranes to levels that have implications in cell viability under starvation conditions. Increased cholesterol content in membranes generally decreases its fluidity that, in cells, are associated with mitochondrial membrane condensation that leads to lower efficiency of the respiratory chain and increased production of ROS [Bosch *et al.*, 2011]. Free cholesterol overload also induced cell death in smooth muscle cells accompanied with increased ROS levels and autophagy induction [Xu *et al.*, 2010]. However, here we observed lower levels of ROS in cells incubated with OA as compared with controls. In addition, treatment of HepG2 cells with OA seems to inhibit autophagy at later steps by affecting fusion of autophagosome with lysosomes. But previous results have found that removal of cholesterol from membranes affect autophagosome-lysosome fusion and lysossomal membrane permeability and pH [Koga et al., 2010; Deng *et al.*, 2009], not corroborating, therefore, the notion that OA is increasing cholesterol levels. But because UA also inhibited autophagy without having the same effect in cell viability as OA, it denotes that we are in the presence of a different effect that is specific of OA.

Interestingly, it was reported recently that OA and UA can integrate in lipid rafts (domains rich in cholesterol and sphingomyelin) decreasing their cholesterol content [Bayer *et al.*, 2011;

Chu *et al.*, 2010; Koga *et al.*, 2010; Prades *et al.*, 2011]. This interference with cholesterol homeostasis and structure of lipid rafts can explain triterpenoid's distinct biological effects by alterations of membrane lateral heterogeneity that will affect pivotal cellular functions, such as membrane-associated proteins related to such domains, signal transduction pathways, fusion of lysosomes with autophagosomes and endocytic pathways. Removal of cholesterol from lipid rafts induced by triterpenoids may indirectly increase the cholesterol content in the remaining membranes fractions (rich in phosphoglycerides) or along the boundaries between both, which will affect also the function of the fluid phosphoglyceride membrane regions [Brown, 1998]. Therefore, an hypothesis for the protection afforded by the MβCD from OA-induced cell death would be that MβCD is removing the harmful high cholesterol content present in the fluid membrane regions that was withdraw from the lipid rafts by OA. But what would explain the difference between UA and OA? Although these two compounds are isomers they differ in a position of a methyl group and recently two different works also found differences in activity between both. OA, but not UA, was able to possess photoprotection against ultraviolet A radiation (UVAR) in human keratinocytes; both compounds integrate in the lipid rafts but only OA inhibited UVAR-induced ceramide formation in lipid rafts [Bayer *et al.*, 2011]. On the other hand, Prades *et al.* (2011) used synthetic model membranes and found that UA are able to segregate cholesterol from lipid rafts but maintains their structural order, whereas OA can have a deeper penetration in membranes reducing the structural organization of lipid rafts by influencing its fluidity and disturbing the presence of the liquid ordered cholesterol-rich domains. Interestingly, we found that OA, but nor UA, induced bigger vacuolar LAMP-2-labbeled structures, which may link to different effects that these compounds have in cholesterol-rich domains of lysosomes. LAMP-2 associates in a dynamic manner with these lipid microdomains at the lysosomal membrane for regulated degradation in these regions [Rodriguez-Navarro and Cuervo, 2010]. Therefore, bigger lysosomal structures with high content of LAMP-2 indicate lower cholesterol levels and disruption of these lipid microdomains by OA, which is in agreement with observations by Prades *et al.* (2011).

But a question remains to be answered: why these effects of OA greatly predispose cells to death under starvation conditions? OA may be compromising the survival of cells under starvation due to inhibition of autophagy. However, UA also inhibits autophagy but did not induce extensive cell death. During metabolic stress autophagy inhibition also leads to apoptotic cell death [Boya *et al.*, 2005], and that was not observed here also. But if HepG2 are apoptotic

defective cells the outcome of autophagy inhibition would be necrosis or necroptosis [Degenhardt *et al.*, 2006; Farkas *et al.*, 2011]. In our case, it may be happening a possible energy fall together with autophagy inhibition and lysosomal dysfunction that will lead to some kind of lysosomal-related cell death, which needs to be explored in further studies.

In conclusion, we have found that OA, but not its isomer UA, are able to induce cell death in HCC HepG2 cells under starvation, probably due to the disruption of cholesterol homeostasis and function in membranes and in cholesterol-rich lipid domains. OA can be viewed in the future as a specific drug for cancer treatment in particular cell physiological conditions, such as under metabolic stress that happens in central areas of tumors, or for sensitizing resistant cancer cells to death during chemotherapy.

Final Remarks and Future Perspectives

1. Final remarks and conclusions

Autophagy is induced in response to both anticancer therapy and in central areas of the tumor as a survival mechanism during these periods of metabolic stress. Inhibition of cellular self-digestion under starvation leads to cell death and is considered a novel approach for the treatment of cancer. In this study, we investigate the effect of two isomers triterpenoids, UA and OA, as potential chemotherapeutic agents of hepatocellular carcinoma cells. Our findings demonstrated that HepG2 cells are more susceptible to death induced by OA than by UA both in normal growth and starvation conditions. In addition, OA sensitized dramatically HepG2 cells to cell death under starvation conditions, a mechanism that seems to be maintained also in other cancer cells, such as in colorectal HCT116 cells. Comparing the effects of triterpenoids with that of Rap in cell death and expression of several autophagic markers indicate that these compounds have opposing effects in modulation of autophagy. Both OA and UA induce remarkable accumulation of acidic vacuoles and the cellular levels of LC3 and p62, suggesting, therefore, that these compounds act as autophagy inhibitors at the later steps of the process. However, only OA, but not UA, caused significant cell death under starvation conditions, which indicates that autophagy inhibition is probably not the cause of toxicity of OA under starvation. That was confirmed by the use of pharmacological inhibitors of autophagosome formation, which also did not prevent OA-induced cell death. Thus, autophagy seems to be not directly involved in cell death induced by OA, but together with other cellular processes may contribute to the fate of death of HepG2 cells under starvation conditions.

In following studies, we also have shown that ROS and JNK signalling seem to not be involved in OA-induced cell death. Also, cell death induced by OA under starvation conditions seems to not be apoptotic related. Considering the structural similarity of cholesterol with triterpenoids and that cholesterol is an essential component of cellular membranes and control important biological functions, we investigated whether OA could be promoting cell death by changing cholesterol homeostasis. Removing cholesterol from membranes we observed that OA-induced cell death was almost entirely reverted. In addition, OA, but not UA, was also able to induce bigger lysosome structures with increased LAMP-2 immunostaining. Based on these results and that of recently published in the literature, we suspect that OA is interfering with cholesterol levels in lipid rafts decreasing their structural organization and fluidity. This needs to

be confirmed in further studies, but that would explain the different cellular effects between OA and UA. The methyl group of UA at C19 in the E-ring of the pentacyclic backbone makes that structure more rigid as compared with OA, which could influence the location of the pentacyclic moiety within the lipid bilayer and its effect on the lipid raft membrane domains.

The specific effect of OA on viability of cells under starvation conditions may, therefore, result from the ability of this triterpenoid to reduce the structural organization of cholesterol-rich domains that, in cells under metabolic stress, will have profound implications in several important cellular structures and activities, such as lysosomes, autophagy, cell signaling, endocytosis and mitochondria activity.

2. Future perspectives

With this study, a novel activity was found for OA that are specific for starvation conditions and with possible applications in cancer treatment. However, many questions remain to be answered, in particular the molecular mechanisms of induction of cell death by OA in HepG2 cells under starvation conditions. Therefore, in following studies experiments should be conducted in order to depict this interesting effect of OA. These cells, although originally isolated from an hepatocarcinoma, are metabolically competent and resemble relatively well the biological activities of normal hepatocytes. UA are usually more toxic than OA, but in these cells the contrary was found. Thus, which are the particularities of HepG2 cells that make them more susceptible to OA as compared with other cells? Would it be that because of their high metabolic activity these cells depend more on functional membrane cholesterol-rich domains, which can be disrupted by OA? Or due to interferences in the cellular lipid metabolism?

Related with the molecular mechanism of induction of cell death by OA much more needs to be investigated. Based in our experiments, OA-induced cell death under starvation conditions seems to not depend on ROS, JNK activity, autophagy inhibition and are not through apoptosis. We should explore in future experiments the possible involvement of programmed necrosis (necroptosis) and/or the lysosomal-type of cell death. In addition, disruption of calcium homeostasis can also be involved and also dependent on pertubation of cholesterol content in membranes. To this regard, the assessment of cholesterol content in membranes and lipid rafts

fractions should be analysed in cells treated or not with OA under starvation conditions. Expression of proteins that localized in microdomains rich in cholesterol, such as AKT and SNAREs should be measured in lipid rafts fractions by western blotting. Other lipid rafts-related proteins, such as caveolin 1 (CAV-1), should also be assessed by immunofluorescence. In addition, the effect of addition of exogenous cholesterol to cells, instead of the removal as done here, on the OA-induced cell death should also be evaluated. The experiments done to assess the mitochondrial membrane potential with TMRM should be repeated, or done with other probe like JC-1, to understand if mitochondria are entering in an energetic failure, which may contribute to cell death. The expression of pAMPK by western blotting should also be evaluated to check the possible energy fall (low ATP values).

The description and understanding of the cellular and molecular mechanisms of action of OA in these particular conditions of metabolic stress will help in the future to the possible use of OA as specific drug for cancer treatment in particular cell physiological conditions.

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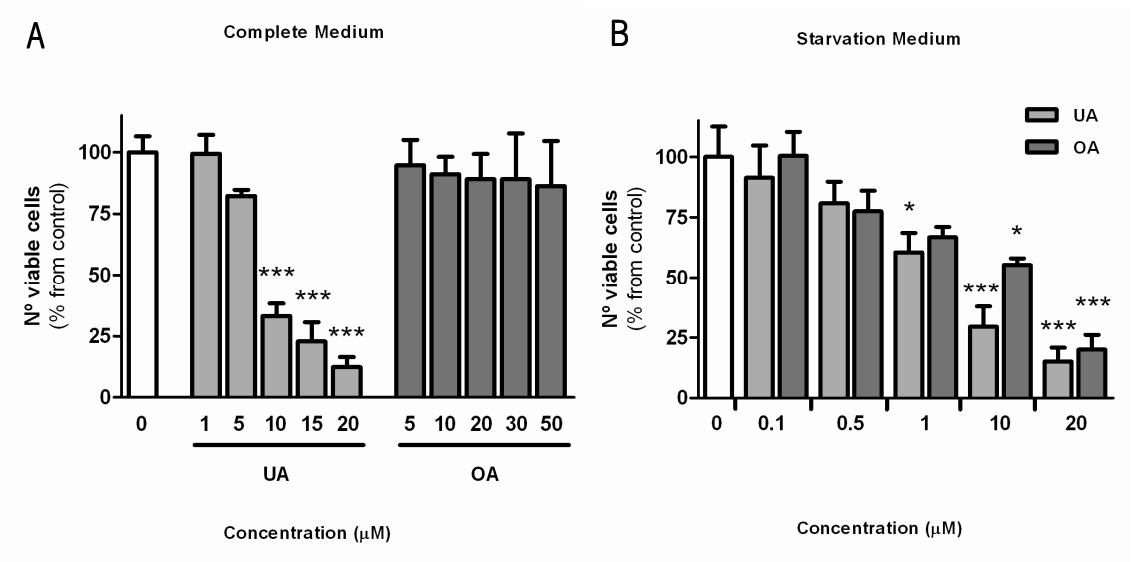
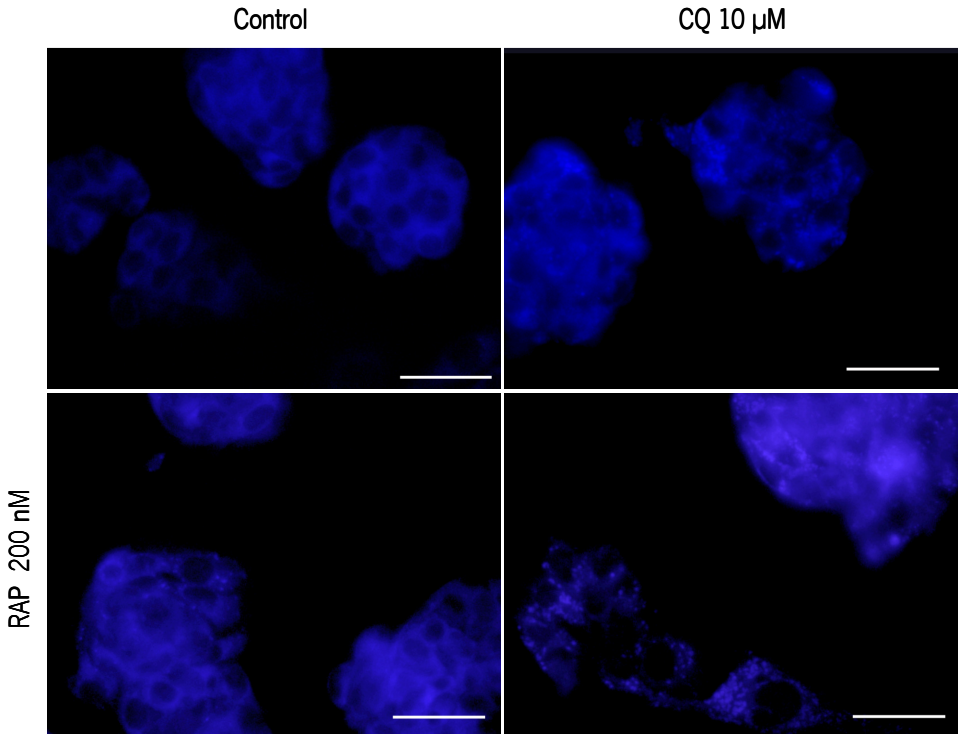
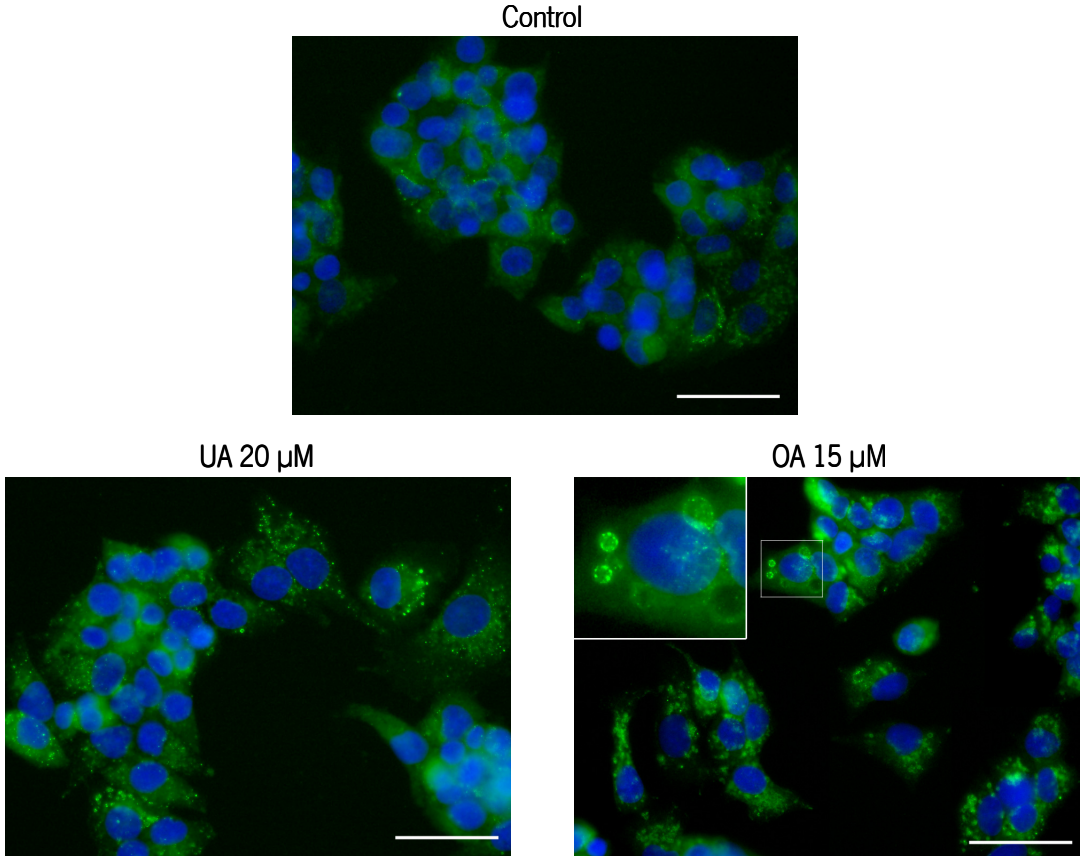
Supplementary Material



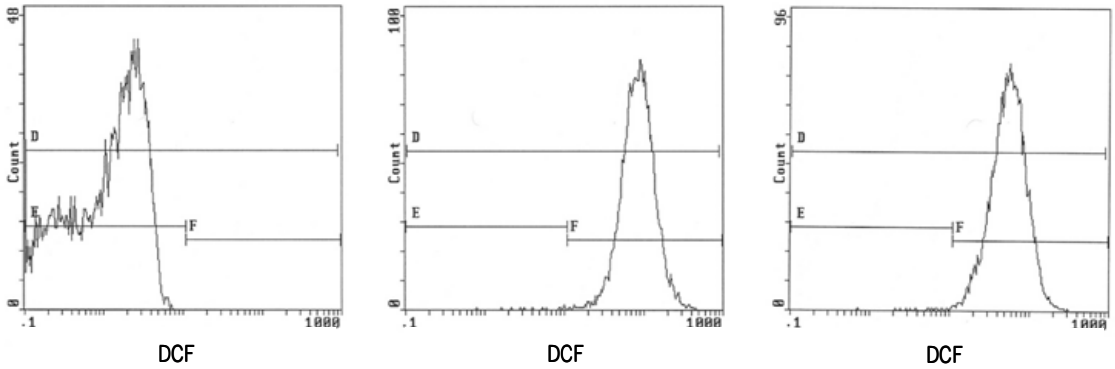
Supplementary Fig. 1. Effect of ursolic acid (UA) and oleanolic acid (OA) in the viability of HCT116 cells, as assessed by MTT reduction assay. Cells were incubated with UA or OA at indicated concentrations for 48 h in complete (A) or starvation (B) medium. Values are mean ± SEM of at least three independent experiments. * $p \leq 0.05$ and *** $p \leq 0.001$ when compared with control by the one-way ANOVA (Newman-Keuls Multiple Comparison Test).



Supplementary Fig. 2. . Effect of rapamycin (Rap) and chloroquine (CQ) in the appearance of cellular acidic vacuoles, as assessed by MDC-staining. HepG2 cells were treated with the indicated compounds for 16 h in complete medium before being stained with 50 μM monodansylcadaverine (MDC) for 10 minutes. Shown are representative images of three independent experiments (bar=50μm).



Supplementary Fig. 3. Effect of ursolic acid (UA) and oleanolic acid (OA) on LAMP-2A levels, as assessed by imunofluorescence. Compounds were incubated 16 h in starvation medium before imunostaining with anti-mouse LAMP-2 antibody (1:200). Images are representative of two independent experiments (bar=50µm).



Supplementary Fig. 4. Effect of oleanolic acid (OA) in the levels of reactive oxygen species (ROS). HepG2 cells were incubated with OA 10 µM (control received vehicle only – DMSO) for 16 h in starvation medium and stained in the end with 10 µM DCF for 30 min, at 37°C, and ROS levels mesuared by flow cytometry. ROS levels expressed as the ratio between fluorescence intensity of each sample and autofluorescence are: control – 86.6; OA – 56.1. Shown are representative distributions of cells labelled with DCF from two independent experiments.