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ANA MARIA FARIA ARAÚJO

NEW TARGET THERAPIES IN HEPATOCELLULAR CARCINOMA - A STUDY IN CELL LINES IN CULTURE

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PROFESSOR DOUTOR JOSÉ MANUEL NASCIMENTO COSTA
PROFESSORA DOUTORA ANA BELA SARMENTO RIBEIRO

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NEW TARGET THERAPIES IN HEPATOCELLULAR CARCINOMA – A STUDY IN CELL LINES IN CULTURE

Araújo A. M.[(1)], Branco J. D.[(1)], Ferreira A. L.[(1)], Neves S.[(1,2,3)], Sarmento Ribeiro A. B. [(1,2,3)], Nascimento Costa J.M. [(1,2,3,4)]

[1]Faculty of Medicine, University of Coimbra (FMUC), Portugal; [2] Center of Investigation in Environment, Genetics and Oncobiology (CIMAGO), FMUC, Portugal; [3] Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Portugal; [4] University Hospital of Coimbra, Portugal;

Correspondence: Ana Bela Sarmento Ribeiro, Applied Molecular Biology/Biochemistry Institute, Faculty of Medicine (pólo 3) - University of Coimbra – Azinhaga de Sta Comba– Celas - 3000-548, Coimbra, Portugal. Email: absarmento@fmed.uc.pt.

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INDEX

Páginas

RESUMO

O carcinoma hepatocelular é uma doença complexa e devastadora que se mantém resistente à quimioterapia convencional. O desenvolvimento de fármacos dirigidos às vias moleculares envolvidas na sobrevivência e proliferação das células neoplásicas, bem como a sua associação aos agentes convencionais poderá constituir uma nova abordagem no tratamento do carcinoma hepatocelular.

O objectivo do presente estudo consiste na avaliação do potencial terapêutico dos novos fármacos dirigidos a alvos moleculares, nomeadamente os inibidores do proteasoma (MG-262), da farnesiltransferase (L-744832) e do mTOR (Everolimus), em monoterapia e em combinação com os agentes da quimioterapia convencional 5-Fluorouracilo e Doxorrubicina, em células de carcinoma hepatocelular em cultura, HUH-7.

Para atingir estes objectivos, as células HUH-7 foram incubadas com concentrações crescentes de MG-262, L-744832 e Everolimus em monoterapia e em associação com 5-Fluorouracilo e Doxorrubicina durante diferentes intervalos de tempo. A viabilidade celular foi avaliada pelo teste Alamar Blue e o tipo de morte celular por microscopia óptica e citometria de fluxo, recorrendo à dupla marcação com anexina V e iodeto de propídio. Os mecanismos moleculares envolvidos na acção e citotoxicidade dos fármacos, nomeadamente, a expressão de conjugados de ubiquitina, lamininas A/C, ciclina D1 e de proteínas relacionadas com a morte celular (BAX e BCL2) foram também analisados por citometria de fluxo através de anticorpos monoclonais ligados a fluorocromos.

Os resultados obtidos indicam que o MG-262, o L-744832 e o Everolimus têm um efeito antiproliferativo e citotóxico em monoterapia que varia em função da dose utilizada e do tempo de incubação. Induzem morte celular principalmente por apoptose, a qual poderá estar relacionada com o aumento da razão BAX/BCL-2. Às 48h, o IC50 é, respectivamente, 100nM (MG-262), 77µM (L-744832) e 18µM (Everolimus). A combinação destes fármacos com os

agentes terapêuticos convencionais demonstra aumento do efeito antiproliferativo para doses inferiores ao IC50 (sinergismo de adição). Além disso, o efeito citotóxico foi observado apenas nas associações do MG-262 com os fármacos da quimioterapia convencional.

Este estudo sugere que os inibidores do proteasoma, os inibidores da farnesiltransferase e os inibidores do mTOR poderão constituir uma alternativa terapêutica no tratamento do carcinoma hepatocelular tanto em monoterapia como em associação com os fármacos convencionais. A escolha de um esquema terapêutico adequado será também crucial para o sucesso do tratamento.

PALAVRAS-CHAVE

Carcinoma hepatocelular, vias de sinalização, linha celular HUH-7, inibidor do proteasoma, inibidor da farnesiltransferase, inibidor do mTOR

ABSTRACT

Hepatocellular carcinoma is a complex and devastating disease which remains highly resistant to commonly used systemic chemotherapy. Alternative treatment strategies focus on specific pathways involved in malignant cell survival and proliferation, as well as combination of different targeted therapies with conventional anticarcinogenic agents may open new horizons in hepatocellular carcinoma therapy.

The aim of the present study is to test the therapeutic potential of new targeted drugs in hepatocellular carcinoma, namely the proteasome (MG-262), farnesiltransferase (L-744832) and m-TOR (Everolimus) inhibitors, in monotherapy and in combination with conventional anticarcinogenic agents, 5-Fluorouracil and Doxorrubicin, in the hepatocellular carcinoma cell line, HUH-7.

To attaint this purpose, the HCC cell line, HUH-7 cells, were incubated with increasing concentrations of MG-262, L-744832 and Everolimus, both as single agents and in association with 5-Fluorouracil and Doxorrubicin, during different periods of time. Cell viability was evaluated by the Alamar Blue assay and cell death by optic microscopy and flow citometry using annexin V and propidium iodide incorporation. The molecullar mechanisms involved in drug citotoxicity, namely the expression of ubiquitin conjugates, laminin A/C, cyclin D1 and proteins related to cell death (BAX and BCL2), were analysed by flow cytometry using monoclonal antibodies labeled with fluorochromes.

The obtained results show that MG-262, L-744832 and Everolimus have an antiproliferative and cytotoxic effect in monotherapy, depending on the applied dose and incubation period, inducing cell death mainly by apoptosis that may be related with the increase in BAX/BCL-2 ratio. The IC50 at 48h for each drug is: MG-262=100nM, L-744832=77μM, Everolimus=18μM. The combination of MG-262, L-744832 and Everolimus with conventional anticarcinogenic drugs reveals an increase in antiproliferative effect for

lower doses than the IC50 (addition synergism). However, the cytotoxic effect is only observed with MG-262 in association with the conventional anticarcinogenic drugs.

These results suggest that proteasome, farnesiltransferase and mTOR inhibitors may constitute a new potential therapeutic approach in HCC not only in monotherapy, but also in association with conventional therapies. The choice of an optimal drug schedule will also be crucial to the success of the therapy.

KEYWORDS

Hepatocellular carcinoma, molecular pathways, HUH-7 cell line, proteasome inhibitor, farnesyltransferase inhibitor, mTOR inhibitor

ABBREVIATIONS LIST

AV: annexin V

DMEM: Dulbecco's Modified Eagle's medium

FC: flow cytometry

FBS: fetal bovine serum

FICT: fluorescein isothiocyanate

FTI: farnesyltransferase inhibitors

HCC: hepatocellular carcinoma

IC50: half-maximal inhibitory concentration

MIF: mean intensity of fluorescence

PBS: phosphate buffer solution

PE: phycoerythrin

PI: propidium iodide

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worlwide, being responsible for 80% of the primary malignant liver tumors (Avila *et al.*, 2006). It is the sixth most common neoplasm in the world, and the third most common cause of cancer-related death (Lachenmayer *et al.*, 2010).

Geographical differences in incidence reflect variations of the main causal factors. In Asia and Africa, hepatitis B virus infection and aflatoxin B1 intake from contaminated food are common. In the West and Japan, hepatitis C virus infection is the main risk factor, as well as other causes of cirrhosis, such as alchool and haemochromatosis. The role of tobacco is not clearly established (Llovet *et al.*, 2003). On the other hand, HCC is more frequent in certain genetic diseases including Wilson disease, porphyria and α-antitrypsin deficiency (Wang *et al.*, 2002*).*

Hepatocarcinogenesis is a mutifactorial process that might explain the complex molecular pathogenesis of HCC. This neoplasm arises in normal livers, abnormal but noncirrhotic livers, and in cirrhotic liver (80% of cases) as a result of different environmental risk factors. Each of these scenarios involves different genetic and epigenetic alterations, chromossomal aberrations, gene mutations and altered molecular pathways (Llovet and Bruix, 2008), that result in the deregulation of key oncogenes and tumor-suppressor genes involved in several signalling pathways (Figure 1) (Farazi *et al.*, 2006).

At the time of presentation less than 40% of patients in the western world fulfill criteria for curative treatment (resection, transplatation, local ablation) and only 20% are eligible for chemoembolization. Alternative or palliative treatment options are very limited due to resistance to conventional chemotherapy and radiotherapy. Therefore, the knowledge of the molecular pathogenesis of HCC can provide new opportunities for target therapies (Lachenmayer *et al.*, 2010).

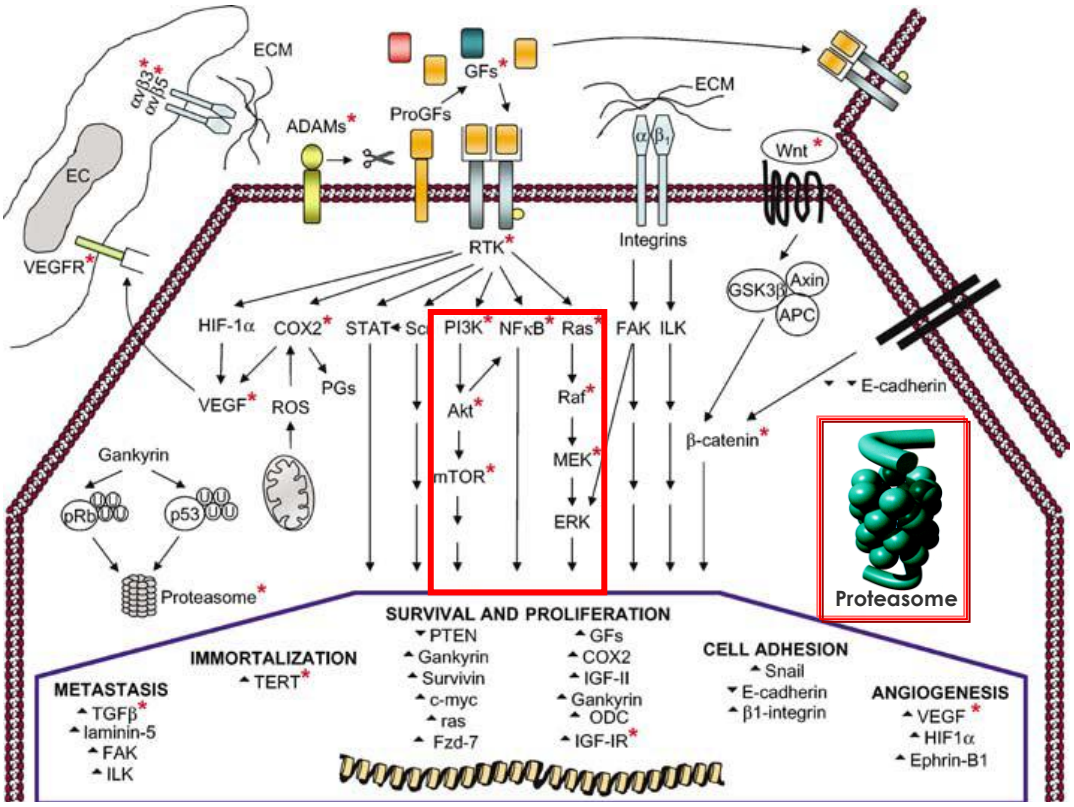

Figure 1: **Signaling pathways involved in HCC**, (Adapted from Avila *et al*., 2006).

Since the inhibition of the ubiquitin-proteasome pathway in tumor cells results in accumulation of tumor suppressor and pro-apoptotic proteins, the possibility of targeting this pathway in cancer therapy is a viable option (Landis-Piwowar *et al.*, 2006). Proteasome inhibition has already been established as a strategy for multiple myeloma and non-Hodgkin's lymphoma affected patients (Baiz *et al.*, 2009).

Furthermore, activated RAS interacts with downstream effectors that mediate several signaling pathways involved in cell proliferation and survival (Haluska *et al.*, 2002). The discovery that the transforming activity of oncogenic RAS depends upon its post-translational farnesylation has led to the development of farnesyltransferase inhibitors (FTIs) (Mazieres *et al.*, 2003). FTIs have already entered several phase I/II/III clinical trials in haematological malignancies and solid tumors, such as pancreas, lung, liver, prostate and bladder (www.clinicaltrials.gov). However, other studies show that the therapeutic efficacy of FTI

may be independent of *RAS* mutations (Song *et al.,* 2000; Appels *et al.,* 2005; Sarmento Ribeiro *et al.*, 2007; Ana Oliveira, 2008; Costa *et al.*, submitted).

Another signaling cascade that is frequently overactive in hepatic carcinogenesis is the PI3k/AKt/mTOR pathway (Piguet *et al.,* 2008). mTOR is one of the most important regulatory element of protein synthesis, considered as a central controller of cell growth. It has a key position which is on the cross road of various signalling pathways, such as RAS/RAF, PI3/AKt, TSC, NF-kB (Strimpakos *et al.*, 2009). Inhibition of mTOR have already been evaluated in haematological malignancies and breast, prostate, bladder, kidney and neuroendocrine tumors (Strimpakos *et al.,* 2009 and Garcia *et al.*, 2008).

In this study we intend to evaluate the therapeutic efficacy and the possible synergistic effect of new targeted drugs, in particular the proteasome inhibitor, MG-262, the farnesyltransferase inhibitor, L-744832 and the mTOR inhibitor, Everolimus, in monotherapy and in combination with conventional chemotherapy, using a HCC cell line in culture.

MATERIALS AND METHODS

1- Chemicals

MG-262 and L-744832 were obtained from BiomoL (USA). Everolimus, Doxorrubicin, 5-Fluorouracil, Resazurin, May-Grünwald solution and Giemsa solution were purchased from Sigma (St. Louis, MO, USA). FBS and DMEM medium were purchased from GIBCO (Barcelona,Spain). The kit FITC-labelled annexin V (AV) and propidium iodide (PI) were obtained from Immunotec (Canada). Antibodies anti-lamin A/C, anti-BAX, anti-ubiquitin conjugates, anti-cyclin D1 and anti-BCL2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2- Cell line culture conditions and evaluation of cell viability

The human HUH-7 cell line, derived from a HCC expressing high levels of mutated p53 (Carloni *et al.*, 2005), offered by Professora Doutora Maria Conceição Pedroso Lima (CNC) were maintained in DMEM medium supplemented with 10% FBS, L-glutamine 2mM, $NaHCO_3$, penicilin 100U/mL and streptomycin 100µg/mL at 37ºC in a humidified incubator containing 5% CO_2. HUH-7 cells were seeded at a density of 50000 cells per cm^2.

To determine the drug dose-dependent changes, cells were cultured in the absence (control) and presence of MG-262 (concentration range: 10nM to 250nM), L-744832 (concentration range: 1µM to 100µM), Everolimus (concentration range: 1µM to 75µM) , 5-Fluorouracil (concentration range: 10µM to 500µM) and Doxorubicin (concentration range: 10ng/mL to 500ng/mL) for up to 72h. No further addition of drug was made after the first dose.

To check for possible synergistic effects, combination treatments of the new drugs (MG-262, L-744832, Everolimus) plus conventional chemotherapy (5-Fluorouracil and Doxorrubin) were compared to those of each drug alone.

The antiproliferative effect was assessed by Alamar Blue assay each 24h, during 72h and the IC50 (drug inhibition concentration to attaint 50% of cell vianility) was calculated from three independent experiments using GraphPad Prism 4.00.

3- Morphological analysis

After incubation for 48h, in the conditions described in 2.2 section, the HUH-7 cells, were stained with May-Grünwald-Giemsa solution and their morphology was analysed by light microscopy using a Leitz Dialux 20 microscope associated with a Moticam 2300 digital camera.

4- Flow cytometry assays

4.1 Cell death analysis

HUH-7 cells cultured in the absence or in the presence of the drugs as previously described were trypsinized, centrifuged at 300xg for 5min and incubated for 10min at 4ºC with 440µL annexin buffer containing 5µL FITC-labelled AV and 2µL PI. Cells were then washed twice with PBS, resuspended in the same buffer and analysed in a FACScalibur cytometer (BD Biosciences, Heildelberg, Germany) equipped with an argon ion laser emiting at 488nm. The fluorescence of AV-FITC and PI was evaluated at 525 and 610nm, respectively.

The results were expressed as percentage of viable, early apoptotic, late apoptotic/necrotic and necrotic cells (Darzynkiewicz *et al.*, 1997; Neves *et al.*, 2006).

4.2 Molecular mechanisms related with the cytotoxic effect of new drugs

Some of the mechanisms involved in the cytotoxic effect were analysed by flow cytometry (FC) through the expression and ratio of the apoptotic regulators BAX and BCL2, ubiquitin-conjugates formation, lamin A/C and cyclin D1 levels.

Cells cultured in the absence or in the presence of the drugs described above were incubated with 100µL fixing solution during 10min at room temperature, in dark. Cells were then washed with 1mL PBS, centrifuged at 300xg for 5min and finally incubated with 1µg of the antibody anti-BCL2 labelled with FITC, 1µg of antibody anti-BAX labelled with PE and 100µL cell-permeable solution during 15min at room temperature, in dark. Cells were washed with 1mL PBS, centrifuged at 300xg for 5min, ressuspended in the same buffer and analysed in the flow cytometer.

The same protocol was made with lamin A/C, ubiquitin conjugates and cyclin D1 levels.

Results were plotted using normalized mean intensity of fluorescence (MIF) arbitrary units, which is proportional to the number of molecules labeled by the antibody.

For all the assays, negative controls were established with isotype IgG, IgG1 and IgG2b, and submitted to the same procedures.

5- Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 4.0 (GraphPad Prism software, Inc., San Diego, CA). Statistically significant differences ($p<0,05$) between the experimental groups were determined by Student's t test.

RESULTS

1- Analysis of cell viability

1.1 Effect of MG-262, L-744832 and Everolimus treatment in monotherapy on the viability of HUH-7cels

In order to evalute the therapeutic effect of the new targeted drugs, HUH-7 cells were cultured in the absence and in the presence of MG-262, L-744832 and Everolimus for up to 72h and the antiproliferative effect was evaluated by the Alamar Blue assay

Our results show that MG-262 induced a decrease in HUH-7 cells viability in a time and dose dependent manner. In Figure 2 we can observe a decrease in cell viability only after 48 hours of incubation, with an IC50 at 48 and 72h hours of exposure of 100nM and 50nM, respectively.

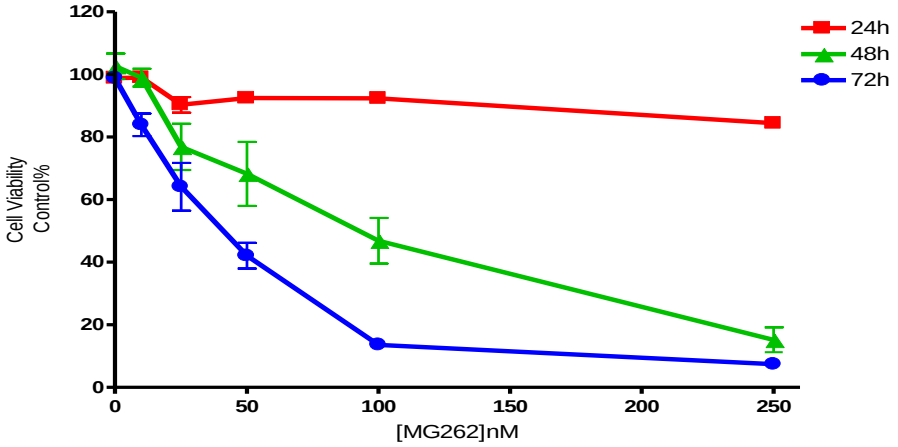

Figure 2: **Dose response curve in HUH-7 cells incubated with MG-262.** Cells were treated with MG-262 in several concentrations during 72h according with described in materials and methods. Cell viability is expressed in percentage (%) of control and represents the mean ± SD of 3 independent experiments.

In the same way, when we treated the cells with the L-744832, we also observed a decrease in cell viability in a dose and time dependent manner, reaching the IC50 at 77µM (48h) and 59µM (72h) (Figure 3). However, and in the opposite with the observed with MG-262, the antiproliferative effect is achieved earlier, after 24h of incubation, for the same concentration range observed in the cells treated during 48h, being the IC50 of 80µM.

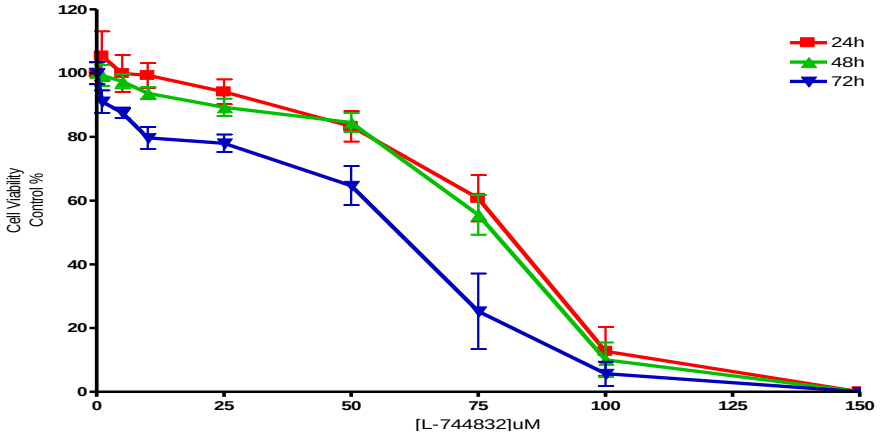

Figure 3: **Dose response curve in HUH-7 cells incubated with L-744832.** Cells were treated with L-744832 in several concentrations during 72 hours according with described in materials and methods. Cell viability is expressed in percentage (%) of control and represents the mean ± SD of 3 independent experiments.

Regarding the cytotoxicity induced by Everolimus, we observed a significant decrease in cell viability after 24h of incubation, being the IC50 similar during all the incubation period (18 to 17μM). However, the lethal dose is only achieved in the cells incubated during periods of 48h or higher (Figure 4).

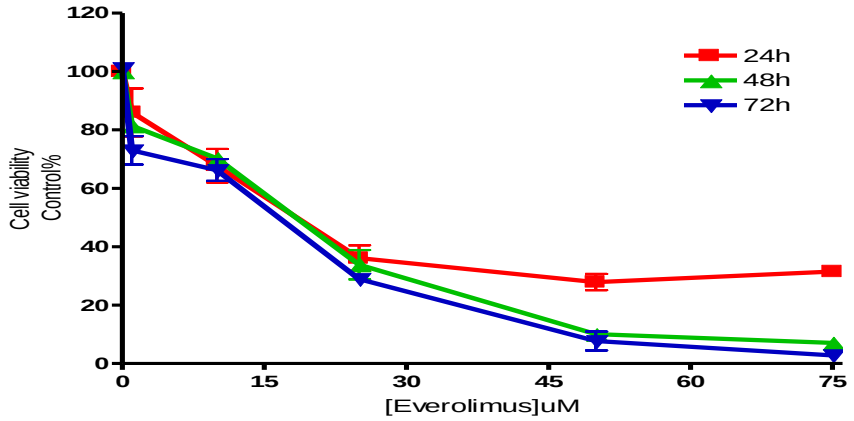

Figure 4: **Dose response curve in HUH-7 cells incubated with Everolimus.** Cells were treated with Everolimus in several concentrations during 72 hours according with described in materials and methods. Cell viability is expressed in percentage (%) of control and represents the mean + SD of 3 independent experiments.

1.2 Effect of MG-262, L-744832 and Everolimus treatment in association with Doxorubicin and 5-Fluorouracil

In order to check for possible synergistic effects, between MG-262, L-744832 and Everolimus with conventional chemotherapy agents, HUH-7 cells were incubated with the targeted drugs in combination with 5-Fluorouracil and Doxorubicin.

As we can see in Figures 5-7, in all tested conditions, a synergistic antiproliferative effect was observed (addition synergism). In fact, the same antiproliferative effect was achieved at lower concentrations than those obtained for all the tested drugs in monotherapy. However the IC50 was achieved earlier in the combinations involving the L-744832 and Everolimus (Figures 6 and 7, respectively) than those involved MG-262 (Figure 5).

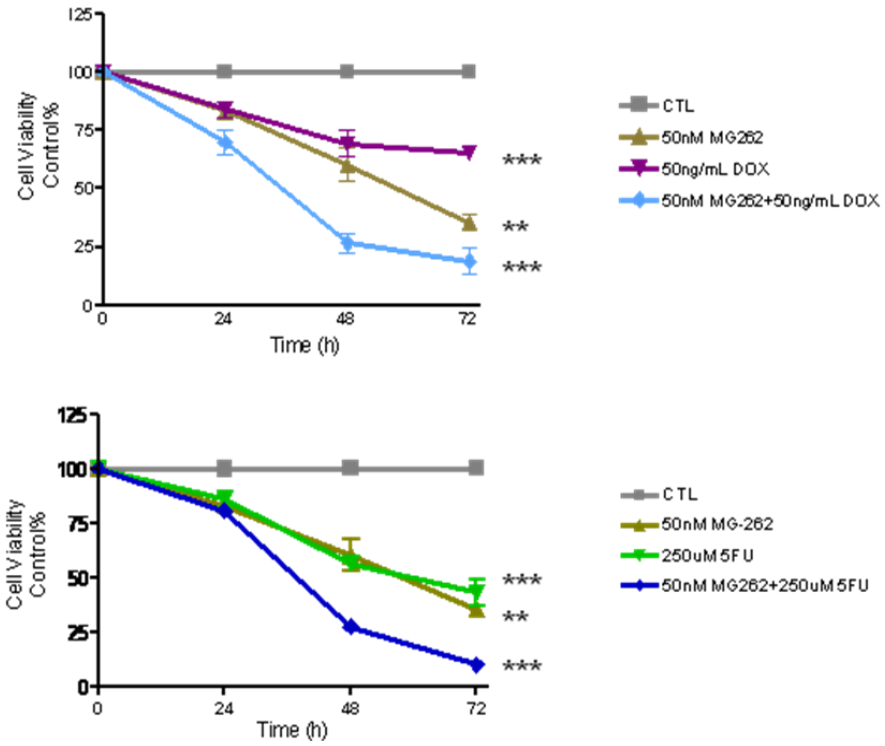

Figure 5: **Dose response curve of MG-262 in association with Doxorubicin (up) and 5-Fluorouracil (below) in HUH-7 cells.** Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 72 hours. Cell viability is expressed in percentage (%) of control and represents the mean ± SD of 3 independent experiments. The difference between experimental groups is statistically significant after 48h of incubation (**p<0,01; ***p<0,001).

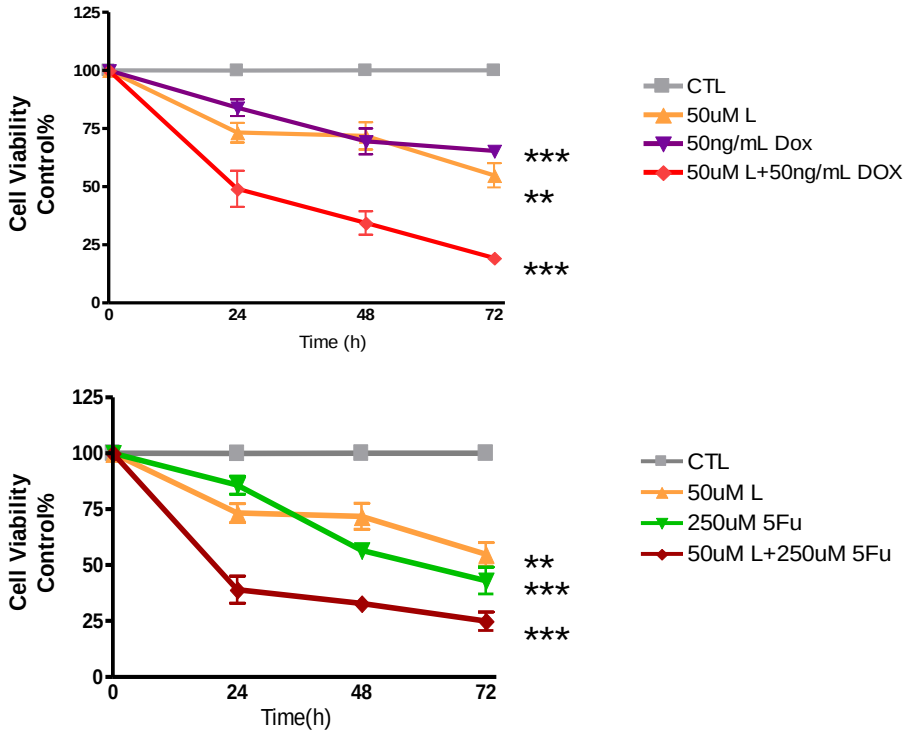

Figure 6: **Dose response curve of L-744832 in association with Doxorubicin (up) or 5-Fluorouracil (below) in HUH-7 cells.** Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 72 hours. Cell viability is expressed in percentage (%) of control and represents the mean ± SD of 3 independent experiments. The difference between experimental groups is statistically significant after 48h of incubation (**p<0,01; ***p<0,001).

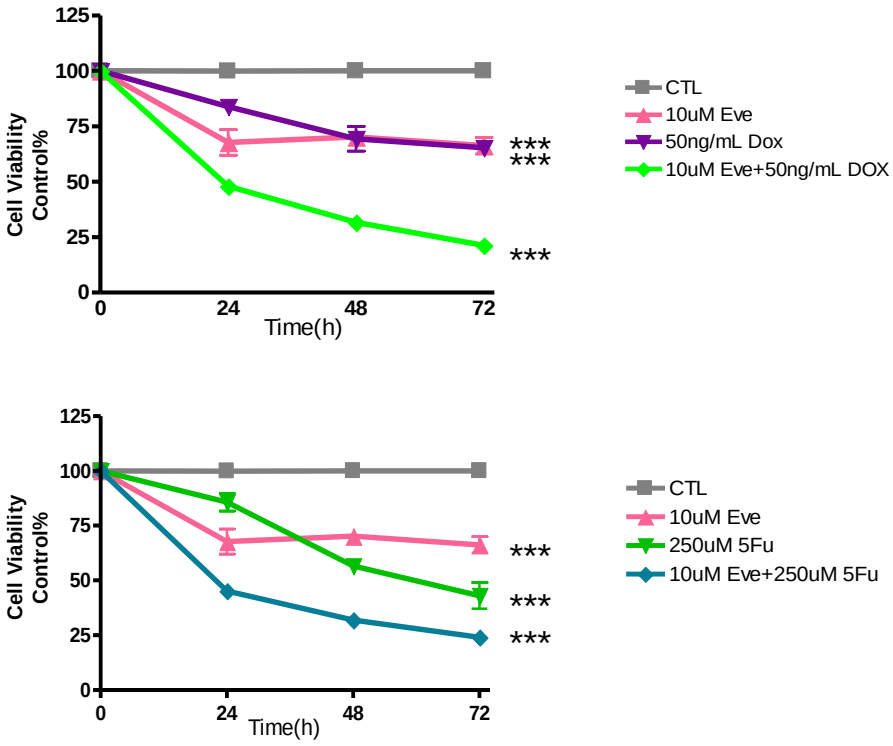

Figure 7: **Dose response curve of Everolimus in association with Doxorubicin (up) or 5-Fluorouracil (below) in HUH-7 cells.** Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 72 hours. Cell viability is expressed in percentage (%) of control and represents the mean ± SD of 3 independent experiments. The difference between experimental groups is statistically significant after 48h of incubation (***p<0,001).

1.3 Evaluation of HUH-7 cells viability treated with new targeted drugs in association

After the combination of new targeted drugs with conventional anticarcinogenic agents we tested the effect of the combinations of MG-262 with L-744832 (Figure 8) and Everolimus (Figure 9) and between L-744832 and Everolimus (Figure 10) on HUH-7 cells viability. As we can observe an addition synergism was achieved in all the tested conditions. In fact, the same antiproliferative effect was obtained with concentrations below the IC50 of the drugs used in monotherapy.

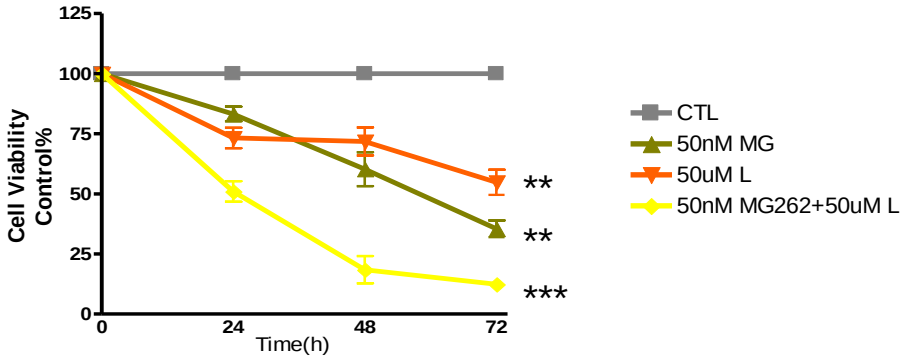

Figure 8: **Dose response curve in HUH-7 cells incubated with MG-262 in association with L-744832.** Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 72 hours. Cell viability is expressed in percentage (%) of control and represents the mean ± SD of 3 independent experiments. The difference between experimental groups is statistically significant after 48h of incubation (**p<0,01; ***p<0,001).

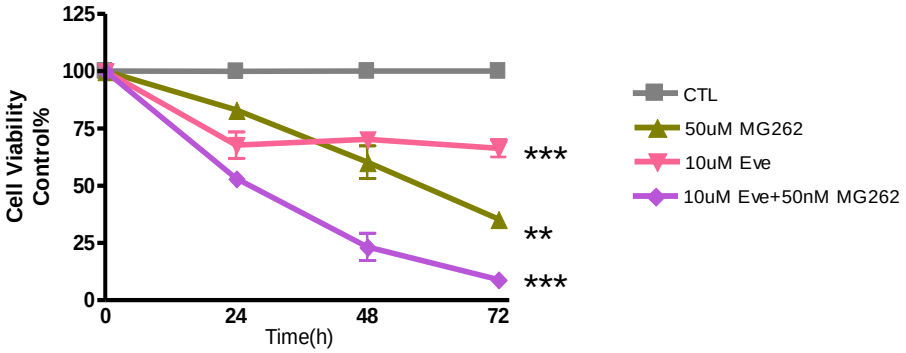

Figure 9: **Dose response curve in HUH-7 cells incubated with MG-262 in association with Everolimus.** Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 72 hours. Cell viability is expressed in percentage (%) of control and represents the mean ± SD of 3 independent experiments. The difference between experimental groups is statistically significant after 48h of incubation (**p<0,01; ***p<0,001).

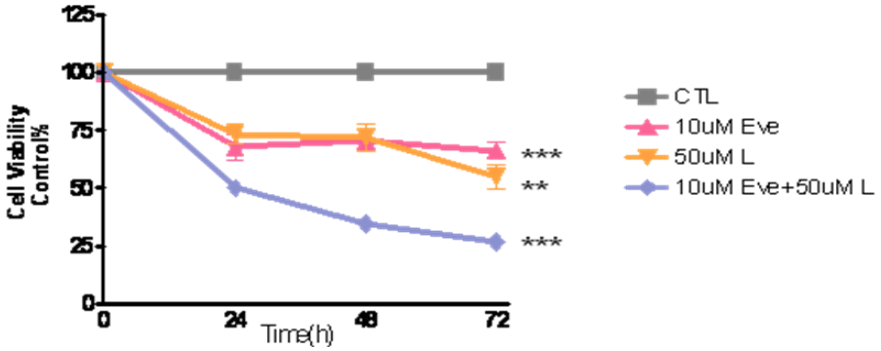

Figure 10: **Dose response curve in HUH-7 cells incubated with L-744832 in association with Everolimus.** Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 72 hours. Cell viability is expressed in percentage (%) of control and represents the mean ± SD of 3 independent

experiments. The difference between experimental groups is statistically significant after 48h of incubation (**p<0,01; ***p<0,001).

2- Morphological analysis

Since tumour cell death mechanisms can interfere with the therapeutic strategy, we also analysed the cytotoxic effect induced by the referred drugs by studying cell death process through morphological analysis by optical microscopy and FC using the AV/PI incorporation.

Figures 11-13 show the morphology of cell smears stained with May-Grünwald-Giemsa before (control) and after treatment with MG-262 (Figure 11), L-744832 (Figure 12) and Everolimus (Figure 13) in monotherapy and in association with Doxorubicin or 5-Fluorouracil during 48h. As it can be seen, cells have morphological characteristics typical of cell death by apoptosis, such as cellular retraction, nuclear fragmentation, blebbing and apoptotic bodies' formation.

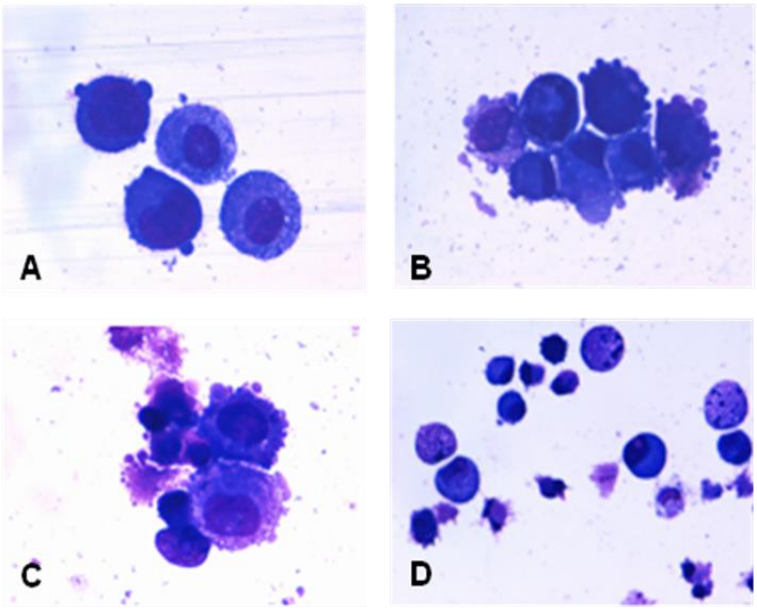

Figure 11: **Morphological analysis of HUH-7 cells before (A) and after treatment with 50nM MG-262(B) and 50nM MG-262 in association with 50ng/mL Doxorubicin (C) or 250µM 5-Fluorouracil (D) by optical microscopy.** There are morphological evidences of cell death by apoptosis, such as cellular contraction, nuclear fragmentation, blebbing and apoptotic bodies' formation**.** Amplification: 500x.

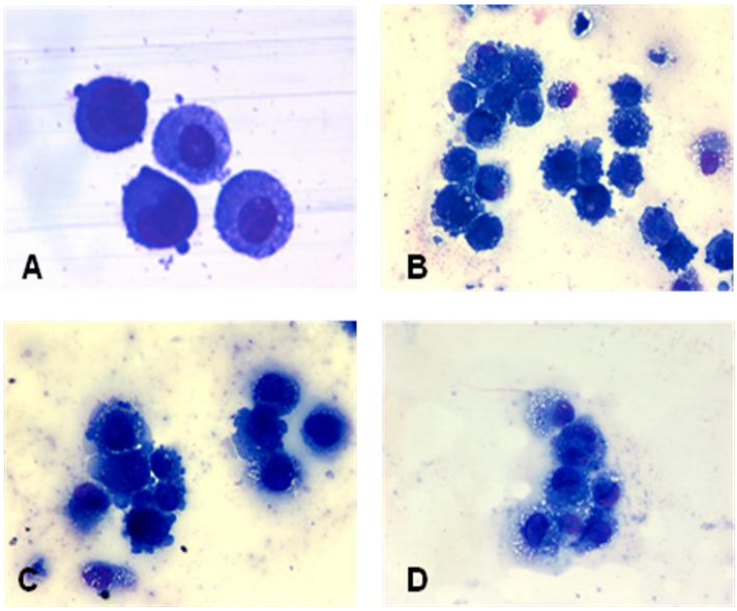

Figure 12: **Morphological analysis of HUH-7 cells before (A) and after treatment with 50µM L-744832 (B) and 50µM L-744832 in association with 50ng/mL Doxorubicin (C) or 250µM 5-Fluorouracil (D) by optical microscopy.** There are morphological evidences of cell death by apoptosis, such as cellular contraction, nuclear fragmentation, blebbing and apoptotic bodies' formation**.** Amplification: 500x.

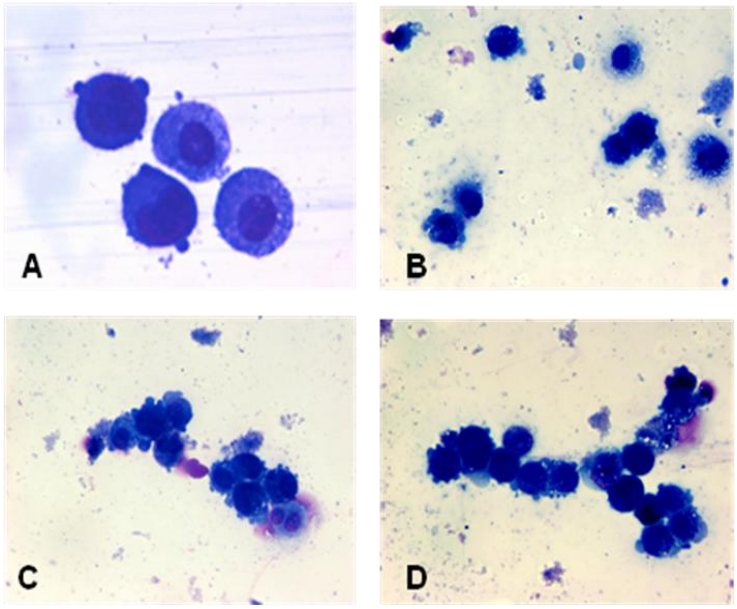

Figure 13: **Morphological analysis of HUH-7 cells before (A) and after treatment with 10µM Everolimus (B) and 10µM Everolimus in association with 50ng/mL Doxorubicin (C) or 250µM 5-Fluorouracil (D) by optical microscopy.** There are morphological evidences of cell death by apoptosis, such as cellular contraction, nuclear fragmentation, blebbing and apoptotic bodies' formation**.** Amplification: 500x.

The morphological characteristics of HUH-7 cells observed after treatment with the new target drugs in association during 48h are also consistent with cell death by apoptosis (Figure 14).

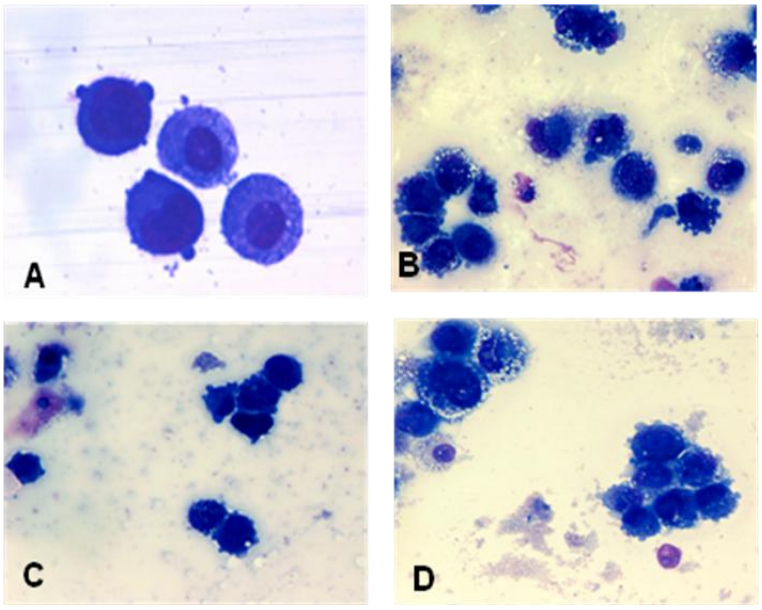

Figure 14: **Morphological analysis of HUH-7 cells before (A) and after treatment with 50nM MG-262 in association with 50µM L-744832 (B), 50nM MG-262 in association with 10µM Everolimus (C) and 50µM L-744832 in association with 10µM Everolimus (D) by optical microscopy.** There is morphological evidence of cell death by apoptosis, such as cellular contraction, nuclear fragmentation, blebbing and apoptotic bodies' formation**.** Amplification: 500x.

3.3 Flow cytometry studies

In order to confirm or results and evaluate the extent of apoptosis and necrosis, we used a FC assay based on staining the cells with AV-FITC and PI incorporation.

The results, shown in figure 15, are almost in agreement with those obtained in morphological studies, as we observed an increase in the percentage of apoptotic cells after the treatment with all the tested conditions. In fact, an increase in the number of cells in early and late stages of apoptosis was observed when cells were treated with MG-262, L-744832 and Everolimus. However, this was more pronounced in cells submitted to the FTI, where a

reduction of 50% in alive cells and a concomitant increase in the percentage of apoptotic cells was observed.

On the other hand, the synergistic effect obtained with the incubation of MG-262 in association with Doxorubicin and 5-Fluorouracil described in figure 5 was translated into an increase in apoptotic cells (Figure 15). Although the concentration of drugs was below the IC50, the apoptotic effect of the associations was superior to the apoptotic effect of each drug alone.

After the incubation of L-744832 and Everolimus in monotherapy and in association with Doxorubicin and 5-Fluorouracil we didn't observe the synergistic effect seen in the dose response curves. Besides apoptosis is the main mechanism involved in HUH-7 cells death upon their treatment with these tested conditions, the number of AV positive cells didn't increase with the drugs administered in association.

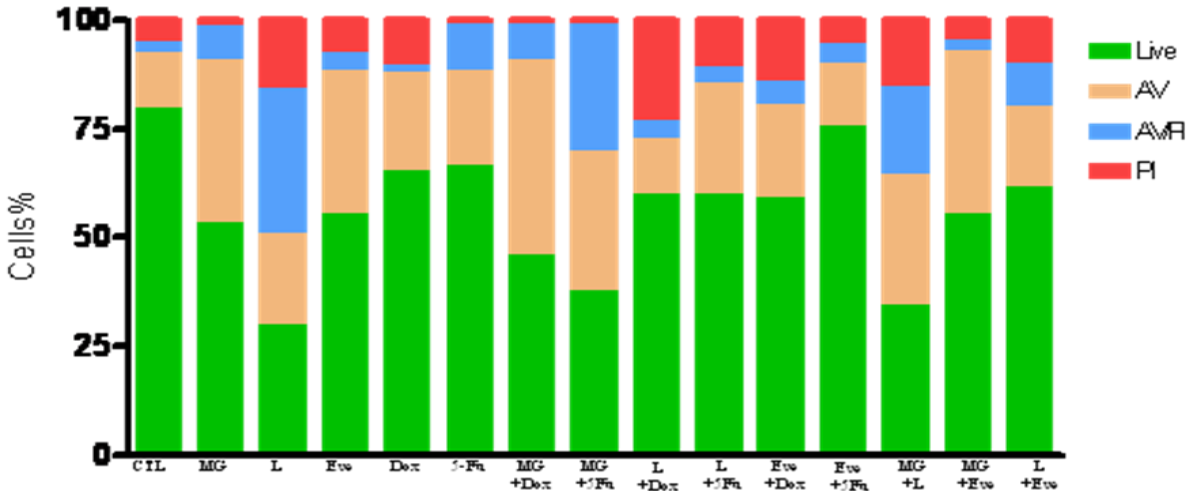

Figure 15: Evaluation of cell death by FC using AV and PI incorporation. HUH-7 cells were incubated in the absence (CTL) and in the presence of 50nM MG-262 (MG), 50µM L-744832 (L), 10µM Everolimus (Eve) in monotherapy and in association with 50ng/mL Doxorubicin (Dox) and 250uM 5-Fluorouracil (5-Fu). HUH-7 cells were also incubated with the new targeted drugs in association. Alive cells are AV/PI negative (green); early stages of apoptosis are AV positive and PI negative (light orange) and cells in late stages of apoptosis are AV/PI positive (blue). Necrotic cells are AV negative and PI positive (red). Results were obtained after 48h of incubation and represent the mean of 2 independent experiments.

In order to characterize some of the molecular events underlying the cell death and the antiproliferative mechanism observed in treated cells, we also chose to study the BAX/BCL2 ratio (Figure 16) and the Cyclin D1 expression (Figure 17).

As we can see in Figure 16, MG-262, L-744832 and Everolimus induced an increase in BAX/BCL2 ratio, whereas when these drugs were tested in association the ratio was higher (about two times).

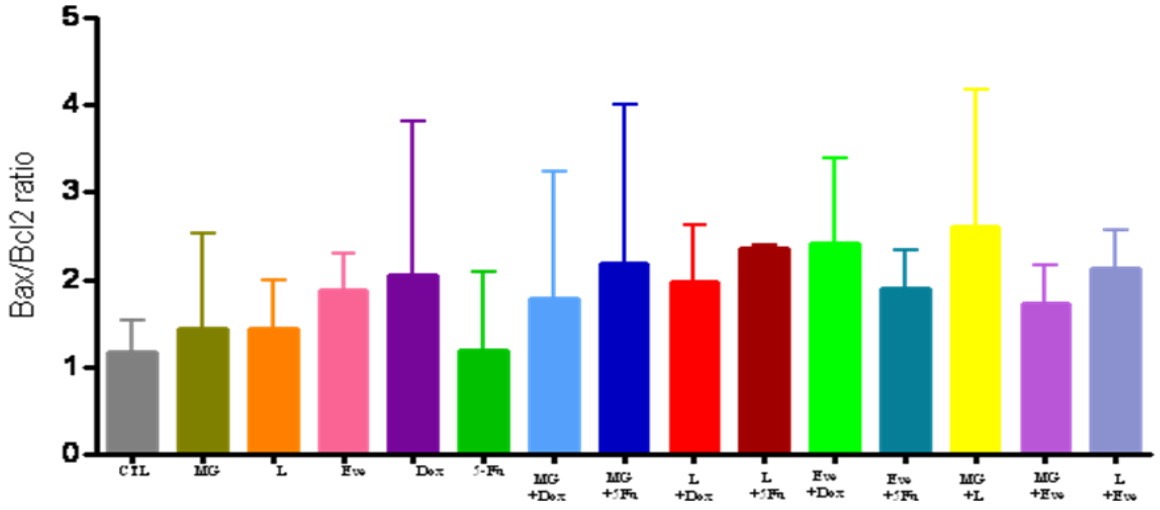

Figure 16: Evaluation of Bax/Bcl2 ratio by FC. HUH-7 cells were incubated in the absence (CTL) and in the presence of 50nM MG-262 (MG), 50µM L-744832 (L), 10µM Everolimus (Eve) in monotherapy and in association with 50ng/mL Doxorubicin (Dox) and 250µM 5-Fluorouracil (5-Fu). HUH-7 cells were also incubated with the new targeted drugs in association.BAX and BCL2 expression was calculated as described in material and methods and the ratio BAX/BCL2 calculated. Results were obtained after 48h of incubation and represent the mean ± SD of 2 independent experiments.

In figure 17 is represented the expression of cyclin D1. As we can observe MG-262 alone didn't induce significative alterations in cyclin D1 levels in HUH-7 cells. However, these levels increase when cells were treated with this proteasome inhibitor in association with Doxorubicin and 5-Fluorouracil. On the other hand, our results also show that after the treatment of HUH-7 cells with L-744832 in monotherapy and in association with MG-262,

there was an increase in cyclin D1 levels. In other way, cyclin D1 levels decreased after the treatment of HUH-7 cells with the other tested conditions.

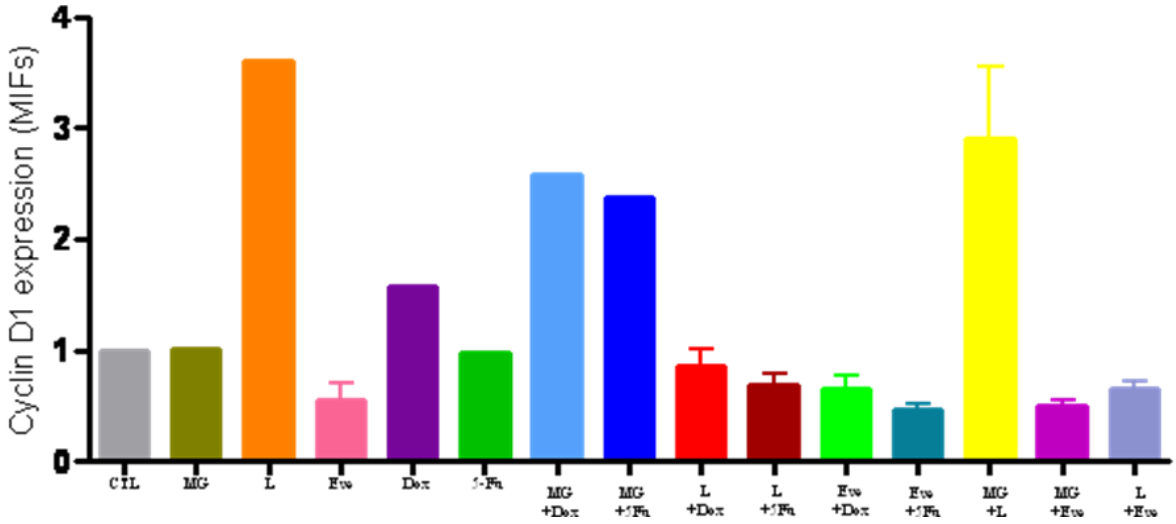

Figure 17: Analysis of Cyclin D1 expression by FC. HUH-7 cells were incubated in the absence (CTL) and in the presence of 50nM MG-262 (MG), 50µM L-744832 (L), 10µM Everolimus (Eve) in monotherapy and in association with 50ng/mL Doxorubicin (Dox) and 250µM 5-Fluorouracil (5-Fu). HUH-7 cells were also incubated with the new targeted drugs in association. Cyclin D1 expression was evaluated as described in material and methods. Results were obtained after 48h of incubation and represent the MFI detected in each cell, which is proportional to the number of cells labelled to the monoclonal antibody anti-cyclin D1. Values are expressed in relation to control and result from 1 or the mean ± SD of 2 independent experiments.

To analyse the efficacy of proteasome inhibition with MG-262 treatment in monotherapy and in association, the expression of ubiquitin conjugates was determined by flow cytometry.

As we can see in Figure 18, when cells were treated with MG-262 in monotherapy and in association with 5-Fluorouracil no significative changes in ubiquitin conjugates formation was seen. However, a decrease in ubiquitin conjugates formation was observed when HUH-7 cells were submitted to the association of MG-262/5-Fluorouracil. On the other hand, the ubiquitin conjugates formation increased after treatment with MG-262/L-744832.

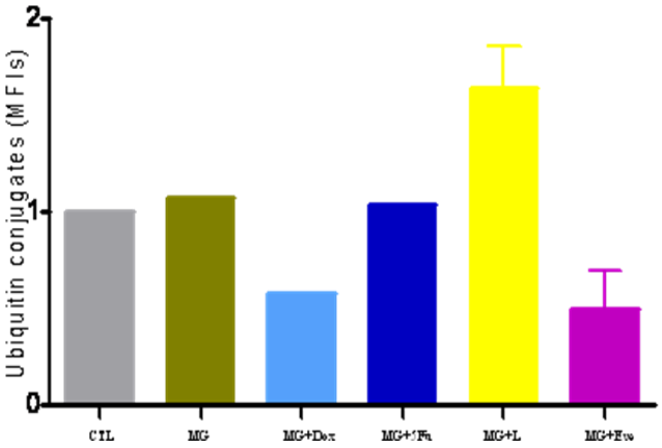

Figure 18: Analysis of Ubiquitin conjugates expression by FC. HUH-7 cells were incubated in the absence (CTL) and in the presence of 50nM MG-262 (MG) in monotherapy and in association with 50ng/mL Doxorubicin (DOX), 250uM 5-Fluorouracil (5Fu), 50uM L-744832 (L) and 10uM Everolimus (Eve). Results were obtained after 48h of incubation and represent the MFI detected in each cell, which is proportional to the number of cells labelled to the monoclonal antibody. Values are expressed in relation to control and result from 1 or the mean ± SD of 2 independent experiments.

The efficacy of inhibition of farnesyltransferase activity with L-744832 in monotherapy and in association was determined through the analysis of Laminins A/C expression.

As we can observe in Figure 19, Laminin expression increased after treatment with L-744832 in monotherapy and in association with MG-262 but decreased after treatment with L-744832 in association with Doxorubicin. On the other hand the association of L-744832 with 5-Fluorouracil or with Everolimus didn't change laminin levels as compared to control.

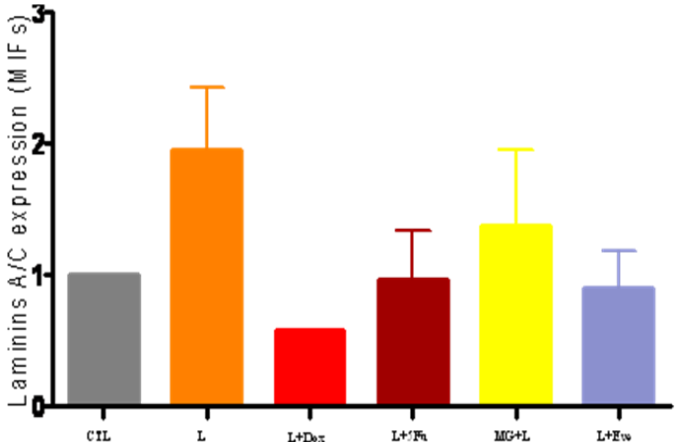

Figure 19: Analysis of Laminis A/C expression by FC. HUH-7 cells were incubated in the absence (CTL) and in the presence of 50uM L-744832 (L) in monotherapy and in association with 50ng/mL Doxorubicin

(DOX), 250uM 5-Fluorouracil (5Fu), 50nM MG-262 (MG) and 10uM Everolimus (Eve). Results were obtained after 48h of incubation and represent the MFI detected in each cell, which is proportional to the number of cells labelled to the monoclonal antibody. Laminins A/C are expressed in relation to control and represent mean ±SD of 2 independent experiments.

DISCUSSION AND CONCLUSION

HCC is a deadly cancer whose incidence has increased dramatically over the past decades. In Europe and the United States this increasing incidence exists mainly due to the rise in hepatitis C virus infection (Lachenmayer *et al.*, 2010).

Several chemotherapeutic protocols have been investigated to treat advanced HCC that is deemed incurable. Despite the lack of encouraging results, chemotherapy is still offered to patients with no other therapeutic alternatives (Piguet *et al.*, 2008).

However, the extensively studying of mechanisms involved in hepatic carcinogenesis, namely genetic and epigenetic alterations, chromosomal aberrations, mutations, and altered molecular pathways, can provide the development of molecular targeted anti-cancer molecules (Lachenmayer *et al.*, 2010).

Wnt-β-catenin, hedgehog, c-Met, IGF, RAS-MAPK, PI3/Akt/mTOR, and apoptosis are the most important intracellular molecular signaling pathways involved in HCC (Lachenmayer *et al.*, 2010).

In this regard, we evaluated, using a HCC cell line (HUH-7 cells), the *in vitro* efficacy of new targeted drugs, namely the proteasome inhibitor, MG-262, the FTI, L-744832, and the mTOR inhibitor, Everolimus, not only in monotherapy but also in combination with each other and with conventional anticarcinogenic agents.

Actually, there are already many clinical trials at various stages using these three classes of molecules in various types of cancer, but in HCC the studies are scarce.

Proteasome inhibition, with Bortezomib, has successfully been evaluated in a number of published and ongoing trials for solid and hematologic malignancies. Besides its prominent role in multiple myeloma, Bortezomib is presently approved by the Food and Drug Administration for use in relapsed and refractory mantle cell lymphoma. Bortezomib also displayed substantial activity in non-small cell lung cancer (Armeanu *et al.*, 2008). The effects of Bortezomib on HCC cell lines have also been studied *in vitro* (Baiz *et al.*, 2009).

FTIs, e.g. Tipifarnib and Lonafarmib, have been shown to inhibit the proliferation of a variety of human tumor cells, both *in vitro* and *in vivo*. FTIs have already entered several phase I/II/III clinical trials in haematological malignancies and solid tumors, such as pancreas, lung, prostate and bladder (Harousseau, 2006; Perabo and Müller, 2007; Li *et al.*, 2009). Lonafarmib has also entered a phase IB clinical study in patients with ressecable primary liver neoplasm (www.clinicaltrials.gov).

mTor inhibitors, e.g Everolimus and Tensirolimus are currently being evaluated in clinical trials for the treatment of many types of cancer such as haematological malignancies and breast, prostate, bladder, kidney and neuroendocrine tumors (Strimpakos *et al.*, 2009 and Garcia *et al.*, 2008). Tensirolimus is presently approved by the Food and Drug Administration for the treatment of advanced kidney cancer. AZD8055 and Sirolimus have also entered a phase I/II and phase III clinical studies , respectively, in patients with HCC (www.clinicaltrials.gov).

As there is only a few work done with these new molecules in HCC, namely in association, in this study we intend to evaluate the therapeutic efficacy and the possible synergistic effect of proteasome inhibitor, MG-262, farnesyltransferase inhibitor, L-744832 and mTOR inhibitor, Everolimus, in monotherapy and in combination with conventional chemotherapy, using an HCC cell line in culture.

Our results show that MG-262, L-744832 and Everolimus have an antiproliferative and cytotoxic effect in monotherapy depending on the applied dose and incubation period in the HCC cell line HUH-7. The combination of MG-262, L-744832 and Everolimus with conventional anticarcinogenic drugs demonstrates an increase in antiproliferative effect for lower doses than the IC50 (addition synergism). On the other hand, a significant synergistic antiproliferative effect was also observed with the combined treatment of MG-262 and L-744832, MG-262 and Everolimus and of L-744832 and Everolimus. In fact the same antiproliferative effect is achieved at lower concentrations than those obtained for all the tested drugs in monotherapy. In all the cases, the reduction of concentrations of new and conventional drugs may decrease the potential side effects of these anti-carcinogenic drugs, suggesting that there is a rational basis to translate into a clinical setting these new targeted drugs.

With our work, we can also conclude that apoptosis is the main mechanism involved in the cytotoxicity induced by the tested drugs. In fact, there are morphological evidences characteristic of apoptotic cell death, such as cellular retraction, nuclear fragmentation, blebbing and apoptotic bodies' formation. These morphological changes observed in microscopic slides are in agreement with those observed by FC analysis using AV/PI incorporation and correlated with the increase in BAX/BCL2 ratio. In fact, there is an increase in percentage of apoptotic cells after treatment with the tested conditions. However, the percentage of apoptotic cells is higher in association than in monotherapy with schemes involving the proteasome inhibitor, particularly when combined with the conventional anticarcinogenic drugs.

The sensibility of the HUH-7 cells to the drug regimens may be related with the increase in BAX/BCL2 ratio, suggesting that intrinsic or mithocondrial pathway may be involved in apoptosis induced by the tested drugs. On the other hand, these results are in agreement with

the increase in proapoptotic molecules, such as BAX, that may be related with proteasome inhibition. In reality, BAX is one protein that is degraded in this enzymatic complex (Milano *et al.,* 2007).

These findings may be relevant for the clinical application of these new target molecular therapies, since therapeutic strategies involving tumor cell death through apoptosis are advantageous as compared to those inducing necrosis, in which case the release of death factors to medium can cause toxicity in healthy tissue surrounding the tumor (Neves *et al.,* 2006).

In order to evaluate the antiproliferative effect of the targeted therapies used in the study we analysed the expression of cyclin D1 by FC. Our results show higher levels of cyclin D1 after the treatment with L-744832, and lower levels after the treatment with Everolimus in comparison with control cells; MG-262 failed to modify cyclin D1 levels.

FTIs are potent modulators of cell cycle, depending on the cell line. They can induce accumulation of cells in G0/G1 or G2/M phase cells, in some human tumour cell lines, while in others no effect is observed on cell cycle distribution depending on the cell line (Mazieres *et al,* 2004). This can explain the up-regulation of the cyclin D1 levels shown in our cell line in opposition to other studies in haematological cell lines (Costa *et al.*, submitted). In order to confirm and clarify the importance of FTIs on HUH-7 cell cycle, it will be also important to study the different phases of cell cycle in the tested conditions.

The ubiquitin-mediated proteasome pathway is the main mechanism of degradation for short-lived cellular regulatory proteins, including p53, cyclins and the cyclin-dependent kinase (CDK) inhibitors p21 and p27, the oestrogen receptor, and the inhibitor of the nuclear transcription factor kappa B (IkB). Expression of specific cyclins is regulated differently by proteasome degradation during each phase of the cell cycle. In addition, the activity of CDKs is regulated further by a variety of inhibitor factors, such as p21 and p27, which are also

proteasomal substrates. Activated p53 arrests cells in the G1-phase and promotes apoptosis through induction of BAX, which, in turn, is also a proteasomal substrate. Taken together, these findings suggest that proteasome inhibition results in the stabilisation of p53, p21, p27 and BAX, dysregulation of cell-cycle progression and, finally, apoptosis (Milano *et al.*, 2007). This stabilization can also explain the unchanged levels of cyclin D1 after treatment with MG-262.

On the other hand, we observed lower levels of cyclin D1 after treatment with Everolimus, in comparison with control cells. mTOR permits translation of proteins that drive cell growth, cell proliferation, and the production of angiogenic growth factors; thus, the inhibition of mTOR delays cell cycling at G1-S phase interface, inhibiting cell growth and angiogenesis (Amato *et al.*, 2009). Consequently, mTOR inhibitors may down-regulate cyclin D1 levels.

We also intend to clarify the molecular mechanisms related with the mechanism of action of the proteasome and the farnesiltransferase inhibitors through the analysis of the expression of ubiquitin conjugates and laminin A/C, respectively.

The ubiquitin-proteasome system is the major catabolic pathway for degradation of short-lived and misfolded proteins. Proteins to be degraded through this pathway first undergo polyubiquitination followed by recognition and proteolysis by proteasome *(Wu et al., 2010)*. Since inhibition of the ubiquitin-proteasome pathway in tumor cells results in accumulation of ubiquitinated proteins, we were expecting an increase of ubiquitin conjugates. In our study we only observe an increase in ubiquitin conjugates levels when cells are incubated with PI in association with 5-Fluoracil and L-744832.

However we have also to consider that the lysosome-autophagy pathway serves complementary with the ubiquitin-proteasome system are the two major protein degradation systems (Wu *et al.*, 2010). Simultaneously, it is already described that mTOR inhibitors

activate the autophagic process (Easton and Houghton, 2006) and that Doxorrubicin, at clinical relevant dose range, activates directly the proteasome (Liu *et al.,* 2008). It has also been reported that proteasome inhibitors induce macroautophagy in breast, colon and glioma cancer cells (Wu *et al.,* 2010). In this way, these findings could be the explanation for the obtained results.

In our study the activity of farnesyltransferase was evaluated following the markers of farnesylation described by Adjei *et al.,* 2000: prelamin A and HDJ-2. Franesyltransferase is essential for the processing of prelamin A into mature lamin A (Kilic *et al.,* 1997) and the use of monoclonal antibodies to measure the intracellular levels of both these molecules may serve as marker of the activity of farnesyltransferase.

Prenylation of proteins is carried out by FTase, GGTase-1 and GGTase-2. FTase and GGTase-1 can act on Ras proteins, but physiologically only FTase is involved (Brunner *et al.,* 2003). The exact mechanism of action of FTI is still currently unknown. FTIs inhibit farnesylation not only of protein RAS but also of a wide range of target proteins, including RHOB, nuclear laminins A and B, transducin, centromeric proteins CENP-E and CENP-F (Appels *et al.,* 2005). Under FTI treatment there is alternative prenylation of certain proteins including RHO-B, K-RAS and N-RAS by GGTase-1 (Brunner *et al.,* 2003).

Our results failed to show a decrease in nuclear laminins. However, this can be related with the dose of L-744832. In fact it was already reported that the farnesyltransferase inhibition isn't immediately correlated to the decrease of cell viability. In fact, the concentrations necessary to obtain biological effects were consistently superior to the concentrations need to inhibit farnesylation (Appels *et al.,* 2005; Costa *et al.,* submitted).

Overall, the present study suggests that proteasome, farnesiltransferase and mTOR inhibitors may constitute a new potential therapeutic approach in HCC not only in monotherapy, but also in association with conventional therapies.

These new molecular targeted therapies and the increasing knowledge of hepatic carcinogenesis will, consequently, contribute to personalize medicine in HCC treatment. However, the choice of an optimal drug schedule will also be crucial to the success of the therapy.

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