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# THE RAS/RAF AND AKT/MTOR PATHWAYS AS THERAPEUTIC TARGETS IN DIFFUSE LARGE B-CELLS LYMPHOMA

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# The RAS/RAF and AKT/mTOR pathways as therapeutic targets in diffuse large B-cells lymphoma

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## ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is a common type of non-Hodgkin lymphoma, accounting for about 25% to 30% of all lymphoma cases in Western countries. It is a clinically aggressive lymphoma in which the patients have a five-year survival rate of 50%. Several intracellular pathways are related to lymphomagenesis, two of the most frequently involved being: the BCR/PI3K/AKT/mTOR (B-cell receptor/phosphatidylinositol-3-kinase/AKT protein kinase B/mammalian target of rapamycin) pathway and the RAS/MAPK (rat sarcoma/mitogen-activated protein kinase) pathway. RAS proteins are activated by farnesylation mediated by the farnesyltranferase enzyme.

The aim of this study is to evaluate the therapeutic potential of a mTOR inhibitor – Everolimus – and a RAS/RAF (rat sarcoma/rat fibrosarcoma) pathway inhibitor – L744,832 – in DLBCL. For the purpose we used a DLBCL cell line, the Farage cells, cultured both in the absence and presence of several concentrations of Everolimus and L744,832 in monotherapy and in combination with each other, as well as in association with the conventional chemotherapy drug Vincristine. Cell growth and viability were evaluated by the rezasurin assay. Drug effectiveness was determined by a dose-response curve and $IC_{50}$ determination. Cell death was investigated by optical microscopy using May-Grünwald staining and by flow cytometry through annexin-V and propidium iodide double staining. The mechanisms involved in the antiproliferative effect and cell death were analyzed by flow cytometry (through the expression of Ki-67 and lamin A/C) and by proteins involved in apoptotic pathways (i.e. Caspase 3), respectively.

Our results show that Everolimus and L744,832 induce cell death in a time- and dose-dependent manner, with $IC_{50}$ values of 25 µM after 24 hours for Everolimus and values ranging from 50 µM to 75 µM after 24 hours for L744,832. A synergistic effect was observed when the drugs were used in combination, since the $IC_{50}$ was attained at lower doses than in monotherapy. In all tested conditions, besides an antiproliferative effect, these compounds also induced cell death, mainly by apoptosis, confirmed by optical microscopy and flow cytometry that may be mediated by Caspase 3.

The inhibition of farnesyltransferase induced by L744,832 is confirmed by a dose-dependent decrease in the expression of lamin of about 40% and 62% for 12.5 µM and 75 µM, respectively, compared with untreated cells (control cells). The decrease was also significant in the association with Vincristine, as well as in combination with Everolimus and Vincristine, amounting to values of 64% and 47%, respectively. The antiproliferative effect observed in cells treated with the mTOR inhibitor Everolimus is confirmed by a dose-dependent decrease in the expression of Ki-67, compared with control cells, of about 37% and 51% for 15 µM and 25 µM, respectively. In the association with just Vincristine and with both L744,832 and Vincristine, the decrease was slightly less.

In summary, our results suggest that Everolimus and L744,832, alone or in combination with Vincristine, could be used as a new therapeutic approach in DLBCL. However, other schemes of drug administration, as well as new associations need to be tested prior to advancing to other study phases.

## RESUMO

O linfoma difuso de grandes células B (LDGC-B) é um subtipo comum de linfomas não-Hodgkin, representando cerca de 25 a 30% de todos os casos destas neoplasias hematológicas nos países ocidentais. É um linfoma clinicamente agressivo, no qual os pacientes têm uma sobrevivência aos 5 anos de 50%. Diversas vias de sinalização intracelulares estão relacionadas com a linfomagénese e duas das mais frequentemente envolvidas são a via BCR/PI3K/AKT/mTOR (*B-cell-receptor/phosphatidylinositol-3-kinase/AKT protein kinase B/mammalian target of rapamycin*) e a via RAS/MAPK (*rat sarcoma/mitogen activated protein kinase*). De notar que as proteínas RAS são activadas por farnesilação mediada pela enzima farnesiltransferase.

O objectivo deste estudo é avaliar o potencial terapêutico de um inibidor do mTOR (Everolimus) e de um inibidor da via RAS/MAPK (L744,832) no LDGC-B. Com este propósito, usamos uma linha celular de LDGC-B, a linha celular Farage, incubada na ausência e na presença de várias concentrações de Everolimus e de L744,832, em monoterapia e em associação entre si e com um fármaco de quimioterapia convencional, a Vincristina. O crescimento e a viabilidade celular foram avaliados pelo método da rezasurina. A eficácia dos fármacos foi determinada pela curva dose-resposta e pela determinação do $IC_{50}$. A morte celular foi investigada usando microscopia óptica com coloração de May-Grünwald e por citometria de fluxo, através da dupla coloração com anexina-V e iodeto de propídio. Os mecanismos envolvidos no efeito antiproliferativo e na morte celular foram analisados por citometria de fluxo, através da expressão de Ki-67 e de laminas A/C, e por proteínas envolvidas em vias de apoptose (i.e. a Caspase 3), respectivamente.

Os nossos resultados mostram que o Everolimus e o L744,832 induzem a morte celular de uma forma dependente do tempo e da dose, com valores de $IC_{50}$ de 25 µM após 24 horas para o Everolimus e valores entre 50 µM e 75 µM após 24 horas para o L744,832. Um efeito sinérgico foi observado quando os fármacos foram usados em associação, pois o $IC_{50}$ foi obtido com doses mais baixas relativamente aos obtidos em monoterapia. Em todas as condições testadas, para além do efeito antiproliferativo, estes agentes também induziram morte celular, principalmente por apoptose, confirmada por microscopia óptica e citometria de fluxo, que poderá ser mediada pela Caspase 3.

A inibição da farnesiltransferase induzida pelo L744,832 é confirmada por uma diminuição dependente da dose da expressão de laminas, comparada com as células não tratadas (células de controlo), de cerca de 40% e 62% para concentrações de 12,5 µM e 75 µM, respectivamente. A diminuição também foi importante na associação com a Vincristina e com o Everolimus e a Vincristina, atingindo valores de 64% e 47%, respectivamente. O efeito antiproliferativo observado nas células tratadas com o inibidor do mTOR (Everolimus) é confirmado por uma diminuição dependente da dose da expressão de Ki-67, em comparação com as células do controlo, de cerca de 37% e 51% para concentrações de 15 µM e 25 µM, respectivamente. Na associação com a Vincristina e com o L744,832 e a Vincristina, a diminuição foi ligeiramente inferior.

Concluindo, os nossos resultados sugerem que o Everolimus e o L744,832, em monoterapia ou em associação com a Vincristina, podem ser usados como uma nova abordagem terapêutica no LDGC-B. No entanto, outros esquemas de administração e novas associações devem ser testados previamente ao avanço para outras fases do estudo.

## Abbreviations:

4E-BP – 4E-binding protein

ABC – activated B-cell like

AKT – protein kinase B

AV – annexin V

BCL-2 – B-cell lymphoma 2

BCL-XL – B-cell lymphoma-extra large

BCR – B-cell receptor

BSA – bovine serum albumin

BTK – Bruton tyrosine kinase

cIAP – cellular inhibitors of apoptosis

CTL – control (cells)

DLBCL – diffuse large B-cell lymphoma

ERK – extracellular signal-regulated kinase

EVE – Everolimus

FC – flow cytometry

FKBP12 – FK506 binding protein

FKBP51 – FK506-binding protein 51

FTI – farnesyltransferase inhibitor

$G_0/G_1$ – gap 0/gap 1

$G_2/M$ – gap 2/mitosis

GAP – GTPase-activating protein

GCB – germinal center B-cell-like

GDP – guanosine diphosphate

GEF – guanine nucleotide exchange factor

GTP – guanosine triphosphate

HIF-1 – hypoxia-inducible factor 1

IKK – IκB kinase

IP3 – inositol triphosphate

JAK – Janus kinase (*just another kinase)*

MAPK – mitogen-activated protein kinase

MCL-1 – myeloid cell leukemia sequence 1

MEK/MAPKK – mitogen-activated protein kinase kinase

MIF – mean intensity fluorescence

mTOR – mammalian target of rapamycin

mTORi – mTOR inhibitor

NF-κB – nuclear factor kappa B

NHL – non-Hodgkin lymphoma

PBS – phosphate-buffered saline

PE – phycoerythrin

PI3K – phosphatidylinositol-3-kinase

PKCβ – protein kinase C beta

PLC – phosphosfolipase C

PMBL – primary mediastinal B-cell lymphoma

RAF – rapidly growing fibrosarcoma or rat fibrosarcoma

RAS – rat sarcoma

RTK – receptor tyrosine kinase

S/G$_2$/M – gap 2/mitosis/synthesis

S6K1 – S6 ribosomal protein kinase

SD – standard deviation

STAT – signal transducers and activators of transcription

STAT3 – signal transducer and activator of transcription 3

SYK – spleen tyrosine kinase

TP53 – tumor protein 53

VCR – Vincristine

VEGF – vascular endothelial growth factor

# 1. INTRODUCTION

Lymphomas are lymphoproliferative malignancies arising at multiple stages of normal lymphoid cell development. They can be divided in Hodgkin or non-Hodgkin lymphoma (NHL) and, according their clinical evolution, they can be indolent, aggressive and very aggressive.

Diffuse large B cell lymphoma (DLBCL) is the most common type of NHL, accounting for approximately 25% to 30% of these hematological neoplasias in Western countries (Stein, H. *et al*., 2008) and for 30% to 40% of newly diagnosed lymphomas (Lenz, G. *et al*., 2010).

It can appear at any age, being more frequent after 60 years old. The five- year survival rate is 50%. Though it can to involve T cells and natural killer cells (NK cells), the DLBCL is more frequently a B-cell neoplasia, in particular of germinal center centroblasts. It is a clinically aggressive lymphoma with a high rate of cellular proliferation, being able to progressively infiltrate the lymph nodes, the bone marrow, the gastrointestinal tract, the central nervous system, the kidneys and other organs (Stein, H. *et al*., 2008).

DLBCL is a heterogeneous entity in terms of morphological, biological and clinical features. Gene expression profile studies allowed for the division in three molecular subtypes: germinal center B-cell like (GCB-LDGC), activated B-cell like (ABC-LDGC) and primary mediastinal B-cell lymphoma (PMBL) (Lenz, G., 2010).

Several intracellular pathways are related to lymphomagenesis such as the BCR/PI3K/AKT/mTOR (B-cell receptor/phosphoinositide-3-kinase/AKT-protein kinase B/mammalian target of rapamycin) pathway, the RAS/MAPK (rat sarcoma/mitogen-activated protein kinase) pathway (Figure 1), the IP3/PLC

(Inositol 1,4,5-triphosphate/phospholipase-C) pathway (Figure 1) and, less often, the JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathway (Parcells, B.W. *et al.*, 2006; Small, D., 2006; Oliveira, A.R.C., 2008; Morschhauser, F. *et al.*, 2010).

The RAS/RAF/MEK or MAPK pathway is a potential target for the development of therapeutic agents in NHL. The RAS proteins are located at the internal surface of the cell membrane by addition of a farnesyl group at the carboxylic terminal. This allows the interaction of the protein with the hydrophobic layer of the cell membrane which is critical to the conversion of RAS into a biologically active form. These modifications are essential to their function by binding and hydrolyzing guanosine triphosphate (GTP), transforming RAS proteins from their active form, when bound with GTP to the inactive guanosine diphosphate (GDP) form (Figure 1). Through the receptor tyrosine kinases (RTKs) and other membrane receptors, several extra-cellular ligands promote the active state coupled with GTP, regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs).

The MAPK pathway is involved in the regulation of proliferation and differentiation of normal hematopoietic cells. The role of this pathway in the pathogenesis of lymphomas is indicated by studies in cells lines derived from lymphomas. Moreover, some studies have shown that this pathway may provide a successfully therapeutic target at two major levels, the RAS and RAF proteins (Hachem, A. and Ronald, B.G., 2005).
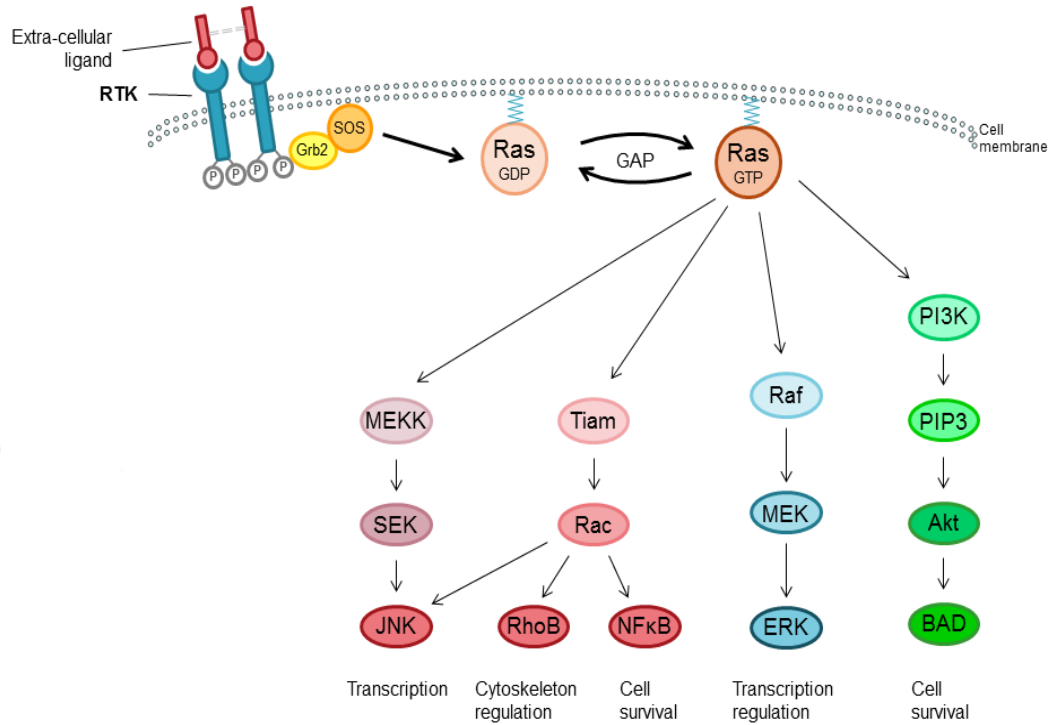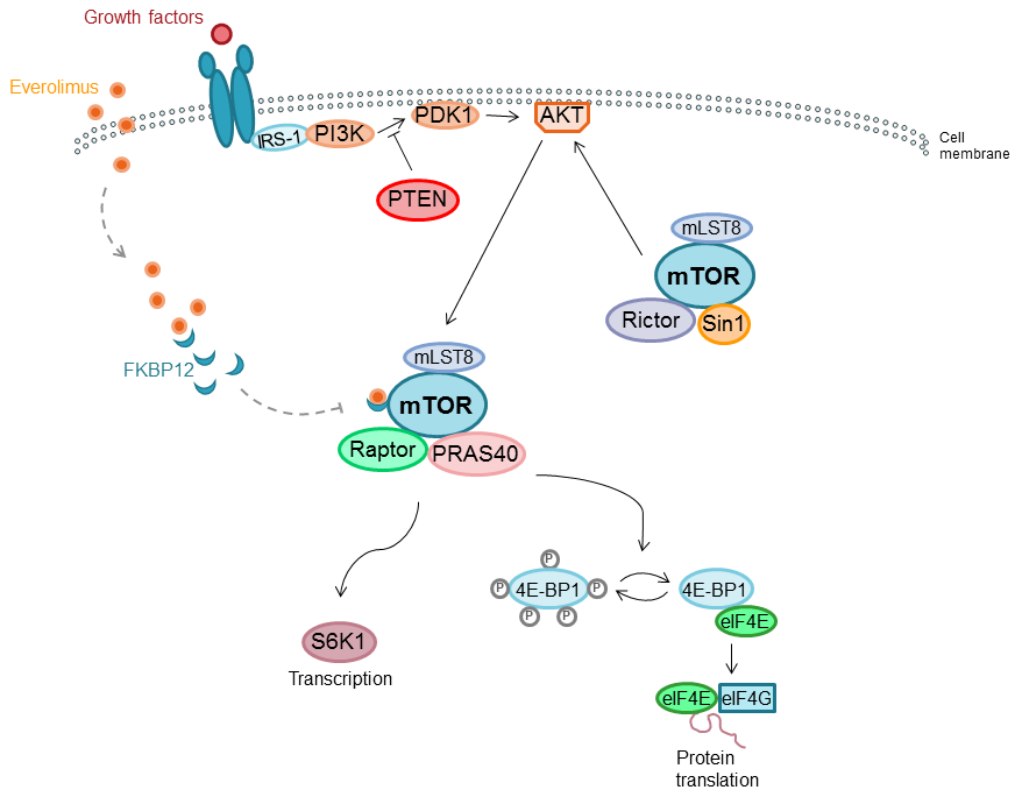
**Figure 1: Signaling pathways downstream of RTK and RAS protein activation**.
Activation of the receptor induces activation of several signaling pathways,
including PI3K/AKT and MAPK pathways, where the protein RAS plays an
essential role (adapted from Stirewalt, D.L. and Radich, J.P., 2003).

The mTOR signaling pathway (Figure 2) is deregulated in many cancers, such as the
DLBCL. Everolimus is an inhibitor of mTOR, a serine-threonine kinase, downstream of
the PI3K/AKT pathway. This drug binds to an intracellular protein, the FKBP12,
resulting in the formation of a complex that inhibits the mTOR kinase activity.
Consequently, a decrease of two effectors involved in protein synthesis, the S6
ribosomal protein kinase (S6K1) and the eukaryotic elongation factor 4E-binding
protein (4E-BP), occurs. In addition to this effect, it has been demonstrated that
Everolimus inhibits the expression of the hypoxia-inducible factor 1 (HIF-1) and
vascular endothelial growth factor (VEGF) (Mead, H. *et al.*, 2010). It has also been

proven that inhibition of mTOR by Everolimus reduces cell proliferation (Easton, J.B. *et al.*, 2006).



**Figure 2: AKT/mTOR signaling pathway**. The inhibition of mTOR downstream may be achieved by binding Everolimus/FKBP12 to the mTOR complex. The arrows represent activation and the bars represent inhibition (adapted from Yang, Q. and Guan, K.L., 2007).

Nowadays, DLBCL conventional treatment differs between patients with localized or advanced disease and consists of chemotherapy doxorubicin regimens followed by radiotherapy. The protocol consisting of Cyclophosphamide, Hydroxydaunorubicin (also called Doxorubicin), Oncovin (Vincristine) and Prednisolone (also known as CHOP) is indicated as a first-line therapy in NHLs. More recently, the association of CHOP with Rituximab (CHOP-R), an anti-CD20 monoclonal antibody, has shown more positive results. In certain cases, it is possible to resort to bone marrow transplantation

(Vose, J.M. *et al*., 2002; Hennessy, B.T. *et al*., 2004; Hoffbrand, A.V. *et al*., 2006; Castagna, L. *et al*., 2007; Michallet, A.S. *et al*., 2009).

Although progress has been made in the development of new drugs, particularly new formulations of conventional anticancer drugs, these continue to show reduced effectiveness in certain types of cancer and serious adverse effects, owing in part to the reduced drug specificity. It should be noted that cancer cells may often be resistant to the action of drugs, either right from the beginning of treatment or after an initial period of response (Begonha, R. and Azevedo, I., 2006). Therefore, the knowledge of the molecular mechanisms involved in proliferation, differentiation and cell death may contribute to the development of therapeutic strategies aimed at neoplastic cells and thus less toxic.

Given that the inhibition of mTOR by Everolimus may interrupt downstream the PI3K/AKT pathway and that the activation of the RAS protein is dependent on the addition of a farnesyl group to the terminal carboxylic of the protein by the farnesyltransferase enzyme, the inhibition of this enzyme may interrupt the RAF/MEK/ERK pathway and could constitute a new therapeutic target in this type of lymphoma.

## 2. AIMS

The aim of this study is to evaluate the therapeutic potential of a RAS/RAF pathway inhibitor – L744,832 – and a mTOR inhibitor – Everolimus – through monotherapy and/or in combination with each other and in association with conventional antiproliferative drugs in a DLBCL cell line.

# 3. MATERIALS AND METHODS

## 3.1 Cell culture conditions

We used a DLBCL cell line, the Farage cells, provided by American Type Culture Collection (ATCC). The cell line was routinely grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640), containing 2 mM L-glutamine, 25 mM HEPES-Na, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen) supplemented with 20% heat-inactivated foetal bovine serum (Gibco, Invitrogen). Cells were seeded at a density of 500000 cells/ml and kept in culture at 37ºC in a humidified atmosphere with 5% $CO_2$.

In order to evaluate the antiproliferative and cytotoxic effect of Vincristine (VCR), L744,832 and Everolimus (EVE), cells were cultured for 72 hours, in monotherapy, in the absence and presence of VCR (Sigma-Aldrich) in concentrations ranging from 1 nM to 100 nM, L744,832 (Enzo Life Science) in concentrations ranging from 1 µM to 150 µM, and EVE (Sigma-Aldrich) in concentrations ranging from 500 nM to 100 µM. Afterwards, the cells were incubated for 72 hours with L744,832 and EVE, in combination with VCR and with VCR and each other.

## 3.2 Cell viability evaluation

Cell viability was evaluated by the resazurin assay, based on mitochondria enzymes activity. Resazurin was prepared as a stock solution of 100 µg/ml in phosphate-buffered saline (PBS). Stock solution was filtered with a sterile 0.20 µm pore filter and stored in the dark at -20°C. After treatment, a final concentration of 10 µg/ml of resazurin solution was added to the cells, which were then incubated at 37°C for 4 hours. Following this, we collected 200 µl from each well and transferred to 96 well plates. We measured the absorbance at 570 nm and 600 nm colorimetrically, using a

Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments), and calculated cell viability as a percentage of the control cells according to the formula:

$$\frac{[(A_{570} - A_{600})\text{sample}] - [(A_{570} - A_{600})\text{blank}]}{[(A_{570} - A_{600})\text{control}] - [(A_{570} - A_{600})\text{blank}]} \times 100$$

## 3.3 Cell death analysis

Cell death was examined through morphological analysis using optic microscopy and by flow cytometry using annexin and propidium iodide double staining.

### 3.3.1 Optic microscopy

After an incubation period of 48 hours in the conditions described in section 3.1, cells were collected and ressuspended in serum in order to obtain a density of 500000 cells/ml. Then, cells were stained upon incubation for 3 minutes with May-Grünwald solution (0.3% v/v in methanol) (Sigma, St. Louis, MO, USA) diluted in a 1:1 ratio with distilled water, followed by staining with Giemsa solution (0.75% p/v in glycerol/methanol 1:1) (Sigma, St. Louis, MO, USA) diluted 1:8 in distilled water for 15 minutes. After rinsing with distilled water, smears were left to dry at room temperature and cell morphology was analyzed by light microscopy using a Nikon Eclipse 80i microscope equipped with a Nikon Digital Camera DXm 1200F.

### 3.3.2 Flow cytometry analysis

After an incubation period of 48 hours in the conditions described in 3.1, cells were washed with PBS and centrifuged at 400 xg for 5 minutes, in order to obtain a density of 1 x $10^6$ cells/ml. Untreated and treated cells were resuspended in 100 μl of binding buffer and then in 5 μl of annexin V-FITC (AV) and 2 μl of propidium iodide (PI) staining solution (ImmunoStep, Salamanca, Spain) were added. Cells were gently

stirred in a vortex and incubated for 15 minutes at room temperature (25ºC) in the dark. Finally, we add 300 µl of binding buffer to each tube.

Cells were then analyzed in a FACS Calibur (Becton Dickinson) flow cytometer equipped with an argon laser. Green fluorescence of AV was collected with a 525 nm band pass filter and red fluorescence of PI with a 610 nm band pass filter. CellQuest software (Becton Dickinson) was used for the acquisition of data and these were analyzed with the Paint-a-Gate software. Results were expressed in percentages of viable cells ($AV^-/PI^-$), early apoptotic ($AV^+/PI^-$), late apoptotic/necrotic ($AV^+/PI^+$) and necrotic cells ($AV^-/PI^+$).

## 3.4 Evaluation of the mechanisms of cell death

### 3.4.1. Apoptosis-regulating molecules

The expression levels of the apoptosis-regulating molecule (Caspase 3) were assessed by flow cytometry (FC) using monoclonal antibodies labeled with fluorescent probes. Cells cultured in the absence and the presence of VCR, L744,832 and EVE, were incubated with a monoclonal antibody anti-caspase 3-phycoerythrin (PE) (BD Pharmingen, Becton Dickinson), according to manufacturer's protocol. In short, cells were fixed with 100 µl of fix solution (IntraCell; Immunostep) for 15 minutes and washed by centrifugation at 300 xg for 5 minutes. Cells were then permeabilized and incubated for 15 minutes with 100 µl of permeabilization solution (IntraCell; Immunostep) and 1 µg anti-Caspase 3-PE (BD Pharmingen, Becton Dickinson). After washing, cells were analyzed by FC. The results are presented as mean intensity fluorescence (MIF) arbitrary units and represent the medium of fluorescence intensity detected in the cells, which is proportional to the protein concentration in each cell.

## 3.4.2 The molecular mechanisms of drug action

To evaluate the specific mechanisms of the action of each drug, namely farnesyltransferase and mTOR pathway inhibitors, the expression levels of the molecules lamin A/C and Ki-67 were assessed, respectively for L744,832 and EVE, using monoclonal antibodies labeled with fluorescent probes, in a similar way as described in section 3.4.1. Cells cultured both in the absence and presence of VCR, L744,832 and EVE were incubated with monoclonal anti-lamin A/C PE and anti-Ki-67 PE (Santa Cruz Biotechnology) antibodies (Santa Cruz Biotechnology), according to the procedure describe in section 3.4.1.

## 3.4.3 Cell cycle analysis

Cell cycle analysis was performed by FC. Farage cells were incubated in the absence and presence of VCR, L744,832 and EVE. The cells were harvested in order to obtain a density of $1 \times 10^6$ cells/ml and then centrifuged for five minutes at 300 xg. The supernatant was removed and the pellet was resuspended in the residual liquid. Cells were fixed by adding 200 µl of 70% ethanol in the cell suspension slowly while vortexing. Cells were left in ethanol at 4ºC for 30 minutes and then washed in 2 ml PBS + 2% bovine serum albumin (BSA). Cells were then centrifuged again for five minutes at 300 xg and the supernatant was removed and the pellet was resuspended in the residual liquid. Afterwards, 0.5 ml of propidium iodide solution (PI/RNase) was added to the cell pellet and mixed. Cells were incubated for 15 minutes at room temperature. Cell cycle distributions were determined using a FACS Calibur (Becton Dickinson) flow cytometer. The data obtained was analyzed with the ModFit software (Becton Dickinson).

## 3.5 Statistical analysis

All data was statistically analyzed using one-way ANOVA and unpaired Student's t-test and is reported as mean ± standard deviation (SD). Differences were considered statistically significant when $p<0.05$.

## 4. RESULTS

## 4.1 Evaluation of the therapeutic potential of FTIs and EVE – dose and time response curves

With the purpose of evaluating the therapeutic potential of inhibitors of RAS/RAF and AKT/mTOR pathways, Farage cells were cultured in the absence and presence of Everolimus (EVE) and L744,832 for 72 hours, and the antiproliferative effect was evaluated by the resazurin metabolic assay.

Our results show that all of tested drugs caused a decrease in cell viability, in a time and dose-dependent manner. The cytotoxic effect of the farnesyltransferase inhibitor (FTI) L744,832, the mTOR inhibitor (mTORi) EVE and of Vincristine (VCR) are shown in Figures 3, 4 and 5, respectively.

As it can be seen in Figure 3, the FTI induces a decrease in cellular proliferation dependent on the concentration and exposure time. Indeed, the $IC_{50}$ is achieved when the Farage cells are exposed to 50 µM to 75 µM L744,832 for 24 hours, while if exposure is longer (48 hours) the reduction of cell viability by 50% is achieved with lower concentrations (25 µM to 50 µM). However, the cytotoxic effect observed seems to be reversible since cell viability increased slightly after 72 hours of exposure to this inhibitor.
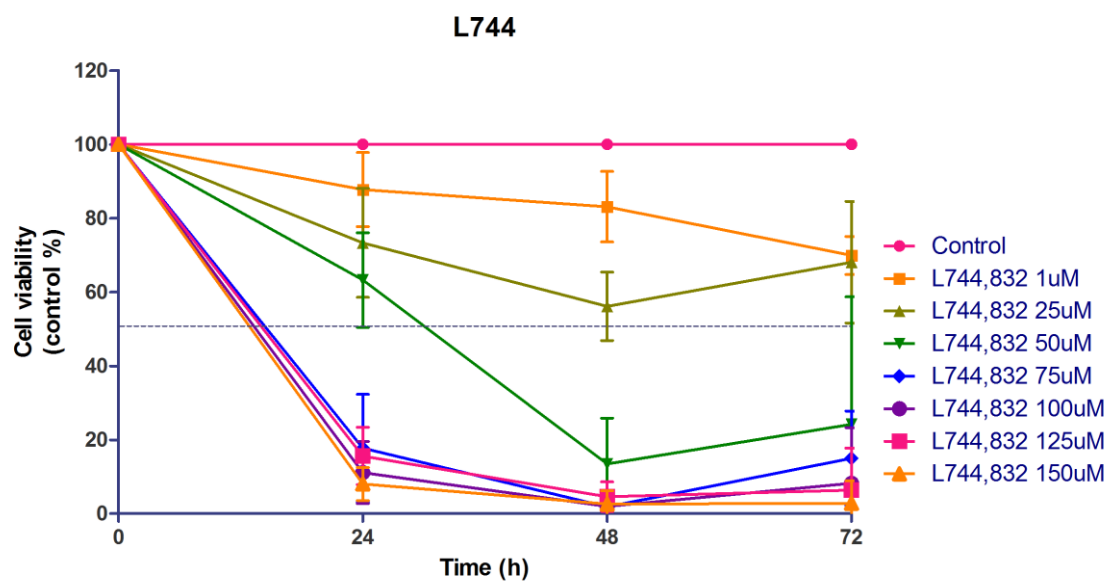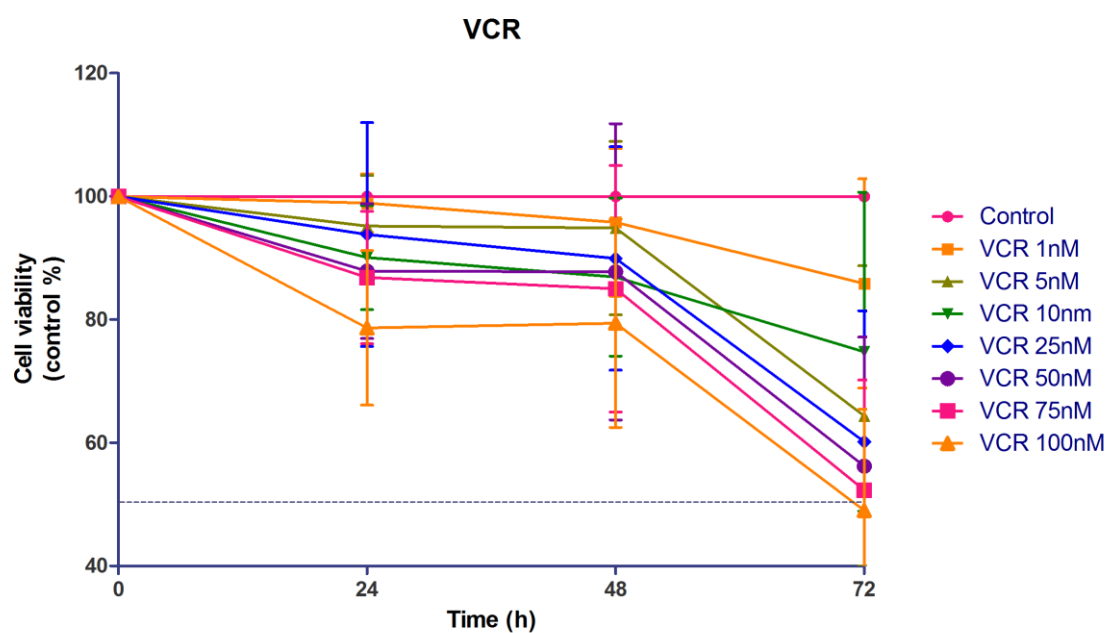
**Figure 3 : L744,832 dose-response curves**. Farage cells were incubated for 72 hours (h) in the absence (control) and in the presence of the L744,832 in the concentrations indicated in the Figure. Cell viability was assessed as described in "Materials and Methods". The results are expressed as percentages (%) compared to control (100%) and represent the average of seven independent experiments ± SD.

As shown in Figure 4, the mTORi EVE induces a decrease in cellular proliferation dependent on the concentration and exposure time. The $IC_{50}$ is achieved when the Farage cells are exposed to 25 μM EVE for 24 hours, while if exposure is longer (48 hours) the reduction of cell viability by 50% is achieved with lower concentrations (500 nM). Nevertheless, the cytotoxic effect observed seems also to be reversible since cell viability increased slightly after 48 hours of exposure to this inhibitor, particularly with lower concentrations (500 nM, 5 μM and 15 μM).
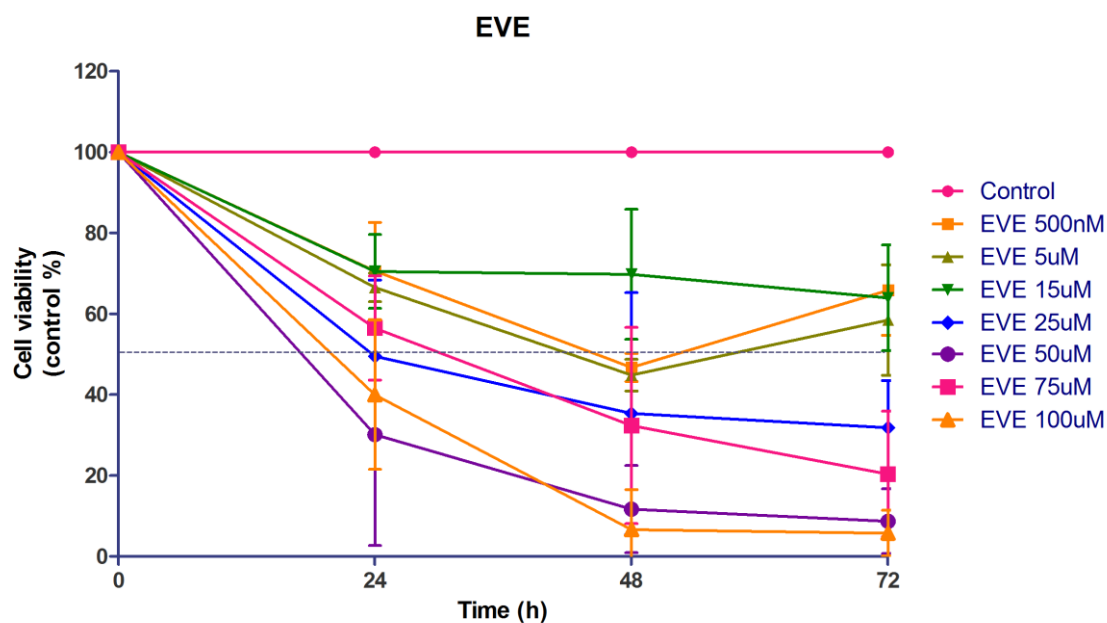
**Figure 4: Everolimus dose-response curves**. Farage cells were incubated for 72 hours (h) in the absence (control) and presence of the mTORi EVE in the concentrations indicated in the Figure. Cell viability was assessed as described in "Materials and Methods". The results are expressed as percentages (%) compared to control (100%) and represent the average of seven independent experiments ± SD.

Similarly, the cytotoxic effect of VCR is dependent of the concentration and time of exposure of cells to this compound. As can be seen in Figure 5, the $IC_{50}$ is achieved when the Farage cells are exposed to 100 nM for 72 hours. However, unlike with the FTI and mTORi, the effect is not reversible, since the decrease in viability is enhanced after 72 hours of incubation.
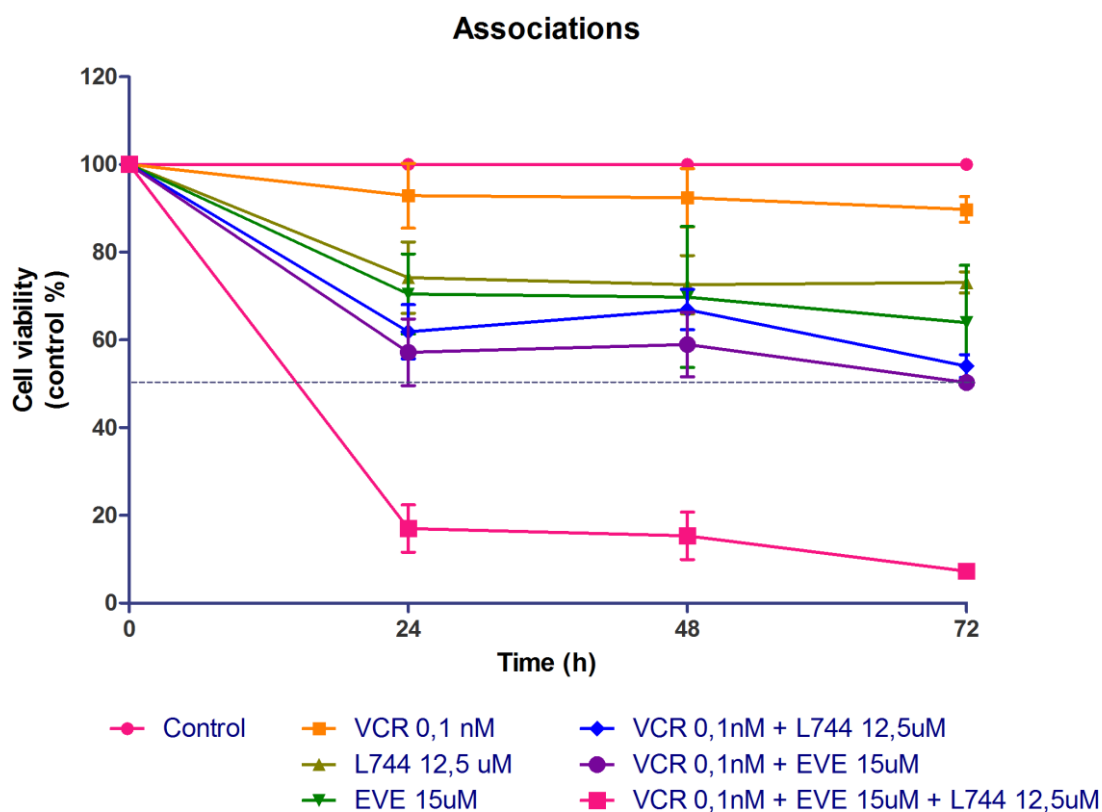
**Figure 5: Vincristine dose-response curves**. Farage cells were incubated in the absence (control) and presence of VCR, at the concentrations indicated in the Figure. Cell viability was assessed as described in "Materials and Methods". The results are expressed as percentages (%) compared to control (100%) and represent the average of eight independent experiments ± SD.

In order to investigate the possibility of a synergistic antiproliferative effect of the drugs tested in monotherapy (L744,832 and EVE) and in combination with the conventional drug (VCR) and with each other, association experiments were performed as represented in Figure 6.

Our results show an increase in the antiproliferative effect when cells are treated with drugs in association, compared with when cells are treated with one drug alone. More precisely, an additive synergistic effect is observed in cells treated with the combination of each targeted drug with VCR and a potentiation synergistic effect in the combination between targeted drugs and VCR. The highest effect is observed when the three drugs are administered in combination.

**Figure 6: Dose-response curves of the association of Vincristine, L744,832 and Everolimus**. Farage cells were incubated for 72 hours (h) in the absence (control) and presence of VCR, L744,832 and EVE in monotherapy, and VCR associated with EVE and/or L744,832, at the concentrations indicated in the Figure. Cell viability was assessed as described in "Materials and Methods". The results are expressed as percentages (%) compared to control (100%) and represent the average of six independent experiments ± SD.
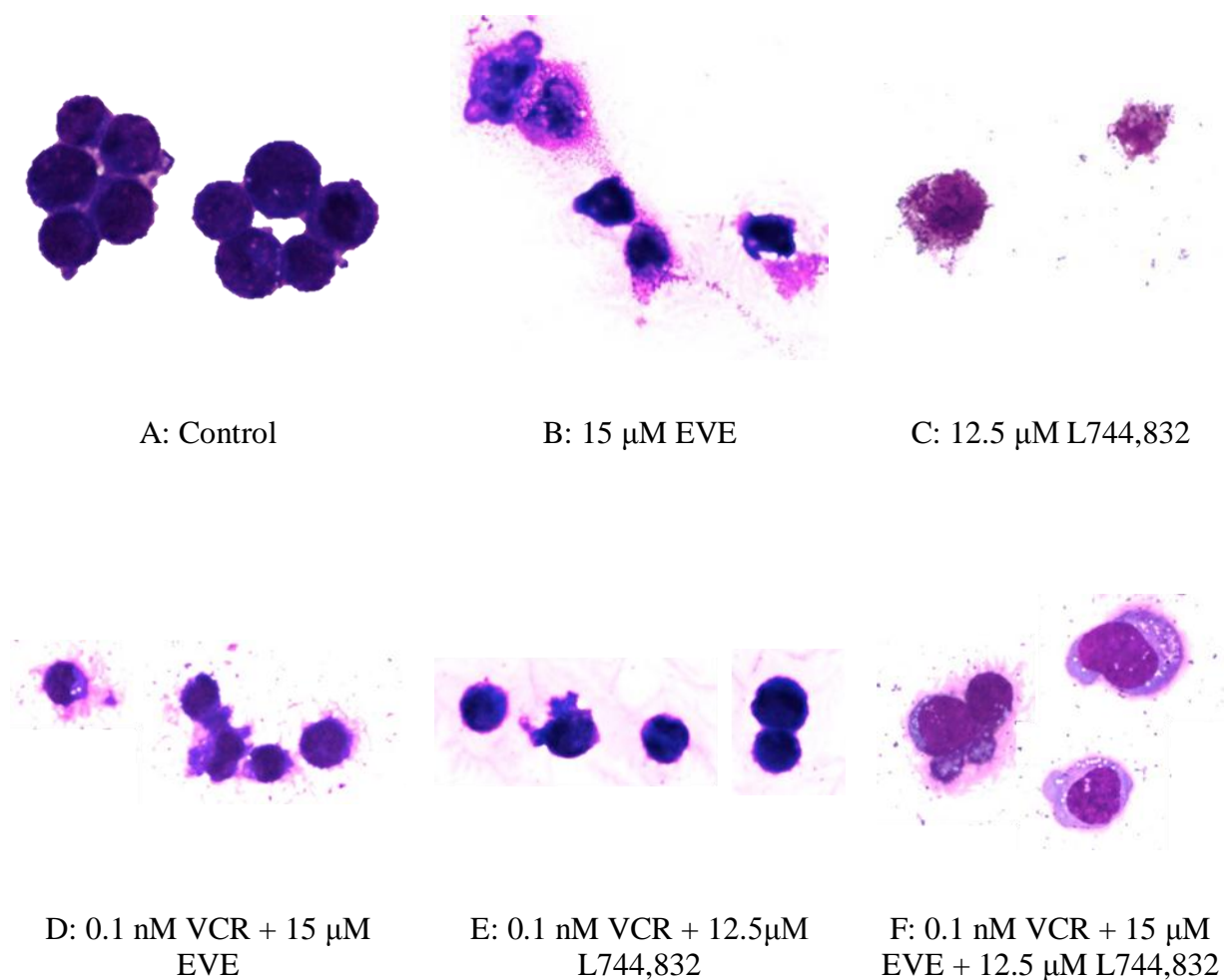
## 4.2. Cell death analysis

As cell death mechanism can influence the therapeutic strategy, it was necessary to analyze the type of cell death induced by the drugs through morphological analysis by optical microscopy and also through FC, using the AV/PI double staining.

### 4.2.1. Evaluation of cell death by optical microscopy: morphological analysis

Figure 7 shows the the morphology of cell smears stained with May-Grünwald-Giemsa before (A: control) and after treatment with 15 μM EVE (B), 12.5 μM L744,832 (C), 0.1 nM VCR and 15 μM EVE (D), 0.1 nM VCR and 12.5 μM L744,832 (E) and with

the association of 0.1 nM VCR, 15 µM EVE and 12.5 µM L744,832 (F). As it can be seen, cells display mostly morphological evidence of cell death by apoptosis, such as cellular contraction, nuclear fragmentation, blebbing and apoptotic bodies formation.



| A: Control | B: 15 µM EVE | C: 12.5 µM L744,832 |



| D: 0.1 nM VCR + 15 µM EVE | E: 0.1 nM VCR + 12.5µM L744,832 | F: 0.1 nM VCR + 15 µM EVE + 12.5 µM L744,832 |

**Figure 7: Morphological characteristics of Farage cells.** Untreated cells (control cells) are shown in A and cells treated with drugs indicated in the cell smears are shown from B to F. Cell smears were stained with May-Grünwald-Giemsa as described in "Material and Methods". Amplification: 500x.

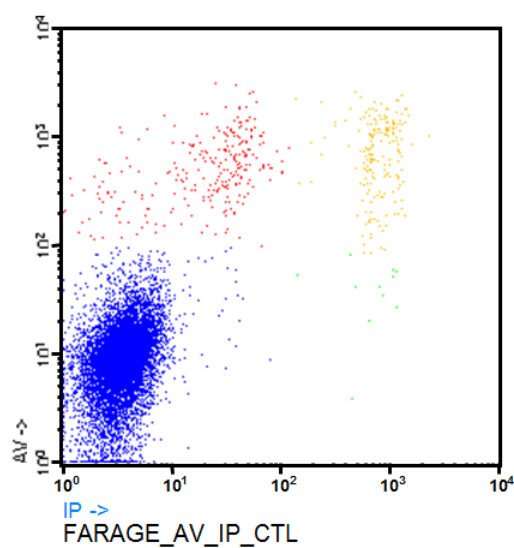## 4.2.2 Evaluation of cell death by flow cytometry

In order to confirm our results and evaluate the proportion of apoptosis and necrosis, we used a flow cytometry assay based on cell double staining with AV-FITC and PI.

As it can be observed in Figure 8, in all of the incubation conditions, there was a decrease in the percentage of viable cells and an increase in the percentage of apoptotic cells that are dose dependent. These results are consistent with the morphological analysis, as morphological evidences of cell death by apoptosis were also observed.
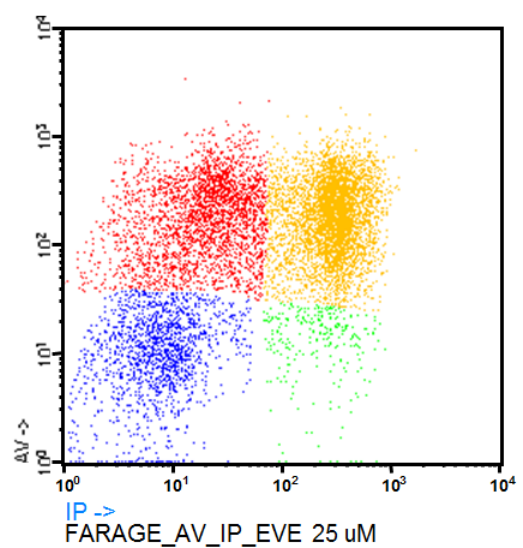
**A**



**B**



FARAGE_AV_IP_CTL

**C**



FARAGE_AV_IP_EVE 25 uM
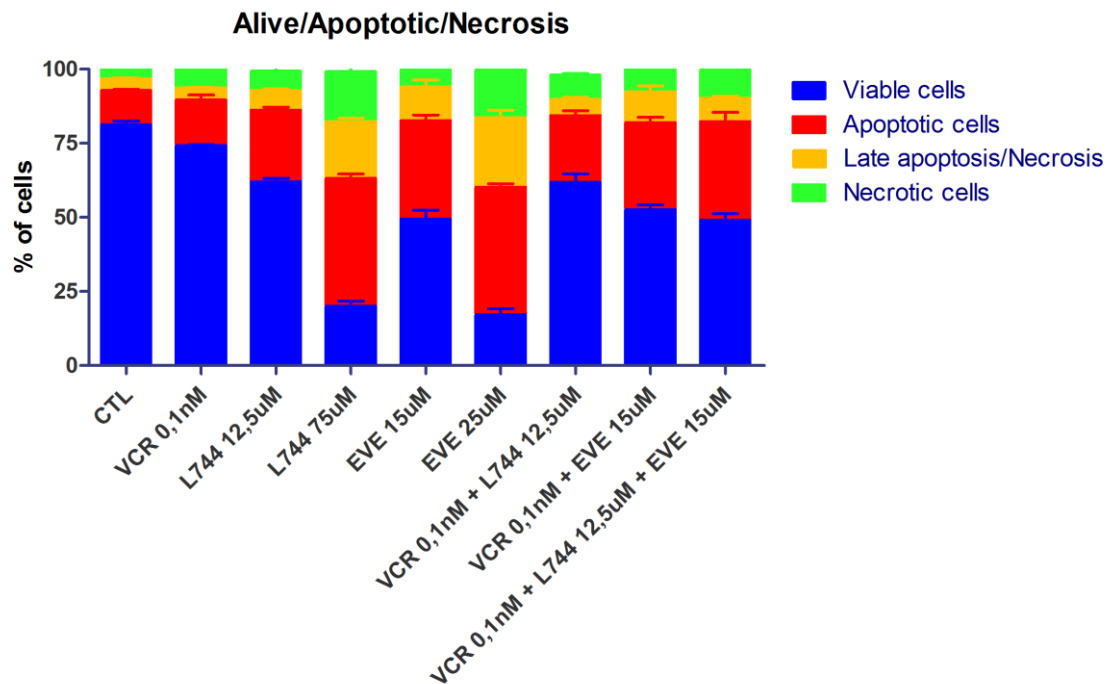
**Figure 8: Evaluation of cell viability and death by flow cytometry.** Farage cells were incubated in absence (control cells – CTL) and presence of 0.1 nM VCR,

12.5 μM and 75 μM L744,832, 15 μM and 25 μM EVE, in monotherapy, and in association with each other and with VCR using 0.1 nM VCR, 12.5 μM L744,832 and 15 μM EVE. Viability and cell death were assessed by FC using AV and PI staining as described in "Material and Methods". Alive cells are AV/PI negative (blue); early stages of apoptosis are AV positive and PI negative (red) and cells in late stages of apoptosis are AV/PI positive (yellow). Necrotic cells are AV negative and PI positive (green). These results were obtained after 48 hours of incubation and correspond to the mean of the three independent experiments. Results represented in A were obtained after 48 hours of incubation and represent the mean of four independent experiments ± SD. B shows the dot plot obtained from control cells and C the dot plot obtained from cells treated with EVE.

## 4.3. Evaluation of the mechanisms involved in cytotoxicity induced by drugs

### 4.3.1. Analysis of apoptosis-regulating molecules expression

In order to evaluate the mechanism involved in the apoptosis induced by the tested drugs, we measured the expression of Caspase 3, a frequently activated apoptotic cell death protease.

As shown in Figure 9, VCR, L744,832 and EVE used in monotherapy and in association with each other and with Vincristine, induced an increase in the expression of Caspase 3 compared with control cells. This increase was dose dependent, as can be verified by comparing Caspase expression in cells treated with L744,832 in a concentration of 12.5 μM and in a concentration of 75 μM. This effect is also observed in the cells treated with EVE in monotherapy.
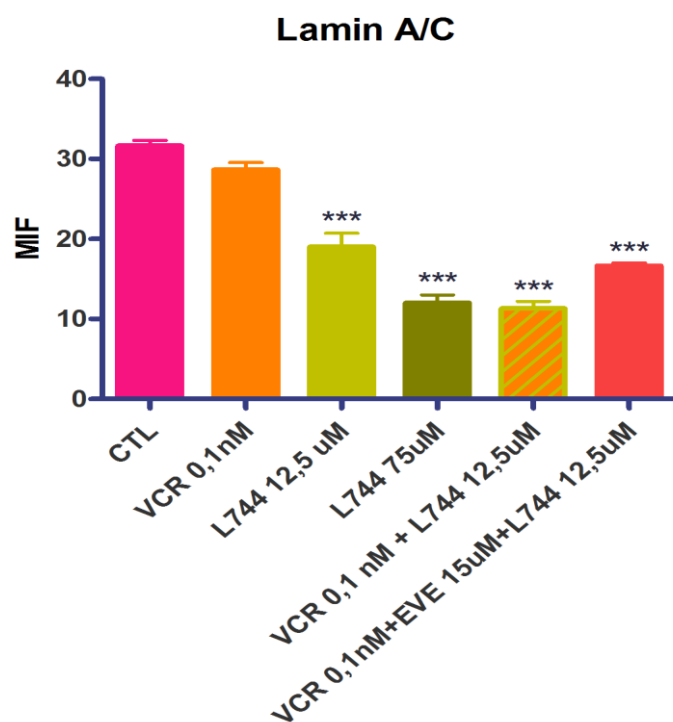
**A**
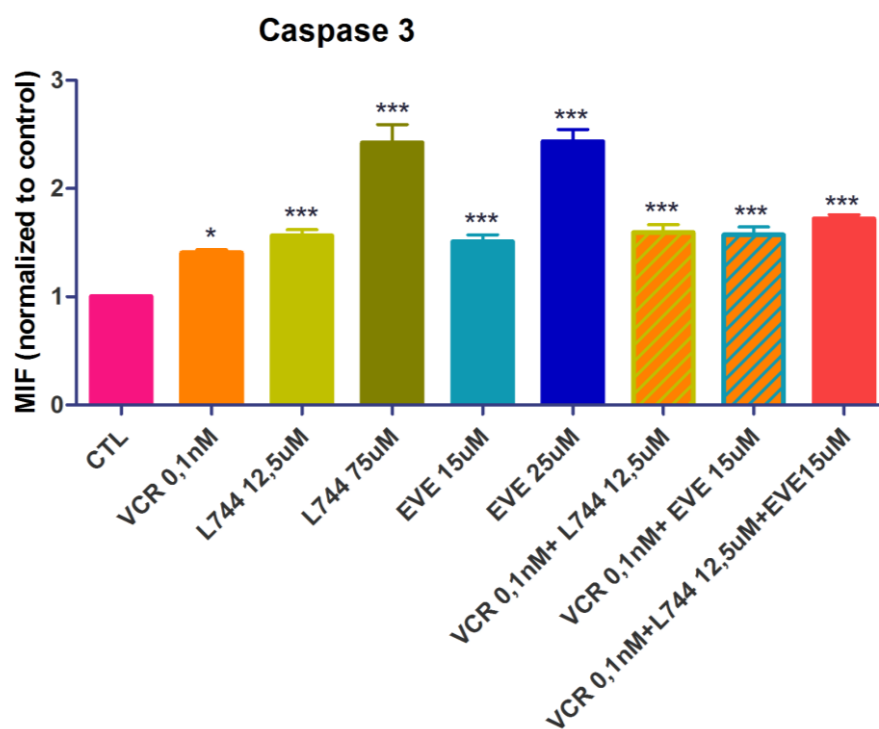


**B**
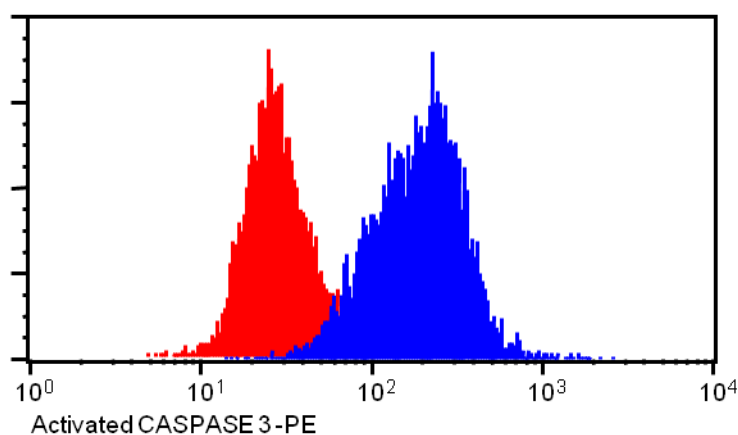


**Figure 9: Evaluation of Caspase 3 expression by flow cytometry.** Farage cells were incubated in absence (control cells – CTL) and presence of 0.1 nM VCR, 12.5 μM and 75 μM L744,832, 15 μM and 25 μM EVE, in monotherapy and in association with each other and with VCR using 0.1 nM VCR, 12.5 μM L744,832 and 15 μM EVE. Results represented in A were obtained after 48 hours of incubation and correspond to the mean of three independent experiments $\pm$ SD. (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$). B shows an

histogram of the expression of activated Caspase 3 in control cells (red) compared to cells treated with 75µM L744,832 (blue).

## 4.3.2. Analysis of the effect of L744,832 and Everolimus on farnesylation and Ki-67 expression

To analyze the effect of L744,832 on farnesylation, Farage cells were incubated with selected concentrations of FTI, below the half maximal inhibitory concentration ($IC_{50}$) found at 48 hours in viability studies. The results, as shown in Figure 10A, demonstrate a statistically significant decrease of lamin A/C levels. This effect is dose dependent, as can be verified by comparing lamin expression in cells treated with L744,832 in a concentration of 12.5 µM and with 75 µM (reduction about 40% and 62%, respectively, compared to control cells). The association of L744,832 with VCR resulted in a statistically significant decrease (around 64%) when compared with L744,832 alone. On the other hand, the decrease in lamin A/C expression in cells treated with the association of L744,832, VCR and EVE was not statistically significant when compared to L744,832 in monotherapy.
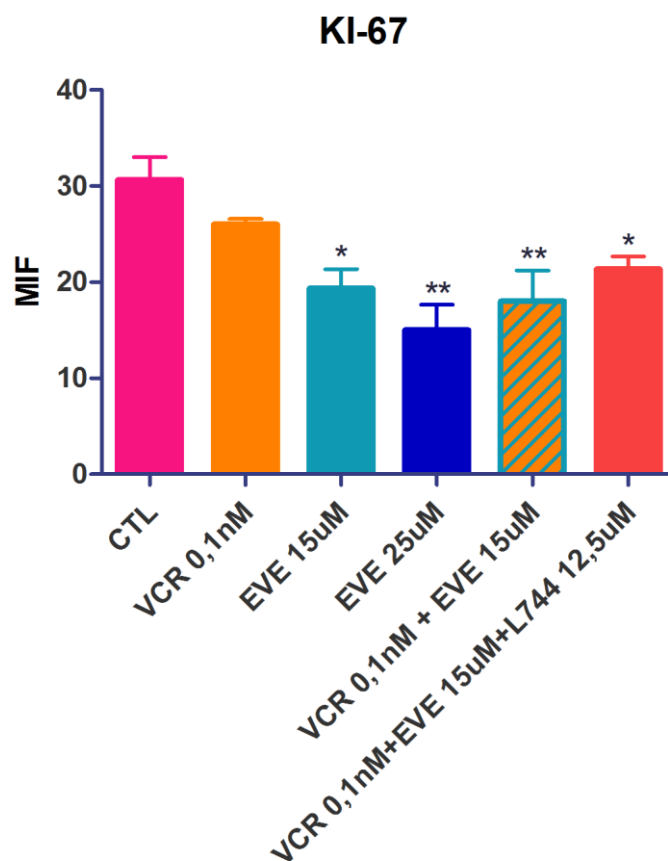
**A**



**B**
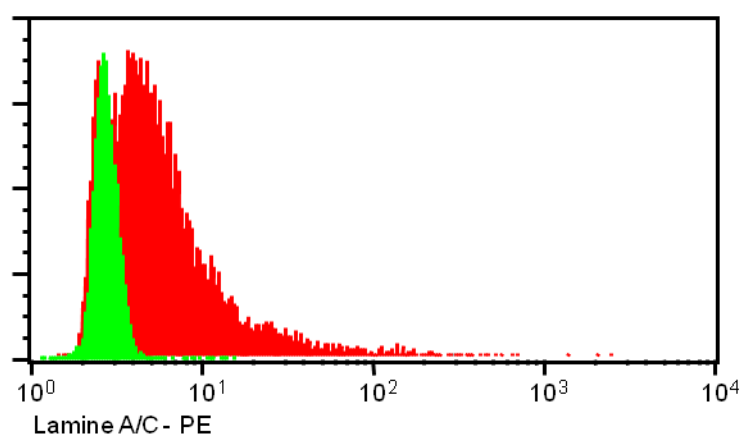


**Figure 10: Evaluation of lamin A/C expression by flow cytometry**. Farage cells were incubated in absence (control cells – CTL) and presence of 0.1 nM VCR, 12.5 μM and 75 μM L744,832, in monotherapy and in association with each other and with VCR using 0.1 nM VCR, 12.5 μM L744,832 and 15 μM EVE. In A, results were obtained after 48 hours of incubation and correspond to the

mean of three independent experiments ± SD. (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$). B shows the histogram of the expression of lamin A/C in control cells (red) compared to cells treated with 75µM L744,832 (green).

In order to determine the antiproliferative effect of EVE by mTOR pathway inhibition, we analyzed the expression of Ki-67 in Farage cells incubated with EVE, in monotherapy and in association with the other tested drugs, as represented in Figure 11. The results display a decrease in the expression of Ki-67 in treated cells compared with control cells (reduction of about 37% for 15 µM EVE and 51% for 25 µM EVE). This decrease was apparently dose dependent, although not statistically significant, comparing 15 µM EVE and 25 µM EVE. The associations of EVE and VCR and EVE with both VCR and L744,832 resulted in a decrease of Ki-67 expression when compared to the control cells (approximately 41% and 30%, respectively) but there was not a statistically significant difference when compared to cells treated only with EVE.

**Figure 11: Evaluation of Ki-67 expression by flow cytometry**. Farage cells were incubated in absence (control cells – CTL) and presence of 0.1 nM VCR, 15 μM EVE and 25 μM EVE, in monotherapy and in association with each other and with VCR using 0.1 nM VCR, 15 μM EVE and 12.5 μM L744,832. These results were obtained after 48 hours of incubation and correspond to the mean of three independent experiments ± SD. (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

### 4.3.3 Cell cycle analysis

To confirm the antiproliferative effect of the tested drugs we performed cell cycle analysis. As can be observed in Figure 12, the majority of control cells (A), had a DNA diploid content, with a small peak representing dividing cells transiently in $G_2/M$ phase (2n DNA content). In comparison, L744,832 treated cells (B) showed a reduction in the percentage of cells in the $G_0/G_1$ phase and also of the cells in the $G_2/M$ phase.

Furthermore, there is a peak pre-$G_0/G_1$ (presented in light blue) which corresponds to apoptotic cells, in line with the previous studies. In the EVE treated cells (C), a higher effect is observed. An increase in percentage of cells in the peak pre-$G_0/G_1$ is detected ($32.5 \pm 2.12\%$) accompanied with an increase in cells in $G_0/G_1$ phase ($91 \pm 2.83\%$), and a decrease in $S/G_2/M$ phase ($4 \pm 2.83$; $5 \pm 0$).
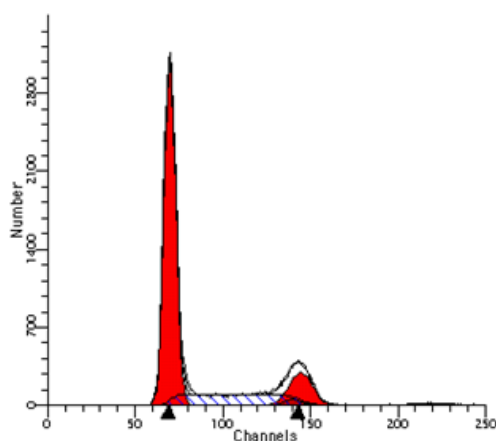


| Apoptotic peak | 4.5% ± 0.71 |
|---|---|
| $G_0/G_1$ | 46% ± 5.66 |
| S | 36% ± 4.24 |
| $G_2/M$ | 18% ± 1.41 |

**A:** CTL
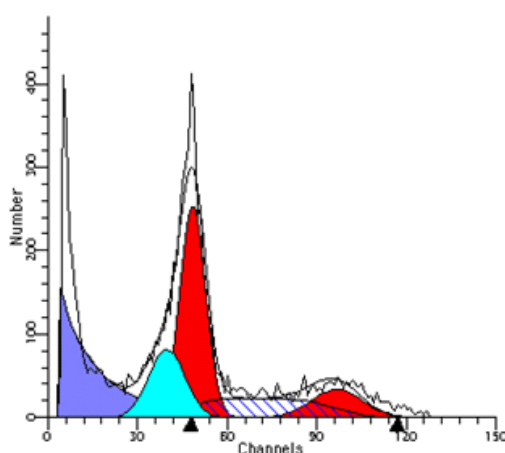


| Apoptotic peak | 22.5% ± 4.95 |
|---|---|
| $G_0/G_1$ | 59% ± 1.41 |
| S | 34.5% ± 4.95 |
| $G_2/M$ | 6.5% ± 3.54 |

**B**:75 μM L744,832
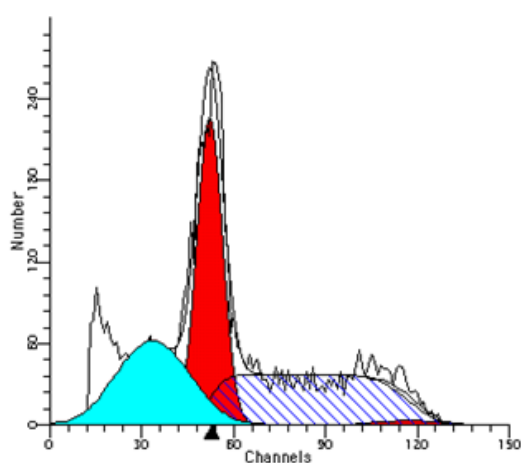
| Apoptotic peak | 32.5% ± 2.12 |
|---|---|
| $G_0/G_1$ | 91% ± 2.83 |
| S | 4% ± 2.83 |
| $G_2/M$ | 5% ± 0 |

**C:** 25 μM EVE

**Figure 12: Analysis of Farage cell cycle by flow cytometry.** Untreated cells (A), cells treated with 75 μM L744,832 (B) and with 25 μM EVE (C), were stained with PI for DNA content analysis. Arrowheads indicate peaks of cells at the $G_1$ and $G_2/M$ phases. The percentage of cells is indicated in the upper right corner of each graph image. These results were obtained after 48 hours of incubation and correspond to the mean of three independent experiments ± SD.

# 5. DISCUSSION AND CONCLUSIONS

Treatment options for diffuse large B-cell lymphoma (DLBCL) differ between patients with localized and advanced disease and the prognosis is extremely good for patients with no adverse risk factors. However, in patients with advanced disease the prognosis is unfavorable and there are no therapeutic options. More than half of the cases of aggressive NHLs can be cured with first-line therapy but for patients with primary refractory disease or early relapse therapeutic failure is a problem.

The signaling pathways involved in lymphomagenesis are well documented and it is known that the BCR/PI3K/AKT/mTOR and RAS/MAPK pathways are two of the most frequently involved. Therefore, drugs that act in the various steps of these pathways can theoretically be used as potential anticarcinogenic agents.

Several studies show that chronic active B-cell receptor signaling, in patients with the ABC subtype, can be interrupted by inhibiting the SRC family kinases BTK, SYK, and PKCβ or the PI3K/mTOR pathway (Lenz, G. *et al.*, 2010).

In this study, we evaluated the therapeutic potential of drugs that act on RAS/MAPK and BCR/PI3K/AKT/mTOR pathways, the L744,832 (a RAS/RAF pathway inhibitor, in particular a farnesyltransferase inhibitor) and Everolimus (a mTOR inhibitor), in monotherapy and in combination with each other and the conventional anticarcinogenic drug Vincristine.

Our results suggest that both drugs, L744,832 and Everolimus, induce an antiproliferative and cytotoxic effect in a DLBCL cell line, in a time- and dose-dependent manner, when used in monotherapy, inducing cell death mainly by apoptosis.

In fact, regarding the FIT L744,832, the $IC_{50}$ is achieved at 24 hours, which is earlier than in an acute lymphoblastic leukemia cell line treated with other FTIs (Costa, C. *et al.,* 2012). However, if cells are exposed to FTI for a longer period (48 hours), the reduction of cell viability by 50% is achieved with lower concentrations. Nonetheless, the cytotoxic effect observed seems to be reversible as cell viability increased slightly after 72 hours of exposure to this inhibitor.

The reduction in cell viability is achieved by a decrease in the percentage of viable cells and an increase in the percentage of apoptotic cells as observed in other studies (Costa, C., et al., 2012), which may be mediated by the observed increase in Caspase 3 levels. The apoptotic process is recognized analyzing the cell death by FC based on cell double staining with AV and PI and also by observation of the morphological characteristics by optic microscopy.

In order to assess the mechanism involved in apoptosis we evaluated the expression of Caspase 3. This protease plays a key role in the apoptotic process, ensuring the efficient accomplishment of the process once the cell has been targeted to die (Lavrik, I.N. *et al*., 2005). The results confirmed that the main way of cell death is by apoptotic pathway.

In addition to the observed cytotoxic effect, L744,832 induces an antiproliferative effect in Farage cells, which is confirmed through cell cycle analysis by flow cytometry. In fact, cells treated with this FTI stopped cell cycle progression through $G_0/G_1$ phase and the apoptotic peak observed is in line with our previous results.

Song and collaborators (2000) showed that the FTI L744,832 leads to an increase in the number of tetraploid cells in pancreatic adenocarcinoma cells by stopping progression of the cell cycle through $G_2/M$. These observations suggest that the effect of this FTI on cell cycle is dependent on the type of the targeted cell. However, other studies using

hematological cell lines show a decrease in the expression of cyclin 1 (Costa, C. *et al.*, 2012), a protein involved in cell cycle progression from $G_1$ to S phases.

To evaluate the mechanism of action of the L744,832 we used lamin A/C levels as a marker of FTI efficacy, as described by Adjei, A.A. *et al.* (2000). Lamins are nuclear membrane structural molecules that are important for certain cellular functions such as cell cycle control, DNA replication and chromatin organization (Beck, L. *et al.,* 1990). Lamins are activated from prelamin and this activation is dependent on the enzyme farnesyltransferase (Kilic, F. *et al.*, 1997). The use of monoclonal antibodies to measure the intracellular levels of both these molecules may serve as a marker of the activity of the FTI (Costa, C. *et al.*, 2012).

Using flow cytometry and analyzing the expression of lamin in Farage cells treated with L744,832, we were able to assess the specific action mechanism and efficacy of this drug.

In all conditions of incubation, a lower expression of lamin was observed in comparison with the control cells, suggesting inhibition of farnesyltransferase by L744,832. This effect is dose dependent, because a higher dose of the FTI resulted in a lower expression of lamin A/C. Associating L744,832 and VCR and L744,832, VCR and EVE we can also see a decrease in expression, although not statistically significant in the case of the combination of all three drugs.

These results are consistent with the existing literature (Brunner, T.B., *et al*., 2003), in which tests of association with other drugs and using other cell lines have shown a synergistic effect. This effect was evident in FTI associations with microtubule-stabilizing drugs. However, it should be noted that the cell cycle block by FTI could inhibit the activity of certain cytotoxic agents that are cell cycle dependent.

As mentioned, the decrease in cell proliferation caused by L744,832 is accompanied by the activation of cell death by apoptosis. Although not yet fully understood, one possible mechanism of apoptosis may involve the inhibition of the FAS gene expression, thereby increasing the cell vulnerability to apoptosis induced by FAS ligands (Brunner, T.B. *et al*., 2003). It is also important to note that there are several pathways which promote cell proliferation and increase cell survival (Appels, N. *et al*., 2005), controlled by the activation of RAS proteins, including the PI3K/AKT pathway, and the apoptotic effect may be associated with any of these pathways.

In relation to the mTOR inhibitor, Everolimus, the antiproliferative effect observed seems to be reversible, since cell viability increased slightly after 48 hours of exposure to the drug, particularly at lower concentrations. This effect is likely due to the regimen of drug administration, as this is observed in particular when drugs are administrated in a single administration. Indeed, in a daily dose administration scheme, the cell proliferation would probably continue to decrease even after 48 hours incubation. Similarly to the FTI L744,832, besides the antiproliferative effect, Everolimus induced cell death mainly by apoptosis. These results are confirmed by Farage cell cycle analyses which show a stopped through the $G_0/G_1$ phase, while an apoptotic peak also happens. In order to assess the action mechanism of the mTORi EVE, we measured the expression of Ki-67, a nuclear protein expressed in proliferating cells that may be required for maintaining cell proliferation (Scholzen, T. *et al*., 2000). However, the exact mechanism remains unknown. This protein has been used as a marker for cell proliferation of solid tumors and some hematological malignancies. Ki-67 is expressed in all proliferating cells which are in the active phases of the cell cycle, but absent in resting cells. Therefore, a reduction in Ki-67 can define an antiproliferative effect. In all conditions of incubation, a lower expression of Ki-67 was observed comparing with the

control cells, suggesting that Everolimus has an antiproliferative effect. This effect is apparently dose dependent, because a higher dose of EVE resulted in lower expression of Ki-67, although not statistically significant. Associating EVE and VCR and EVE, VCR and L744,832 we can also recognize a decrease in Ki-67 expression, though also not statistically significant.

The pro-apoptotic effect of mTORi is related to several mechanisms, including down-regulation of anti-apoptotic proteins (MCL-1, BCL-2, BCL-XL, cIAP, STAT3) (Yang, Q. *et al*., 2007), anti-angiogenic effects and down-regulation of cell cycle proteins regulators such as cyclin D1 and D2 or p34cdc2 (Kelly, R.K. *et al.*, 2011 and Récher, C. *et al.*, 2005). Everolimus on particular has been shown to sensitize cisplatin-induced apoptosis through the inhibition of p21 translation in tumor cells with TP53 gene mutations (Beuvink, I. *et al*., 2005). These mutations are associated with poor response to chemotherapy and short survival (Ichikawa, A. *et al*, 1997). Another effect of mTORi, independent of mTOR, is the negative regulation of the NF-κB pathway, by inhibiting the FKBP51 immunophillin, a cofactor of the IKK complex that positively regulates NF-κB activity. This inhibition of FKBP51 promotes apoptosis induction. (Kelly, R.K. *et al*., 2011).

Several agents to block this important pathway have been investigated in search of new drugs for cancer therapy. While some agents showed disappointing results, other proved promising. In phase 1 and 2 trials, the mTOR inhibitors demonstrated clinical activity against a variety of solid tumors (Chang, S. *et al.,* 2004; Wolpin, B. *et al*., 2009). Preclinical studies in human multiple myeloma cells using a xenograft model confirmed the activity of an mTOR inhibitor in myeloma cells through inhibition of proliferation, angiogenesis, and induction of apoptosis (Frost, P. *et al*., 2004). In a B-cell lymphoma mouse model, Wendel and colleagues demonstrate that activated AKT promoted

lymphomagenesis by disabling apoptosis. The phenotype was resistant to conventional chemotherapy agents when used alone. However, in combination with the mTOR inhibitor rapamycin, it restored the cells sensitivity to these drugs (Hachem, A. *et al.*, 2005).

Given the emergence of many new therapeutic agents that affect essential regulatory pathways in lymphomas, the challenge is to identify rational combinations that kill lymphoma cells synergistically. Since both PI3K and NF-κB signaling supply survival signals to ABC cells, combination therapies that block both pathways may prove synergistic in patients with chronic active B-cell receptor signaling.

The understanding of the molecular pathogenesis of non-Hodgkin's lymphomas (NHL) has improved significantly in recent years. Advances in molecular biology and genetics led to the identification and characterization of several oncogenic pathways involved in lymphomagenesis. This knowledge will ultimately lead to improved diagnostic and therapeutic strategies for patients with NHL.

In conclusion, our results suggest that the tested drugs may be used as a new therapeutic approach in diffuse large B-cell lymphoma. However, other schemes of drug administration, as well as, new associations should be tested prior to advancing to other study phases.

## 6. ACKNOWLEDGMENTS

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