FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

TRABALHO FINAL DO 2° ANO DO MESTRADO EM INVESTIGAÇÃO BIOMÉDICA

MARTA DE JESUS RIBEIRO

TERAPIA GÉNICA – AVALIAÇÃO DO POTENCIAL TERAPÊUTICO EM CÉLULAS DE CARCINOMA HEPATOCELULAR EM CULTURA.

ÁREA CIENTÍFICA DE BIOLOGIA MOLECULAR/ONCOLOGIA

TRABALHO REALIZADO SOB A ORIENTAÇÃO DE:

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SETEMBRO 2012

GENE THERAPY - EVALUATION OF THE THERAPEUTIC POTENTIAL IN HEPATOCELLULAR CARCINOMA CELL LINES

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RESUMO

O carcinoma hepatocelular (CHC) é uma doença com mau prognóstico cuja incidência tem aumentado dramaticamente nas últimas décadas. Muitos estudos têm vindo a ser realizados e variados protocolos investigados para o seu tratamento. Apesar disso, a taxa de mortalidade dos doentes com CHC avançado ainda é muito elevada, havendo assim a necessidade de desenvolvimento de novos fármacos, e de novas opções de tratamento, que poderão ser utilizados como agentes únicos ou em combinação. Esta falta de opções terapêuticas seguras e eficientes contra muitos tipos de cancro tem vindo a incentivar o desenvolvimento de novas aplicações para a terapia génica.

A terapia génica suicida envolve a entrega de um gene suicida às células-alvo, tornando-as sensíveis a determinado pró-fármaco sendo os sistemas mais utilizados a timidina cinase do Vírus Herpes Simplex (HSV-TK)/ ganciclovir (GCV) e a citosina desaminase bacteriana (CD)/ 5-fluorocitosina (5-FC). A entrega do material genético pode ser feita por vectores virais ou não virais. Devido aos problemas que têm surgido com a utilização de vectores virais, principalmente relacionados com questões de segurança, os lipossomas catiónicos surgiram como sistemas promissores, devido à sua baixa toxicidade e imunogenicidade, à falta de patogenicidade e versatilidade.

O objectivo do presente estudo consiste na avaliação do potencial terapêutico *in vitro*, em linhas celulares de carcinoma hepatocelular (HUH-7 e Hep-G2), das estratégias de terapia génica suicida (HSV-TK / GCV ou CD / 5-FC), em monoterapia ou em terapia combinada com agentes de quimioterapia convencional (doxorrubicina) e novos fármacos dirigidos a alvos moleculares (inibidor proteasoma MG-262), utilizando para a transfecção lipossomas associados a transferrina. A viabilidade foi avaliada pela técnica do Alarmar Blue e os mecanismos de morte verificados por citometria de fluxo com AV/IP, morfologia em esfregaços corados por May-Grünwald-Giemsa e análise do ciclo celular.

Os resultados obtidos indicam que a aplicação da terapia génica suicida utilizando os sistemas HSV-TK/GCV ou CD/5-FC, tem um efeito antiproliferativo e citotóxico dependente da dose de pro-fármaco utilizada e do tempo de incubação. A combinação destas abordagens terapêuticas com doses inferiores ao IC50 de MG-262 ou doxorrubicina apresenta aumento do efeito antiproliferativo e citotóxico, indicando que a aplicação combinada de doses mais baixas de outros fármacos poderá levar a uma potenciação dos resultados com níveis de toxicidade e efeitos secundários reduzidos. Este efeito antiproliferativo foi verificado através das alterações observadas no ciclo celular das células (acumulação na fase G2 / M). Por citometria de fluxo e por estudos de morfologia, verificou-se que o mecanismo de morte celular predominantemente observado após aplicação dos diferentes tratamentos foi a apoptose.

Estes resultados sugerem que a terapia de génica suicida com HSV-TK/GCV ou CD/5-FC pode constituir uma nova abordagem com potencial terapêutico no CHC não só em monoterapia, mas também, em associação com as terapias convencionais ou novos fármacos dirigidos a alvos moleculares.

PALAVRAS-CHAVE

Carcinoma hepatocelular, linha celular HUH-7, linha celular Hep-G2, terapia génica suicida, TK/GCV, CD/5-FC, inibidor do proteasoma MG262, lipossomas catiónicos, transferrina, apoptose.

ABSTRACT

HCC is a deadly cancer whose incidence has increased dramatically over the past decades. Although many studies have been made and protocols investigated to treat HCC, the mortalilty rate of patients with advanced HCC is still high, so, there is an unmet need for new drugs, or new treatment options either as a single agents or in combination. This lack of safe and efficient therapeutic options against many types of cancer is encouraging the development of new gene therapy applications.

Suicide gene therapy involves the delivery of a suicide gene into target cells, making them sensitive to an appropriate prodrug being the most commonly used Herpes Simplex Virus thymidine kinase (HSV-TK)/ ganciclovir (GCV) and the bacterial cytosine deaminase (CD)/ 5-fluorocytosine (5-FC) systems. This delivery can be made by viral or non-viral vectors. However, due to the problems involving the use of viral vectors, namely regarding safety issues, cationic liposomes emerged as promising systems due to their low toxicity and immunogenicity, lack of pathogenicity and versatility.

The aim of the present study is to test the in vitro therapeutic potential in HCC cell lines (HUH-7 and Hep-G2), of suicide gene approaches (HSV-TK/ GCV or CD/ 5-FC), in monotherapy and in combination with conventional anticarcinogenic agents (doxorrubicin) and new targeted drugs (proteassome inhibitor MG-262), using liposomes coupled with transferrin. Viability was assessed by Alamar Blue assay and death mechanisms were verified by flow cytometry with AV/PI, morphology in smears stained with May-Grünwald-Giemsa and cell cycle analyses.

Our results showed that suicide gene therapy using HSV-TK/GCV or CD/5-FC systems, resulted in a decreased viability, depending on the respective pro-drug dose and incubation time in HCC cell lines in culture. Combinations of these systems with lower doses than the IC50 of MG-262 or Doxorubicin enhanced the antiproliferative and cytotoxic effect

meaning lower doses of other drugs with potentiation of the results and reduced toxicity levels and side effects. This antiproliferative effect was in agreement with the observed cell cycle alterations (accumulation in G2/M phase) upon applications of the suicide gene systems alone or in combination with doxorubicin and proteasome inhibitor. By flow cytometry and morphology studies, we observed that the mechanism of cell death was apoptosis upon application of the different treatments.

These results suggest that suicide gene therapy with HSV-TK/GCV or CD/5-FC may constitute a new potential therapeutic approach in HCC not only in monotherapy, but also in association with conventional therapies or new targeted drugs.

KEYWORDS

Hepatocellular carcinoma, HUH-7 cell line, Hep-G2 cell line, suicide gene therapy, TK/GCV, CD/5-FC, proteassome inhibitor, cationic liposomes, transferrin, apoptosis.

ABBREVIATIONS LIST

5FC: 5-fluorocytosine

AV: annexin V

CD: cytosime desaminase

DMEM: Dulbecco's Modified Eagle's medium

DOX: doxorubicin

FBS: fetal bovine serum

FC: flow cytometry

FICT: fluorescein isothiocyanate

GCV: ganciclovir

HCC: hepatocellular carcinoma

IC50: half-maximal inhibitory concentration

MIF: mean intensity of fluorescence

PBS: phosphate buffer solution

PI: propidium iodide

TK: tirosine kinase

INTRODUCTION

1- Hepatocellular carcinoma (HCC)

Liver cancer is one of the most common malignant tumors worldwide. In men, liver cancer is the fifth most common neoplasm in the world and the second most common cause of cancer-related death. In women, it is the seventh most common cancer and the sixth leading cause of cancer death. An estimated 748,300 new liver cancer cases and 695,900 deaths occurred worldwide in 2008 (Jemal A, 2011).

Hepatocellular carcinoma (HCC) is a major health problem, representing the major histological subtype among primary malignant liver tumors, accounting for 70% to 85% of the total liver cancer burden worldwide, (Avila MA, 2006; Jemal A, 2011).

The highest liver cancer rates are found in East and South-East Asia and in Middle and Western Africa, with an incidence of 50–150 cases per 100,000 population and year, whereas in South-Central and Western Asia, as well as in Northern and Eastern Europe, the rates are lower. A higher incidence, of 10 cases per 100,000 population year, is found in North America and Western Europe (Jemal A, 2011; Spangenberg HC, 2008).

These geographical differences in incidence may reflect variations in the main causal HCC factors.

In some parts of Asia and Africa the high HCC incidence rates reflect the elevated prevalence of chronic hepatitis B (HBV) or C (HCV) virus and aflatoxin B1 intake from contaminated food. Their interaction has also been noted to increase liver cancer (Jemal A, 2011; Severi T, 2010).

In the USA and other low-risk Western countries, HCV infection is, as well as other causes of cirrhosis, such as alcohol and haemochromatosis and possibly nonalcoholic fatty

liver disease, associated with obesity, account for the majority of liver cancer (Jemal A, 2011; Llovet JM B. A., 2003). Being the rise in its incidence and mortality possibly due to epidemic obesity and the rise in HCV infection (Severi T, 2010 ; Jemal A, 2011),. In contrast, in some historically high-risk areas rates decreased, possibly due to the HBV vaccine (Jemal A, 2011).

In a study performed from 1993 to 2005, in Portugal, the conclusions of the demographic data were that the rate of mortality by Chronic Liver Disease and Cirrhosis are similar to that by HCC. Besides that, one quarter of patients admitted to hospitals in Portugal for HCC died and the most common cause of cirrhosis in Portugal is alcoholic cirrhosis, with two-thirds of the total number of cases (Marinho RT, 2007).

In more than 80% of cases, HCC is associated with cirrhosis or with advanced fibrosis. In the absence of cirrhosis or advanced fibrosis HBV infection, aflatoxin B1, some genetic disorders such as Tyrosinosis and drugs (e.g. anabolic steroids) are the main risk factors (Severi T, 2010 ; Lachenmayer A, 2010). However in patients with liver cirrhosis, the risk to develop HCC depends on the activity, duration, and the etiology of the underlying liver disease.

Some clinical and biological variables as age, anti-HCV positivity, partial prothrombin time value, and platelet count, may enable identification of a subset of patients with the highest risk of HCC development. Coexistence of various etiologies, as HBV and HCV infection, aflatoxin B1 exposure, alcohol and/ or tobacco use, diabetes mellitus, liver steatosis and obesity, has also to be considered and further increases the relative risk of HCC development (Spangenberg HC, 2008).

HCC is phenotypically (morphology, microscopy) and genetically very heterogeneous, possibly in part due to the heterogeneity of etiologic factors implicated in HCC development, the complex functions of the liver cell, and the advanced stage at which HCC usually are becoming clinically symptomatic and diagnosed (Spangenberg HC, 2008).

As for most types of cancer, hepatocarcinogenesis is a multifactorial multistep process (Figure 1) involving different genetic and epigenetic alterations, chromosomal aberrations, mutations, and altered molecular pathways that result in the deregulation of key oncogenes and tumor-suppressor genes involved in several signaling pathways. These events accumulate in a cell and ultimately lead to malignant transformation of the hepatocyte and to clinical liver cancer. The molecular contribution of the different factors, their interaction and exact sequence of hepatocarcinogenesis, including the development of preneoplasic lesions and progression to HCC, are still poorly understood (Lachenmayer A, 2010; Llovet JM B. J., 2008).

The previously mentioned risk factors for HCC can cause initial damage to the liver, but several additional external or internal influences are needed to initiate the multistep process leading to HCC development (Lachenmayer A, 2010).

In general, any condition leading to chronic inflammation in the liver causes genomic and mitochondrial cells damage facilitating the development of neoplasms. Malignant transformation of hepatocytes may occur, regardless of the etiologic agent, through a pathway leading to increased liver cell turnover, induced by chronic liver injury and regeneration in a context of inflammation, immune response and oxidative DNA damage. So, in populations of both mature hepatocytes or liver stem cells this increased turnover renders liver cells more sensitive to the adverse effects of other mutagenic agents and genetic and epigenetic changes may occur, leading to the subsequent formation of dysplastic nodules and finally to HCC (Spangenberg HC, 2008 ; Lachenmayer A, 2010; Severi T, 2010).

Chronic hepatitis B, C, and D, alcohol, metabolic liver diseases such as hemochromatosis and α-1-antitrypsin deficiency may act predominantly through this pathway of chronic liver injury, regeneration, and cirrhosis (Spangenberg HC, 2008).

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Figure 1: Schematic representation of the multistep process of hepetocarcinogenesis (Adapted from Severi T, 2010).

From an epidemiological and clinical point of view HBV, HCV and alcoholic cirrhosis are clearly the most important diseases for the development of HCC. From a pathophysiological perspective, oxidative stress represents an important pathway by which viruses or others risk factors exert their carcinogenic properties (Severi T, 2010).

The tumor environment also plays an important role in tumorigenesis and tumor progression. Cancer cells are not as autonomous as once thought, they depend on angiogenesis, inflammatory cells and fibroblasts. Most HCC cases occur after many years of chronic liver disease that provides a mitogenic and mutagenic environment with abundance of fibroblasts, some of them cancer associated fibroblasts (Severi T, 2010). While there is evidence that HBV and HCV may, under certain circumstances, play an additional direct role in the molecular hepatocarcinogenesis, aflatoxins have been shown to induce mutations of the p53 tumor suppressor gene, thus pointing to the contribution of an environmental factor to tumor development at the molecular level (Spangenberg HC, 2008).

Current therapeutic strategies for HCC can be divided into established therapies as surgical interventions (tumor resection and liver transplantation), percutaneous interventions (ethanol injection, radiofrequency thermal ablation), transarterial interventions (embolization, chemoperfusion, or chemoembolization), and experimental strategies such as radiation therapy and drugs, including gene and immune therapy. Potentially curative therapies are tumor resection, liver transplantation, and percutaneous interventions that can result in complete responses and improved survival in a large proportion of patients. However, surgical resection is limited by tumor size, the presence of multiple lesions, and impaired function in the case of cirrhotic livers and liver transplantation is limited by a shortage of organ donations and occurrence of transplant relapse (Spangenberg HC, 2008). In advanced disease, not eligible to the use of potentially curative therapies, systemic treatment has been suggested as beneficial to some patients, especially with the use of tamoxifen and doxorubicin (Alves RC, 2011).

Until recently, there wasn't any available therapy that prolonged overall survival in patients with advanced HCC, indicating the need for new therapies. In 2007, with the advent of new targeted drugs hope has been offered to patients. One of this new targeted drug is Sorafenib, a multikinase inhibitor, that block the VEGF and PDGF-dependent angiogenesis, showed an increase on overall survival in patients with unresectable HCC. This drug is now considered the standard of care in patients with advanced HCC and preserved liver function. Unfortunately, all patients with advanced HCC still die from the disease and there is an unmet need for other drugs, or treatment options either as a single agent or in combination (Severi T, 2010).

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 KR, 2006). Proteasome

inhibition has already been established as a strategy for multiple myeloma and non-Hodgkin's lymphoma patients (Baiz D, 2009).

Treatment of patients with HCC is based on staging, which includes assessment of tumor extent, liver function, portal pressure and clinical performance status.

Several systems that address the extent and prognosis of the disease have been proposed for HCC staging, being the more used the Barcelona Clinic Liver Cancer (BCLC) classification (Spangenberg HC, 2008). So far, HCC molecular classification has not yet found its place in the staging systems and selection of therapy. However, the active search and analysis for biomarkers in all current phase III studies may rapidly change the field (Severi T, 2010).

The widely used Barcelona-Clinic-Liver-Cancer (BCLC) staging system links staging of HCC in cirrhosis with treatment modalities. The system identifies patients with early HCC (stage 0 and A) who may benefit from curative therapies (resection, radiofrequency ablation, liver transplantation), patients at intermediate or advanced stage (stage B, C) who may benefit from palliative treatments (such as transarterial chemo- or radioembolisation), and the patients with a very poor life expectancy (stage D) where supportive care is the only option (Severi T, 2010).

In 2010 less than 40% of patients in the western world fulfilled criteria for curative treatment (resection, transplantation, local ablation) and only 20% were eligible for chemoembolization. Alternative or palliative treatment options are very limited due to resistance to conventional chemotherapy and radiotherapy (Lachenmayer A, 2010). Therefore, there is an urgent need for new therapeutic strategies for HCC and the knowledge of its molecular pathogenesis can provide the opportunity to indentify new biomarkers and establish a molecular classification for HCC and for the development of new experimental strategies, including target therapies, gene and immune therapy.

2- Gene therapy

Gene therapy represents a promising therapeutic strategy based on the transfer of genetic material into cells in order to generate a beneficial effect against the diseases. The main purpose of gene therapy involves the inserting of "functional" genes into patient's cells in order to replace the "defective" genes but, other manipulation approaches, involving the repair of defective genes, turning off defective genes or introducing normal genes into the target cells, in order to obtain a therapeutic effect, are mostly used . The genetic material can be packaged and delivered, using a variety of viral and non-viral vectors (*in vivo* gene therapy), or delivered into isolated cells *in vitro* that are subsequently introduced into the organism (*ex vivo* gene therapy) (Hernandez-Alcoceba R, 2006; Hwang, 2006; Tani J, 2011).

Recent studies investigated various potential applications for gene therapy, and clinical applicability has been successfully demonstrated in several diseases, including the treatment of Leber's congenital amaurosis and Severe Combined Immunodeficiency. Most studies explored the potential role of gene therapy in correcting genetic diseases as certain muscular distrophies, Huntington's, Haemophilia, Parkinson's and Alzheimer's disease, or treating malignant disorders but there are also other studies on cardiovascular diseases or AIDS (Tani J, 2011).

So although the initial aim for the field of gene therapy was the correction of inherited genetic diseases it can also be applied to the treatment of acquired genetic deficiencies, in particular cancers (Touchefeu Y, 2010).

3- Cancer gene therapy

Despite the advances that have been achieved for the treatment of neoplastic diseases, as in terms of specificity, efficiency and decreased side effects of radiation therapy,

chemotherapy, as in the development of drugs directed to new molecular targets, many tumors remain resistant to existing therapies especially those who are diagnosed in advanced stage.

This lack of safe and efficient therapeutic options against many types of cancer, gives rise to the necessity to develop alternative therapies that can achieve more effective results, encouraging in this way, the development of new gene therapy applications for these diseases (Sangro B, 2010).

Gene therapy for treatment of cancer is a rapidly growing field and the most frequent application of experimental gene therapy approaches (Tani J, 2011). Besides the mentioned lack of therapeutic options, some of the reasons why the applicability of gene therapy in cancer is a rapidly growing field, are related to the genetic alterations that give rise or contribute to the malignant transformation of cells being unraveled. These provide multiple candidate targets for gene therapy intervention. (Hernandez-Alcoceba R, 2006).

Cancer gene therapy involves the manipulation of intracellular DNA in order to control or destroy cancer cells. Like cytostatic chemotherapies, it is assumed that there are certain biochemical, molecular or environmental characteristics of tumour cells which distinguishes them from normal tissues, that are able to be exploited for gene therapy (Hughes, 2004; Dachs GU, 2009). In this way gene therapy has the potential to provide highly selective, curative cancer treatment without systemic toxicity.

Strategies for cancer gene therapy include inhibition of activated oncogenes, transfer or activation of tumor suppressor and cytokine genes (immunotherapy), inhibition of angiogenesis and selective prodrug activation by suicide genes (Neves S, 2009).

3.1 Tumor suppressor gene replacement

Tumor suppressor genes encode a variety of proteins that regulate the cell cycle and mediate DNA repair pathways. These genes control cell proliferation and apoptosis in order to

maintain an equilibrated cell turnover in each tissue. The progression from normal tissue to tumor involves the presence of mutations or loss of function of some of these genes and by inhibiting the expression of tumor suppressor genes. Cancer cells can continue to proliferate, acquire new gene mutations, become less differentiated, and avoid apoptosis. One gene therapy approach is, thus, to restore tumour-suppressor gene expression (Hughes, 2004), (Hernandez-Alcoceba R, 2006).

In fact transfection of tumor suppression genes into cancer cells has been effective at arresting growth and inducing apoptosis. Among the various tumor suppressor genes capable of triggering apoptosis, the gene encoding the p53 protein has been the more studied, since the functional inactivation of this protein occurs in over 50% of tumors (Sherr, 2004). The goal of this approach is to restore the function of p53, which is involved in the regulation of DNA repair processes and apoptosis in abnormal or damaged cells, and therefore, in inhibiting tumor growth. Under experimental conditions, it has been demonstrated that the restoration of tumour suppressor genes can revert the malignant phenotype of cells. The transfer of p53 tumour suppressor gene has shown effect in several cancer animal models, including HCC (Hernandez-Alcoceba R, 2006).

About 11% of transferred genes in gene therapy clinical trials are tumour-suppressor genes and many trials have been performed in cancer gene therapy using the p53 gene, mostly including patients with lung or head and neck cancers (Touchefeu Y, 2010).

3.2 Oncogene inactivation

The progression from normal tissue to tumor also involves the presence of mutations in proto-oncogenes that are silenced after fetal development to prevent abnormal tissue growth. Cancer cells often propagate by activating and amplifying these proto-oncogenes (Hughes, 2004)

One form of cancer gene therapy is targeted disruption of tumor oncogenes that are active in the tumours like tumours like *RAS*, *c-MYC*, *ERB-2* or *BCL-2* genes. This can be accomplished by several strategies : (1) inhibition of the oncogene transcription into mRNA, using DNA oligonucleotides designed to bind to specific oncogene promoter regions; (2) reduction of mRNA translation into protein, by the transfer of antisense nucleotides, artificial sequences complementary to the mRNA corresponding to the gene whose inhibition is attempted or, more recently, with RNA interference, a posttranscriptional gene silencing mechanism based on the production of double-stranded stretches of RNA complementary to the target mRNA or (3) interference with the oncoprotein transportation and function by intracellular expression of single chain antibody-based molecules against the oncoprotein (Hughes, 2004; Hernandez-Alcoceba R, 2006).

It is anticipated that the inhibition of oncogene expression will decrease cell proliferation, but also restore sensitivity of cells to apoptotic stimuli. For instance it is known that the inhibition of the *RAS* oncogene, blocks a cascade of mitotic signals, but also relieves the repression exerted on the p53 pathway and predisposes cells to apoptosis In the case of HCC, the inhibition of several genes had shown potential antitumor effect with *in vitro* studies showing growth inhibition or induction of apoptosis, and *in vivo* with tumours growth retardation (Hernandez-Alcoceba R, 2006).

3.3 Immunotherapy

Cancer immunotherapy or immunopotentiation consists in the enhancement of the immune system's ability to destroy cancer cells.

Passive immunopotentiation involves boosting the natural immune response to make it more effective by delivering into tumor cells genes that encode immunomodulators such as cytokines, TNF-α, interleukins (IL-2, IL-12), interferon (INF-γ) and growth factor

granulocyte macrophage (GM-CSF), to enhance the antitumoral immune response (Touchefeu Y, 2010; Cross D, 2006).

Active immunopotentiation requires the initiation of an immune response against a previously unrecognized or poorly antigenic tumor, being a promising antitumor strategy, also designated genetic vaccine. This strategy aims to immunize patients specifically against their own tumors through genetic modification of target cells with a gene, that usually encodes tumor-specific antigens, and which product increases the immune reactivity anti-tumor (Rice J, 2008; Cross D, 2006). Currently a "dendritic cell vaccine" for recurrent prostate cancer has been recommended for approval by US FDA (Tani J, 2011).

3.4 Inhibition of angiogenesis

The sustainable growth of a solid tumor depends on its ability to develop and maintain an adequate blood supply that meets demands for nutrients and oxygen. Experimental and clinical studies have shown that primary tumors as well as metastases can remain dormant due to a balance between proliferation and apoptosis unless the angiogenesis is switched on. The onset of tumor angiogenesis, required for the rapid growth of solid tumors and tumor metastases, is likely to be triggered by an upregulation of angiogenic factors such as basic fibroblast growth factor (bFGF) or vesicular endothelial growth factor (VEGF), or by a down-regulation of antiangiogenic factors such as angiostatin, endostatin, or thrombospondin (Hwang, 2006).

Realization that tumor growth requires intense neovascularization is the basis for a series of approaches aimed to specifically block the cancer-induced angiogenesis and tumor adaptation to hypoxia. Expression of anti-angiogenic factors such as endostatin have demonstrated the ability to inhibit tumor growth *in vivo* and gene therapy may play an important role in this field, because anti-angiogenic factors need to be delivered for long

period of times to control the progression of tumors. Other anti-angiogenic approaches are focused on blocking angiogenic factors as the VEGF receptor, an important mediator of angiogenesis, or the endothelium-specific receptor Tie2, which affects direct tumor growth and neovascularization (Hernandez-Alcoceba R, 2006; Hughes, 2004).

3.5 Suicide gene therapy

Suicide gene therapy is another gene therapy strategy to treat cancer by selective tumor destruction without inducing significant systemic toxicity. Opposing to the conventional drugs that usually have toxicity both in malignant and non-malignant cell the aim of this approach is to improve conventional cancer chemotherapy by selectively activating the prodrug at the tumor site. This allows higher active drug concentrations at the tumor, minimizing tumor burden, by limiting the exposition of the patient's normal tissues and organs to potentially harmful quantities of drugs (Hwang, 2006; Silke Schepelmann, 2008).

Suicide-gene therapy, or prodrug activation therapy, is based in the introduction into tumor cells of a viral, bacterial or fungal gene that encodes enzymes able to metabolize a nontoxic prodrug into a cytotoxic drug. These strategies are based on the fact that some viruses, bacteria and fungi use enzyme systems not found in humans and the prodrugs have been developed in a way that for them to be converted into a toxic form, it is necessary the digestion performed by an enzyme expressed from the exogenous gene added (Portsmouth D, 2007).

This approach involves two steps. In the first step, the gene for a foreign enzyme is targeted to the tumor cells. After expression of the foreign gene at the tumor site, a relatively nontoxic prodrug is administered, which is converted into an active, cytotoxic drug by the foreign enzyme (Figure 2) (Silke Schepelmann, 2008).
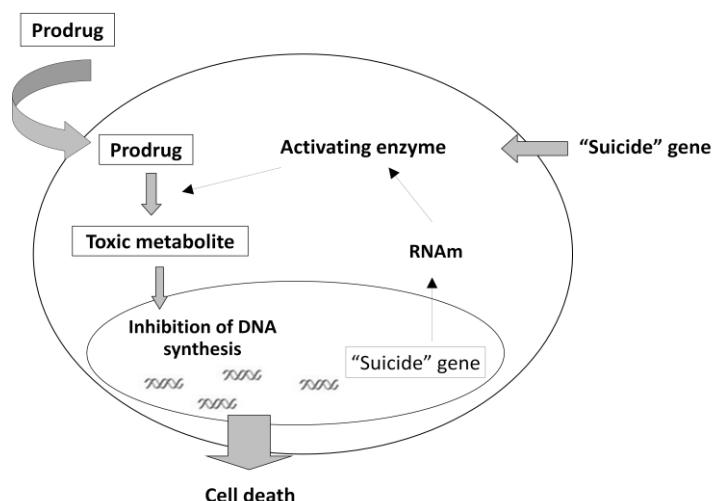
Figure 2: Schematic representation of suicide gene therapy. Suicide gene therapy consists in the delivery of a suicide gene to the target cell that encodes an enzyme which will activate a subsequently administered prodrug, into a toxic metabolite leading to cell death. (Adapted from Neves, 2009)

A critical problem to overcome in cancer gene therapy is the delivery of genes to a sufficient number of tumour cells to cause tumour regression, which is especially important since gene transfer efficiencies in the clinic, are unlikely to exceed 10% of the target tissue. Suicide gene therapy is associated with bystander effects which cause the death of nontransgenic cells, due to indirect effects of neighbouring transgenic cells treatment (Dachs GU, 2009). The local bystander effect is known to induce tumor regression, although only a fraction of tumor cells express the suicide gene and several hypotheses have been proposed to explain killing of neighboring untransfected tumor cells, as the passive diffusion of the drug; passage of the drug through gap junctions; endocytosis of apoptotic vesicles; release of soluble factors; or stimulation of the immune system *in vivo* (Duarte S, 2012).

Among the different suicide systems the herpes simplex virus thymidine kinase gene (HSV-tk) with ganciclovir (GCV) as prodrug, and the cytosine deaminase gene (CD) of Escherichia coli, which converts the non-toxic antifungal agent 5- fluorocytosine (5-FC) into 5-fluorouracil (5-FU), are the most extensively studied (Duarte S, 2012).

3.5.1 Thymidine kinase and ganciclovir

The thymidine kinase from Herpes Simplex Virus (HSV-TK) is the best characterized suicide gene and in combination with the prodrug GCV constitutes the most used suicide gene therapy (Figure 3).
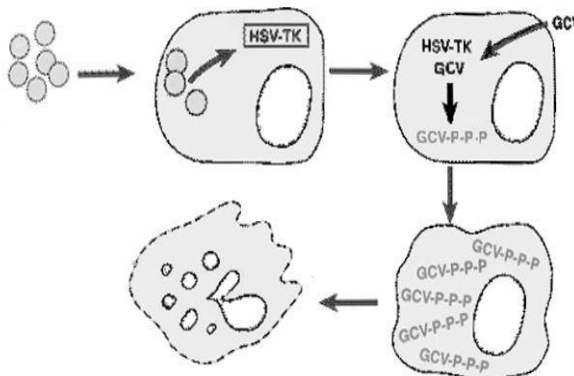

Figure 3: Schematic representation of suicide therapy HSV-TK/GCV. HSV-TK gene is delivered to cells by a vector. The thymidine kinase enzyme expressed converts ganciclovir, an analogue of deoxyguanine subsequently administered, into his monophosphorylated form. The host cell endogenous kinases promote the addition of two more phosphates to form ganciclovir triphosphate, highly toxic, which is incorporated into DNA during replication, resulting in the inhibition of the DNA strand synthesis, causing cell death (Adapted from Neves, 2009).

GCV is a synthetic analogue of 2'-deoxy-guanosine first synthesized in 1980 as an antiviral agent. GCV is phosphorylated by the thymidine kinase from HSV-1 (HSV-TK) to a monophosphate, and cellular kinases complete the conversion to the active triphosphate (Dachs GU, 2009). Although human cells express cytosolic and mitochondrial TK enzymes, these endogenous enzymes have much lower ability to convert GCV compared to HSV-TK, so the GCV phosphorylation by the HSV-TK to GCV monophosphate is the limiting step of the prodrug transformation in a toxic metabolite (Balfour, 1999). The triphosphate form is structurally similar to 2'-deoxyguanosine triphosphate (dGTP) and is erroneously incorporated into DNA during replication, causing inhibition of DNA polymerase and rapid chain termination in both nuclear and mitochondrial DNA synthesis, mainly by the induction of errors in the synthesis of a new DNA strand which subsequently leads to DNA

fragmentation and activation of apoptosis mechanisms, leading to cell death (Dachs GU, 2009; Duarte S, 2012).

Anti-tumor activity of the TK/GCV system has been demonstrated *in vivo* in several carcinoma animal models, including leukemia, glioma, bladder cancer, intrahepatic metastasis of liver cancer, colon adenocarcinoma, and oral cancer (Duarte S, 2012).

The promising results achieved in the pre-clinical studies led to the HSV-TK/GCV system application in several clinical trials in different types of cancer as glioblastoma (Rainov, 2000; Voges J, 2003), prostate cancer (Nasu Y, 2007), head and neck cancer (Xu F, 2009) and hepatocellular carcinoma (Li N, 2007; Sangro B M. G., 2010).

3.5.2 Cytosine deaminase and 5-fluorocytosine

Another suicide gene strategy widely used envolves the gene coding for cytosine deaminase (CD), an enzyme found only in bacteria and fungi, followed by systemic use of 5-fluorocytosine (5-FC) which will be converted in 5-fluorouracil (5-FU) (Figure 4).

5-FC was initially synthesized in the early 1960s as an cytosine antimetabolite in the search for antileukaemic drugs, but it has been used to treat fungal infections since 1968. 5-FU is the standard chemotherapeutic agent for advanced colorectal cancer, and has been widely in cancer chemotherapy but usually there are toxic side effects associated to its systemic administration. This suicide system results in tumor targeted chemotherapy and may allow bypassing this toxic side effects (Duarte S, 2012; Dachs GU, 2009).
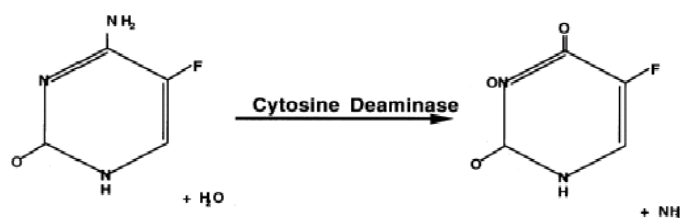

Figure 4: Conversion of 5-fluorocytosine to 5-fluorouracil by cytosine deaminase.

CD enzyme catalyses the deamination of cytosine to uracil converting 5-FC into the toxic anabolite 5-FU, which is subsequently processed by intracellular enzymes leading to the production of either to 5-fluorouracil triphosphate, which is incorporated into the RNA and interferes with RNA processing, or to 5-fluoro-2'-deoxyuridine 5'-monophosphate that irreversibly inhibits thymidylate synthase, a key enzyme in pirimidine biosynthesis, and thus interferes with the DNA synthesis (Kuriyama S, 1999).

In vivo anti-tumor activity of the CD/5-FC combination has been demonstrated in several animal models, including fibrosarcomas, colo-rectal carcinomas, hepatocellular carcinomas (Kanai F, 1997), gliomas and metastatic formations of different origin (Duarte S, 2012).

Several clinical trials have been reported using the CD/5-FC system in patients with breast cancer (Pandha HS, 1999), prostate cancer (Freytag SO, 2003), liver cancer (Crystal RG, 1997; Cunningham C, 2001), and lung cancer (Zarogoulidis P, 2012).

4- Vectors

In order to deliver therapeutic gene into target cells, the gene of interest must be inserted into a vector adjacent to a promoter that induces transcription. Then, the construct must be packaged and delivered to a specific target cell, transcribed and expressed in high enough concentration to have an effect. The effectiveness of gene therapy is highly dependent on the efficient and selective delivery of the therapeutic genes to tumor cells (Silke Schepelmann, 2008).

All of the current methods of gene delivery have some limitation. So, the choice of vector will often be dictated by the need. If expression of the gene is required for only a short time (for example, expression of a toxic gene-product in cancer cells) the adenoviral vectors are ideal. But if sustained expression is needed (such as for most genetic diseases), then an

integrating vector without attendant immunological problems is more desirable (Somia N, 2000).

An ideal vector may have to borrow properties from both viral and synthetic systems, and should have: (1) capability to protect the genetic material from degradation in the extracellular environment and unlimited packaging capacity; (2) a simple and reproducible production at high concentration on a commercial scale allowing many cells to be infected; (3) a long-term expression, once delivered, it should be able to express its genetic cargo over a sustained period or expression should be regulable in a precise way; (4) the ability to repeat delivery if needed, since none of its components elicit an immune response; (5) efficient gene transfer to the desired type of cell to achieve specificity of action; (6) the ability to infect both dividing and non-dividing cells so that is possible the transduction of post-mitotic cells as neurons and hepatocytes; (7) the ability to allow integration of the exogenous gene in the host cell at a specific location, to eliminate the uncertainty of random integration into the host chromosome causing mutations or activating oncogenes (Al-Allaf FA, 2010; Somia N, 2000).

Various vector systems have been proposed for gene therapy. Which can be generally grouped in viral or non-viral gene delivery vectors.

4.1 Viral vectors

Due to their higher efficiency of gene transfer, compared to non-viral techniques, viral vectors have been used in the majority of gene therapy studies. Viral vectors may be RNA or DNA virus-based. RNA viruses include the retroviruses, the lentiviruses and the spumaviruses. The DNA viruses include adenovirus, adeno-associated viruses, vaccinia virus, and HSV. These vectors are designed by replacing non-essential genes involved in viral replication or pathogenic protein production with foreign therapeutic genes (Hughes, 2004; Silke Schepelmann, 2008).

In table 1, are presented some vantages and disadvantages of the most commonly viral vectors used for gene delivery.

Table 1 - Some vantages and disadvantages of most common viral vectors.

Vector	Advantages	Disadvantages
Retrovirus	Stable transgene expression Only infects dividing cells High transfection efficiency *ex vivo* Low imunogenicity	Small transgene insert size capacity Low transfection efficiency *in vivo* Possible insertional mutagenesis (integration) and oncogene activation Difficult production and storage
Lentivirus	Infects both dividing and non-dividing cells	Possible insertional mutagenesis (integration)
Adenovirus	Large transgene insert capacity Biologically safe High transfection efficiency *ex vivo* and *in vivo* Infects both dividing and non-dividing cells	High immunogenicity Transient transfection efficiency Difficult production and storage Low transport capacity
Adeno-associated viruses	Stable transgene expression *in vivo* Reduced immunogenicity Efficient transfection of a wide cell variety *in vivo*	Small transgene insert size capacity Very difficult production and storage Possible insertional mutagenesis and oncogene activation Neutralization by antibodies in the case of successive doses
Herpes simplex virus	Large transgene insert capacity Availability of anti-herpetic drugs	Immunogenicity Transient and low transfection efficiencies
Vaccinia virus	Long history of safe human use Large transgene insert capacity High transfection efficiency	High immunogenicity Productive infection in immune suppressed patients Replication in skin lesions

Despite their high potential for gene delivery, this vectors present some disadvantages related to their safe application, as immune recognition, mutagenic integration (retroviral and lentiviral vectors), and inflammatory toxicity (adenoviral vectors), which appear as limitations for their use and led to a renewed interest in non-viral methods (Silke Schepelmann, 2008).

4.2 Non-viral vectors

Over the past decade, employing non-viral gene delivery systems in gene therapy has attracted a lot of attention. Comparing with viral vectors these non-viral delivery systems offer several advantages such as low immunogenicity and toxicity, easier and less expensive large scale production; they are also relatively safer, and have the potential for more tissue specificity. But, even with recent technological advances, the transfection efficiency of non-viral vectors still needs to be improved so has their targeting ability. Nonetheless, recent studies had shown that non-viral vectors indeed hold great promise for their future development (Ding B, 2012; Al-Dosari MS, 2009).

The numerous non-viral methods for gene transfer that had been proposed can be divided in physical methods and chemical methods.

In physical methods, researchers attempt to enhance gene delivery by exerting physical forces. Methods such as needle and jet injection, hydrodynamic gene transfer, gene gun delivery, electroporation and sonoporation had been described. Chemical methods mainly involve the employment of chemical vectors with cationic components, currently in use include are cationic lipids (forming lipoplexes upon mixing with DNA), cationic polymers as PEI, peptides or dextrans (forming polyplexes upon mixing with DNA) and inorganic nanoparticles (Al-Dosari MS, 2009).

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4.2.1 Cationic liposomes

Cationic liposomes are presented as the most promising vectors among the non-viral systems and have been extensively used. This widespread application is due to a number of important advantages, including their capacity to transport large amounts of genetic material; their physico-chemical versatility, allowing innumerous modifications; their easy and inexpensive large scale production; and their low immunogenic response. Since cationic liposomes were first described for gene delivery, an increasing number of new cationic lipids have been produced and used in transfection protocols of different cell lines, animal models and patients submitted to gene therapy clinical trials. At present, 6.4% of the protocols approved for gene therapy trials involve lipoplexes, these being mainly applied in the treatment of cancer and cystic fibrosis (Duarte S, 2012).

Cationic liposomes are positively charged lipid bilayers that can form vesicles and interact with negatively charged DNA, structures called lipoplexes. In this lipoplex structure, DNA molecules are surrounded with positively charged lipids which grant them protection against nucleases. Lipoplexes, due to their positive charges, tend to electrostatically interact with the negatively charged molecules of the cell membrane that may facilitate their cellular uptake (Touchefeu Y, 2010; Al-Dosari MS, 2009) (Figure 5).
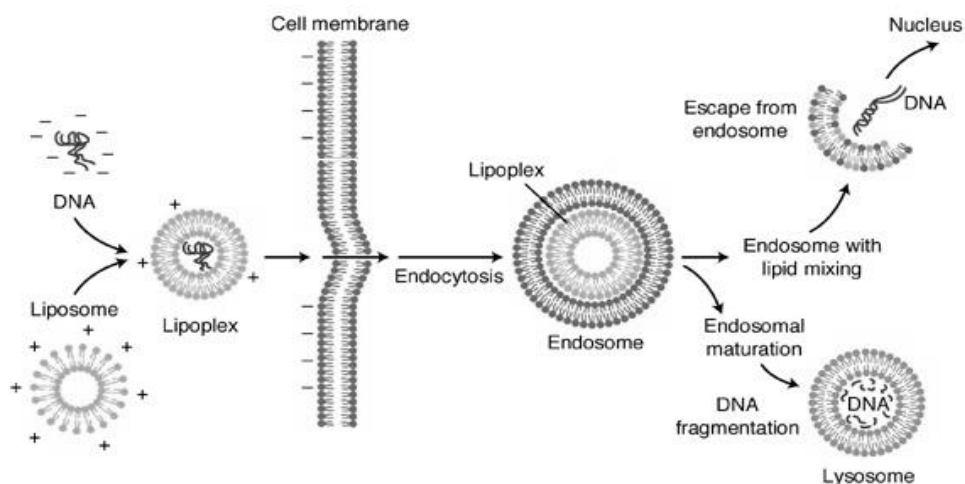

Figure 5: Esquematic lipoplex-mediated transfection and endocytosis.

For the formation of liposomes for gene transfection a solution of cationic lipids with neutral helper lipids is often used and liposome formulations used in genetic therapy seek to find a compromise between stability and toxicity (Balazs DA, 2011; Al-Dosari MS, 2009). Well-characterized and widely used for transfection cationic lipid include DOTMA, DOTAP, DC-Chol and DOGS. DOTAP (Figure 6A) shows little to no cytotoxic effect which is hypothesized to be due to ester bonds, which are hydrolysable and can render the lipid biodegradable and reduce cytotoxicity. But the use of formulation containing only DOTAP for gene delivery is inefficient due to the density of positive charges on the liposome surface, which possibly prevents counter ion exchange. Thus, for DOTAP to be more effective in gene delivery, it should be combined with a neutral helper lipid (Balazs DA, 2011). The most commonly used co-lipids are cholesterol and DOPE. The presence of cholesterol (Figure 6B) stabilizes the cationic lipidic membrane structures, better than DOPE, against the destructive activity of serum components and can provide better activity for *in vivo* transfection when serum components are present (Al-Dosari MS, 2009).
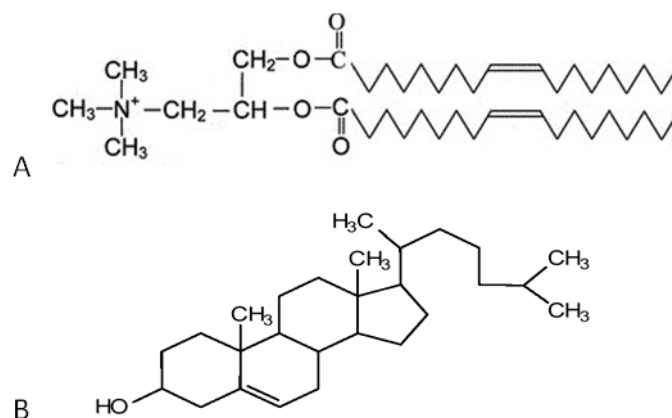

Figure 6: Representative structure of: A – Cationic lipid DOTAP; B – Neutral helper lipid cholesterol.

Despite all the progress made in recent years, efficiency of gene transfer and expression is still low and a variety of ligands have been examined for their ability to enhance the biological activity and specificity of the lipoplexes to target cells. These targeting ligands are

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selected based upon specific target cell receptors and include, folates, haloperidol and transferrin (Balazs DA, 2011). Transferrin is a popular ligand for delivery of anticancer drugs to solid tumors *in vivo* since cancer cells presents elevated levels of transferrin receptor (TfR) expression, attributed to the vigorous proliferation and requirement of high iron level for their growth. There is also indication of higher affinity of the TfR to transferrin (Tf) which can be useful for a targeted therapy (Lu Q, 2008). Some studies showed that using Tf as ligand increases gene transfection efficiency (Lu Q, 2008; Zhong ZR, 2007).

In this study we intend to test the therapeutic potential of gene therapy using suicide genes (HSV-TK/ GCV or CD/ 5-FC) in monotherapy and in combination with conventional anticarcinogenic agents (Doxorrubicin) and new targeted drugs (proteassome inhibitor MG-262) in the hepatocellular carcinoma cell lines, HUH-7 and Hep-G2 in culture. The mechanisms involved in the toxicity will be also studied, namely the mechanisms involved in cell death and changes in cell cycle.

MATERIALS AND METHODS

1- Materials

MG-262 was obtained from BiomoL (USA). Doxorubicin, Resazurin, May-Grünwald solution and Giemsa solution were purchased from Sigma (St. Louis, MO, USA). 5-Fluorocitosine and Ganciclovir were purchased from Invivogen (Toulouse, France).

FBS and DMEM medium were purchased from GIBCO (Barcelona, Spain).

The plasmid pCMVTK that encodes the therapeutic gene thymidine kinase, the pCDβGEO plasmid that encodes the therapeutic gene cytosine deaminase gene under the control of the CMV promoter and the plasmid pCMVGFP, that encodes the reporter gene GFP, were kindly provided by Professora Doutora Maria da Conceição Pedroso de Lima from Centre for Neuroscience and Cell Biology of Coimbra.

Plasmids were amplified according to the manufacturer's instructions using the kit GenElute™ Endotoxin-free Plasmid Midiprep Kit purchased from Sigma (St. Louis, MO, USA).

The cationic lipid DOTAP, cholesterol (Chol), and the human holo-transferrin were obtained from Sigma (St. Louis, MO, USA).

The kit FITC-labelled annexin V (AV) and propidium iodide (PI) were obtained from Immunotec (Canada). PI/RNase kit used for cell cycle analyses for flow cytometry was purchased from Immunostep (Salamanca, Spain).

2- Cell line culture conditions

The human HUH-7 cell line, offered by Professora Doutora Maria Conceição Pedroso Lima (Center for Neuroscience and Cell biology), was established by Nakabayashi *et al.,* (1982) from hepatoma tissue of a 57-yr-old Japanese male with well differentiated hepatocellular carcinoma which expressed high levels of mutated p53 (Carloni V, 2005).

The human Hep-G2 cell line, offered by Professora Doutora Filomena Botelho, from *Biophysics/*Biomathematics of the Faculty of Medicine, University of Coimbra, was established by Aden *et al.,* (1979) from a liver tumor biopsy, with normal p53 expression, obtained from a 15-yr-old Caucasian male. The morphological characteristics and epithelial cell shape were compatible with that of liver parenchymal cells. Histology of the liver biopsy revealed well differentiated hepatocellular carcinoma with a trabecular pattern.

The cell lines were maintained in DMEM medium supplemented with 10% FBS, L-glutamine 2mM, $NaHCO_3$, penicillin 100U/mL and streptomycin 100μg/mL at 37ºC in a humidified incubator containing 5% CO_2. Before the assays, both cells were seeded 24h before. at a density of 50000 cells per cm^2.

3- Cationic liposomes and lipoplexes preparation

Cationic liposomes composed of DOTAP and Chol (1:1 molar ratio) were prepared by the ethanol injection method (Cardoso AL, 2007), (Penacho N, 2009). The lipids, from stock solutions in chloroform, were mixed in a glass tube to a final concentration of 6mM and then dried under nitrogen flow in order to obtain a thin lipid film. The dried lipid film was dissolved in 100μL of pure ethanol and the resulting solution injected, using a Hamilton syringe, under vortex, into 900μL of HBS buffer (10mM NaCl, 20mM HEPES, pH 7.4). The

resulting MLVs (multilamellar vesicles) were then sonicated for 10 minutes to obtain SUVs (small unilamellar vesicles). The suspension was stored at 4ºC until use.

Transferrin-associated lipoplexes (Tf-lipoplexes) were prepared by adding liposomes (the amount required for the different charge ratio) to 100µl of a HBS solution containing 320 µg/ml of transferrin (to obtain a final concentration of 32µg of transferrin per µg DNA). This mixture was incubated at room temperature for 15 minutes to allow interaction between transferrin and liposomes. Then 100µl of HBS buffer containing 1µg plasmid DNA (pCMVTK, pCDβGEO or pCMVGFP) was added and the solution was further incubated at room temperature for 15 minutes (Neves S, 2009).

For complexes with different charge ratios (+/-), amount of DNA remained constant (1mg DNA corresponds to 3.03nmol of negative charges) and only varying the amount of liposomes. The complexes were used immediately after being prepared.

4- *In vitro* gene transfer

Cells, plated in 48 well plates 24h before assay, were rinsed with serum-free medium and then covered with 0.3ml of DMEM (without serum) before lipoplexes were added. The complexes were gently added to cells in a volume of 0.2ml per well. After incubation for 4 h (in 5% CO_2 at 37 °C), the medium was replaced with DMEM supplemented with 10% FBS, L-glutamine 2mM, $NaHCO_3$, penicillin 100U/mL and streptomycin 100µg/mL, and the cells were further incubated under different experimental conditions.

5- Transfection activity

Transfection efficiency mediated by the complexes was evaluated using the reporter gene green fluorescent protein (GFP). For this purpose, HUH-7 and Hep-G2 cells were plated in 6

well plates and treated as previously described, using the appropriate volumes. 48h or 72h after transfection the percentage of transfected cells was accessed by flow cytometry. For that, cellswere trypsinized, centrifuged at 300xg for 5min, washed twice with PBS, ressuspended in FACSflow buffer and analyzed in a FACScalibur cytometer (BD Biosciences, Heildelberg, Germany) equipped with an argon-ion laser emitting at 488nm which recorded forward scatter (FSC), side scatter (SSC) and green fluorescence (FL1). FSC and SSC data were used to identify viable cells, gates were set to exclude cellular debris and results were evaluated to determine the number of transfected cells (percentage of cells expressing GFP).

6- Cytotoxicity studies, evaluation of cell viability

To evaluate the cytotoxicity induced by suicide gene therapy in these cell lines, HUH-7 and Hep-G2 cells were transfected with transferrin associated lipoplexes in the absence or presence of GCV, in a range of concentrations from 0,05mM to 0,5mM, or 5-FC, in a range of concentrations from 5mM to 50mM, during 168h.

To check for possible synergistic effects, we performed combination treatments of the suicide gene therapy (TK/GCV or CD/5-FC) with Doxorubicin or MG262, using doses below the IC50 of both the drugs and pro-drugs during 168h.

Cell viability, under the different experimental conditions, was assessed by Alamar Blue assay. In this assay the metabolic activity of cells is measured through the conversion of the active compound of the Alamar Blue resazurin (oxidized form) to resorufin (reduced form) by viable cells (O'Brien J, 2000). This results in colorimetric (absorbance) and fluorescence changes. Resazurin is blue and non-fluorescent whereas resorufin is red and highly fluorescent and the difference between the two compounds can be evaluated by spectroscopy.
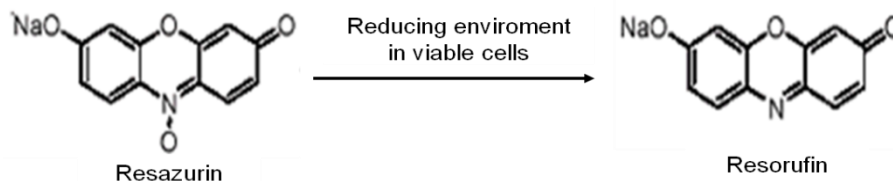
Figure 7: Alamar blue. Convertion of resazurin to resorufin.

The medium was removed and 300µl of 10% (v/v) resazurin in DMEM were added to each well. After 2 h to 4 h incubation at 37 °C, 200µl of supernatant were collected from each well and transferred to 96-well plates. The absorbance at 570 nm and 600 nm was measured using a Mediators PhL luminometer (Mediators Diagnostika, Vienna, Austria). Cell viability (as a percentage of control) was calculated according to the formula (A570−A600) of treated cells×100/ (A570−A600) of control cells (Neves SS, 2006).

Readings were performed at 48h, 72h, 144h and 168h and after each reading the culture medium was replaced by fresh one with the appropriate drugs concentration.

7- Morphological analysis

After incubation for 48h, under the different experimental conditions, the cells were trypsined, centrifuged at 300xg for 5min and ressuspended in serum in order to obtain a density of 50000cells/mL. Then, cells were placed on a slide and stained for 5min with May-Grünwald solution (0.3% v/v in methanol) (Sigma, St. Louis, MO, USA), diluted in 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 8x in distilled water for 20 min. After rinsed with distilled water, cell morphology was analyzed by light microscopy using a Motic AE31 microscope associated with a Moticam 2300 digital camera

8- Flow cytometry assays

8.1 Cell death analysis

HUH-7 and Hep-G2 cells were added in triplicate to a 6-well culture plate, 24 h later they were transfected and treated as described previously. After 48h incubation cells were trypsinized, centrifuged at 300xg for 5min and incubated for 10min at 4ºC with 440μL annexin buffer containing 5μL FITC-labelled annexin V (AV) and 2μL propidium iodide (PI).

Annexin V binds with high affinity to phospholipids negatively charged including phosphatidylserine which is exposed in outer leaflet of the plasma membrane during apoptotic process. PI is a non-specific DNA marker which is internalized by cells killed due to the membrane integrity loss associated with necrosis (Darzynkiewicz Z, 1997). With this technique, it is possible to distinguish non-apoptotic live cells (AV-FITC and PI negative), early apoptotic cells (AV-FITC positive and PI negative), late apoptotic (positive for FITC-AV and PI) and necrotic cells (positive for PI and AV-FITC negative).

Cells were then washed twice with PBS, ressuspended in the same buffer and analyzed in a FACScalibur cytometer (BD Biosciences, Heildelberg, Germany) equipped with an argon ion laser emitting at 488nm. FSC and SSC data were used to identify viable cells, and gates were set to exclude cellular debris. 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 Z, 1997; Neves SS, 2006).

8.2 Cell cycle analysis

The effect of the HSV-Tk/GCV or CD/5-FC suicide gene therapy and the described combinations on the cell cycle phase distribution of the HCC cell lines was analyzed by flow

cytometry using the PI/RNase kit (Immunostep, Salamanca, Spain) according to the manufacturer's instructions.

PI binds to DNA by intercalating into the double stranded macromolecule, it also binds to RNA and is necessary to remove the RNA with a nucleases treatment (RNase) for optimal DNA resolution. Mammalian cells are characterized for having three populations or definite regions, cells in G2 and M phases of the cell cycle that have double DNA content, those in G0 and G1 phases, and a region correspond to cells in phase S. The quantity of binded PI is proportional to the quantity of DNA and quantification of the DNA content permit us to know the distribution of cells along the different phases of the cell cycle represented in fluorescence intensity.

Briefly, cells were added in triplicate to a 6-well culture plate, 24 h later they were transfected and treated as described previously. After 48h incubation, cells were detached using trypsin/EDTA after previous wash with PBS. Cells were centrifuged at 300xg for 5min, fixed with 200 μl of 70% ethanol by vortexing and incubating 30 minutes at − 4ºC and, then, washed once with PBS + 2% BSA. Finally, 0,5 mL of propidium iodide solution (PI/RNase) were added and after mixing well the cells were incubated for 15 min at room temperature before analyzed by flow cytometry.

9- Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 5.0 (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- Transfection efficiency

Lipoplexes charge ratio is an important factor to take into account in the efficiency of this type of transfection since it changes the characteristics of the lipoplexes, particularly in terms size and surface charge. So, in order to choose the most effective formulation to transfect our hepatocellular carcinoma cells, we examined the biological activity of complexes prepared at different charge ratios cationic lipid / DNA (+ / -), from variable amounts of DOTAP: Chol liposomes and 1 µg of pCMVGFP. Also, all formulations were made in the presence of transferrin because it facilitates the internalization of the liposome by endocytosis with the aid of the transferrin receptor increasing transfection efficiency.

Determination of the transfection efficiency using green fluorescence protein (GFP) fluorescence can be noninvasively detected in living cells. Flow cytometric analysis of GFP expression technique permits the measurement of transfection efficiency without the requirement for cell fixation or sample preparation. For this purpose, cells suspended in PBS were analyzed by flow cytometry 48 and 72h after transfection in order to determine the percentage of positive cells (fluorescent cells that express GFP).

Figure 8 shows the transfection efficiency of ternary lipoplexes prepared in different charge ratios in Hep-G2 and HUH-7 cells. As we can observe, although charge ratio of 1/1 presents the highest values of GFP protein expression at 48h highest values of GFP protein expression were observed in the cationic lipid / DNA (+/-) prepared at charge ratio of 3 / 2 at 72h, so this was the chosen ratio for the following tests because it permits a more sustained by transfection.
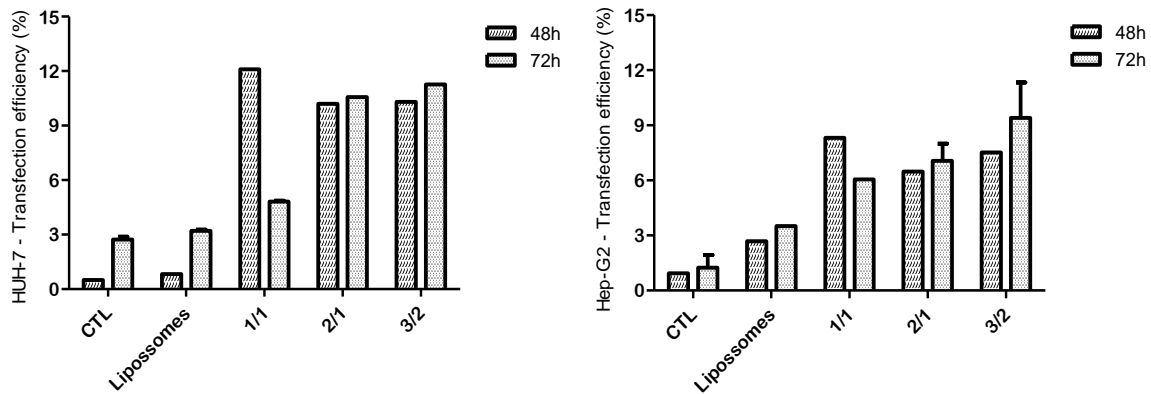
Figure 8: Transfection efficiency in HUH-7 and Hep-G2 using different lipoplex cationic lipid / DNA (+/-) charge ratios. Cells were transfected with cationic liposomes containing DOTAP and cholesterol complexed with 1 µg of pCMVGFP in the indicated charge ratios (+/-) in the presence of 32 µg of transferrin. After incubation of cells with the complex for a period of 4 hours at 37 ° C, the culture medium was replaced with medium enriched with 10% FBS and the cells were maintained in culture for another 48 or 72 hours. The transfection efficiency was assessed in terms of percentage of cells expressing the GFP protein ± SD, measured by flow cytometry in2 independent experiments.

2- Analysis of cell viability

2.1 Effect of HSV-TK/GCV or CD/5-FC treatment on the viability of HUH-7 and Hep-G2 cells

In order to evalute the therapeutic effect of gene therapy using suicide genes (HSV-TK/ GCV or CD/ 5-FC) in hepatocellular carcinoma cell lines, transfected and non transfected HUH-7 and Hep-G2 cells were cultured in the absence and in the presence of the appropriate prodrug (GCV or 5-FC) for up to 168h. The antiproliferative effect was evaluated by the Alamar Blue assay.

Our results represented in Figures 9 and 10 show that CD/5-FC treatment induced a decrease in cell viability in both cell lines, in a time and dose dependent manner. However, HUH-7 cells seems to be more sensitive. In fact, we can observe a decrease in cell viability after 48 to 72 hours of incubation, with an IC50, for HUH-7 cells, at 144h hours of exposure of 24mM 5-FC and of 33mM 5-FC for Hep-G2 cells.
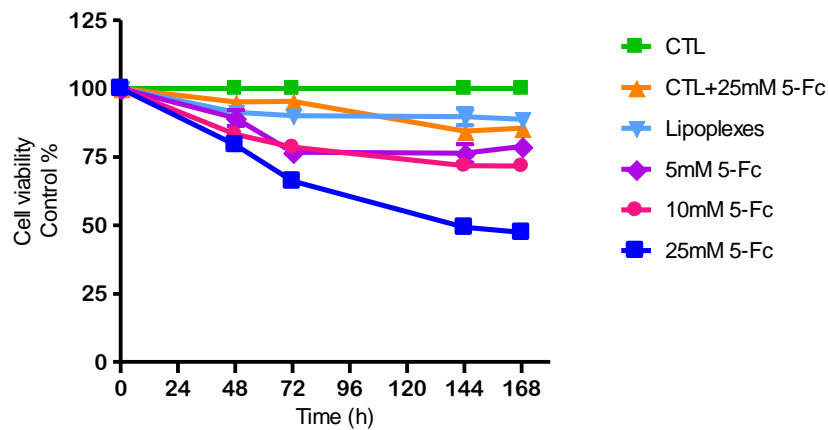
Figure 9: Dose response curve in HUH-7 cells treated with CD/5-FC. Cells were submitted to suicide gene therapy (CD/5-FC) mediated by the Tf-lipoplexes, using several concentrations of 5-FC, up to a non toxic concentration of 25mM to the non-tranfected cells, during 168h, according with described in materials and methods. Cell viability is expressed in percentage (%) of control and represents the mean + SD of 3 independent experiments.
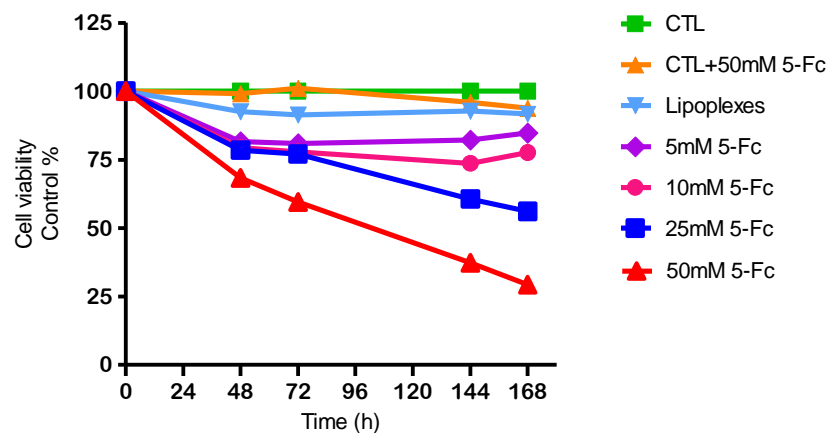

Figure 10: Dose response curve in Hep-G2 cells treated with CD/5-FC. Cells were submitted to suicide gene therapy (CD/5-FC) mediated by the Tf-lipoplexes, using several concentrations of 5-FC, up to a non toxic concentration of 50mM to the non-tranfected cells, during 168h, according with described in materials and methods. Cell viability is expressed in percentage (%) of control and represents the mean $\pm$ SD of 3 independent experiments.

When cells are treated with HSV-TK/GCV we also observed a decrease in cell viability in both cell lines in a time and dose dependent manner as represented in Figures 11 and 12. We can observe a decrease in cell viability only after 48 hours of incubation, with an IC50 at

144h hours of exposure of 0,3mM GCV for HUH-7 cells, and of 0,8mM for Hep-G2 cell line, which presents a lower sensitivity to this system.

Although both suicide gene therapy systems decreases the viability in both cell lines, HUH-7 cell line appeared to be more sensitive to both treatments than Hep-G2 cell line which shown higher IC50 values. The most effective approach in HUH-7 cell line was with the TK/GCV system and Hep-G2 cell showed little sensitivity to the TK/GCV system and appeared more sensitive to the approach using the CD/5-FC system.
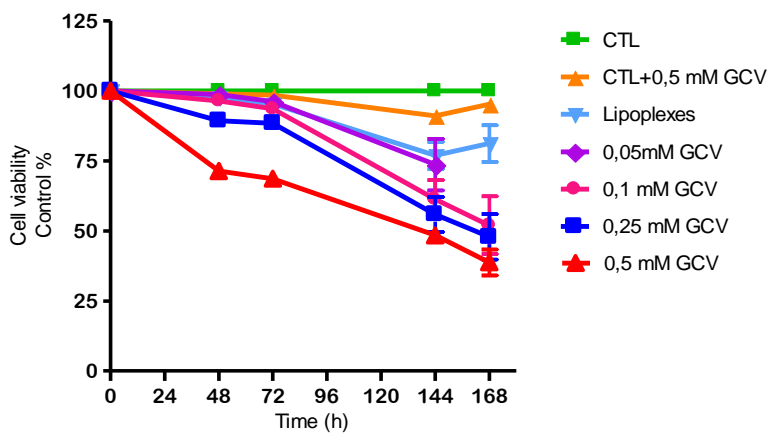

Figure 11: Dose response curve in HUH-7 cells treated with HSV-TK/GCV. Cells were submitted to suicide gene therapy (HSV-TK/GCV) mediated by the Tf-lipoplexes, using several concentrations of GCV, up to a non toxic concentration of 0,5mM to the non-tranfected cells, during 168h, according with described in materials and methods. Cell viability is expressed in percentage (%) of control and represents the mean $\pm$ SD of 3 independent experiments.
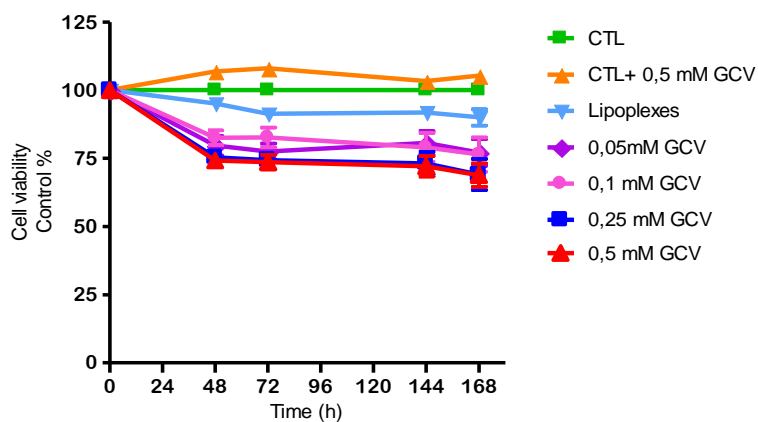

Figure 12: Dose response curve in Hep-G2 cells treated with HSV-TK/GCV. Cells were submitted to suicide gene therapy (HSV-TK/GCV) mediated by the Tf-lipoplexes, using several concentrations of GCV, up to a non toxic concentration of 0,5mM to the non-tranfected cells, during 168h, during 168h according, with

described in materials and methods. Cell viability is expressed in percentage (%) of control and represents the mean + SD of 3 independent experiments.

2.2 Effect of HSV-TK/GCV or CD/5-FC treatment in association with Doxorubicin and MG262 on the viability of HUH-7 and Hep-G2 cells

In order to evaluate the cytotoxicity of suicide gene therapy associated with a conventional chemotherapeutic agent and a drug directed to novel molecular targets HUH-7 and Hep-G2 cells were incubated with the pro-drugs in combination with Doxorubicin and MG262. The IC50 for these drugs were previously determined (Data not shown) and in these associations were used concentrations below the IC50.

As we can see in Figures 13 and 14, a greater reduction in cell viability in both cell lines, was observed when associated the CD/5-FC system with Doxorubicin or MG262 showing an addition synergism between the two therapies.

Using TK / GCV system in combination with Doxorubicin or MG262 (Figures 15 and 16), this effect was even more pronounced for Hep-G2 cell line especially in the combination with Doxorubicin in which it can be observed an addition synergism.
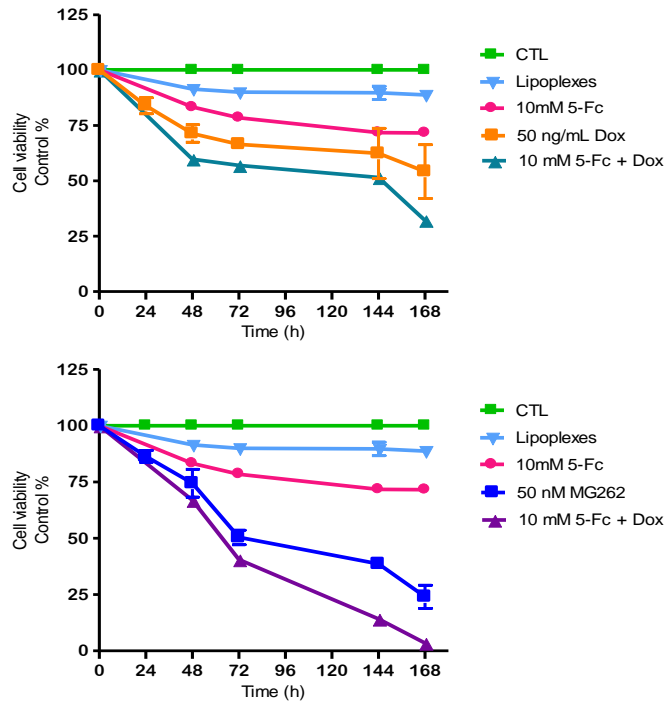
Figure 13: Dose response curve of CD/5-Fc in association with Doxorubicin (up) and MG262 (below) in HUH-7 cells. Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 168 hours. Cell viability is expressed in percentage (%) of control and represents the mean + SD of 3 independent experiments.
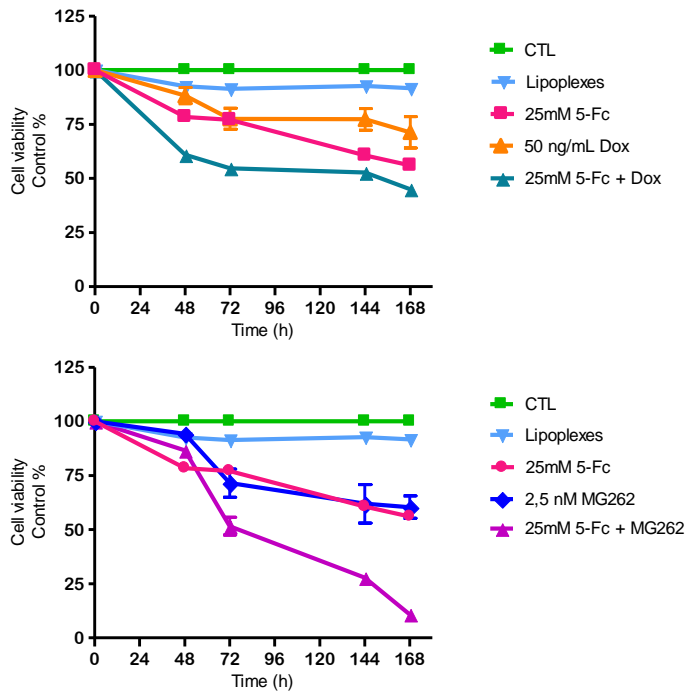

Figure 14: Dose response curve of CD/5-Fc in association with Doxorubicin (up) and MG262 (below) in Hep-G2 cells. Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 168 hours. Cell viability is expressed in percentage (%) of control and represents the mean ± SD of 3 independent experiments.

Figure 15: Dose response curve of HSV-TK/GCV in association with Doxorubicin (up) and MG262 (below) in HUH-7 cells. Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 168 hours. Cell viability is expressed in percentage (%) of control and represents the mean $\pm$ SD of 3 independent experiments

Figure 16: Dose response curve of HSV-TK/GCV in association with Doxorubicin (up) and MG262 (below) in Hep-G2 cells. Cells were treated with concentrations below the IC50 of the drugs used in monotherapy during 168 hours. Cell viability is expressed in percentage (%) of control and represents the mean $\pm$ SD of 3 independent experiments.
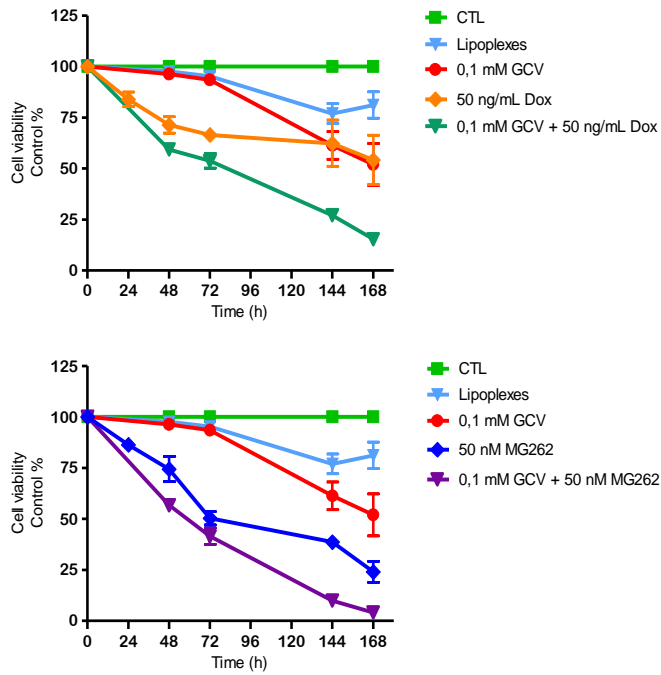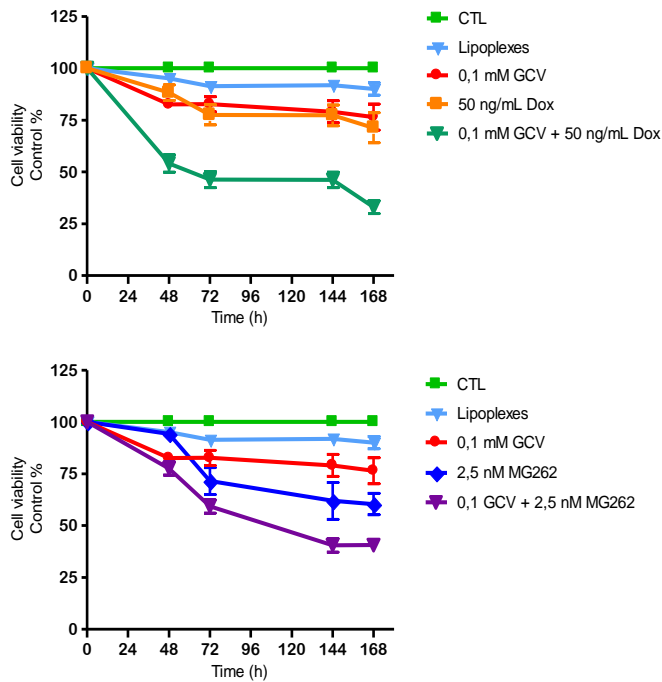
3- Morphological analysis

Since tumour cell death mechanisms can interfere with the therapeutic strategy, we also analysed the cytotoxic effect induced by the suicide gene therapy strategies associated with MG262 and Doxorubicin in both cells lines by studying cell death process through morphological analysis by optical microscopy and flow cytometry using the AV/PI staining.

Figures 17 and 18 show the morphology of cell smears stained with May-Grünwald-Giemsa before (control) and after treatments during 48h in HUH-7 cell lines (Figure 17) and Hep-G2 cell line (Figure 18).

As it can be seen, both in Figure 17 and Figure 18, cells treated with liposomes are morphologically similar the control, the same happening with cells treated with prodrugs (GCV or 5-FC) alone, which present only few morphologically apoptotic cells. Cells treated with the suicide systems (TK/GCV or CD/5-FC) alone or in combination with doxorubicin or MG262 have mainly typical morphological characteristics of apoptosis, such as cellular retraction, nuclear fragmentation, blebbing and apoptotic bodies' formation. The presence of some necrotic cells with characteristics as the swelling of the cell, cell lysis or release of cytoplasmic contents were also observed.

Figure 17: Morphological analysis of HUH-7 cells by optical microscopy. A- Control, B- Lipossomes, C- 5-FC, D- GCV, E- CD/5-FC, F- TK/GCV, G- CD/5-FC+MG262, H- TK/GCV+ MG262, I- CD/5-FC+ Dox, J- TK/GCV+ Dox. There are morphological evidences of cell death by apoptosis, such as cellular contraction, nuclear fragmentation, blebbing and apoptotic bodies' formation. Amplification: 400x.
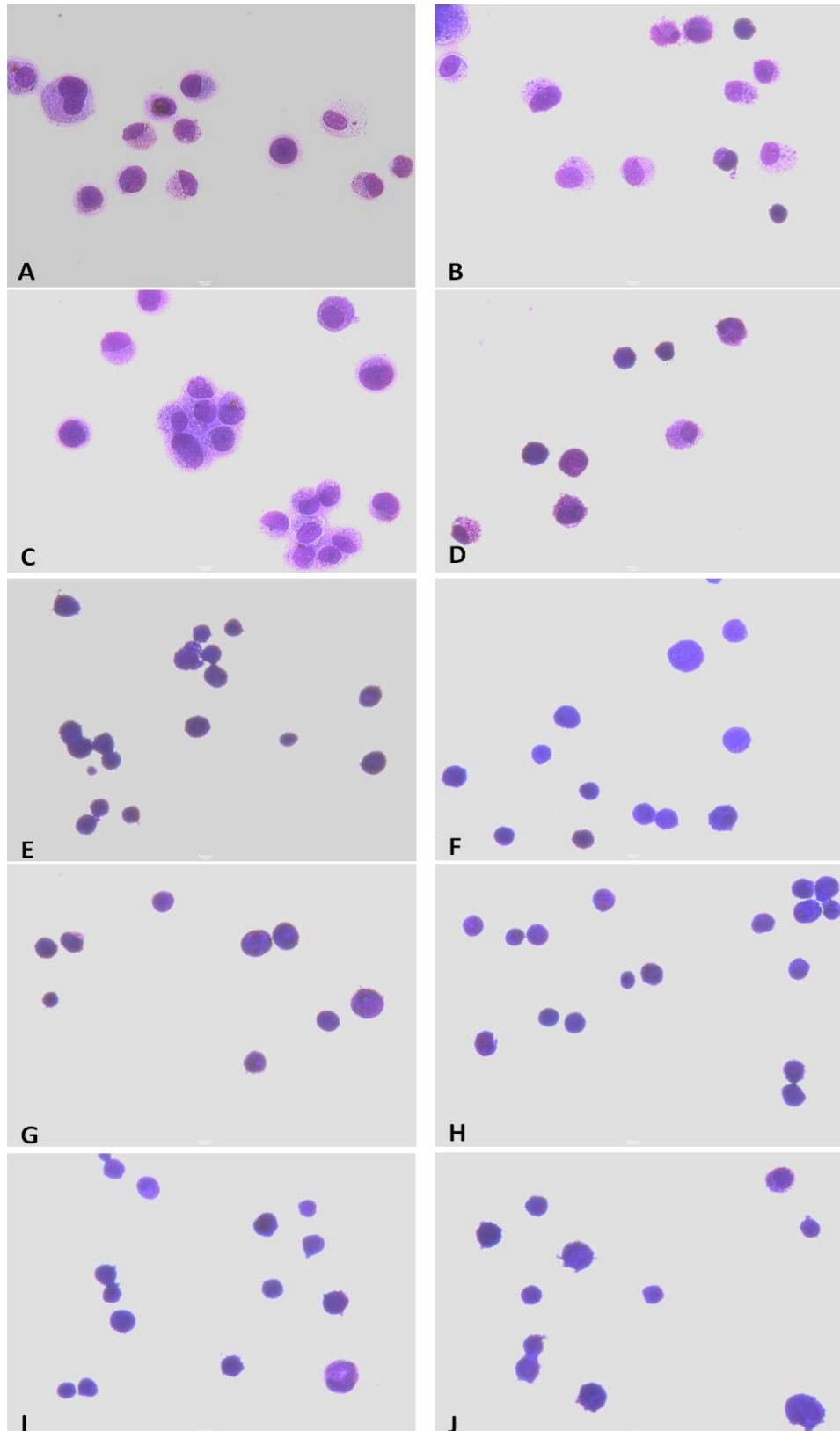
Figure 18: Morphological analysis of Hep-G2 cells by optical microscopy. A- Control, B- Lipossomes, C- 5-FC, D- GCV, E- CD/5-FC, F- TK/GCV, G- CD/5-FC+MG262, H- TK/GCV+ MG262, I- CD/5-FC+ Dox, J- TK/GCV+ Dox. There are morphological evidences of cell death by apoptosis, such as cellular contraction, nuclear fragmentation, blebbing and apoptotic bodies' formation. Amplification: 400x.
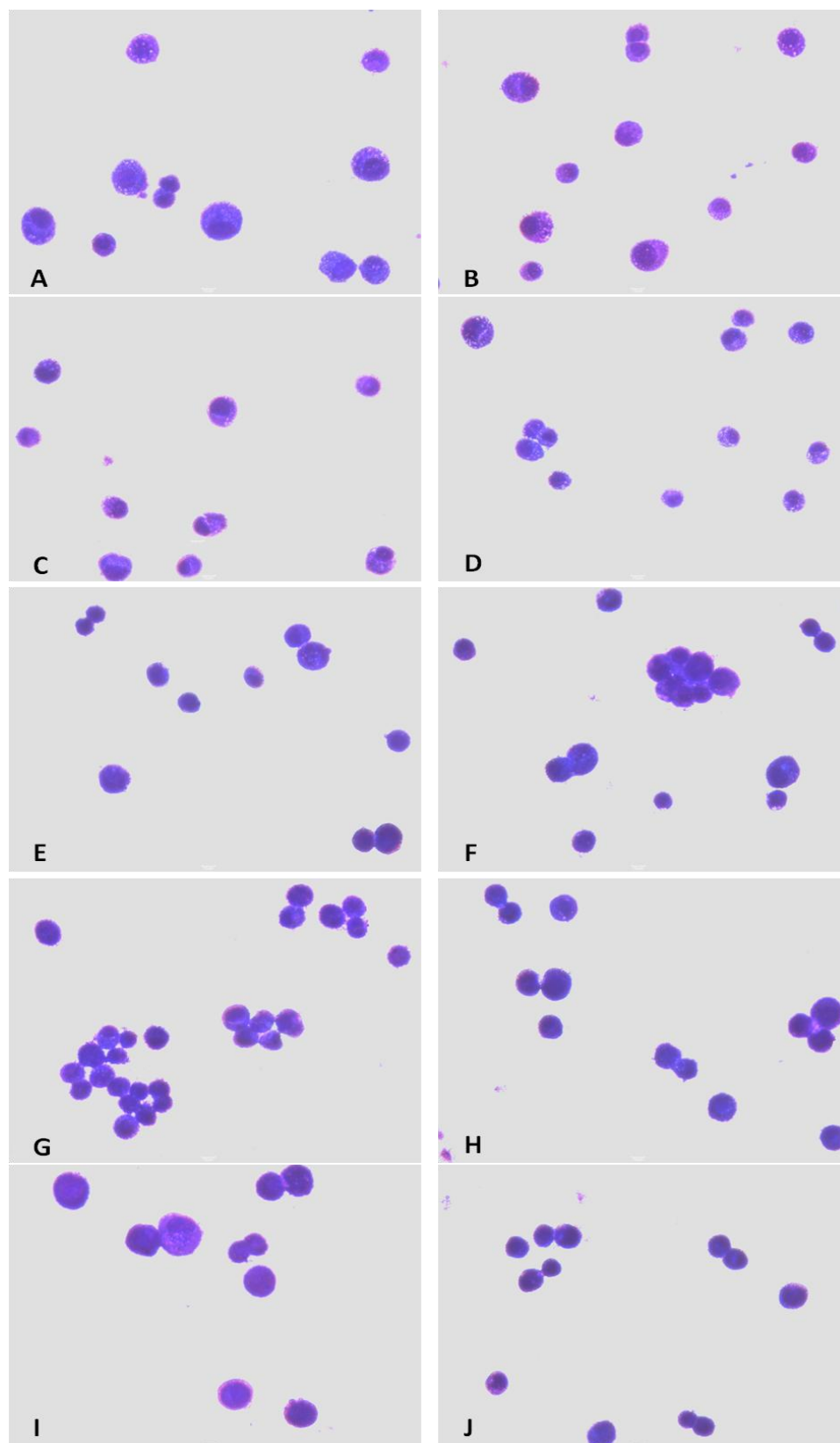
4- Flow cytometry studies

4.1 Analysis of cell death

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

Figure 19: Evaluation of cell death in HUH-7 by FC using AV and PI incorporation. HUH-7 cells were incubated in the absence (CTL) and in the presence of 50nM MG-262, 50ng/mL Doxorubicin (Dox), 10 mM 5-Fluorocitosine (5-FC) and 0,1 mM Ganciclovir (GCV) in monotherapy and associations. 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
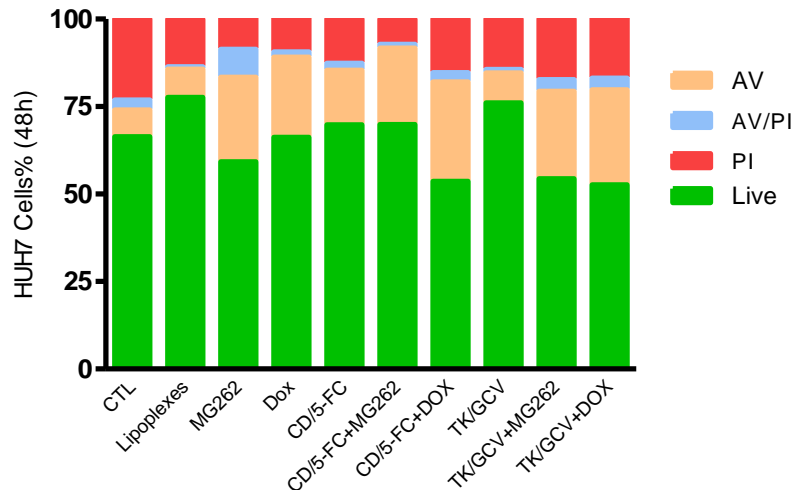

Figure 20: Evaluation of cell death in Hep-G2 by FC using AV and PI incorporation. Hep-G2 cells were incubated in the absence (CTL) and in the presence of 2,5nM MG-262, 50ng/mL Doxorubicin (Dox), 25mM 5-Fluorocitosine (5-FC) and 0,1mM Ganciclovir (GCV) in monotherapy and associations. Alive cells are

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
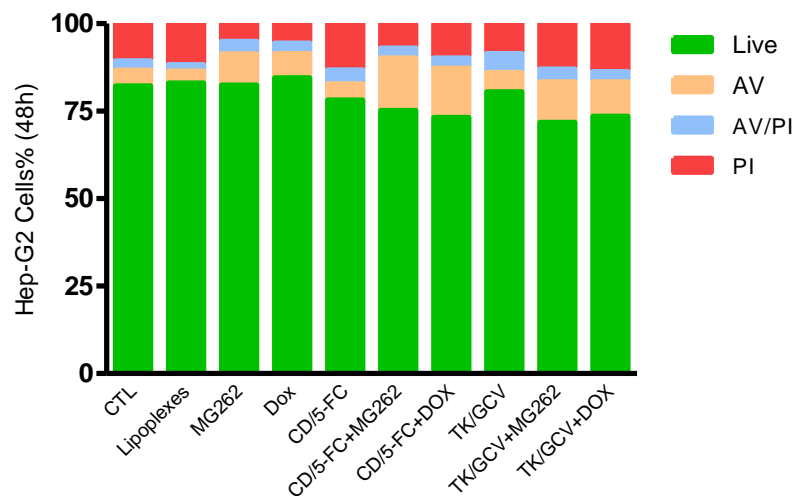
The results, shown in figures 19 and 20 are almost in agreement with those obtained in morphological studies, as we observed an increase in the percentage of apoptotic cells after the treatment in almost all the tested conditions. In fact, an increase in the number of cells in early and late stages of apoptosis was observed when cells were treated with CD/5-FC and TK/GCV and 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 in most of the tested combinations.

4.2 Analysis of cell cycle

In order to evalute the effect of HSV-TK/GCV or CD/5-FC suicide gene therapy and the described combinations on the cell cycle phase distribution of the HCC cell lines, the cells transfected and non transfected were analyzed by flow cytometry using PI/RNase kit as described previously (Figures 21 to 24).

Our results show a normal the cell cycle in the control and the condition using only lipoplexes and an anti-proliferative effect with cell cycle arrest and accumulation of cells in the G2 phase for cells treated with the suicide gene approaches (TK/GCV and CD/5-FC) and their combinations with doxorubicin and MG262 as it can be seen by the G2 peak and presented frequencies, in comparison with the ones in the control. This is more evident, with even an inversion of the cell cycle, in most of the combinations with doxorubicin which also functions by cessation of DNA synthesis.

Figure 21: Evaluation of cell cycle in HUH-7 cell line using PI/RNase kit by flow citometry. A – CTL. B – Lipoplexes. C – TK/GCV. D – TK/GCV + Dox. E – TK/GCV + MG262.

Figure 22: Evaluation of cell cycle in HUH-7 cell line using PI/RNase kit by flow citometry. A – CTL. B – Lipoplexes. C – CD/5-FC. D – CD/5-FC + Dox. E – CD/5-FC + MG262.
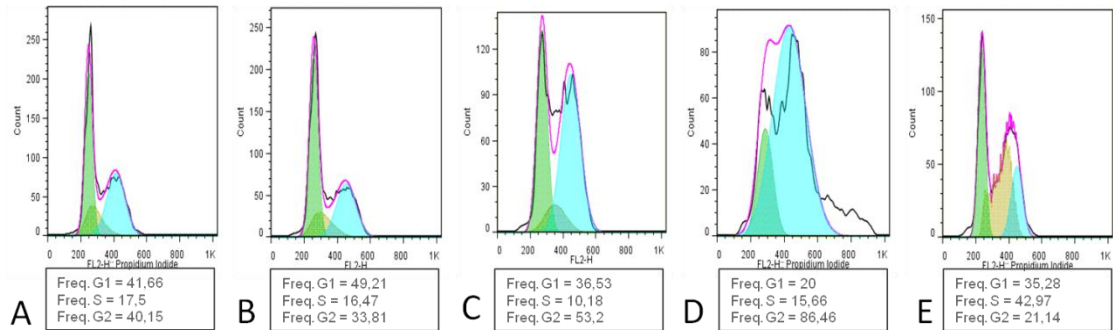

Figure 23: Evaluation of cell cycle in Hep-G2 cell line using PI/RNase kit by flow citometry. A – CTL. B – Lipoplexes. C – TK/GCV. D – TK/GCV + Dox. E – TK/GCV + MG262.
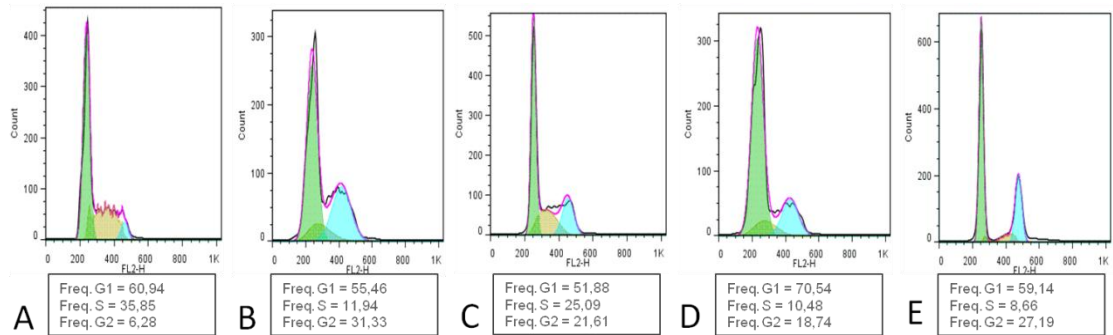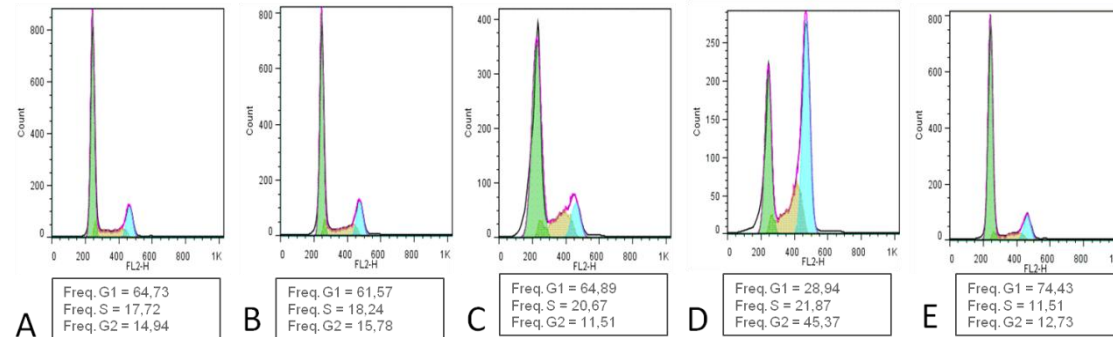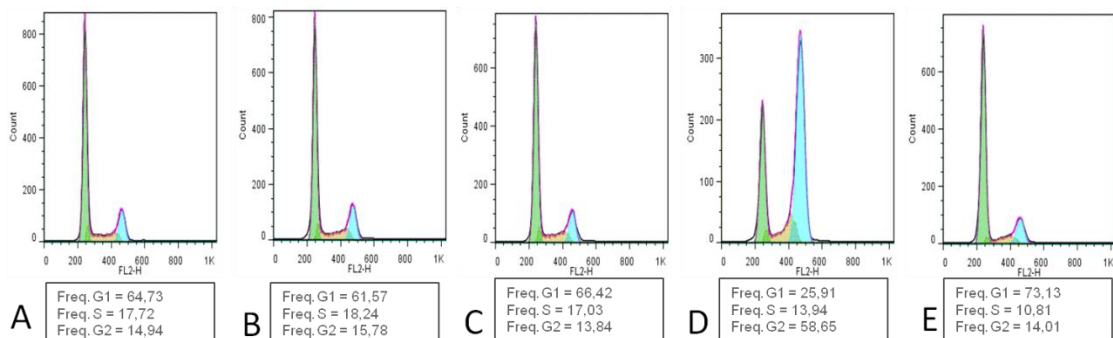

Figure 24: Evaluation of cell cycle in Hep-G2 cell line using PI/RNase kit by flow cytometry. A – CTL. B – Lipoplexes. C – CD/5-FC. D – CD/5-FC + Dox. E – CD/5-FC + MG262.

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 epidemic obesity and rise in hepatitis C virus infection (Jemal A, 2011).

Although many studies have been made and protocols investigated to treat HCC, the majority of patients with advanced HCC still die from the disease and there is an unmet need for other drugs, or treatment options, either as a single agents or in combination (Severi T, 2010). This lack of safe and efficient therapeutic options against many types of cancer, as in liver cancer, gives rise to the necessity to develop alternative therapies that can achieve more effective results and encouraging the development of new gene therapy applications for these diseases (Sangro B, 2010).

Suicide gene therapy, opposing to the conventional antiproliferation drugs, that usually have toxicity both in malignant and non-malignant cells, aims to improve conventional cancer chemotherapy by selectively activating the prodrug at the tumor site, thus permitting higher concentrations of active drug at the tumor and minimizing tumor burden, by limiting the exposition of the patient's normal tissues and organs to potentially harmful quantities of drugs (Hwang, 2006), (Silke Schepelmann, 2008).

Insufficiency of gene transfection and target-orientated gene therapy are main obstacles to the application of gene therapy. The selection of an appropriate vector system for transferring the desired therapeutic gene into the target is an important issue in developing a safe and efficient gene therapy.

Viral vectors generally facilitate highly efficient transfer but they lack desirable targeting ability and can be immunogenic, cytopathic or recombinogenic (Silke Schepelmann, 2008). This safety concerns led to the exploration of non-viral vectors, as the cationic liposomes, which have been tested for safety with little or no toxicity

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reported (Lu Q, 2008) and are simple to prepare, can complex with large amounts of DNA, and are reliable to transfect even into non-dividing cells, without the risk of random integration (Al-Dosari MS, 2009), (Balazs DA, 2011). Though, cationic liposomes lack tumor specificity and have relatively low transfection efficiencies a variety of ligands as transferrin is used to enhance the biological activity and specificity of the lipoplexes to target cells. (Balazs DA, 2011)

With this regards, we evaluated, using HCC cell lines (HUH-7 and Hep-G2 cells), the *in vitro* therapeutic potential, using liposomes coupled with transferrin, of suicide gene approaches (HSV-TK/ GCV or CD/ 5-FC), in monotherapy and in combination with conventional anticarcinogenic agents (Doxorrubicin) and new targeted drugs (proteassome inhibitor MG-262).

In fact there are already several clinical trials using HSV-TK/ GCV or CD/ 5-FC systems, in various types of cancer, but not many are in HCC and either with the proposed treatment combinations or formulation of vectors used for the gene therapy (Rainov, 2000; Voges J, 2003; Nasu Y, 2007; Xu F, 2009; Li N, 2007; Sangro B M. G., 2010 ; Pandha HS, 1999; Freytag SO, 2003; Crystal RG, 1997; Cunningham C, 2001; Zarogoulidis P, 2012)

We first evaluated the transfection efficiency of several formulation DOTAP:Chol associated with transferrin prepared at different (+/-) charge ratio. Our results showed that, even in the chosen charge ratio of 3/2, the transfection efficiency levels were low (approximately 10% of transfected cells). Similar results were obtained by Neves *et al.* (2006) where the highest values of transgene expression in the Tf-lipoplexes was found for the 3/2 lipid/DNA (+/−) charge ratio at which the zeta potential of the lipoplexes is close to neutrality and large aggregates formed.

Our results on the toxicity mediated by suicide gene therapy showed decreases in viability in both CD/5-FC and HSV-TK/GCV treatment, with both cell lines, depending on the applied prodrug dose and incubation period. HUH-7 cell line appeared to be more sensitive than Hep-G2 cell line to both treatments. The most effective approach in HUH-7 cell line was with the TK/GCV system and Hep-G2 cell appeared more sensitive to the approach using the CD/5-FC system although there were not remarkable differences.

Having into account the transfection efficiency levels the cytotoxicity results can be explained by the bystander effect and probably the characteristics of the different cell lines. The CD/5-FC system has a significant local bystander effect that does not require direct cell contact, since 5-FU can move through the cell membrane by non-facilitated diffusion. 5- FC also accumulates after multiple doses, unlike GCV (Dachs GU, 2009). *In vitro* experiments showed that 1– 30% of CD-expressing cells exposed to 5-FC could generate sufficient 5-FU to inhibit the growth of the untransfected neighboring cell (Al-Dosari MS, 2009). *In vivo* studies on tumours containing only 2% CD transduced cells were shown to produce significant tumour reductions (Huber BE, 1994). Since GCV triphosphate cannot passively diffuse to neighboring cells, the bystander effect of the TK/GCV system was shown to be mediated by different mechanisms. It was demonstrated it requires cell-to-cell contact and the requirement of gap junctions was evidenced by comparing the bystander effect in tumor cell lines transfected or not with connexin genes. It can also be mediated via apoptotic bodies generated from dying TK-expressing cells and phagocytosed by unmodified neighboring cells which was demonstrated with flow cytometry and electron microscopic analysis and a gap junction-independent local bystander effect mediated by soluble factors, likely corresponding to phosphorylated GCV metabolites was also reported. (Duarte S, 2012). More recently, the expression of E-cadherin, which is involved in the formation and function of gap

junctions, was shown to correlate strongly with the TK/GCV bystander effect (Garcia-Rodríguez L, 2011).

The combination of CD/5-FC system with doxorubicin or MG262 demonstrates a greater reduction in cell viability even with the use of doses lower than the IC50 with a more pronounced effect in the combinations using MG262. With the TK/GCV system in combination with doxorubicin or MG262 this effect was even more pronounced especially for Hep-G2 cells in the combination with MG262 in which can be observed an addition synergism. In fact the same or higher antiproliferative effect is achieved at lower concentrations than those obtained for all the tested conditions in monotherapy. And the reduction of concentrations of new or conventional drugs and even the ones of the prodrugs, may decrease their potential side effects.

In our work, we also concluded 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 where there is an increase in percentage of apoptotic cells after treatment with the tested conditions, although in this study the differences were not pronounced, probably due to the short period of incubation of 48 hours.

Our results are in concordance with other studies. In fact, some authors found activation of caspases (Tomicic MT, 2002), and the aggregation of receptors associated with cell death FAS (FADD) in cells submitted to the action of HSV-TK/GCV system (Beltinger C, 1999). Other studies showed that, upon application of this system GCV-induced apoptosis occurred mainly by activating the mitochondrial damage pathway, with cytochrome C release, accumulation of p53 protein and decline in BCL-2 levels (Tomicic

MT, 2002; Beltinger C, 1999; Fischer U, 2005). And similarly to HSV-TK/GCV system, apoptosis is also involved in the mechanism of cytotoxicity induced by the CD/5-FC suicide system. It was reported that in glioma cells, the mitochondrial pathway is involved in the process of cell death induced by both suicide gene systems, while p53 and death receptors are not implicated in such process. But even though mitochondrial pathway is involved, the mechanisms of modulation of BCL-2 proteins were found to be different (Fischer U, 2005).

In the analyses of the effect of HSV-TK/GCV or CD/5-FC suicide gene therapy and the described combinations on cell cycle, our results showed an anti-proliferative effect with a cell cycle arrest and accumulation of cells in the G2 phase, which is consistent with the cessation of DNA synthesis effect of suicide gene therapy. This results were even more evident in most of the combination with doxorubicin.

Some studies demonstrated that erroneous GCV triphosphate incorporation into DNA, results in S-phase delay, as well as G2-phase arrest by the activation of 3' exonuclease and post-replicative endonuclease repair mechanisms (Halloran PJ, 1998). As a result of GCV-induced cell cycle arrest, Wei *et al.*(1998) found that apoptosis rather than a direct chemical effect, was involved in HSV-TK-transduced B16F10 melanoma cell death. In a recent study, the relevance of the cell cycle control towards the sensitivity of pancreatic tumor cells to the cytotoxicity induced by the HSV-TK/ GCV system was demonstrated, since a Chk1 activation was associated with a greater HSV-TK/GCV extent of cell death (Abate-Daga D, 2010). Results in combinations with doxorubicin are justifiable through doxorubicin mechanisms of action because it acts as an intercalating agent and wedges between the DNA bases, blocking DNA synthesis and transcription. It also inhibits the activity of topoisomerase type II and this leads to breaks in the genomic

DNA. Both of these mechanisms result in DNA disruption which also justifies the observed effect in the cell cycle (Kim HS, 2009).

In overall in the present study suicide gene therapy using HSV-TK/GCV or CD/5-FC resulted in decreased viability of HCC cell lines in culture, depending on the respective pro-drug dose and incubation time, inducing cell death by apoptosis. Combinations of these systems with lower doses than the IC50 of MG-262 or Doxorubicin enhanced the antiproliferative and cytotoxic effect meaning that lower doses of other drugs with the potentiation of the results and reduced toxicity levels and side effects. This antiproliferative effect was in agreement with the observed cell cycle alterations (accumulation in G2/M phase).

These results suggest that suicide gene therapy HSV-TK/GCV or CD/5-FC may constitute a new potential therapeutic approach in HCC not only in monotherapy, but also in association with conventional therapies or new targeted drugs.

In the future the systems and combinations used should be tested in pre-clinical trials in an animal model of hepatocellular carcinoma.

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