



Universidade do Minho

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Ursolic and Oleanolic acid effects on cellular cholesterol and impact on signaling pathways of cell death and proliferation

Dissertação de Mestrado

Mestrado em Bioquímica Aplicada – Biomedicina

Trabalho efectuado sob a orientação do
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Título da dissertação:

Ursolic and Oleanolic acid effects on cellular cholesterol and impact on signaling pathways of cell death and proliferation

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Ano de conclusão: 2015

Mestrado em Bioquímica Aplicada, ramo de Biomedicina

DE ACORDO COM A LEGISLAÇÃO EM VIGOR, NÃO É PERMITIDA A REPRODUÇÃO DE QUALQUER PARTE DESTA TESE/TRABALHO

Universidade do Minho, 30 de Janeiro de 2015

Assinatura: _____

AGRADECIMENTOS

“No meio da dificuldade encontra-se a oportunidade”

Albert Einstein

Em primeiro lugar gostaria de agradecer à Prof^a. Doutora Cristina Pereira-Wilson, orientadora deste trabalho, pela oportunidade de trabalhar neste projeto e integrar a sua equipa de investigação, pela orientação, pelo apoio e tempo disponibilizado e acima de tudo por partilhar o seu conhecimento científico comigo.

Gostaria de agradecer também ao Dr. Cristóvão Lima, meu coorientador, pelo apoio e incentivo, e pelas sugestões construtivas ao longo deste trabalho.

Um especial agradecimento á Dalila, Carla, Cristina e Ana Rita por todo o tempo e paciência que tiveram comigo, pelos ensinamentos, e principalmente pelas conversas, simpatia, amizade, e momentos de descontração.

Aos restantes colegas de laboratório, pelo bom ambiente de trabalho e boa disposição, pelo suporte e pelo espírito de equipa demonstrado.

Aos meus pais e irmã um agradecimento muito especial pelo apoio incondicional, por me darem a oportunidade de estar aqui e por nunca duvidarem das minhas capacidades. Graças a eles e ao excelente exemplo de luta e garra que me mostraram ao longo da minha vida sou a pessoa que sou hoje. Foi com eles que aprendi que a vida não é fácil, que as coisas não nos aparecem de mão beijada e que se lutarmos conseguimos ultrapassar todas as nossas dificuldades! Obrigada!

Por fim mas não menos importante, um sincero obrigado ao meu marido, Hernani, pelo apoio incondicional que me deste durante este longo percurso. Por nunca me teres deixado desistir nos momentos que mais me fui abaixo e por teres sempre um sorriso nos lábios e um abraço apertado quando mais precisei. Obrigado por teres depositado toda a confiança em mim e por seres o meu porto seguro! Obrigado por tudo! Amo-te!

ABSTRACT

Cancer is a disease characterized by the uncontrolled growth of abnormal cells that beyond the limits of a cell division in normal conditions. Colorectal cancer (CRC) is the third most common tumors and is a major cause of cancer related death worldwide. Cholesterol metabolism has been established as possible source of therapeutic targets in cancer progression, because the cholesterol synthesis pathway provides farnesyl pyrophosphates groups essential to prenylation of proteins involved in cell proliferation and cancer growth. For this reason, targeting cholesterol synthesis with statins (HMG CoA reductase inhibitors) has been explored but with limited results in the clinic due to the toxicity of high dose statin treatment. Additionally, cholesterol is involved in proliferative cell signalling through receptors activation upstream of RAS and PI3K because it is a major constituent of lipid rafts. Thus, the aim of this project is to test if natural compounds with structure similar to cholesterol, such ursolic acid (UA) and oleanolic acid (OA) can alter the lipid composition and influence signalling pathways in order to inhibit the proliferative activity of carcinoma colorectal cells (HCT116). For this we evaluated the effect of the triterpenoids in the amount of cellular cholesterol by cholesterol quantification assay and found that the UA reduces and OA increase cholesterol cellular levels. These effects may be affect AKT signaling pathway induced by insulin because our results indicate that UA, but not OA, causes a decrease in p-AKT. Furthermore, by the method of nuclear condensation we evaluated apoptosis, and both the UA and OA increased tumor necrosis factor alpha (TNF- α) induced apoptosis. These results help to take another step in understanding the mechanisms of action of these natural compounds, helping control the progression of cancer.

RESUMO

O cancro é uma patologia caracterizada pelo crescimento descontrolado de células anormais, que ultrapassam os limites de uma divisão celular em condições normais. O cancro colorrectal representa mundialmente o terceiro cancro mais comum e a maior causa de morte relatada por cancro. O metabolismo do colesterol tem sido proposto como possível alvo terapêutico na progressão desta neoplasia, porque a via do mevalonato responsável pela síntese deste composto proporciona grupos farnesil pirofosfatos essenciais à prenilação de proteínas que ativam vias relacionadas com a proliferação e o crescimento celular. Por esta razão, o combate da síntese de colesterol com estatinas (inibidores da HMG CoA Reductase) tem sido explorado mas com resultados limitados na prática clínica devido a toxicidade causada pelo tratamento com elevadas doses de estatinas. Adicionalmente verifica-se que o colesterol está também envolvido na proliferação através da ação de recetores de membrana que ativam a via da RAS e do PI3K, por ser o maior constituinte dos *lipid rafts*. Assim este projeto visa testar se compostos naturais com estrutura semelhante à do colesterol, como o ácido ursólico (AU) e ácido oleanólico (AO), conseguem alterar a composição lipídica e influenciar vias de sinalização de maneira a inibir a atividade proliferativa em células do carcinoma colorrectal (HCT116). Para tal avaliamos o efeito dos triterpenoides na quantidade de colesterol celular através do método de quantificação de colesterol, e verificamos que o AU diminui os níveis celulares e o AO provoca o seu aumento. Estes efeitos podem afetar a via de sinalização AKT induzida pela insulina, porque os nossos resultados indicam que o AU, mas não o AO, provoca uma diminuição da proteína p-AKT. Por outro lado, pelo método de condensação nuclear avaliamos a apoptose, e tanto o AU como AO aumentam a apoptose induzida pelo Fator tumoral de necrose alfa (TNF- α). Estes resultados ajudam a dar mais um passo na compreensão dos mecanismos de ação destes compostos naturais, ajudando no controlo da progressão do cancro.

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ABBREVIATIONS LIST

- AKT – Protein kinase B
- APC – Adenomatous polyposis coli
- BSA – Bovine serum albumin
- Cav-1 – Caveolin-1
- Chol – Cholesterol
- COPII – Coatamer protein complex II
- CRC – Colorectal cancer
- DISC – Death-inducing signaling complex
- DMAPP – Dimethylallyl pyrophosphate
- DMSO – Dimethyl sulfoxide
- DNA – Deoxyribonucleic acid
- EDTA – Ethylenediamine tetraacetic acid
- EGF – Epidermal growth factor
- EGFR – Epidermal growth factor receptor
- ER – Endoplasmic reticulum
- ERK – Extracellular-signal-regulated kinase
- FADD – Fas-associated protein with death domain
- FAP – Familial adenomatous polyposis
- FAS – Fatty acid synthase
- FBS – Fetal bovine serum
- GDP – Guanosine diphosphate
- GTP – Guanosine triphosphate
- HMG CoA – 3-hydroxy-3-methylglutaryl CoA
- HNPCC – Hereditary nonpolyposis colorectal cancer

Icmt – Isoprenylcysteine carboxyl methyltransferase

Insig – Insulin-induced gene product

IPP – Isopentanyl pyrophosphate

IR – Insulin receptor

LDL – Low density lipoproteins

MAPK – Mitogen-activated protein kinase

MMR – Mismatch repair

MTT – 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

M β CD – Methyl- β -cyclodextrin

Na₃VO₄ – Sodium orthovanadate

NaF – Sodium fluoride

NF- κ B – Nuclear factor kappa-light-chain-enhancer of activated B cells

OA – Oleanolic acid

PBS – Phosphate-buffered saline

PDGF – Platelet derived growth factor

PFA – Paraformaldehyde

PI3K – Phosphatidylinositol 3 kinase

PMSF – Phenylmethylsulfonyl fluoride

PVDF – Polyvinylidene difluoride

Rcel – Ras converting enzyme

Rip – Regulated intramembranous proteolysis

ROS – Reactive oxygen species

RPMI – Roswell park memorial institute médium

RTK – Receptor tyrosine kinase

S1P – Site-1-protease

S2P – Site-2-protease

SCAP – SREBP cleavage-activating protein

Simv – Simvastatin

SRE – Sterol regulatory element

SREBP – Sterol regulatory element-binding protein

SSD – Sterol sensing domain

TCC – Total cellular cholesterol

TNFR – Tumor necrosis factor receptor

TNF- α – Tumor necrosis factor alpha

TRADD – Tumor necrosis factor receptor 1-associated death domain protein

TRAIL – TNF-related apoptosis-inducing ligand

UA – Ursolic acid

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1. INTRODUCTION

1.1-Cancer

Cancer is a disease that affects millions of people worldwide. According to the World Health Organization, in 2012 there were 8.2 million deaths caused by cancer, while there were 32.6 million people living with cancer. Also 14.1 million new cases of cancer were estimated worldwide, 7.4 million of these in men and 6.7 million in women. This number is expected to increase to 24 million by 2035 (Worldwide cancer statistics|WCRF, 2010),(GLOBOCAN 2012).

Cancer is a disease characterized by the uncontrolled growth of abnormal cells, which break the rules of cell division. Normal cells are subject to a set of signals that indicate if they have to divide, differentiate or die. Cancer cells acquire autonomy with respect to these signals, resulting in uncontrolled growth and proliferation. The disease involves modifications, and/or mutations in the genome of the cell, which yields abnormal proteins responsible for cell imbalance, resulting in a slow progression and accumulation of abnormal cell masses, the tumors (Hejmadi, 2010).

Generally, multiple mutations in key genes are required to convert a normal cell into a cancer cell. This is the principle of the so-called “Multi-Hit Model” of Cancer. In this model, cell suffers a mutation that would give it a tenuous growth advantage. The progeny cells could then suffer a second mutation that could lead uncontrollably growth and form a small benign tumor. If a third mutation occurs in these cells could allow it to outgrow the others and overcome constraints imposed by the tumor microenvironment, its descendants may form a mass of cells, each one with three mutations. Finally, an additional mutation in one of these cells could permit its progeny to invade the blood system and create progeny colonies at other sites, forming metastasis (Lodish, 2013).

The specific characteristics that distinguish cancer cells from normal cells are so called “hallmarks of cancer”. Originally six: self-sufficiency in growth signals; insensitivity to antigrowth signals; capacity to evading apoptosis; limitless replicative potential; sustained angiogenesis; tissue invasion and metastasis (Hanahan and Weinberg, 2000). In 2011, *Hanahan and Weinberg* added two additional hallmarks: the capacity to modify cellular metabolism to support neoplastic proliferation and capacity to evade immunological destruction. The authors added also two new characteristics: genome instability and

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mutation; and promotion of tumor inflammation (figure 1.1) (Hanahan and Weinberg, 2011).

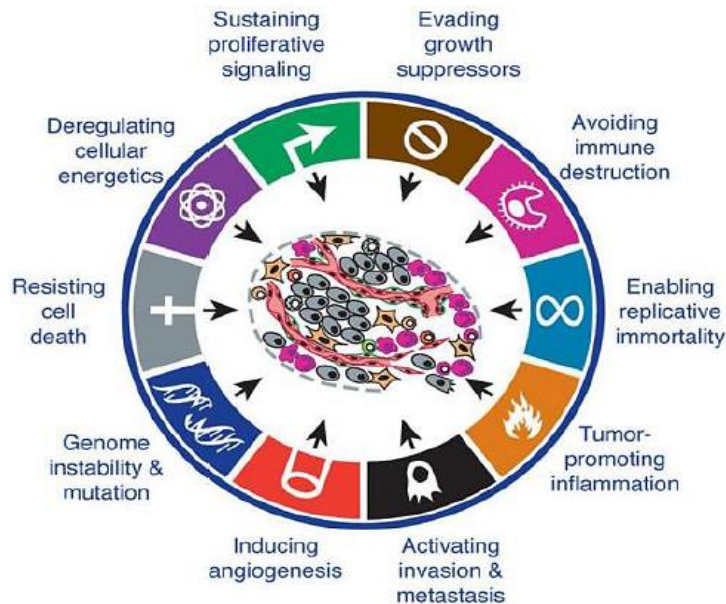


Figure 1.1 – The hallmarks of cancer. Adapted from (Hanahan and Weinberg, 2011)

The origin of cancer cells is associated with defects in cell cycle. Changes in the functioning of cell cycle regulatory genes are responsible for the development of cancer. Two classes of genes, oncogenes and tumor suppressor genes are related to cell cycle regulation. The oncogenes are responsible for the production of proteins that act in the stimulation of the cell cycle, while tumor suppressor genes are responsible for the production of proteins that act by inhibiting the cell cycle (Osborne et al., 2004).

1.2-Colorectal cancer

Colorectal cancer (CRC) is one of the most common cancers and is a major cause of cancer death related worldwide. According *Globocan 2012*, CRC was the third most common cancer with nearly 1.4 million (9.7%) new cases in 2012. This type of cancer is the third most common in men (746,000 cases, 10.0% of the total) and the second in women (614,000 cases, 9.2% of the total) worldwide. Most cases of CRC (55%) occur in more developed

regions of the world. In less developed regions the incidence is lower with more deaths, this reflect a low survival in these regions (GLOBOCAN 2012). After 5 years of the diagnosis, survival rates are 90% for localized disease, 65% for regional disease and 10% for metastatic CRC (Deschoolmeester et al., 2010). Its mortality rate can be reduced if the diagnosis is performed in the early stages of the disease, so it is necessary that prevention screenings are made, including simple tests to the stool or colonoscopy, recommended from 50 years in healthy individuals.

The development of CRC result from the progressive transformation of normal epithelium to adenocarcinoma and may have two origins: genetic or environmental (Kheirelseid et al., 2013). At genetic level it is known that individuals who suffer from Crohn's disease or chronic colitis have a higher probability for developing CRC as well as individuals who suffer from inflammation of the intestines, which corresponds to 1-2% of CRC cases (Stolfi et al., 2012). It is also known that 15% of CRC are originated from familial. The hereditary CRC can be divided depending on whether there are or not colonic polyps: familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). The FAP is characterized by the development of many adenomas in the colon and rectum during the second decade of life and is caused by a germline mutation in the adenomatous polyposis coli (APC) gene (figure 1.2) (Fearon, 2011; Half et al., 2009).

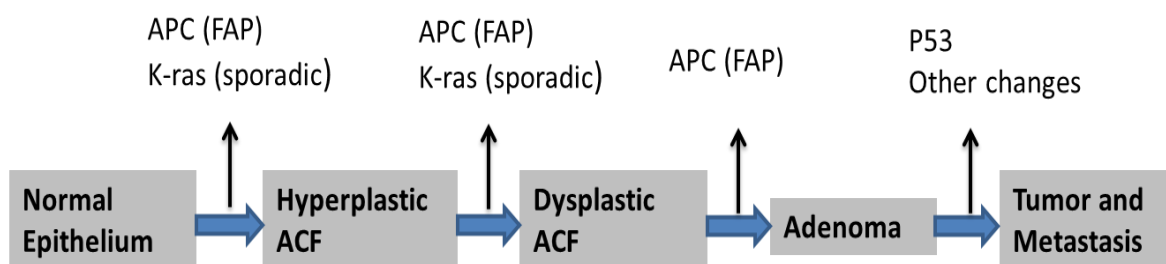


Figure 1.2 - Schematic representation of some key genetic alterations during colorectal cancer (CRC) carcinogenesis. Mutations in APC gene occur in the initiation process of CRC carcinogenesis, while mutations in the *KRAS* and *TP53* genes appear during the adenoma evolution.

APC is a tumor suppressor gene situated on the extensive arm of chromosome 5 in band q21. The region that encodes is divided into 15 exons and codifies a large protein with 309kD. The APC protein has many domains that mediate oligomerization and the binding to

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a diversity of intracellular proteins, which have an significant role in initiation of transduction, transcriptional activation and cell adhesion (Half et al., 2009).

The HNPCC, also known as Lynch syndrome, is the most common form of hereditary CRC that is initiated by germline mutations in mismatch repair (MMR) system associated with DNA-methylation changes and somatic inactivation of the wild-type parental allele. The loss of functional MMR system in HNPCC patients occurs by mutations or silencing of MMR genes that leads to genomic instability and thus to the development of CRC (Markowitz and Bertagnolli, 2009; Kheiriseid et al., 2013)

However the majority of cases occur spontaneously in individuals with no family history or genetic predisposition. In these cases there is an influence of environmental factors. Age is a considerable risk factor, because even though the cancer occurs at all ages it is more common (60% of cases) among elderly people (over 65), which occurs due to hormonal and immune changes (Hejmadi, 2010); the absence of exercise associated with obesity rate, is also considered a potential carcinogen. Other factors to consider are the consumption of alcohol and tobacco because when consumed in excess and combined can cause irreparable damage in DNA. Worldwide, 3.6% of all cancers derive from chronic alcohol consumption. The alcohol acts as a solvent allowing other substances, notably from smoke tobacco, to penetrate cell membranes. In addition, the alcohol leads to an increase in blood acetaldehyde produced by alcohol dehydrogenase and cytochrome P4502E1 (Seitz and Stickel, 2010), leading to a slower metabolism by the enzyme acetaldehyde dehydrogenase (Fedirko et al., 2011). These compound (acetaldehyde) is highly toxic and carcinogenic when in circulation because it interferes with the synthesis and repair of DNA causing mutations. It is also believed that activation of cytochrome P4502E1 leads to formation of reactive oxygen species (ROS), and activation of various pro carcinogens present in the diet and in tobacco smoke, which facilitates the carcinogenic process (Seitz and Stickel, 2010).

A contributing factor to the development of the CRC is the diet. Individuals who have a fat diet rich in animal protein and high consumption of meat, especially red meat, have a

higher risk of developing the disease. The fatty acid composition of meat, heterocyclic amines formed during food preparation, the nitrosamines present in processed products and iron from the meat have been suggested as potential factors that may explain the increased risk of CRC (Järvinen and Knekt, 2001). Moreover diets rich in animal fat leads to increased levels of triglycerides and cholesterol which may also be a risk factor. Several epidemiological studies have shown that a diet rich in fruits, vegetables and whole grains with high amounts of bioactive phytochemicals are associated with the decrease risk of development chronic diseases and the carcinogenesis process. Therefore, these foods have strong antioxidant and anti-proliferative effects helping in the body healthy maintenance (Liu, 2004; Pan et al., 2008; Lima et al., 2006).

1.3-Lipid Rafts

The lipid rafts are defined as low density microdomains and resistant to detergents within the lipid bilayer of the plasma membrane with a relatively small size (50 nm). They are constituted by transmembrane proteins, GPI-anchored proteins and lipids, namely cholesterol and sphingolipids (figure1.3) (Staubach and Hanisch, 2011). This composition has impact on their characteristics, namely fluidity and stability, and allows building platforms rich in diverse signaling molecules such as EGFR, Src, AKT, heat shock protein 90 (Hsp90), and Fas (CD95) (Lee et al., 2014; Sharma et al., 2002). Lipid rafts have been associated with many biological functions of sorting and signaling to regulate cell survival, proliferation, migration, adhesion and in apoptotic pathway (Onodera et al., 2013; Lee et al., 2014). The heterogenic and dynamics composition of these lipid rafts contributes to the

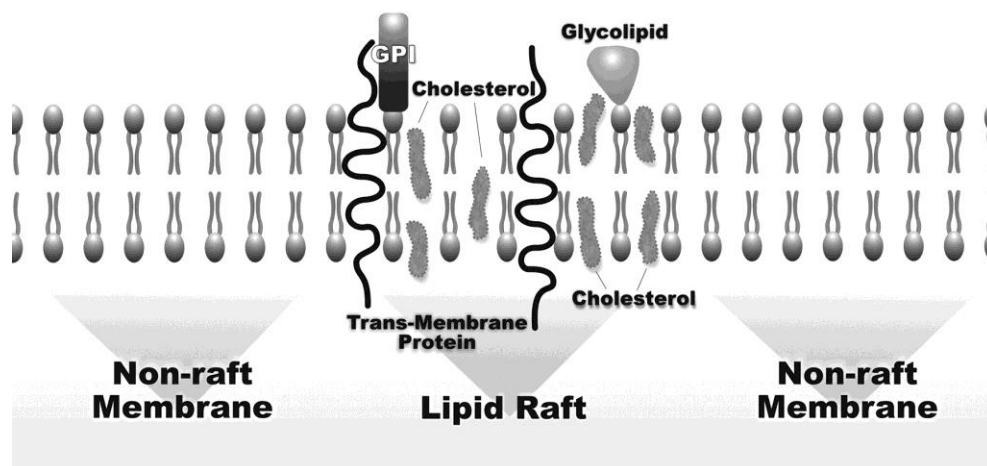


Figure 1.3 - Structure and composition of membrane lipid rafts. From (Chichlowski and Hale, 2008)

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large number of signals capable of being transmitted from the external cell membrane to cytoplasmic organelles or even to the nucleus (Mollinedo and Gajate, 2006).

The lipid rafts can be divided into two subclasses: the planar lipid rafts, which do not correspond to invaginate microdomains and which possess morphological flaws; and the caveolar rafts, a subclass specialized in tubular invagination of the plasma membrane with specific proteins, caveolins, including caveolin-1 (Cav-1). This is a classical protein hairpin that plays a key role in processes of endocytosis and transport. Cav-1 also controls signal transduction events and serve as a scaffolding protein in the organization of signaling molecules. Cav-1 serves as an adapter that initiates the signaling pathway-specific RAS-ERK and can block other routes involved in the apoptotic process (Staubach and Hanisch, 2011). Moreover, act as stabilizer of rafts on the cell surface by recruiting receptors or sequestering cholesterol to these regions of the membrane (Lajoie and Nabi, 2007).

The increase of total cholesterol levels in circulation automatically leads to an increase of its concentration in lipid rafts (Mollinedo and Gajate, 2014). Thus, a progressive increase in cholesterol content in the membrane contributes to the expansion of lipid rafts and oncogenic pathways progression (Patra, 2008). Cholesterol is the most abundant lipid component of these raft domains and its concentration levels are essential for the formation, stabilization and organization of these structures. This sterol is a molecule that has more affinity for sphingolipids than unsaturated phospholipid. Therefore, the cholesterol acts as a barrier of hydrocarbon chains of sphingolipids allowing to maintain the raft attached and functioning as “dynamics glue”. Without cholesterol the membranes become fluid and permeable to some molecules (Simons and Ehehalt, 2002).

Thus cholesterol is crucial to maintain cellular homeostasis and provide platforms capable of activating pro and anti - apoptotic or proliferative pathways of signaling (George and Wu, 2012; Li et al., 2006). Recent studies show that changes in cholesterol levels of these rafts can directly induce the activation of multiple pathways, activation or deactivation of kinases, activation of apoptotic membrane receptors and increased levels of intracellular calcium. Some of these membrane proteins can be activated in these microdomains in the absence of their ligands. This type of activation of them occurs by aggregation of receptor molecules in lipid rafts. This occurs due to changes in lipid and

protein contents of these domains, which consequently results in the formation of signaling platforms (George and Wu, 2012).

The observation that many receptors after ligand stimulation are able to move inside or outside lipid rafts suggests that the association of these compartments is not continuous and is subject to regulation. The association of receptors with rafts is an equilibrium process that can be moved to one side or other based on exterior conditions (Pike, 2005).

1.3.1- Apoptosis and lipid rafts dependent membrane receptors

Lower rate of death cells than of proliferation is one of the essential factors for development of cancer, and consequently the capacity for the cells resist to apoptotic stimuli is a hallmark of cancer. Lipid rafts have been recently involved in the triggering of death receptor-mediated apoptosis.

Apoptosis or programmed cell death is a cellular mechanism that allows the cell to defend and protect from neoplastic development by eliminating genetically damaged cells or excess cells. Apoptosis is therefore responsible for maintaining cellular homeostasis and it is characterized by marked changes in cellular morphology, including plasma membrane blebbing, DNA fragmentation, chromatin condensation, nuclear breakdown and the presence of membrane associated apoptotic bodies (Patra, 2008).

This programmed cell death in mammalian cells is regulated by two major signaling pathways: the intrinsic pathway which is activated by intracellular signals from the mitochondria and the extrinsic pathway which is activated by the binding of apoptotic ligands to death receptors on the cell surface (Gajate et al., 2009; Fouqué et al., 2014). The intrinsic pathway is activated when there is accumulation of DNA damage, viral infections or by dysregulation of mitochondrial function. These stimuli lead to release of cytochrome c into the cytoplasm by mitochondria, where cytochrome c binds the adapter Apaf-1 and pro-caspase 9 to form the apoptosome. This complex leads to the activation of caspase 9 and hence the pathway of caspases (caspase -3, -6, and -7) that allows cell death. On the other hand the extrinsic pathway of apoptosis occurs when there is activation of death receptors of the membrane. Membrane death receptors belong to the tumor necrosis factor (TNF) superfamily, which includes the Fas/CD95, the Tumor necrosis factor receptor (TNFR), and

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the TNF-related apoptosis-inducing ligand (TRAIL) receptors (Wu, 2009). Death receptors consists of more than 20 proteins with a broad variety of biological functions that have two regions of high homology: extracellular cysteine-rich domains and a cytoplasmic domain called “death domain” with 80 amino acids, which have an important role in transmitting the death signal from the cell surface to intracellular signaling pathways (Mollinedo and Gajate, 2006). When activated, these receptors recruit adaptor proteins such as Fas-associated protein with a death domain (FADD) or tumor necrosis factor receptor 1-associated death domain protein (TRADD) for the membrane microdomains and procaspase 9 to form the death-inducing signaling complex (DISC). This complex activates caspase 8, resulting in the subsequent activation of caspases -3, -6 and -7, or activates the intrinsic pathway through caspase-8-mediated Bid cleavage, which activates the release of cytochrome c from mitochondria via interaction with Bax and Bak and indirectly activating caspases-3, -6, -7 and inducing the apoptosis (Wu, 2009; Gajate et al., 2009).

The redistribution of tumor necrosis factor family receptors into and out of lipid rafts may dynamically regulate the competence and outcomes of signaling by these receptors. The integrity of membrane rafts is required for the initiation of apoptosis, because these domains provide a signaling platform for the activation of pro-apoptotic membrane receptor molecules, and this occurs via receptor oligomerization by agents that promote the integrity of the membrane rafts in the absence of receptor ligand molecules (Hryniewicz-Jankowska et al., 2014). An example of this phenomenon is Fas (CD95) receptor, a member of the family of TNF receptors, which converts apoptotic signals in the presence of its ligand FasL and plays a crucial role in apoptosis. However, this receptor can be also activated in the absence of its ligand (Mollinedo and Gajate, 2006). FAS-mediated apoptosis involves the translocation of Fas into the lipid rafts. This recruitment of Fas and its downstream effectors into membrane rafts may provide a mechanism for amplifying Fas signaling by reorganizing membrane microdomains and bringing molecules together for this domains reducing the space, and facilitating protein-protein interactions among different signaling molecules and pathways. It is well documented that Fas oligomerization in lipid raft microdomains results in successive recruitment of FADD and then procaspase-8, allowing formation of DISC. The activation of this receptor also results in recruitment of additional pro-apoptotic proteins into the lipid raft and/or activation of the JNK apoptotic pathway (George and Wu, 2012).

The disruption of lipid rafts by agents such as methyl- β -cyclodextrin (M β CD) may, therefore, negatively interfere with death receptors mediated apoptosis (figure 1.4).

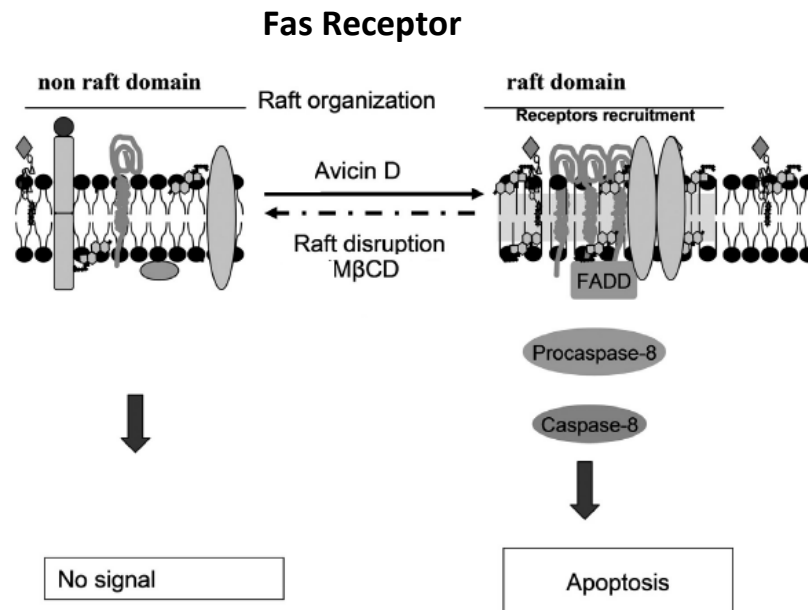


Figure 1.4 – Influence of disruption of membrane rafts domains in Fas signaling pathway. Reproduced from (Hryniewicz-Jankowska et al., 2014).

A study conducted by *Xu et al.* using the triterpene Avicin D, showed that this compound enhances Fas translocation to lipid rafts, where it associates with FADD, inducing apoptotic signals. On the other hand the use of M β CD, which is a cholesterol-depleting compound that extracts cholesterol from the plasma membrane and perturbs lipid raft organization, blocks the clustering of this receptor and its DISC complex to rafts domains thereby inhibiting apoptosis (figure 1.4) (*Xu et al.*, 2009). This example demonstrates the importance of the stability of lipid rafts for the clustering of the death receptor Fas which is crucial to trigger apoptotic signal transduction. Fas-mediated apoptosis can be induced not only by FasL but also by TNF- α (*Du et al.*, 2014). TNF- α is a major pro-inflammatory cytokine, synthesized as a 26 kDa transmembrane type II protein of 233 amino acids, involved in inflammation and initiation of the acute phase reaction by promoting survival/inflammatory signaling or inducing cell death (*Fouqué et al.* 2014). TNF binds to their membrane receptor TNFR, another death-inducing receptor that belongs to the tumor necrosis factor superfamily, which mediates biological fates from inflammation to apoptosis and other forms of programmed cell death. The binding of TNF- α to its receptors initiates a complex array of signaling events, mainly through the activation of the MAPK stress signaling cascade

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and of the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) transcription factor.

After binding of TNF- α to TNFR, this is moved to lipid rafts where there is the formation of Complex I, constituted by RIP1, TRAF2, TRADD and cIAP1 proteins, which translates signals that lead to activation of NF- κ B responsible for inflammation. When the activity of NF- κ B is not necessary for the cell the RIP1, TRADD and TRAF2 dissociates from TNFR to recruit FADD and the caspase-8 to form a new complex (Complex II) and initiate cell death. NF- κ B inhibits cell death through increasing the expression of anti-apoptotic genes that directly inhibit caspase-8 activation in Complex II (Legler et al., 2003; Muppidi et al., 2004).

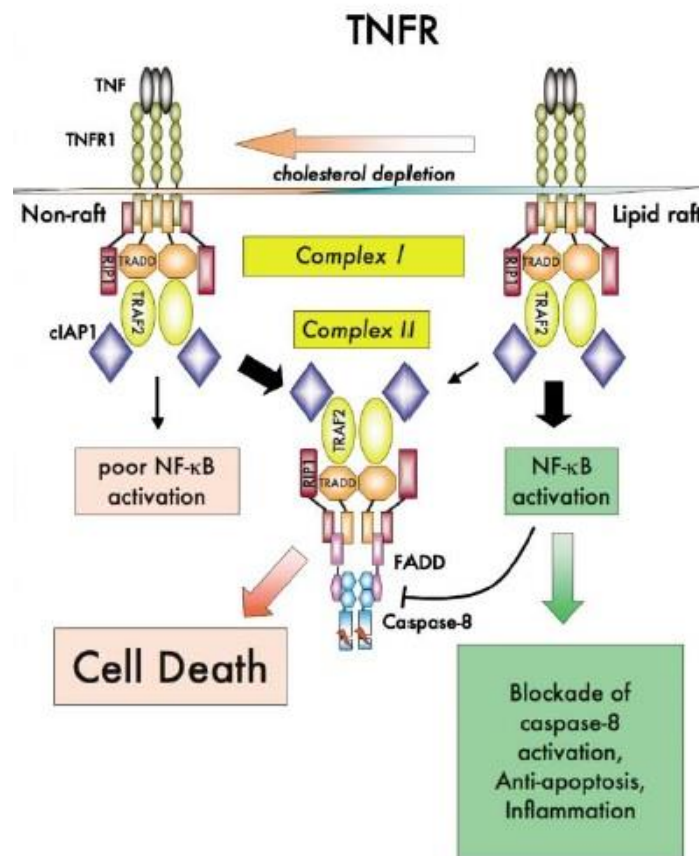


Figure 1.5 – Example how depletion of cholesterol in lipid rafts can modify early events in TNFR family signaling. Reproduced from (Muppidi et al., 2004)

Cholesterol depletion (by M β CD for example), disrupted the structure of lipid rafts and the formation of the complex I occurs outside of lipid rafts and can't signal efficiently to NF- κ B. So in conditions produced by cholesterol depletion, occurs a change of the

inflammation signaling for apoptosis (figure 1.5) because lipid rafts disruption blocks the ubiquitination of the adaptor proteins RIP1 and TRADD in the TNFR signaling complex and prevents NF- κ B induction by complex I. Can also inhibit the synthesis of growth factors resulting in increased sensitivity of the cells to apoptosis mediated by TNF- α (Muppidi et al., 2004).

The both Fas and TNF receptors are examples that disruption of lipid rafts integrity can lead to anti (Fas) or pro-apoptotic (TNFR) effects, depending on the stimulation or apoptotic factors involved. For example, for TNF receptor the M β CD, mevastatin and cholesterol oxidase have all demonstrated pro-apoptotic effects after disruption of lipid rafts in keratinocytes. For Fas receptor the M β CD is able to inhibit Avicin D-induced apoptosis in Jurkat cells (George and Wu, 2012).

1.3.1- Cell proliferation and lipid rafts dependent membrane receptors

In many types of cancer, proliferation and cell growth are the result of an abnormal signaling by growth factor receptors. Lipid rafts also act as signaling platforms for proliferative growth factor mediated signaling. Many receptor tyrosine kinases including the EGF receptor (EGFR), the PDGF receptor and Insulin receptor (IR), have been shown to be localized in these microdomains, and in all cases the alteration on cholesterol content of rafts controlled the signaling by these receptors (Hryniewicz-Jankowska et al., 2014; Pike, 2005).

EGFR is a member of the family of receptor type tyrosine kinases (RTK's), consisting of three domains: an extracellular domain of 620 amino acids that recognizes and binds to the EGF; a single transmembrane lipophilic domain of 24 amino acid penetrating through the membrane alpha helix shaped; and a cytoplasmic domain that include an intrinsic tyrosine kinase domain. The stimulation of the EGFR receptors present in lipid rafts induces a conformational change in the extracellular domain of the receptor, which consequently promotes homo or heterodimerization with other RTK's. This dimerization activates the intrinsic activity of the receptor, resulting in auto phosphorylation of tyrosine residues at the C-terminal (Brand and Wheeler, 2012; Pike, 2005).

The lowering of cholesterol levels in lipid rafts or their disruption by using M β CD stimulates ligand activation and EGFR phosphorylation (Wang and Paller, 2006; Tekpli et al.,

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2013). Moreover several groups have shown that cholesterol depletion causes an increase in the amount of bound EGF, and on the other hand increased cholesterol levels leads to a reduction in binding of EGF to EGFR. This seems to indicate that the receptor location in lipid rafts impairs the ability of EGF binding, because restriction of spatial character. The depletion of cholesterol is still associated with an increase in receptor dimerization induced by EGF, suggesting that cholesterol changes in lipid rafts affect not only receptor binding but also the signaling (Lambert et al., 2009; Pike, 2005; Ringerike et al., 2002). But this effects is still controversial in the literature because a study performed by *Zhuang et al.* showed that caveolin-negative prostate cancer cell line LNCaP treated with an agent that disrupts the rafts suppressed ligand-dependent EGFR phosphorylation in the rafts fraction of the membrane and re-formation of this microdomains with cholesterol restored EGFR/AKT pathway (Zhuang et al., 2002).

The insulin signaling cascade is also proposed to function via lipid rafts. Insulin is a major anabolic hormone controlling critical steps such as cellular glucose uptake and lipid metabolism. Insulin plays an important role in vesicle trafficking, stimulation of protein kinases and phosphatases, promotion of cellular growth and differentiation and activation or repression of transcription. Insulin exerts its effects via binding to the IR (Saltiel and Pessin, 2002). The IR is a heterotetrameric bifunctional complex, consisting of 2 extracellular α subunits that bind insulin and 2 transmembrane β subunits which contain tyrosine kinase

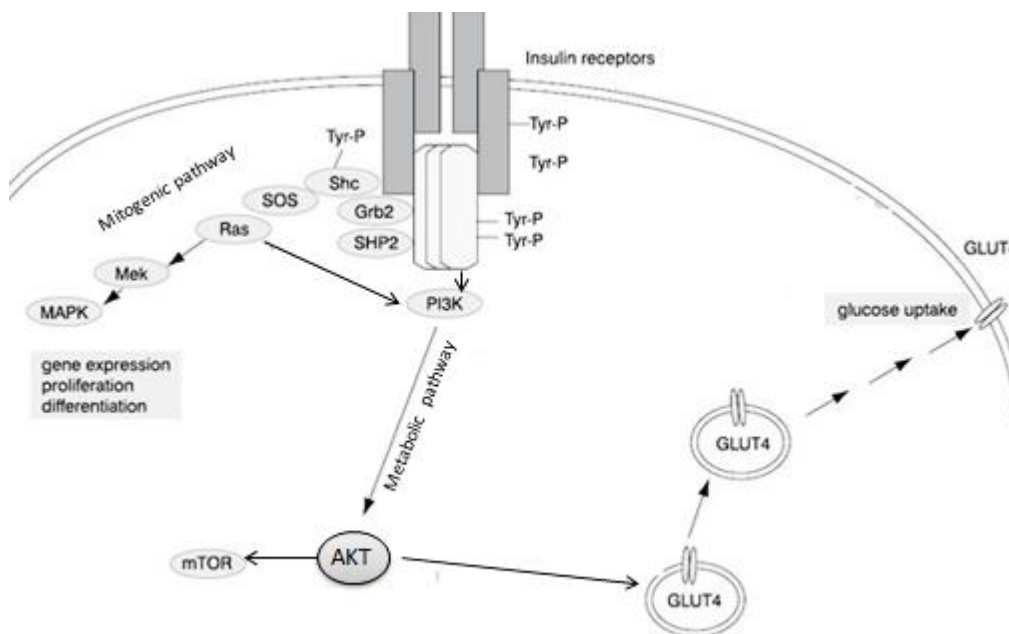


Figure 1.6 – Schematic representation of Insulin receptor pathways: PI3K mediating metabolic responses and Ras mediating mitogenic signaling. Adapted from (Mlinar et al., 2006)

activity. After insulin binding to the α subunit, the phosphorylation of one β domain is induced as a result of the increase catalytic activity of the receptor kinase activity (Chang et al. 2005) that phosphorylates and recruits different substrate adaptors of IRS family causing activation of several signaling pathways. Insulin uses the PI3K/AKT signaling pathway to exert most of their metabolic activities such uptake of glucose. Insulin leads to an increased phosphorylation of AKT but also induces mitogenic signaling transmitted via RAS/MAPK pathway (figure 1.6) (Sánchez-Wandelmer et al., 2009).

In cells that have caveolae the IR are constitutively sequestered in caveolae, but in case of caveolae-negative cells the receptors are recruited into rafts only by the addition of insulin (Pike, 2003). IR directly interacts with caveolin-1 and stimulates their tyrosine phosphorylation (Saltiel and Pessin, 2003). Cholesterol depletion and loss of lipid raft structures in plasma membrane did not reduce the quantity of IR or their affinity for insulin. A study by *Parpal et al.* reported that the interaction with the immediate downstream mediator molecule IRS-1 and metabolic control (such uptake of glucose) is, however, inhibited. As a consequence of cholesterol depletion, the further downstream propagation of the signal to AKT phosphorylation was being blocked, while insulin signaling via the MAPK pathway remains intact (Parpal et al., 2001; Karlsson et al., 2004). When cholesterol levels were then reestablished in these cells, protein phosphorylation mediated by insulin returned to normal, and lipid rafts were again morphologically recognizable (Cohen et al., 2003).

The activation of these RTK's leads to the activation of the signaling pathway of the RAS protein. This protein is one the most studied oncoproteins, because is one component of the cascades RAS-RAF-MAPK and RAS-PI3K-AKT-mTOR. RAS plays an important role in the initiation of cell proliferation. The RAS oncogene is mutated in approximately 25% of human cancers (Hejmadi, 2010), and are noted in 50% of CRC cases (Bos, 1989). This mutation changes the RAS protein into an oncoprotein, characterized by constitutive activation, which in turn triggers an uncontrolled growth, making the cells fail to turn off their proliferative state (Hejmadi 2010). Under normal conditions, the RAS protein is activated by growth factors (Bos, 1989), through binding one molecule of guanosine triphosphate (GTP), thereby increasing affinity for subsequent effector molecules in the cascade. This is later disabled when the hydrolysis of GTP to guanosine diphosphate (GDP) occurs (Brand & Wheeler

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2012). When there is a mutation in RAS the hydrolysis of GTP does not occur and the molecule is constantly active and with sufficient autonomy to induce proliferation independently of receptors signaling and extracellular ligands (Swanson and Hohl, 2006).

RAS activation causes a conformational modification in the RAF kinase, which leads to activation of MAPK nuclear transporter that is responsible for cellular proliferation. RAS also recruits PI3K to the membrane leading to activation of AKT protein, and consequently mTOR, which induces protein synthesis and other metabolic effects, and inhibition of several pro-apoptotic signals (figure 1.7) (Brand and Wheeler, 2012).

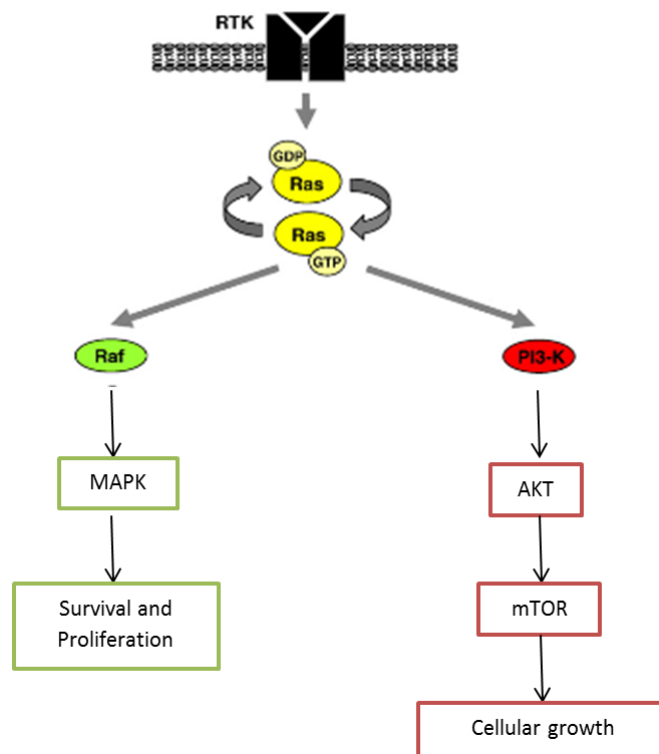


Figure 1.7 – Activation of protein RAS after hydrolysis of GTP to GDP and their signaling pathways. Adapted from (Van der Weyden and Adams, 2007)

Post-translational modifications of RAS, namely prenylation, are important for RAS signaling. RAS protein begins as a hydrophilic globular protein exhibiting a C-terminal region with the sequence - CAAX (C a cysteine, A an aliphatic acid - valine, leucine, isoleucine - and X any amino acid), which corresponds to a mark for post-translational modifications, including farnesylation, proteolysis and methylation (Fehrenbacher et al., 2009; Hancock and Parton, 2005). RAS is synthesized in the cytoplasm, where its CAAX sequence is recognized by farnesyltransferase enzyme that adds a farnesyl group to the terminal

cysteine. Then farnesylated proteins are sent to the ER where they find Ras converting enzyme (Rce1) that remove the AAX amino acids and the isoprenylcysteine carboxyl methyltransferase (Icmt) that subsequently add a methyl group to the carboxyl cysteine (Fehrenbacher et al., 2009). The RAS is ultimately sent to the plasma membrane, especial for rafts domains and may also be regulated by local accumulation of anionic phospholipids (Rocks et al., 2006). The post-translational modification of RAS protein, required for its membrane insertion and subsequent activation depends on the synthesis of lipid molecules by the mevalonate pathway. Therefore RAS signaling depends on cholesterol homeostasis because it depends on prenylation for activating and on lipid rafts composition for assembling its signaling targets.

1.4-Cholesterol biosynthesis

1.4.1-Mevalonate pathway

This pathway, which takes place in the cytoplasm, is a highly conserved metabolic cascade responsible for the synthesis of various products. In the first step of the pathway 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase converts HMG CoA to mevalonate (Thurnher et al., 2013). HMG CoA reductase is the enzyme that catalyzes the limiting step of

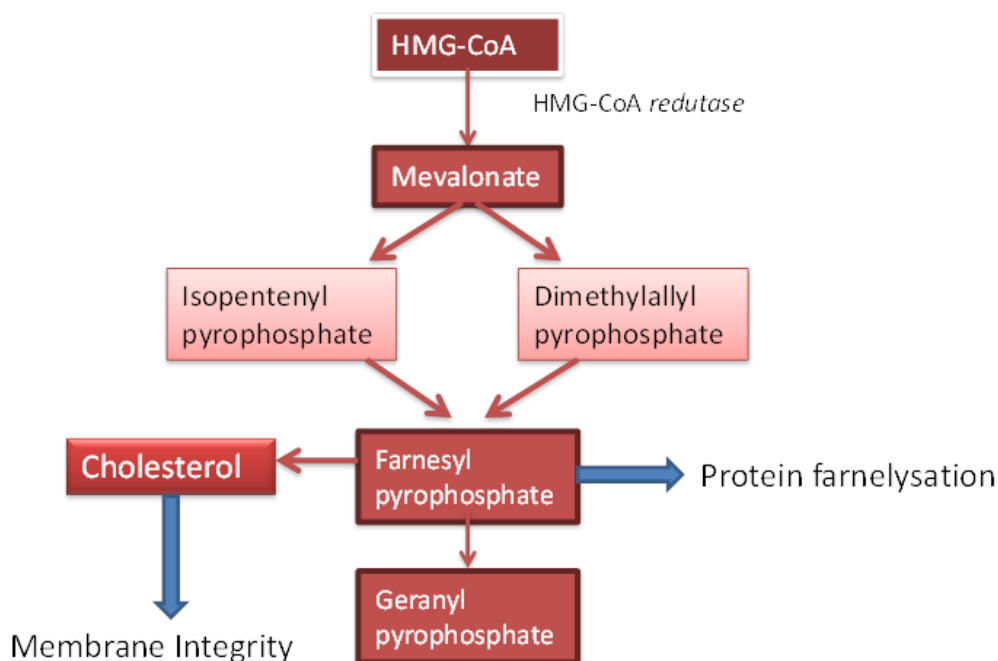


Figure 1.8 - Schematic representation of the Mevalonate pathway. Adapted from (Thurnher et al., 2013)

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the mevalonate pathway, and it is essential for the synthesis of isoprenoid compounds and cholesterol, dolichol and ubiquinone (figure 1.8).

The isoprenoids (farnesyl pyrophosphate and geranyl pyrophosphate) are essential for the post-translational modifications of many proteins, including RAS (Winiarska et al., 2008), and are synthesized by condensation of isopentenyl pyrophosphate (IPP), a compound of five carbon atoms, and its isomer dimethylallyl pyrophosphate (DMAPP) (Thurnher et al., 2013).

The regulation of mevalonate pathway and cholesterol homeostasis is regulated by a family of transcription factors called sterol regulatory element-binding protein 2 (SREBP2) (Yellaturu et al., 2009).

1.4.2 - Sterol regulatory element binding protein

SREBP-2 function as transcription factor that activate specific genes involved in cholesterol biosynthesis and endocytosis of low density lipoproteins (LDL) (Edwards et al., 2000).

In humans there are two SREBP genes: SREBP-1 and SREBP-2 that encode three different proteins. The SREBP-1 gene encodes two proteins, the SREBP1a and SREBP1c derived from distinct promoters. The gene SREBP-2 encodes the SREBP2 protein. In humans, the SREBP1c primarily regulates FA metabolism, such as fatty acid synthase (FASN) gene and SREBP2 is responsible for the activation of genes of cholesterol biosynthesis: genes such as HMG CoA reductase and LDL receptor (Krycer et al., 2010). Human SREBP2 contains 1141 amino acids and is 47% identical to SREBP1a with its 1147 amino acids (Xiao and Song, 2013); (Sato, 2010).

The SREBPs are synthesized as 125kDa membrane proteins of the ER in the form helix-loop-helix-leucine zipper structure: an N-terminal with 480 amino acids; an intermediate hydrophobic region of 80 amino acids and a regulatory domain in the C-terminal with 590 amino acids (Daemen et al., 2013; Xiao and Song, 2013). The N-terminus of SREBP2 region is rich in glutamine, proline, serine, and glycine (Shimano, 2001).

After synthesis of the pre-SREBP, this protein binds to the SREBP cleavage-activating protein (SCAP) in ER and then this complex is displaced from the ER to the Golgi apparatus. The sequence of amino acids MELADL, found in cytosolic loop 6 of SCAP, is required for coatamer protein complex II (COPII) binds to SREBP-SCAP and shall transport (Xiao and Song, 2013). The protein SCAP has two different domains in each molecule: the N-terminal has eight transmembrane helices and contains the sterol sensing domain (SSD) and that that recognize the SREBPs; the C-terminal on the other hand contains five WD repeats, with 40 amino acids each one, which allows the interactions protein-protein. This is the region of SCAP that forms a complex with C-terminal domain of SREBP (Sato, 2010). When cellular cholesterol levels are low, pre SREBP binds to SCAP, and after entering the Golgi complex is cleaved by site-1-protease (S1P) that causes a conformational change in the sterol and allows this binding to site-2-protease (S2P). S2P cleavage occurs within the transmembrane region, a process called Regulated intramembranous proteolysis (Rip). This cleavage results in the liberation of terminal domain from membrane (Edwards et al., 2000). Thus the N-terminus of SREBP enters the nucleus by binding to ATCACCCAC sequence of cis-element called sterol regulatory element (SRE) promoting gene expression (figure 1.9).

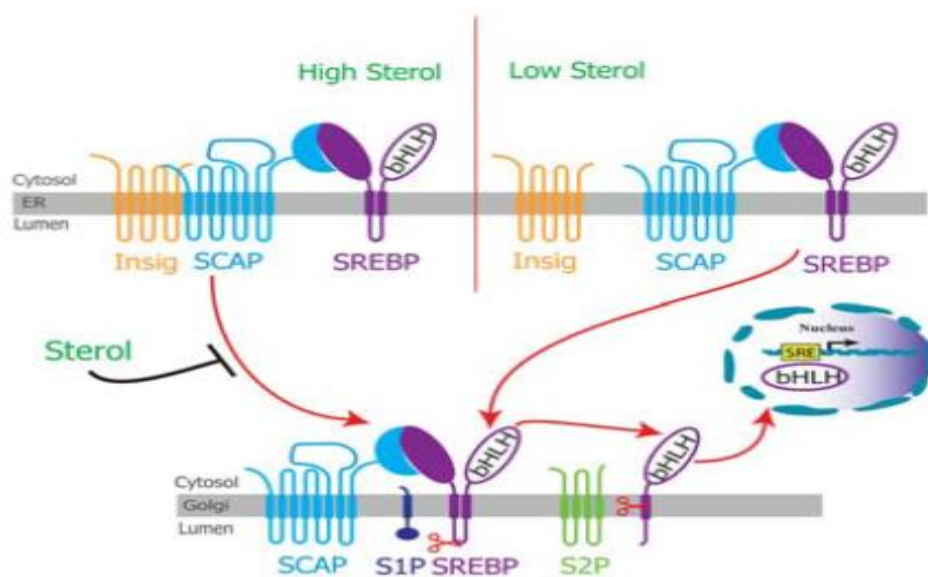


Figure 1.9 – Mechanism of action of SREBPs. Reproduced from (Xiao and Song, 2013).

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When cholesterol levels are elevated the SCAP directly binds to cholesterol promoting a conformational change in protein. This conformation allows the binding of SCAP to Insig (insulin-induced gene product), a protein residing in the ER, preventing movement of the SCAP-SREBP complex to the Golgi apparatus and thus blocking activation of SREBP's target genes. The binding of Insig prevents the COPII from recognizing the MELADL sequence (Shimano, 2001; Daemen et al., 2013). In this case Insig can still bind to the N-terminus of HMG CoA reductase in order to recruit E3 ubiquitin ligase and allow access to the proteasome, extracting the enzyme from the membrane and allowing its degradation (Xiao and Song, 2013).

There are two genes, *INSIG1* and *INSIG2* that codify two proteins: the Insig-1 and Insig-2, respectively, which are 59% identical. These genes control tissue and signal specific regulation of SREBP. Insig-1 is the dominant isoform and the only that are subject to proteasome dependent degradation; high levels of cholesterol inhibit ER to Golgi transport of SREBP by stabilization of the Insig-1. On the other hand Insig-2 is more stable, is expressed at low levels and its suppressed by insulin (Shao and Espenshade, 2012; Bengoechea-Alonso and Ericsson, 2007). Some studies indicate that SREBP1/SCAP complex is usually associated with Insig-2 and the SREBP2/SCAP complex interacts with Insig-1 (Sato, 2010).

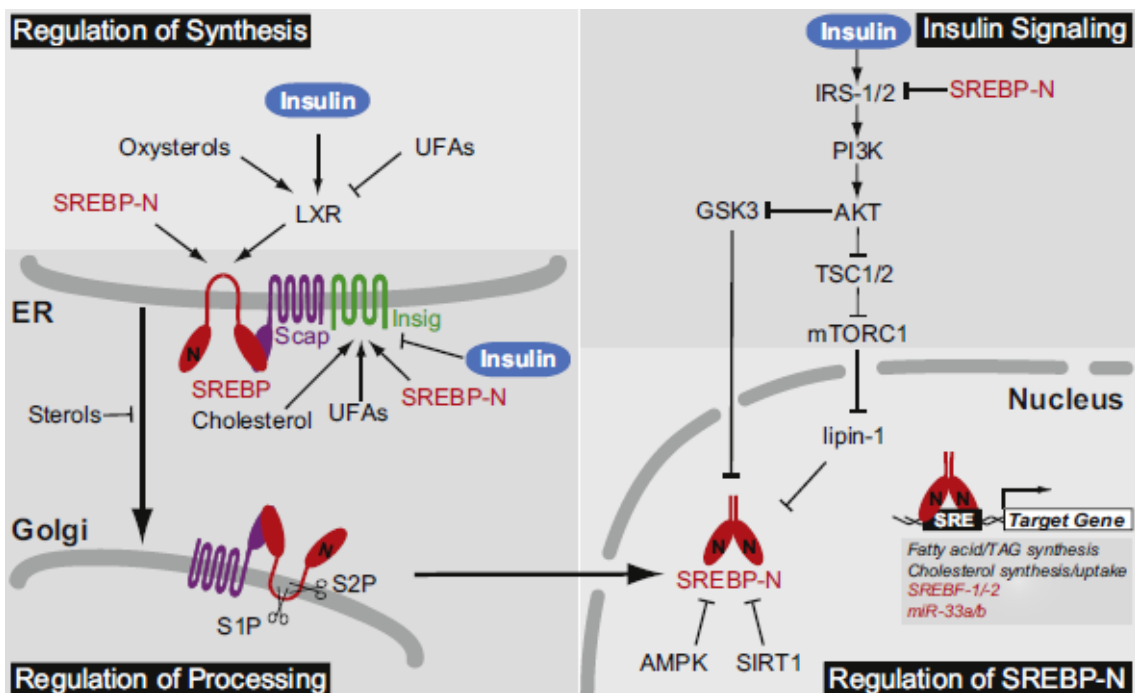


Figure 1.10 – Regulation of SREBP by insulin signaling. Reproduced from (Shao and Espenshade, 2012) .

Recent studies demonstrated that formation of mature SREBPs is controlled in various levels, namely in response to changes in the levels of insulin, oxysterols and polyunsaturated fatty acids (figure 1.10) (Edwards et al., 2000).

Insig-1 is one of the most readily inducible genes by insulin but gene expression of Insig-2 is suppressed. This because insulin stimulates proteolytic cleavage of mature SREBP1c increasing the affinity these for the SCAP through COPII vesicle enhancing the activation of SREBP targets genes and reduces the amount of Insig-2 protein by quickly degradation of the cognate mRNA (Yellaturu et al., 2009).

SREBP only not involving in sterol regulated events but are also targets of intracellular signaling pathways such MAPK kinase and PI3K/AKT. This insulin stimulated SREBP1c activation is PI3K/AKT dependent, because the induction of this pathway potentiates the ER to Golgi transport and the proteolytic activation (Rochira et al., 2013; Shao and Espenshade, 2012).

1.4.3-Statins

HMG CoA reductase inhibitors, known as statins, were originally identified in 1970 by the Japanese Biochemical *Akira Endo* and received commercial approval for the treatment of hypercholesterolemia in 1980 because these were capable of reducing endogenous cholesterol synthesis, leading to a decrease of LDL in circulation. Furthermore, statins appear in various processes involved with pleiotropic effects including inhibition of cell proliferation, induction of apoptosis, modulation of inflammation and angiogenesis (Lochhead and Chan, 2013).

In the context of cancer therapy, inhibitors of the enzyme HMG CoA reductase have been proposed as anti-tumor agents due to evidence that farnesylation by farnesyl group resulting from the use of this pathway is the crucial step for anchoring the protein RAS to membrane and also because the cells of colorectal cancer show high levels of expression and activity of HMG CoA reductase compared to normal tissue (Calleros et al., 2009). Some antitumor effects of statins could also result from the capacity of these drugs to interrupt the formation of lipid rafts (Winiarska et al., 2008).

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The first statins were of natural origin, deriving from fungal fermentation - lovastatin, simvastatin, pravastatin, mevastatin – but there are now many synthetic statins – fluvastatin, atorvastatin, rosuvastatin, pitavastatin. All have a lactone ring: when in the inactive form this ring is closed, when in the active form the ring it's open. Just the form with the open ring has the ability to block the catalytic activity of HMG CoA reductase, because it mimics an intermediate of the mevalonate pathway and binds to its active site with more affinity than the natural substrate (Wong et al., 2002). Studies conclude that the use of statins in the long term actually reduce the risk of pancreatic cancer in 59%, prostate cancer in 50% and 49% the risk of CRC compared to patients not undergoing treatment using to statins (Takahashi et al., 2006).

Despite their beneficial effects, many studies have also reported cytotoxic effects exerted by statins, however although direct toxicity to normal muscle cells and endothelium have been described, other normal cell types are unaffected by statins. In contrast, strong toxicity is induced in many tumor cells, which may consequently result in programmed cell death (Corcos and Le Jossic-Corcos, 2013). In other studies, it is observed that use of simvastatin, the most widely used because it is of natural origin and with high stability, significantly decreased the concentration of cholesterol in the lipid rafts, thereby inhibiting AKT cascade and inhibits proliferation (Lochhead and Chan, 2013; Miraglia et al., 2012). However, the dose and time required is still to be determined, because unfortunately the anti-proliferative effects in vitro are observed at concentrations too high for the clinical reality (Osmak, 2012)

1.5-Natural compounds and chemoprevention

Epidemiological studies show that cancer correlates with the diets poor in fruits and vegetables. Therefore, during the last decade several studies have been performed to understand the cellular and molecular mechanisms underlying the process of carcinogenesis and leading to the development of methodologies for dietary prevention of cancer, called chemoprevention. The objective of chemoprevention is the use of noncytotoxic natural (or synthetic) agents to delay, inhibit or reverse the growth and the development of malignant

cells through modulation of metabolic mechanisms and signaling pathways (Yadav et al., 2010), (Pan et al., 2008), (Newman et al., 2000).

The interest and the use of natural compounds extracts derived from fruits, vegetables and whole grains, increased in recent years because they offer a great range of advantages: low toxicity; easier to obtain; and favorable physiological functions (Yi et al., 2010; Pan et al., 2008). For this reason 70% of the drugs used in modern medicine as anti-cancer have their roots in products derivate from nature (Prasad et al., 2011). The National Cancer Institute of the United States has identified around 40 plant foods that exert preventive effects against cancer, including turmeric, soybean, green tea, ginger, cabbage, red wine and cauliflower.

Phytochemicals are defined as bioactive nonnutrient plant compounds, in other words, are plant chemicals (Liu, 2004). Phytochemicals can be grouped in different classes such as: phenolics, alkaloids, carotenoids, organosulfur compounds, and terpenoids (or isoprenoids) (Pan et al., 2008).

Terpenoids are lipophilic phytochemicals which have a multicyclic structure (Thoppil and Bishayee, 2011; Pan et al., 2008). These compounds are derived from isoprene units $[\text{CH}_2=\text{C}(\text{CH}_3)\text{CH}=\text{CH}_2]$ which have oxygen atoms in multiple functional groups. According to the number of carbon units, terpenoids can be classified as: monoterpenes (e.g., carvone, geraniol), diterpenes (e.g. retinol and trans-retinoic acid), triterpenes [e.g., betulinic acid (BA), lupeol, oleanic acid (OA), and ursolic acid (UA)], and tetraterpenes (Thoppil and Bishayee, 2011). More than 40,000 different terpenoids are known to occur in nature and according to various studies on development of drugs for cancer, many of these compounds have been shown to inhibit cell proliferation and metastasis by different mechanisms of action (Bishayee et al., 2011).

1.5.1-Triterpenoids

In particular the triterpenoids are an important class of bioactive phytochemicals in plants synthesized by cyclization of squalene, a triterpene hydrocarbon and precursor of all steroids (Bishayee et al., 2011). They are organic metabolites of isopentenyl pyrophosphate

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oligomers characterized by the basic structure of 30-carbon isoprenoid composed from six isoprene units ($C_{30}H_{48}$), arranged in five rings with several oxygen atoms attached (Yadav et al., 2010; Yan et al., 2013). Triterpenoids are distributed throughout the plant kingdom in the form of free triterpenoids, triterpenic glycosides (saponins), phytosterols and/or their precursors (Patlolla and Rao, 2012).

These compounds have a high variety of benefits for human health, especially antioxidant, antiviral, antimicrobial and hypoallergenic. Recent studies have not only confirmed these proprieties, but also showed that triterpenoids are cytotoxic to cancerous cells without showing any toxicity in normal cells (Bishayee et al., 2011). Studies indicate that these compounds may act at different stages of tumor development: inhibiting initiation and promotion, inducing cell differentiation and apoptosis, regulating various signaling pathways, down regulating of transcription factors (e.g., nuclear factor-kappaB [NF- κ B]), modifying expression of anti-apoptotic proteins (e.g., bcl-2, bcl-xL) or promoters of cell proliferation (e.g., cyclooxygenase-2 [COX-2], cyclin D1, c-myc), as well as suppressing invasion and metastasis (Thoppil and Bishayee, 2011; Yadav et al., 2010).

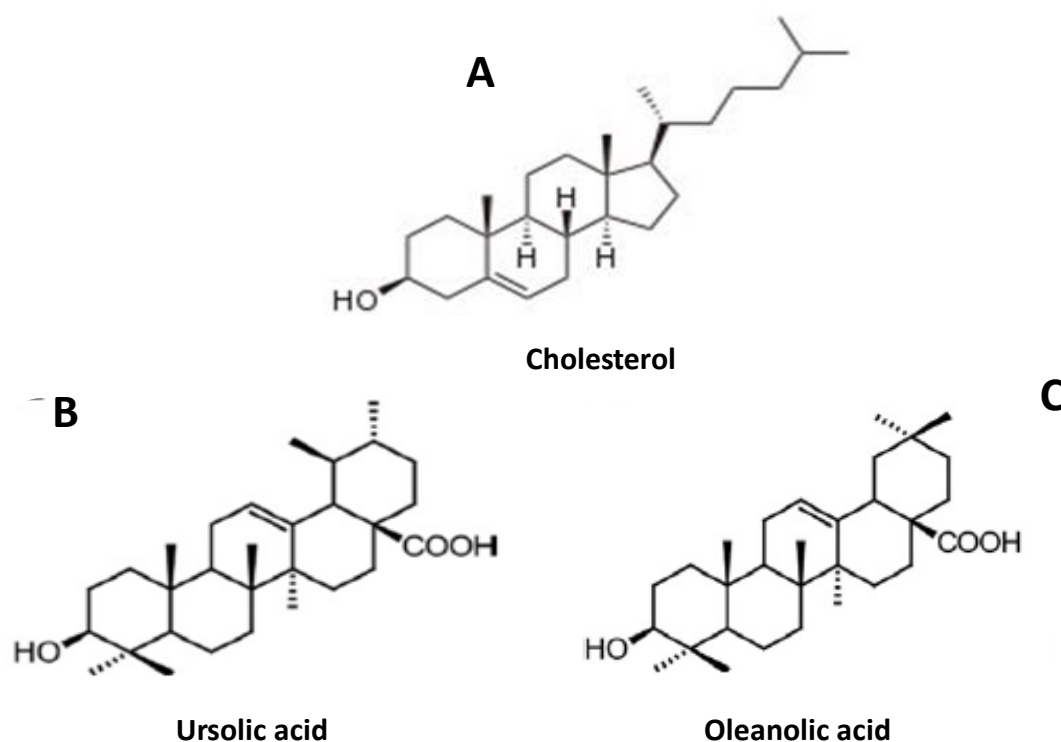


Figure 1.11 – Structure of Cholesterol (A), Ursolic acid (B) and Oleanolic acid (C).

There are about 20,000 triterpenoids from several fruits and medicinal plants, that can be subclassified into several groups, including: lupanes, oleananes, ursanes, holostanes, squalenes, limonoids, dammaranes, (Shanmugam et al., 2013; Bishayee et al., 2011). In the present work we used two pentacyclic triterpenoid isomers – ursolic acid (UA) and oleanolic acid (OA) because they have structure similar to cholesterol (figure 1.11).

1.5.1.1- Ursolic Acid

Ursolic acid (3 β -hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpenic acid (figure 1.11-B) identified in various plants, herbs and fruits namely in *Salvia* species, rosemary, olive, apples, pears and berries. This natural compound may appear in the form of free acid or as aglycone precursor for saponins (Shanmugam et al., 2013; Prasad et al., 2011).

The first study with the UA on anti-mammary cancer effects was conducted by *Es-Saady* and coworkers, and according to this study the UA acts as an inhibitor of proliferation by altering the cell cycle at the G1 phase (Es-Saady et al., 1996). Other studies have shown that UA exhibits a large spectrum of properties, such as anti-inflammatory, antioxidant, hepatoprotective, anti-tumor and anti-metastatic with low toxicity. This effect against proliferation correlates with suppression of EGFR/MAPK pathway by inhibition of EGFR phosphorylation and AKT regulation; low Bcl-2 and Bcl-x and inducing apoptosis (Shan et al., 2009). A study conducted by *Xavier et al.* 2009, shows that UA not affect the expression of p-ERK but inhibits p-AKT in CO115 cells, without affecting the expression of KRAS protein and has PI3K as one of its molecular targets. It also showed that this compound inhibits proliferation and induces apoptosis in HCT15 cells (Xavier et al., 2009). The same author demonstrated that UA could be involved in modulation of autophagy through activation of JNK signaling in HCT15 cells and by inducing the accumulation of p63 levels (Xavier et al., 2013).

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1.5.1.2- Oleanolic Acid

Oleanolic acid (3 β -hydroxyolean-12-en-28-oic acid) is a member of oleanane triterpene family and an isomer of UA (figure 1.11-C). OA and UA have a similar chemical structure varying only in the position of a methyl group, in E ring. When the methyl group in position C19 of UA is moved to the position C20 is formed the OA (Pollier and Goossens, 2012).

This pentacyclic triterpenic acid is isolated from more than 1620 plant species, including many food and medicinal plants, and similarity to the UA, OA have pharmacological activities, such as hepatoprotective effects, and anti-inflammatory, antioxidant, or anticancer activities (Juan et al., 2008; Pollier and Goossens, 2012). OA induces apoptosis in HL-60 and HCT15 cells through the release of cytochrome c into the cytosol and thus leading activation of the caspases pathways. This effect is also verified by PARP cleavage in HL-60 cells and suppression of activation of NF- κ B and Bcl-2 proteins. Several studies have also shown that the antitumor activity of the OA occurs through cell cycle arrest and inhibition of DNA replication (Shyu et al., 2010; Shanmugam et al., 2012; Juan et al., 2008).

2. RESEARCH OBJECTIVES

Hypercholesterolemia has been implicated in cancer development and progression. Cholesterol is an amphipathic molecule present in the cell membranes which plays an important role in the regulation of cellular homeostasis, in particular the integrity, fluidity and membrane permeability. Cholesterol is therefore an important constituent of membrane microdomains called lipid rafts. These specialized microdomains are also rich in proteins involved in anti and pro apoptotic signaling and are determinants of survival and proliferation.

Cholesterol lowering drugs such as statins have been proposed to play a crucial role in the progression of cancer, because the mevalonate pathway responsible for the synthesis of this compound provides farnesyl pyrophosphate groups essential for the prenylation of the RAS oncogene in membrane, which activates related proliferation pathways and cell growth.

The triterpenoids OA and UA are natural compounds which have chemical structures similar to cholesterol. Thus the aim of our study is to verify:

1. If these compounds possess anti-cancer activity by altering cellular cholesterol levels;
2. Death and proliferative signaling pathways of colorectal cancer cells (HCT116)
 - 2.1. Effects on TNF- α signaling.
 - 2.2. Effects on insulin signaling.

3. MATERIAL AND METHODS

3.1 - Cell Culture, conditions and reagents

HCT116 cell line (derived from a human colorectal carcinoma) was kindly provided by Dr. M. Rohde from The Danish Cancer Society and grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 6% fetal bovine serum (FBS) (Biochrom KG, Berlin, Germany), antibiotic-antimitotic 1% solution (Sigma-Aldrich, St. Louis, MO, USA), sodium pyruvate 0.1mM, sodium bicarbonate 2g/L and HEPES 10mM (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained in 25cm², not toxic and transparent tissue culture flasks (TPP), at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. The medium was changed twice a week. Cellular subcultures were performed when confluence reached values close to 80-90%. For subcultures and plating, the adherent cells were detached with trypsin solution 1% (Sigma-Aldrich, St. Louis, MO, USA) and trypsin was neutralized by adding fresh medium. The cell density of the suspension was determined using a haemocytometer and was calculated by the formula: Mean of cells in the four quadrants x dilution factor x 10⁴ = number of cells per mL.

Simvastatin, UA and OA were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in dimethyl sulfoxide (DMSO) to stock solutions, aliquots were kept at -20°C. The methyl-β-cyclodextrin (MβCD), cholesterol and insulin were also obtained from Sigma-Aldrich (St. Louis, MO, USA). The MβCD was dissolved in RPMI medium without serum, and aliquots were stored at -20°C at a concentration of 5mM and for the experiments we used 1.5mM. Furthermore, insulin was dissolved in water and stored at 4°C in concentration of 100μM, for the experiments we used 2μL of stock for 2mL of medium. The stock solution of TNF-α was also stored at -20°C in concentration of 10μg/mL and for the condensation assay we used 2μL of stock for 2mL of medium. The DMSO concentration that is placed in the culture medium in all conditions did not exceed 0.5% (v/v) and the controls were treated only with the vehicle (DMSO).

3. Material and Methods

3.2 - Cell Viability by MTT reduction assay

The MTT assay allows the evaluation of cell viability by dehydrogenases associated metabolic reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow compound, to a purple colored formazan. Only metabolically active cells are capable to reducing this compound and therefore the number of viable cells is directly proportional to the amount of formazan crystals produced (Chang et al. 2013).

HCT116 cells were plated in 24-well plates two days before incubation with compounds to a cellular density of 0.6×10^5 cell/mL and left to adhere for 48h. Following, fresh medium was added to cells containing the test compounds: UA at final concentration of 2.5, 5, 10 and 15 μ M; OA at 5, 10, 20, 30 and 40 μ M; Simvastatin at 10 and 30 μ M for 48h incubation time.

One hour before the end of incubation time, 50 μ L MTT (5mg/mL, diluted in phosphate-buffered saline (PBS) solution and maintained in the dark and at -20°C) (Sigma-Aldrich, St. Louis, MO, USA) were added to each well and cells were incubated at 37°C , with 5% CO_2 during 1h. After this time the supernatant was removed, formazan crystals were dissolved in 1mL of ethanol / DMSO solution (proportion 1:1) and 200 μ L of each condition was transferred for a 96-well plate. The reading of absorbance was done at 570 and 690nm using a micro plate reader (Molecular Devices Spectra Max Plus 384). As a blank, 200 μ L of DMSO/ethanol solution were used. The results were expressed as percentage relative to the control (untreated cells). The IC_{50} value of compounds was calculated as the concentration at which 50% reduction in cell viability was detected relative to untreated controls.

3.3 - Protein extraction and Western Blot analysis

HCT116 cells were plated in 6-multiwell culture at a density of 0.6×10^5 cell/mL. After treatment with compounds at different incubation times or concentrations, the cells were washed with PBS solution, trypsinized and medium was added for neutralizing the trypsin and were collected into a respective falcon. The samples were centrifuged at 500g for 5 min at 4°C. After this the supernatant was discarded up to 500µl, samples were resuspended and transferred to individual eppendorf tubes. The samples were centrifuged at 1500g for 3 min at 4°C, and the pellet was resuspended in lysis buffer (1% Triton in 150mM NaCl, 50mM Tris-base (pH 7.5), 1mM EDTA, 0.1% SDS, 1% Sodium deoxycholate) supplemented with 20mM of sodium fluoride (NaF), 1mM phenylmethylsulfonyl fluoride (PMSF), 20mM of Sodium orthovanadate (Na_3VO_4), and protease inhibitor cocktail (Roche, Mannheim, Germany). After 10 min on ice the samples were centrifuged at 10 000g for 10 min, obtaining in supernatant the total protein.

Protein quantification of each sample was performed using the kit Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and a bovine serum albumin (BSA) used as protein standard. For Western Blot, 20µg of protein were loaded and separated on 8% SDS-polyacrilamide gel and then transferred to Hybond-P polyvinylidene difluoride (PVDF) membranes (Westran Clear Signal, USA). The membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) nonfat dry milk under shaking for at least 2h, washed three times in TPBS solution and incubated with primary antibody (table 3.1) overnight. After being washed again with TPBS, the membranes were incubated with secondary antibody (containing IgG horseradish peroxidase) for 1h at room temperature. The immunoreactive bands were detected using the Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) under a chemiluminescence detection system, the ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA, USA). β -actin protein was used as the control. The area intensity of the bands was quantify using Quantity One software (Bio-Rad) and standardized relative to control.

3. Material and Methods

Table 3.1 – Primary Antibodies and respective secondary antibodies used in the Western blot.

Primary Antibody	Dilution	Secondary antibody	Source
FAS	1:1000	Rabbit	Cell signaling Technology (Danvers, MA, USA)
p-AKT	1:2000	Mouse	Cell signaling Technology (Danvers, MA, USA)
p-ERK	1:2000	Mouse	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
AKT total	1:2000	Rabbit	Cell signaling Technology (Danvers, MA, USA)
SREBP1	1:1000	Mouse	Abcam (Cambridge, UK)
SREBP2	1:1000	Rabbit	Abcam (Cambridge, UK)
β -actin	1:2000	Mouse	Sigma-Aldrich (St. Louis, MO, USA)

3.4 - Nuclear Condensation

The nuclear condensation assay allows detect cells in apoptosis, because demonstrate nuclear condensation of cells. In this work, this technique was used to evaluate the effect of TNF- α in cells treated with M β CD, Simvastatin, UA and OA. After 24h of incubation with compounds, the medium was changed to serum free medium only or serum free medium with TNF- α during 24h. After this time, the medium was collected to harvest dead cells and the attached cells were treated with trypsin solution. The trypsin was then neutralized with respective harvest medium and collected to respective falcons. The collected cells were centrifuged at 2000rpm for 10 min, washed with PBS and resuspended in 500 μ L medium. Then the cells were fixed with 4% paraformaldehyde (PFA) for 20 min, and after this time they were washed with PBS and centrifuged at 2000rpm for 10 min. The supernatant was aspirated up to 500 μ L and the pellet resuspended and transferred to eppendorfs. Posteriorly, the cells

were attached onto a polylysine-treated slide using a Shandon Cytospin at 500rpm for 5 min and washed two times in PBS for 5 min. To visualize apoptotic cells in the different conditions tested, the cells were stained with 20 μ L of Hoechst, which marks the nuclear DNA, for 10 min in a dark chamber. The dye was kept at 4°C in aliquots of 0.5mg/mL (Sigma-Aldrich, St. Louis, MO, USA). This dye was diluted in PBS (1:100) and the final concentration was 5 μ g/mL. After the incubation period was added glycerol 50% in order to fix the cover slip to the blade. Under an inverted microscope equipped with a fluorescent light source (DAPI fluorescence filter) (Olympus IX71), the number of cells in each condition was determined (figure 3.1). The percentage of apoptotic cells is calculated by the ratio between the cells with nuclear condensation and the total number of cells. For condition should be considered at least 500 cells.

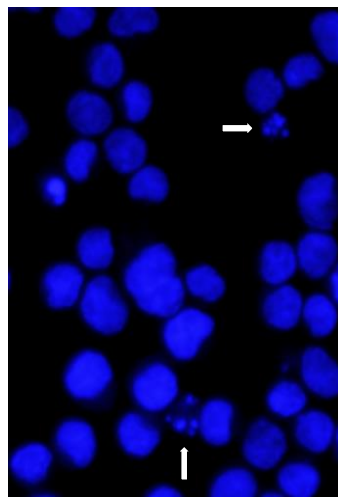


Figure 3.1 - Evaluation of nuclear condensation by Hoechst staining in HCT116 cells exposed to OA treatment after 24h of incubation. Amplification 40x. White arrows indicate cells with nuclear condensation.

3.5 - Cholesterol Assay

The assay used is based on an enzyme-coupled reaction that detects both free cholesterol and cholesteryl esters. Cholesteryl esters are hydrolyzed by cholesterol esterase (CHE) into cholesterol, which is then oxidized by cholesterol oxidase (CHOD)

3. Material and Methods

to yield H₂O₂. The H₂O₂ is detected using peroxidase (POD), 4-Aminophenazone and phenol. The intensity of the color formed is directly proportional to the cholesterol concentration present into the sample. The quantification of cholesterol was performed using the commercial kit Cholesterol- LQ CHOD-POD Liquid (Spinreact, Spain).

HCT116 cells were seeded into 6-well plates at concentration of 1.2×10^5 cell/mL (using two wells per condition) in RPMI medium. After 48h of adhesion, fresh medium containing various compounds (Simvastatin 10 μ M, M β CD 1.5mM, UA 10 μ M, and OA 20 μ M) was added and maintained for 24h and 72h. Control cells were treated with DMSO. After 24h or 72h of incubation, the medium was collected for respective falcons and the wells washed with PBS two times. The attached cells were treated with trypsin solution. The trypsin was then neutralized with PBS and collected to respective eppendorf tube. The samples were centrifuged at 500g for 5 min at 4°C. After this, the supernatant was discarded and the pellet was resuspended in 300 μ L of lysis buffer. After 10 min in the ice with periodic vortexing, 20 μ L of each sample was withdrawn to a new eppendorf tube to quantify protein.

In a fume hood, 200 μ L of chloroform was added to the lysed cells and the organic phase (more dense) collected into new eppendorf tubes (the procedure was repeated three times). After drying in fume hood, the samples were dissolved in 80 μ L of ethanol, exposed to ultrasound 5 min and centrifuged for 2 min at maximum speed. For each sample 25 μ L was transferred for a 96-well plate and mixed with 250 μ L of Cholesterol- LQ reagent for 5 min at 37°C. The absorbance was readed at 505nm using a micro plate reader (figure 3.2). Ethanol was used as a blank and 2.5 μ L of 2000 μ g/mL cholesterol aqueous were used as standard. The quantity of total cholesterol in each condition was calculated by the ratio between the quantity of cholesterol and the quantity of protein (μ g chol/ μ g prot).

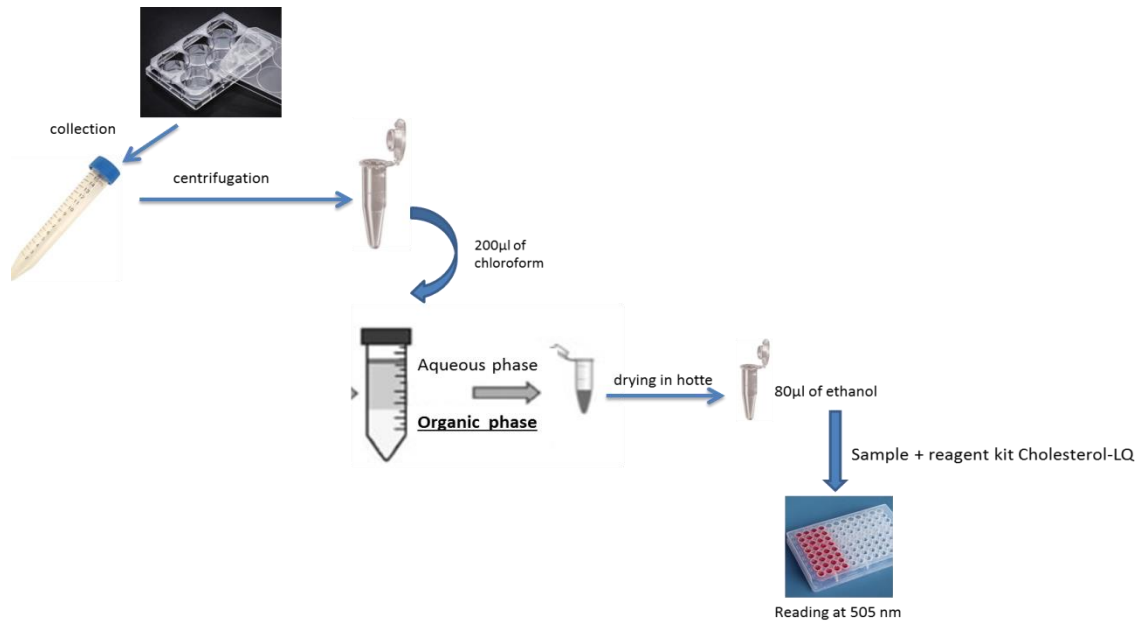


Figure 3.2 - Representative scheme of general steps involved in standard cholesterol assay.

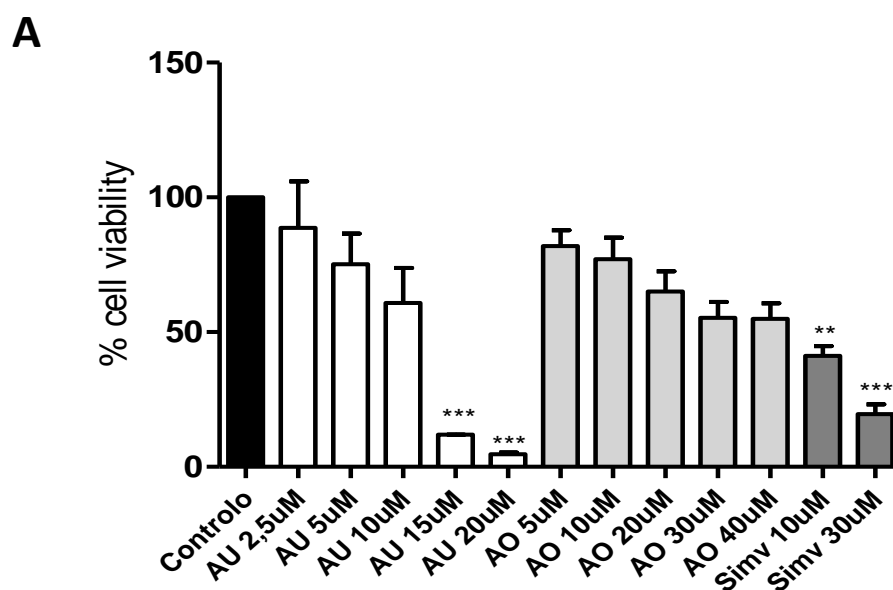
3.6 - Statistical analysis

Data are expressed as the mean \pm SEM of 2 to 3 independent experiments. Statistical significances were analyzed by one-way ANOVA followed by Tukey's Multiple comparison test, using GraphPad Prism 5.0 software (San Diego, CA, USA). Differences between groups were considered statistically significant when $P \leq 0.05$ (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

4. RESULTS

4.1 – Effects of triterpenoids and Simvastatin on cell viability

In order to study the cytotoxic effects of the triterpenoids and simvastatin in cells and define an adequate concentration mean the one that induces 50% of cell death to use in subsequent studies, HCT116 cells were incubated for 48h with different concentrations and cell viability evaluated by the MTT assay. Figure 4.1-A shows that 48h incubation with UA and OA or reference compound (simvastatin), decrease in cell viability in a concentration dependent manner. UA showed to be more antitoxic than OA in these cells. Simvastatin also decreased cell viability. IC_{50} for the different compounds were estimated to be mean 10 μ M to Simvastatin, 10 μ M to UA and 20 μ M to OA. These results show that the UA more significantly sensitizes cells to death as compared with OA.



4. Results

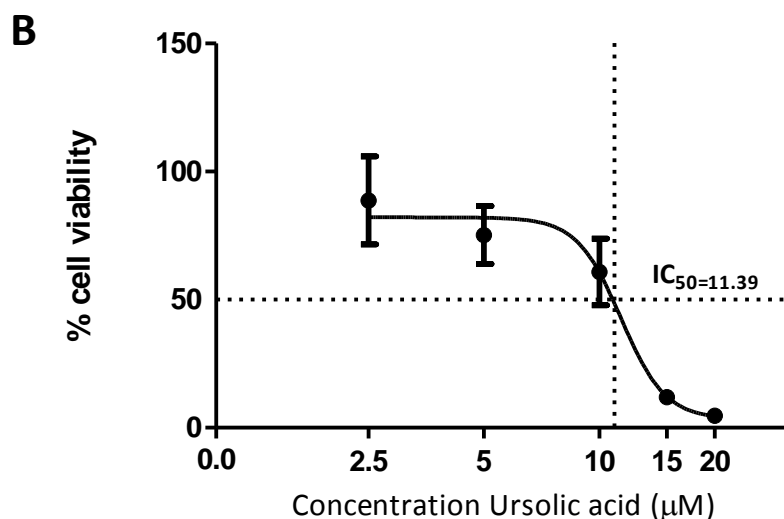


Figure 4.1 – Effects of triterpenoids (UA and OA) and Simvastatin on cell viability in HCT116 cells by MTT assay. (A) Cells were treated with 0.5% of DMSO for the control and with compounds at indicated concentrations for 48h. The percentage of cell viability was calculated by the absorbance relative to the value detected for the control cells that was defined as 100% of cell viability. In (B) is presented data of (A) with a sigmoid mathematical modelling with the program GraphPad Prism for calculation of the IC₅₀ value. For each condition were performed three independent experiments and data are expressed as mean ± SEM. Significant differences were found, by one way ANOVA, when compared with respective control (** P < 0.01; *** P < 0.001).

4.2 – Effects of compounds on cellular cholesterol levels

UA and OA are natural compounds that have a chemical structure similar to cholesterol, which suggest that they may interact with the biomembranes (Prades et al., 2011) and this way change total cellular cholesterol. It was therefore important to evaluate the effect of triterpenoids UA and OA on cellular cholesterol.

For this assay, cells were incubated 24h and 72h with UA, OA, Simvastatin, M β CD or with the M β CD+cholesterol (complex form due to the affinity that M β CD has for cholesterol and its lipophilicity allows the delivery of cholesterol to the cells), M β CD+UA and M β CD+OA. The combination of M β CD with OA or UA in ensured that this triterpenoids were in fact delivered to the cell.

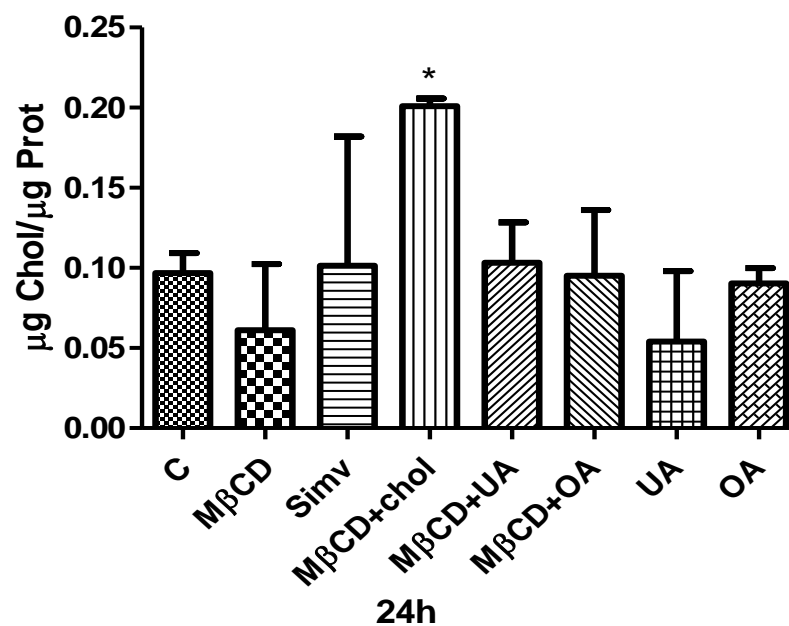
Total cellular cholesterol (TCC) was evaluated and effects of reference compounds, Simvastatin and M β CD, were significant TCC at 72h (figure 4.2B) but not

24h (figure 4.2A). On the other hand, the combination M β CD+cholesterol resulted in a significant increase in total cellular cholesterol both 24h and 72h. These results shown that the kit used was detecting cellular cholesterol and changes induced by agents that are known decrease (simvastatin and M β CD) or increase (M β CD+chol) total cholesterol levels. UA and OA were tested and shown not to interfere with cholesterol quantification (data not shown).

In spite of their structural similarity UA and OA had opposite effects on total cellular cholesterol: UA significantly decreased and the OA increased cholesterol levels after 72h as shown in figure 4.2B. When using the M β CD with OA or UA the effects of total cellular levels were similar to effects of OA and UA alone.

As expected, control cells did not display any significantly changes in cholesterol levels during the experiment.

A



4. Results

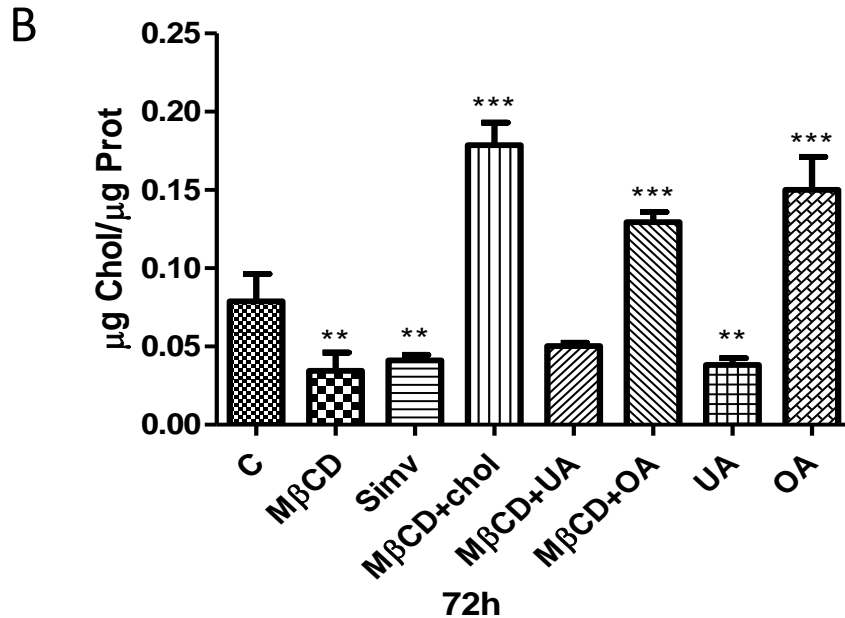


Figure 4.2 – Effect on quantity of cholesterol cellular after incubation of 24h (A) and 72h (B) with the different compounds. The percentage of cholesterol were calculated through to the ratio between quantity of cholesterol (μg) and the quantity of protein (μg) For each condition were performed three independent experiments and data are expressed as mean \pm SEM. Significant differences were found, by One-way ANOVA followed by Tukey's Multiple comparison test when compared with respective control (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

4.3 – Effects of UA and OA on TNF- α induction in cell death

Lipid rafts are known to be abundant in various signaling molecules, such as cell surface receptors and serve as platforms for interaction of many intracellular signaling molecules. Thus, these membrane microdomains have been involved in the regulation of apoptosis and cell proliferation (Li et al., 2006).

If UA and OA through their effects on total cellular cholesterol have an impact on lipid rafts composition then they may interfere with signaling platforms such as cell death receptors mediated apoptosis.

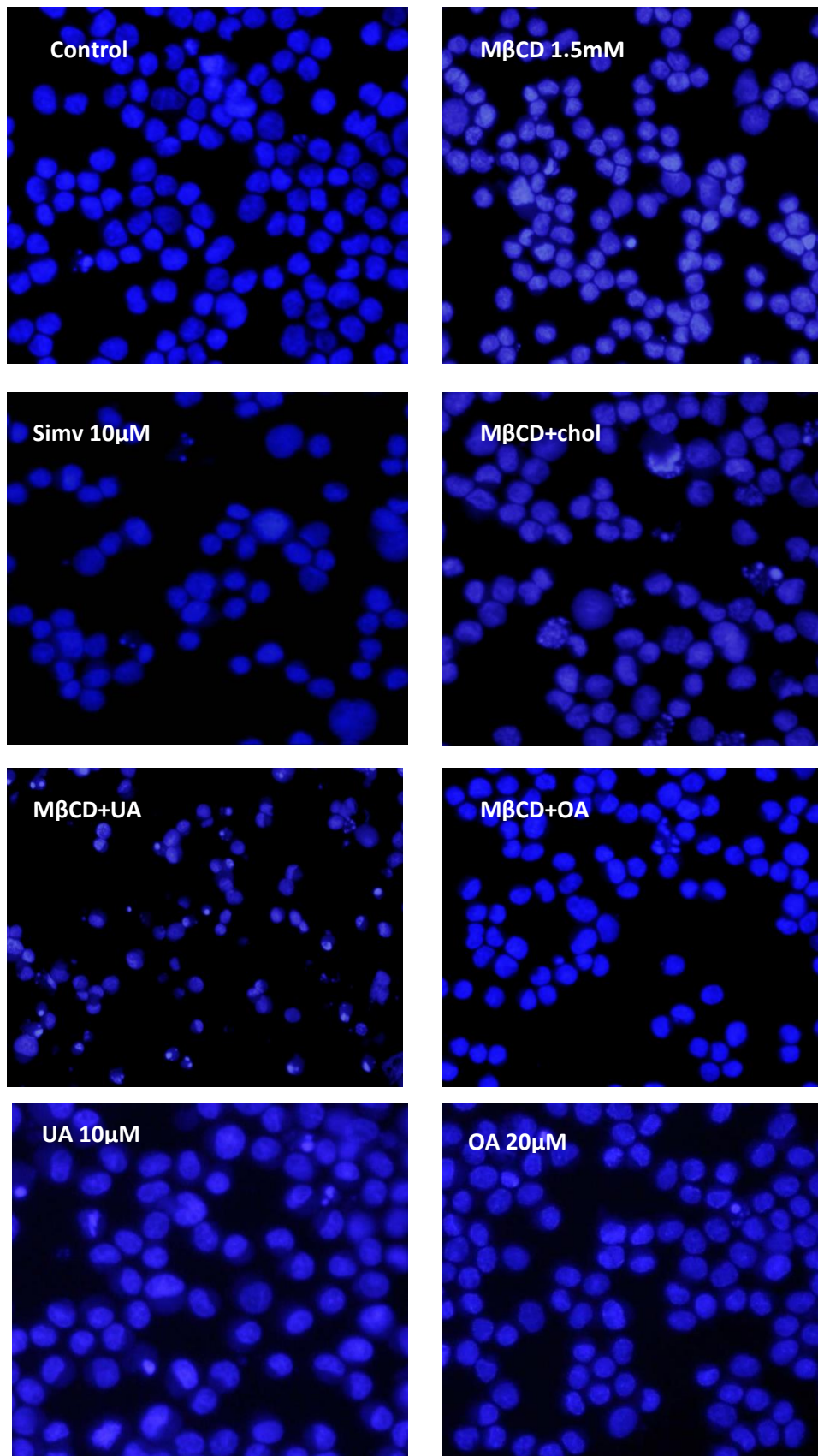
So, the following step was to test the same compounds in 24h incubation with and without TNF- α in order to verify if the alterations observed for cholesterol associate with alterations in apoptosis induction in cells HCT116.

As can be observed in the figure 4.3B, it is clear that TNF- α induces cell death in both control and treated cells. We can also observe that the M β CD+cholesterol complex induces cell death even without TNF- α , and in the presence of this cytokine cell death increases significantly. UA and OA have effects similar to each other in response to TNF- α , significantly increasing the induction of death by TNF- α relative to control.

In M β CD+UA situation, that have more delivery of UA to the cell (even not leading to alterations in total cellular cholesterol), there is a significantly high induction of cell death and further increased in the presence of TNF- α . These effects were not observed for M β CD+OA.

4. Results

A



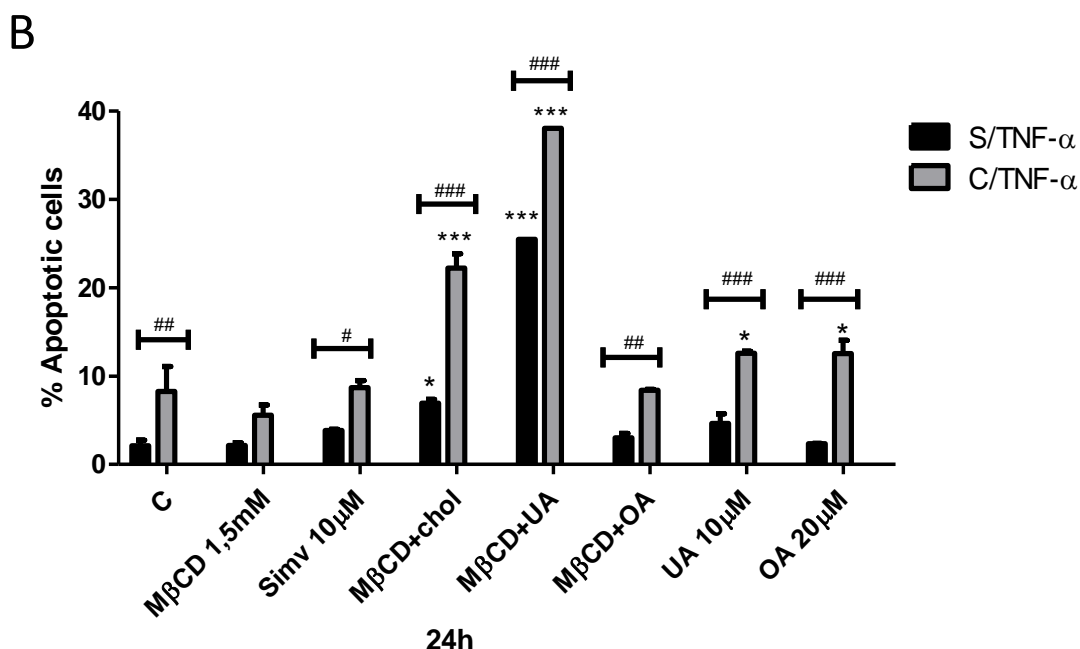


Figure 4.3 - Effect on induction of apoptosis after incubation of 24h with the different compounds plus 24h with and without TNF. The percentage of apoptotic cells were calculated through to the ratio between cells in nuclear condensation and the total number of cells. (A) Representative images of the induction of apoptosis in HCT116 cells by the tested compounds/extracts after 24h incubation without TNF- α (B) For each condition two independent experiments were performed and data are expressed as mean \pm SEM. Significant differences were found, by One-way ANOVA followed by Tukey's Multiple comparison test when compared with respective control (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Significant differences between the same compound but with and without TNF were considered when p-values ≤ 0.05 (# $p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$).

4.4 – Effects of UA and OA on induction of proliferative pathways by insulin

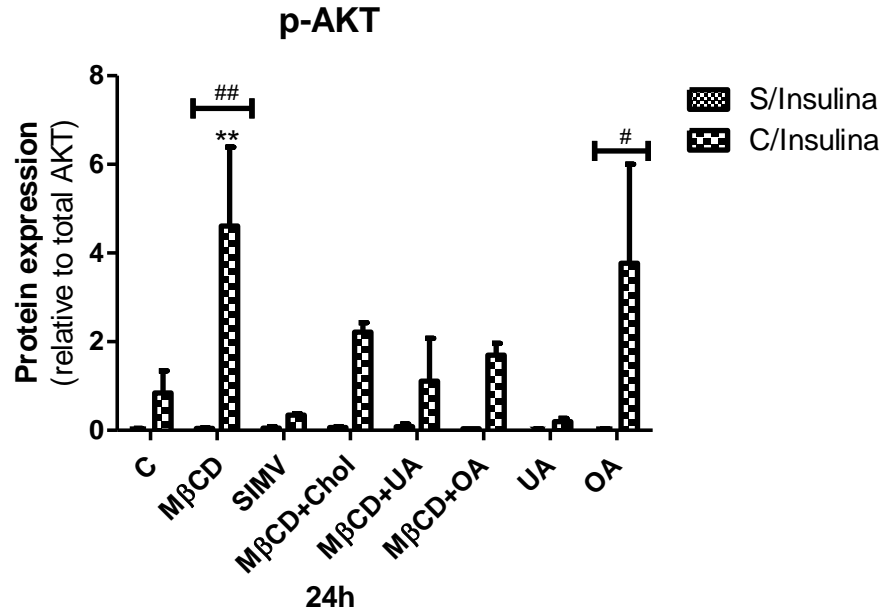
The lipid rafts are, as already noted, plasmatic membrane regions enriched in cholesterol and also in proteins. The growth factor receptors are tyrosine kinases (IR, EGFR, VEGF, and PDGF) and examples of proteins involved in cell signaling that are located in these membrane microdomains.

In order to check if the alterations in the total cellular cholesterol contents induced by UA and OA have an effect on growth factor signaling pathways, we evaluated the protein phosphorylated levels of p-AKT and p-ERK in the different conditions and once inhibits to its receptor. The insulin receptor is present on these domains caveolae-dependent signaling is induced with activation of both the MAPK

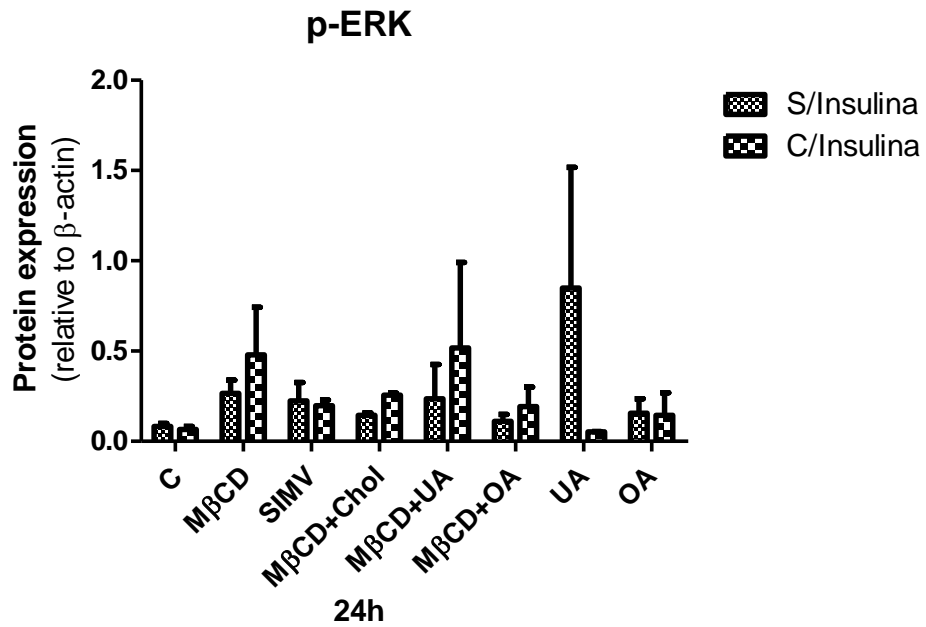
4. Results

and PI3K-AKT pathway. The effects of 24h and 72h exposed to UA and OA to insulin (1h) on both p-AKT and p-ERK levels were quantified by western blot.

A



B



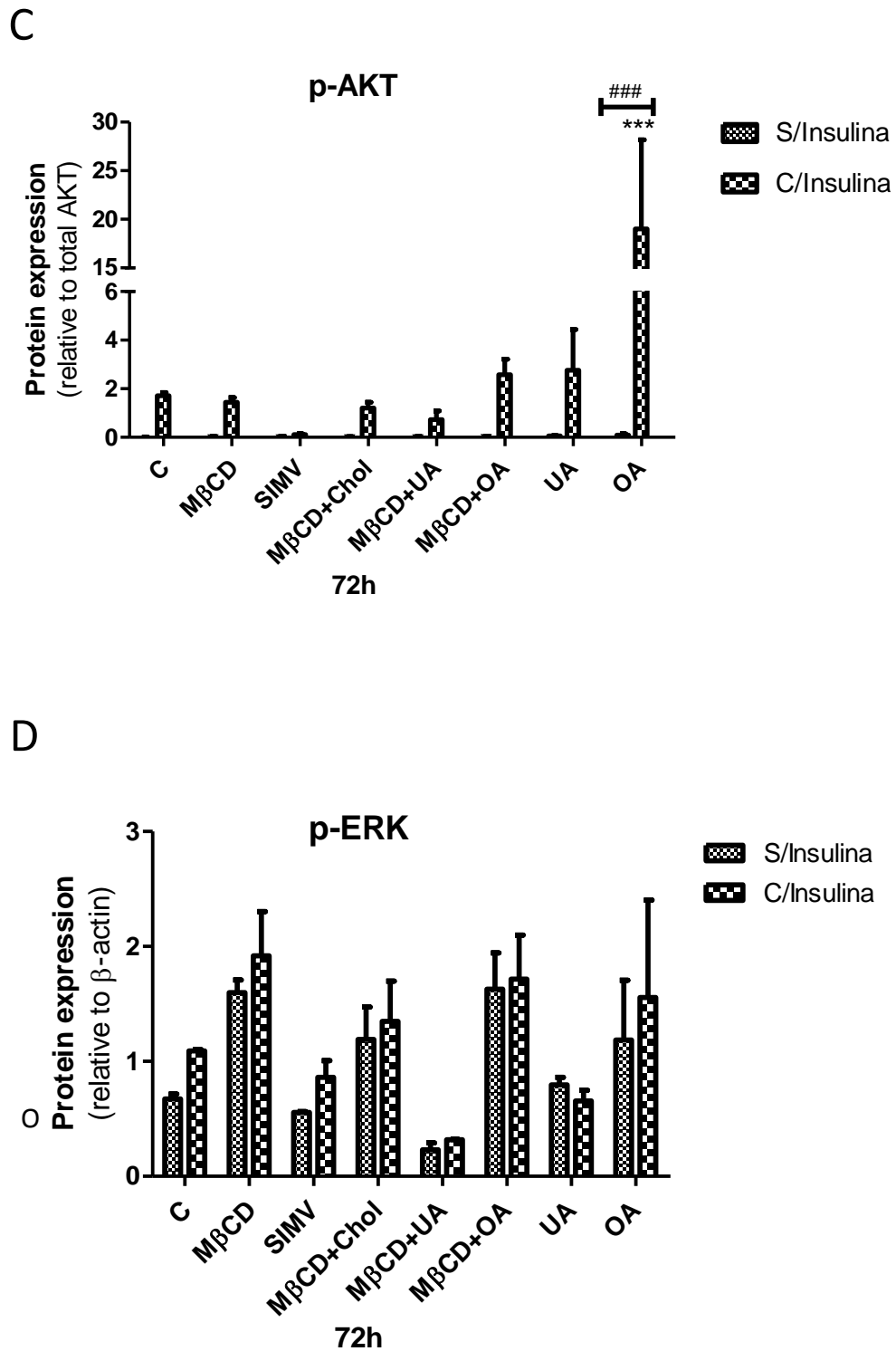


Figure 4.4 - Effect of 24h (A,B) and 72h (C,D) of incubation with triterpenoids, simvastatin and MβCD on p-AKT and p-ERK protein expression, in HCT116 cells. The protein levels were evaluated by Western Blot. As loading control we used β-actin. For each condition two independent experiments were performed and data are expressed as mean ± SEM. Significant differences were found, by one way ANOVA, when compared with respective control control (* P < 0.001). Significant differences between the same compound but with and without Insulin were considered when p-values ≤ 0.05 (# p ≤ 0.05; ## p ≤ 0.01; ### p ≤ 0.001)**

4. Results

Our results show that for both 24h and 72h of incubation with insulin (figure 4.6-B and D) no significant differences on the expression levels of p-ERK protein were obtained compared with controls. However the same does not happen for p-AKT protein.

For the OA our results show a significant increase in the phosphorylation of AKT in a time-dependent, in other words, the levels of p-AKT is increased in OA exposed 24h and in 72h OA exposed insulin induces higher p-AKT levels. While for UA the decrease in p-AKT is only observed for 24 h and for 72h the effect is not found.

Our studies also show (even through not significantly) that simvastatin reduces the levels of insulin induction of p-AKT both for 24h and 72h (figure 4.6-A and C). M β CD at 24h but not at 72h also increased p-AKT protein relative to control.

5. DISCUSSION AND CONCLUSIONS

5.1 – Discussion

Cholesterol is an important component that has as a major role in the structural integrity of the plasma membrane since it maintains its fluidity. It also creates microdomains, named lipid rafts (Scheinman et al., 2013), which provide signaling platforms capable of activating signaling pathways. An alteration in the membrane cholesterol level can result in the disruption of lipid rafts and activate or deactivate raft associated proteins (George and Wu, 2012) with consequences on signal transduction. For example, it has been demonstrated that in prostate cancer prognosis the cholesterol and lipid rafts are important as survival factors and cholesterol depletion results in apoptosis (Scheinman et al., 2013).

The triterpenoids UA and OA are natural compounds found in many fruits and vegetables of the diet and that have a similar chemical structure to cholesterol, which makes them into good candidates to interact with the cell membranes. It is possible that such interactions would modulate the structural properties of the affected membranes and that this trigger, functional changes in membrane-related proteins and signaling (Prades et al., 2011)

The anticancer potential of triterpenoids has been well described in the literature. Several studies (Li et al., 2002; Shyu et al., 2010; Prasad et al., 2011; Shan et al., 2009), demonstrated the anticancer activity of these triterpenoids against various types of tumor cells. The potential of UA to modulate several important signaling pathways related to proliferation and cell death was demonstrated in several types of cancers with few studies reported in CRC. In previous studies by our group it was found the potential anticancer these triterpenoid by several mechanisms of action, namely that decreasing the KRAS signaling, inducing cell death and modulating autophagy through the JNK pathway (Xavier et al., 2009, 2013). In HepG2 cells it was demonstrated that OA but not UA induces cell death under starvation-induced autophagy (Duarte, 2012).

In the present study, we tested the hypothesis that the two isomeric triterpenoids UA and OA possess anti-cancer activity by altering cellular cholesterol

5. Discussion and Conclusions

levels thereby modulating signal transduction associated with cell membrane receptors such as insulin and death receptors (TNFR/Fas).

In the cell viability assay our results showed that UA decreases cell viability in a dose-dependent manner (figure 4.1A) with an IC_{50} of $11.39\mu\text{M}$ (figure 4.1B), but the same was not found for the OA. These results are consistent with those obtained previously for the UA and OA by our group. In the study of *Duarte et al.* the UA showed good anticancer activity with an IC_{50} of $8\mu\text{M}$, whereas OA showed no significant decrease in cell viability until the highest concentration tested ($50\mu\text{M}$) (Duarte, 2012). The small difference observed between the IC_{50} of UA in the study of *Duarte et al.* ($8\mu\text{M}$) and IC_{50} obtained in our results ($11.39\mu\text{M}$) may result from the sensitivity of cells, the number of cell passages or the operator/work condition. Our results show that simvastatin decreased cell viability, this is consistent with a study performed by *Chang et al.* using a statin in the same cell line (Chang et al., 2013), which confirms that simvastatin has anticancer potential. The observed decrease in the number of viable cells induced by the isolated compounds or simvastatin may be the result of inhibition of cell proliferation and/or induction of cell death.

Due to the structural similarity of UA or OA and cholesterol, we believe that these compounds could replace cholesterol and alter membrane and lipid raft composition and lipid raft dependent signaling. Simvastatin was used because, being a statin, it blocks the enzyme HMG CoA reductase that is responsible for the limiting step in the synthesis of cholesterol. M β CD was used because it induces cholesterol efflux from the plasma membrane by binding the molecule with high affinity, disrupting the structure of membrane lipid rafts (George and Wu, 2012). Both the compounds were used because they interfere with the amount of cholesterol, one by decreasing endogenous synthesis and the other through direct removal.

Through quantification of total cellular cholesterol we found that M β CD and simvastatin decrease the TCC as expected. We found also that despite their similarity, UA and OA have opposite effects on TCC levels, because after 72h UA decreases and OA increases TCC (figure 4.2B). In literature it has been described that in Caco-2 cells (24h) both UA and OA are capable of inhibiting acylCoA:cholesterol acyltransferase

(enzyme required for the storage of cholesterol), and in vivo promotes the reduction of cholesterol plasma levels (Lin et al., 2009). In our study only UA decreased TCC. It has also been reported that the UA has the ability to increase the mRNA expression of cholesterol 7-hydroxylase and to accelerate the conversion of cholesterol into bile acid in BRL cells (Xue et al., 2006), which may explain the reduction in TCC levels. The increase of TCC levels in cells treated with OA can be explained based on a study performed by *Wong et al.* which show that treatment of HepG2 cells with OA modulates the expression of several FXR target genes, such as the suppression of CYP7A1, a rate-limiting bile acid biosynthetic enzyme (Liu and Wong, 2010). When using the M β CD with UA or OA the effects of total cellular cholesterol were similar to effects of UA and OA alone. The combination of M β CD with OA or UA in ensured that this triterpenoids were in fact delivered to the cell. It is important to refer that this TCC evaluation is not specific for lipid rafts cholesterol providing, therefore no information specifically on changes in lipid raft cholesterol. We also have investigated whether the UA and OA have some similarity to M β CD and Simvastatin on the expression of proteins involved in regulation of lipid metabolism. In our results, none of the compounds tested appear to interfere with the expression levels of total SREBP1 and total SREBP2 at 16h (supplementary material). The expression of FAS (fatty acid synthase) protein, which is transcriptionally regulated by SREBP1 in response to insulin, also didn't change. Nevertheless, these results may also be related to the time and concentrations tested, since a study by *Changhao Sun* and coworkers showed that only after 6 days of incubation with UA (10 μ M) was there a decrease of the expression of SREBP1 and its target FAS in 3T3-L1 adipocytes cells (He et al., 2013).

Many studies suggest that cancer cells have elevated cholesterol levels when compared with normal tissues, especially in solids tumors (like prostate and breast). The cancer cells requirement of high cholesterol levels may have two explanation: cancer cells have an up regulation activity of the enzyme HMG CoA reductase that catalyzes the cholesterol biosynthetic pathway (Calleros et al., 2009) or that tumor cells depend on more intensive proliferative signaling that are dependent on the stability of the plasma membrane (Li et al., 2006) . Therefore, its important verified if

5. Discussion and Conclusions

the alterations caused by these two triterpenoids in TCC may induce modifications on lipid raft-dependent signaling.

Several authors have studied the effect of the alteration in cholesterol levels in pro and anti-apoptotic signaling pathways (Zhuang et al., 2002; Park et al., 2009; Onodera et al., 2013; Xu et al., 2009). A study by *Scheinman et al.* shows that the addition of cholesterol causes an increase in proliferation compared to the control, while the addition of M β CD blocks the proliferative state of the cells (Scheinman et al., 2013). However, another study showed that disruption of lipid rafts with this compound prevents disruption of the mitochondrial transmembrane potential and apoptosis (Gajate et al., 2009). Our results show that M β CD in 24h of incubation (with and without TNF- α) does not induce apoptosis (figure 4.3B). This result may however be due to the fact that cholesterol quantification also did not show reduction at 24h or because M β CD is fast acting and the 24h incubation time is too long for the compound and the cell restore homeostasis.

TNF- α also induces Fas-mediated apoptosis and this could also be an explanation for these result, since the integrity of lipid rafts is required for proper functioning of Fas receptor. Furthermore, a study by *Xu* and co-workers showed that treatment with a triterpenoid (Avicin D) allowed translocation of Fas to lipid rafts with activation of its apoptotic signaling pathway, whereas treatment with M β CD suppressed the pathway. In same study, the authors showed that Avicin D inhibited NF-KB and its associated functions, which indicated that activation of caspase-8 by Avicin D does not occur via the TNFR1 (Xu et al., 2009). Also for UA, already studies have shown that this compound can inhibit the activation of NF-kB induced by TNF- α in different cell lines dose-dependently (Shanmugam et al., 2013; Shishodia et al., 2003). Since our results show no significant alterations at 24h in total cellular cholesterol (figure 4.2A) and both UA and OA have similar effects in response to TNF- α with a significantly increase in induction of death by TNF- α (figure 4.3B), this increased of apoptosis can be associated with the ability of these compounds has action similar to Avicin D and allow Fas-mediated apoptosis induced by TNF- α . We observed that the UA appears to have no significantly effect on death, but when it has a transporter (M β CD) that takes it to the membrane, the effect increases. But in return, the

M β CD+UA do not induce any changes in total cholesterol levels. This effect may indicate that this complex induces cell death does not increase or decrease in cellular cholesterol, but induce a rearrangement of the membrane which allows a better receptor signaling. The apoptosis verified by the M β CD+cholesterol in our results is consistent with a study performed by *Boch et al.* In this study the authors demonstrated that high levels of cholesterol can increase mitochondrial membrane condensation, due to deficiency in caveolins, proteins that bind cholesterol, promoting an increase of influx and accumulation of free cholesterol. Subsequently, leads a decreased efficiency of the respiratory chain and intrinsic antioxidant defenses leading to accumulation of ROS resulting on cell death (Bosch et al., 2011). Several studies indicate that cholesterol depletion induces apoptosis through the inhibition of such proliferative pathways, for example a study with A431 cells, showed that the use of simvastatin causes a reduction in the formation of rafts, AKT inhibition and apoptosis at 48h (Chang et al., 2013). In our study we found that for the simvastatin apoptosis does not occur at 24h, but again this demonstrates that statins requires more time incubation to inhibit the endogenous pathway and destabilize the cell membrane by depletion cholesterol. Thus, this result was expected. A study by *Calleros et al.* showed that the oncogenic RAS protects apoptosis induced by depletion of cholesterol. These authors showed that oncogenic RAS suppresses JNK pathway activation induced by low cholesterol levels through the constitutive activation of PI3K/AKT pathway (Calleros et al., 2009)

The tyrosine kinase receptors are also localized in lipid rafts and when activated by ligand binding lead to activation of RAS protein, which in turn can induce MAPK and/or PI3K/AKT pathways (Pike, 2005). In most cases, the depletion of cholesterol seems to reduce the activity of these growth factor receptors and thus their effector pathways, which seem to be consistent with the idea that cholesterol in lipid rafts has a role in proliferative signaling (Hryniewicz-Jankowska et al., 2014). The only exception found to this rule is EGFR for which it is clear that cholesterol depletion leads to an increase in receptor binding and kinase activities as well as the enhancement of downstream signaling pathways (Furuchi and Anderson, 1998; Ringerike et al., 2002). However, in our study we could not observe this effect since when we incubated EGF

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to cells treated with M β CD, simvastatin and UA at different times (15, 30 and 60min) we did not observe any change in the levels of the downstream MAPK effector p-ERK and total ERK relative to control (data not shown).

The insulin receptor is also a growth factor receptor present in raft domains. A previous study by *Gustavsson et al.* showed that treated isolated rat adipocytes with M β CD resulted in a decrease in the amount of insulin-stimulated ATP citrate lyase phosphorylation, indicative of decreased insulin signaling. In addition, the authors showed that insulin-mediated glucose uptake was also abrogated by M β CD treatment in both isolated adipocytes and 3T3-L1 cells. When cholesterol content was then restored in these cells, insulin-mediated protein phosphorylation returned to normal, and lipid rafts were again morphologically recognizable (*Gustavsson et al.*, 1999). These findings were confirmed by another group (*Parpal et al.*, 2001) who showed that disruption of lipid rafts by depletion of cholesterol attenuated insulin-mediated glucose uptake in 3T3-L1 adipocytes cells without affecting insulin receptor number or the ability of insulin to bind to its receptor. Furthermore, it was demonstrated that IRS-1 phosphorylation and activation of the downstream kinase AKT was inhibited by M β CD treatment that did not decrease activation of MAPK pathway. Our results confirm this previous study, since the level of p-ERK addition not being changed when induced with insulin (relative to control without insulin) did not differ significantly when exposed to various compounds tested (figure 4.4B and D). However the same does not happen for p-AKT levels. M β CD at 24h but not at 72h has increased p-AKT protein, which may again mean that the cells are trying to regain its homeostasis, due to rapid action of the compound. This result may also be explained by the fact that the cells in insulin response activate the AKT pathway that can control transcription, stability and abundance of SREBPs (*Krycer et al.*, 2010; *Xiao and Song*, 2013). So in consequence leads to cholesterol synthesis for combat the failures caused by the compound in their membrane integrity (figure 5.1). Some studies indicate that simvastatin reduces the AKT phosphorylated by insulin without affecting total AKT in A549 cells at 48h (*Miraglia et al.*, 2012), and that UA decreases in dose and time-dependent the levels of PI3K and p-AKT in SNG-II cells (*Achiwa et al.*, 2007). Our results also show that simvastatin reduces the p-AKT both at 24h and 72h (figure 4.4-A and C).

While for UA the decrease in p-AKT is only observed at 24 h. These results seem to demonstrate that the IR is dependent on the integrity of lipid rafts also in HCT116 cells, once simvastatin is a drug that leads to depletion of cholesterol, and the effect of this drug seem decrease de activation on the AKT pathway may be by for disruption of membrane. Thus we conclude that the UA could possibly have an action method similarly to simvastatin.

For the OA our results show a significant and time-dependent increase in AKT phosphorylation (figure 4.4A and C). This result therefore did not come agree with a study conducted by *Wang et al.* in hepatic cells of mice treated during two weeks with OA. The authors showed that there is a decrease in p-AKT in response to insulin, (*Wang et al.*, 2013). The difference obtained for the levels of p-AKT protein between our results and *Wang et al.* may be related with the experimental model and/or the concentrations used. However in another study performed by *Castellano et al.* indicate that OA is a stimulator of insulin-induced AKT pathway in vascular smooth muscle cells. The same authors showed that OA binds to IR is not the insulin binding site but in the β -subunits of the receptor, and indicating that OA has benefits in the insulin receptor and its subsequent pathways (*Castellano et al.*, 2013). Nevertheless, we can also correlate the increase of p-AKT with membrane stability. As stated, the insulin receptor seems to need the stability of lipid rafts to produce its metabolic processes, and therefore as OA appears to increase total cellular cholesterol in our cells at 72h, it may be the increased p-AKT levels is related to the stability provided by increase cholesterol in cells thus allowing a better insulin signaling. Our results suggest that OA is a potential anti-diabetic agent with pharmacological properties for insulin resistance, but for anticancer potential this result is not satisfactory because the increase of p-AKT levels result an increased energy, subsequent degradation and increased proliferation. AKT phosphorylation is also a signal inhibitory of cell death by apoptosis.

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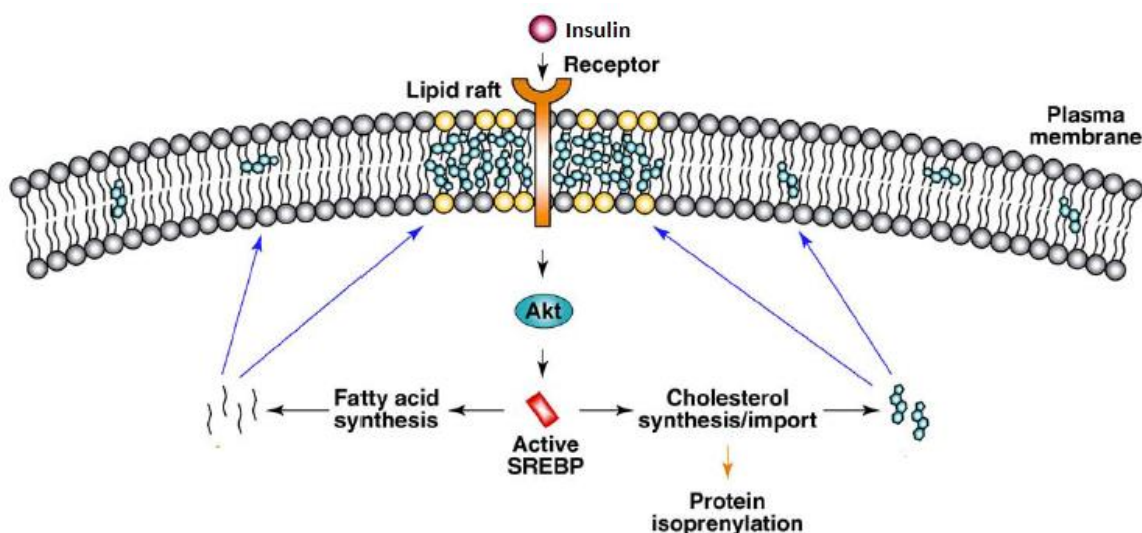


Figure 5.1 – Effect of insulin in AKT signaling pathway. Adapted from (Krycer et al., 2010)

Taken together, our results suggest that UA have more potential anticancer activity than OA. UA decrease the cellular viability in dose dependent manner resulting in IC_{50} lower than OA. UA also inhibit the AKT pathway in response to insulin. These results seem to reflect the effect that UA has decreasing cholesterol quantity. It should also be mentioned that the anticancer potential increases when UA is combined with M β CD, because it increases the apoptotic cells.

5.2 – Conclusions

Triterpenoids such as ursolic acid and oleanolic acid are natural compounds found in many fruits and herbs, and have been attributed many health improving effects including anti-tumor effects. Furthermore they have a chemical structure similar to cholesterol, so our study focused in evaluating the effects on the cholesterol content of the cells and what are their consequences on cell survival and death.

In our studies we found that UA reduce the amount of cholesterol at 72h while the OA increases cellular cholesterol levels at 72h. Our results also indicate that UA sensitizes more the cells to cell death than OA. In many studies is demonstrate that

cholesterol depletion of lipid rafts promote the apoptosis and cholesterol addition stimulate the proliferation in TNFR, but this is dependent of death receptor type because for Fas receptor the effects are antagonistic. In our results we observe that depletion of cholesterol by M β CD and simvastatin not induces apoptosis, but the cholesterol delivery to cells increased apoptosis suggesting that this effect is Fas receptor dependent. The decrease in cell viability found by UA and OA does not seem to be caused by apoptosis. In contrast when combined with TNF- α , both compounds increased cell death by apoptosis relative to control, which may mean that they in some way modify the stability of the membrane in order to enhance the death receptor signaling and that only activated in the presence of ligand. We can also see that the effect of the UA is enhanced when complexed with M β CD, a transporter that leads the compound to regions of cholesterol-rich membrane.

The results obtained for total cholesterol levels may be related to those found for AKT pathway induced by insulin, because OA increases insulin signaling with induction of expression of FAS (data not shown). On the other hand the UA is less effective since it decreases expression of p-AKT to 24h and FAS, which is good to prevent accumulation of fats.

The insulin signaling pathway is involved in enhancing the proliferation of tumor cells, facilitating the uptake of glucose and sending survival signals. Thus, our results show that UA, but not OA, decreases phosphorylation of AKT protein by destabilization of the plasma membrane. This can be beneficial for the treatment of colorectal carcinoma because besides reducing the signs of cell proliferation also increase death.

In conclusion our results suggest that UA have more anticancer potential than its isomer OA. It is possible that UA may be used in combination with statins, because statins also possess anticancer activity due to inhibition of cholesterol synthesis and decrease the anchoring of RAS protein (which is verified by the reduced activity of p-AKT). Thus, the combination of these two compounds can decrease the dose and toxicity associated to statins increasing its effect. All these aspects reveal a certain capacity of triterpenoids to affect cholesterol homeostasis and associated cellular

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functions, which may be explored as a novel approach to disrupt the survival of resistance of tumor cells.

5.3 – Future Perspectives

Our results show that both triterpenoids UA and OA alter the quantity of cholesterol in HCT116 cells, and these alterations may impact changes verified in the insulin-induced AKT pathway. However, it is necessary to investigate whether the decrease in TCC produced by the UA and the increase by OA is caused by these compounds interference directly on endogenous cholesterol pathway or by causing a rearrangement in lipid rafts of the plasmatic membrane. For that more studies are needed, in particular to evaluate if the compounds alter the activity of HMG CoA Reductase responsible for the mevalonate pathway that leads to production of cholesterol and farnesyl pyrophosphate groups. These groups are essential for anchorage proteins to plasmatic membrane, such RAS protein, and therefore the activity of this protein should also be evaluated.

In addition, it is known that cholesterol is the major component of lipid rafts and these microdomains are important for signaling of certain receptors, then it would be appropriate to proceed to the isolation of lipid rafts to verify whether these compounds change the lipid/protein composition and the position of the receptors for domains not rafts.

Our results also show that both UA and OA induce apoptosis only when cells were exposed to TNF- α at 24h, and therefore it would be necessary to determine whether these effects occur by increased caspase-8 activity or if death occurs in another pathway. In posteriors studies it is also necessary repeat the nuclear condensation assay at 72h of incubation time with compounds to compare the results obtained at 24h.

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7. ANNEXES

Annex A

The cells were incubated 16h with UA (10 μ M) and OA (20 μ M) in concentrations obtained from the MTT assay, the Simvastatin with 5 μ M and M β CD with 1.5mM. The effects of 16h exposed to compounds on SREBP1, SREBP2 and FAS levels were quantified by western blot. As noted in figure A, any of the compounds appear to interfere with the expression levels of total SREBP1, total SREBP2 and FAS since the differences are not significant relative to control.

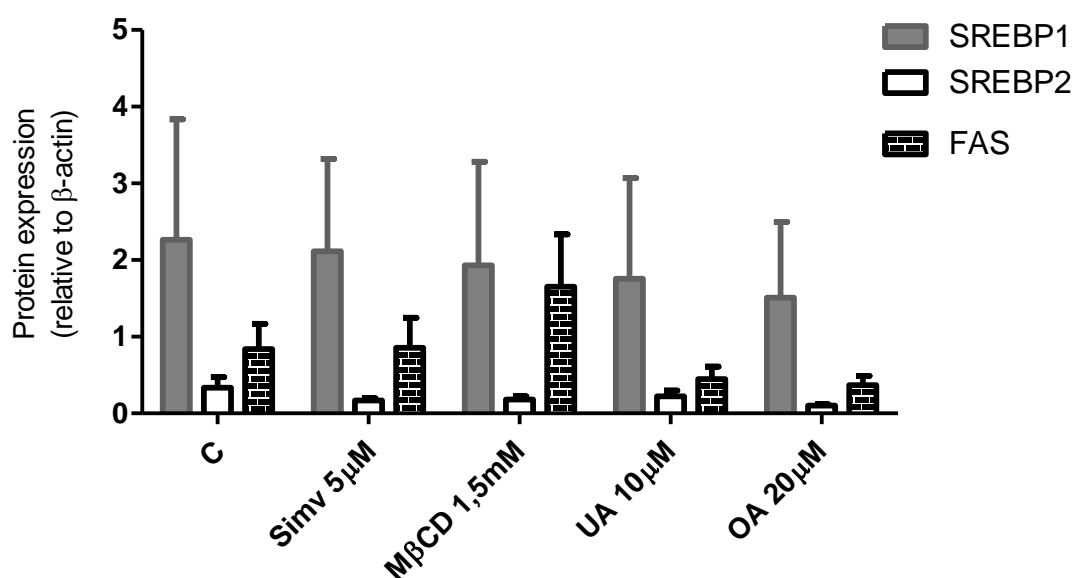


Figure A - Effect of 16h of incubation with triterpenoids, simvastatin and M β CD on SREBP1, SREBP2 and FAS protein expression, in HCT116 cells. The protein levels were evaluated by Western Blot. As loading control we used β -actin. For each condition three independent experiments were and data are expressed as mean \pm SEM. Significant differences were found, by one way ANOVA, when compared with respective control.

