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FACULTY OF SCIENCES AND TECHNOLOGY
UNIVERSITY OF COIMBRA

Development of lipoplex formulations for specific genetic material delivery to pancreatic cancer cells.

Cláudia Marisa Monteiro Saraiva

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Dissertation submitted to the University of Coimbra in partial fulfillment of the requirements for the Master Degree in Biochemistry, carried out under the supervision of Dr. Henrique Faneca (University of Coimbra) and Prof. Dr. Mª Conceição Pedroso de Lima (University of Coimbra).

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“Don't be arrogant, because arrogance

kills curiosity and passion.”

Mina Bissell

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Abstract:

Pancreatic adenocarcinoma is the most lethal solid tumor, being the fourth cause of death in the western world. Pancreatic cancer patients have an extremely poor prognosis with a median survival inferior to 6 months and a survival rate of 3-5%. Surgery remains the only treatment offering an advantage in terms of overall survival but, unfortunately, only 10-20% of the patients present resectable disease at the time of diagnosis. For locally advanced, unresectable, and metastatic disease, treatment is palliative being chemotherapy the standard approach. The genetics of pancreatic cancer makes it one of the most complex malignant diseases, with more mutations than any other common tumor type. An unique miRNA signature was identified in pancreatic cancer distinguishing this cancer from normal and benign pancreas and pancreatitis with 95% of accuracy. Other molecules that are overexpressed in pancreatic tumors present a great potential as tools for diagnose and therapy, namely EphA4 receptors.

The broad field of gene therapy promises a number of innovative treatments that are likely to become important in preventing death from cancer. Cationic liposomes are non-viral vectors and represent a simple and, most importantly, a safe way to deliver therapeutic molecules into the target cells. PEG molecules and ligands (such peptides) are common improvements allowing intravenous applications and conferring specificity, respectively, to the liposomes.

The purpose of this work was the development of a new lipoplex formulation, based on EPOPC:Chol/DNA lipoplexes, that had the ability to specifically deliver genetic material (namely pDNA encoding a therapeutic gene and/or LNAs against miRNAs overexpressed in PDAC) to pancreatic cancer cells, by using an intravenous administration pathway.

The HSA-EPOPC:Chol/DNA 4/1 (+/-) lipoplex formulation showed a high biological activity, even in the presence of serum. The application of a combined strategy involving chemotherapeutic agents and lipoplexes demonstrated that some drugs had the ability to strongly increase the transfection activity of lipoplexes. It was also observed that the HSA-EPOPC:Chol/LNA 4/1 (+/-) formulation is an efficient carrier to deliver LNAs, although this efficiency had been significantly reduced when LNAs were pre-incubated with pDNA. It was also developed a post-pegylation strategy and a targeting approach to the EphA4 receptors using peptides (APY or KYL). From all the developed formulations only the EPOPC:Chol/DNA 4/1 (+/-) lipoplexes with

2mol% of $CerC_8$-PEG presented high biological activity, similar to that obtained with the positive control lipoplexes. The lipoplex formulations containing DSPE-PEG-MAL molecules, with or without peptide, had low transgene expression, most probably due to their entrapment in the endolysomal pathway.

Overall, the obtained results indicate that combined strategies involving chemotherapeutic agents and gene therapy approaches could be of great importance for the development of new antitumor strategies for application in pancreatic cancer. Regarding the gene delivery systems, the EPOPC:Chol/DNA-based lipoplexes incorporating low amounts of Cer-PEG presented high potential for in vivo applications. However, more work should be done in order to develop a formulation that had the ability to specifically and efficiently deliver genetic material into pancreatic cancer cells.

Keywords: Pancreatic cancer; gene therapy; lipoplexes.

Resumo

O adenocarcinoma do pâncreas é a quarta causa de morte do mundo ocidental sendo, dos tumores sólidos, o mais letal. Pacientes diagnosticados com cancro do pâncreas apresentam um prognóstico extremamente pobre com uma sobrevivência média inferior a 6 meses e uma taxa de sobrevivência de 3% a 5%. A cirurgia continua a ser a única forma de tratamento. Infelizmente, apenas 10 a 20% dos pacientes apresentam tumores operáveis aquando do diagnóstico. Para casos de tumores pancreáticos localmente avançados, inoperáveis e metastáticos não existe cura sendo a quimioterapia o tratamento usado. O cancro do pâncreas devido à sua genética é considerado um dos cancros mais complexos tendo mais mutações que qualquer outro tipo de tumor. Esta patologia apresenta ainda um padrão de expressão único de microRNAs que permite distinguir com 95% de precisão tumores pancreáticos de pancreatite e de tecido saudável. Existem ainda outras moléculas que se encontram sobre-expressas neste tipo de tumores, que apresentam um elevado potencial quer terapêutico quer de diagnóstico, nomeadamente os receptores de EphA4.

A terapia génica é uma estratégia terapêutica promissora para o tratamento do cancro. Os vectores não virais, como os lipossomas catiónicos, apresentam-se como uma forma simples e segura de entrega de agentes terapêuticos nas células alvo. A

adição de PEG e de ligandos (como peptídeos) aos lipossomas são melhoramentos que, respetivamente, permitem a sua administração intravenosa e conferem-lhes especificidade.

O objectivo deste projecto consistiu em desenvolver uma nova formulação de lipoplexos, baseada em lipoplexos de EPOPC:Chol/DNA, que possuísse a capacidade de entregar, de forma específica, material genético (nomeadamente pDNA que codifique um gene terapêutico e/ou LNAs contra microRNAs sobre-expressos) às células do cancro do pâncreas, através da sua administração intravenosa.

A formulação de lipoplexos HSA-EPOPC:Chol/DNA 4/1 (+/-) apresentou uma elevada actividade biológica, mesmo na presença de soro. A aplicação de uma estratégia combinada envolvendo agentes quimioterapêuticos e lipoplexos demonstrou que alguns fármacos têm a capacidade de aumentar substancialmente a actividade biológica dos lipoplexos. Além disso, esta formulação demonstrou também uma elevada eficiência de entrega de LNAs às células. Contudo, quando os lipoplexos foram preparados com LNAs pré-incubados com pDNA a eficiência dos LNAs diminuiu significativamente. Foram também desenvolvidas estratégias de pós-peguilação e de direcionamento para os receptores EphA4 usando peptídeos (APY ou KYL), por forma a obter complexos estáveis e específicos. De todas as formulações desenvolvidas apenas a formulação EPOPC:Chol/DNA 4/1 (+/-) contendo 2 mol% de $CerC_8$-PEG apresentou elevada actividade biológica, semelhante à obtida com os lipoplexos controlo. As restantes formulações de lipoplexos, contendo DSPE-PEG-MAL, com ou sem peptídeos, apresentaram uma baixa actividade biológica, muito provavelmente porque estes lipoplexos não são capazes de sair da via endocítica.

Os resultados obtidos neste trabalho indicam que estratégias combinadas, envolvendo agentes de quimioterapia e terapia génica, poderão ser de grande importância para o desenvolvimento de novas estratégias antitumorais para aplicação no cancro do pâncreas. No que diz respeito aos sistemas de transporte e entrega de material genético, os lipoplexos baseados em EPOPC:Chol/DNA, contendo pequenas quantidades de Cer-PEG, apresentam um elevado potencial para aplicação in vivo. Contudo, é necessário fazer trabalho adicional para desenvolver uma formulação que possua a capacidade de entregar, de forma específica e eficiente, o material genético às células do cancro do pâncreas.

Palavras-chave: Cancro do Pâncreas; terapia génica; lipoplexos.

Contents

Abbreviations

Akt	Protein Kinase B
AntimiRs	Anti-sense oligonucleotides
ATP	Adenosine-5'-triphosphate
BRCA2	Breast cancer 2 susceptibility protein
CA19-9	Carbohydrate antigen 19-9
CDK	Cyclin-dependent kinase
$CerC_8$-PEG_{750}	N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)750]}
Chol	Cholesterol
CSCs	Cancer stem cells
C_T	Threshold cycle determination
CDK	Cyclin dependent kinase 4/6 complex
DMEM-HG	Dulbecco's modified eagle medium – high glucose
DNA	Deoxyribonucleic acid
DPC4	Deleted in pancreatic cancer locus 4 (Smad4)
DSPE-PEG	Distearoylphosphatidylethanolamine- poly(ethylene glycol)
DSPE-PEG_{2000}-MAL	(1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N [maleimide(polyethylene glycol)-2000]
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Eph receptors	Ephrin receptors
EPOPC	1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine
EPR	Enhanced permeability and retention effect
FGF	Fibroblast growth factor
FTIs	Farnesyl transferase inhibitors
GT	Gene therapy
HBS	HEPES-buffered saline solution
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
HSA	Human serum albumin
IGF	Insulin-like growth factor
IGF-I receptor	Insulin-like growth factor I receptor
IPMN	Intraductal papillary mucinous neoplasm
iRNA	Interference RNA
LNAs	Locked nucleic acids
LUV	Large unilamellar vesicles
MCN	Mucinous cystic neoplasm
MES	2-(N-morpholino)ethanesulfonic acid
miRISC	miRNA-induced silencing complex
mRNA	Messenger RNA
miRNAs/ miR	Micro RNA
MLV	Multilamellar vesicles
MMPs	Matrix metalloproteinases

Abbreviations

mTOR	Mammalian target of rapamycin
MUC	Mucins
NA	Nucleic acids
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTB	2-nitro-5-thiobenzoate
p16/INK4a	Cyclin-dependent kinase inhibitor 2A
P21/WAF1	Cyclin-dependent kinase inhibitor 1
p27/CIP1	Cyclin-dependente kinase -interacting protein 1
$p27^{kip1}$	p27 cyclin dependent kinase
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate-buffered saline solution
PC	Phosphatidylcholine
PDAC	Pancreatic ductal adenocarcinoma
pDNA	Plasmid deoxyribonucleic acid
PE	Phosphatidylethanolamine
PEG	Poly(ethylene glycol)
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real-time polymerase chain reaction
Rb	Retinoblastoma
Rh-PE	Rhodamine-dioleoylphosphatidylethanolamine
RNA	Ribonucleic acid
RNaseIII	Ribonuclease III
RTKs	Receptor tyrosine kinases
SHH	Sonic hedgehog homolog
siRNA	Small interfering RNA
SUV	Small lamellar vesicles
TGF-β	Transforming growth factor beta
TP53INP1	Tumor protein 53-induced nuclear protein 1
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

This chapter will give a brief overview of the biology of the pancreatic cancer namely the epidemiology, precursor lesions, genetics and abnormalities related with the disease. Diagnostics, available treatments and the research of new therapeutic approaches will also be addressed. A special emphasis will be done to gene therapy strategies, namely non-viral strategies for genetic material delivery.

1.1 Pancreatic Cancer

1.1.1 Epidemiology

Cancer is a major public health problem in the world affecting all age groups **(Siegel et al., 2011)**. Cancer is a group of diseases that is characterized by an abnormal growth of cells which tend to proliferate in an uncontrolled way and, in some cases, to metastasize (spread to other locations in the body via lymph or blood).

The pancreas is a vital organ that is part of the human digestive and endocrine systems. This organ is the key regulator of protein and carbohydrate digestion and glucose homeostasis. The exocrine pancreas (80% of the tissue mass of the organ) is composed of a branching network of acinar and duct cells that produce and deliver digestive zymogens into the gastrointestinal tract. The endocrine pancreas, which regulates metabolism and glucose homeostasis through the secretion of hormones into the blood- stream (insulin and glucagon), is composed of four specialized endocrine cell types gathered together into clusters called Islets of Langerhans **(Hezel et al., 2006)**. Injury or disease of the pancreas can result in severe illness and possibly death.

Pancreatic cancer is one of the most lethal human cancers. It is one of the cancers for which survival has not improved substantially during the past 30 years. The little improvement in the survival rates reflects a combination of earlier diagnosis and better-quality of treatments. However, pancreatic cancer continues to be a major unsolved health problem at the beginning of 21st century **(Siegel et al., 2011; Donghui Li et al., 2004)**. It is the fourth leading cause of cancer related death in the United States with 37.660 deaths and more than 44.000 new cases reported in 2011 **(Siegel et al., 2011)**. In Europe, although the downward trends in rates for the majority of the cancers, pancreatic cancer is an exception, presenting a slight rise in the number of cases with more than 77.000 deaths predicted for 2012 **(Malvezzi et al., 2012)**.

Broadly speaking, there are three basic types of pancreatic tumors: ductal adenocarcinoma (more than 90% of pancreatic cancers); neuroendocrine tumors (rare) and cystic neoplasm (less than 1% of pancreatic cancers) **(Saif, 2011; Hezel et al., 2006)**. Pancreatic ductal adenocarcinoma (PDAC) is the most common epithelial, exocrine pancreatic malignancy. It arises from epithelial cells in the pancreatic ducts or develops from resident stem cells **(Chu et al., 2010)**.

Despite all the progress, the prognosis for patients diagnosed with pancreatic cancer has remained extremely poor. PDAC is characterized by rapid local spread, persistent invasion of surrounding structures and the early creation of distant metastases **(Zakharova et al., 2012; Chu et al., 2010)**. Surgical resection remains the only potentially curative treatment. Unfortunately, merely 10%-20% of patients are resectable at the time of diagnosis. About 40% of patients have locally advanced nonresectable disease and the remaining patients have metastatic disease **(Zakharova et al., 2012; Saif, 2011)**. The prognosis for pancreatic cancer is the worst of all cancers with a mortality/incidence ratio of 0.99 and it has a median survival of less than 6 months and a dismal 5-years survival rate of 5% **(Siegel et al. 2011; Iovanna et al., 2012; Donghui Li et al., 2004; Saif, 2011)**. The fact of incidence rate be almost equal to mortality rate shows the urgent need for novel therapeutic approaches to battle against pancreatic cancer.

1.2Pancreatic Ductal Adenocarcinoma (PDAC)

1.2.1 Risk Factors of PDAC

Pancreatic cancer, like many others malignant diseases, results from accumulation of acquired mutations being associated with demographic, environmental factors (host) and hereditary (genetic) predisposition **(Chu et al., 2010)**. The most significant demographic factor is advancing age (80% of PDAC are diagnosed in the age range of 60-80 years). However, factors as male gender, Ashkenazi Jewish descent, and African-American ancestry are also associated with increased risk of PDAC **(Chu et al., 2010; Donghui Li et al., 2004; Iovanna et al., 2012)**. Host factors include cigarette smoking (estimated to account for 25–29% of pancreatic cancer incidence), obesity and late-onset diabetes mellitus **(Chu et al., 2010; Donghui Li et al., 2004)**.

Another risk factor for pancreatic cancer is family history. Approximately 8% of patients diagnosed with PDAC have a first-degree relative with a history of pancreatic cancer **(Chu et al., 2010)**. Familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, hereditary breast/ovarian cancer, hereditary pancreatitis, Peutz-jeghers syndrome and hereditary melanoma are genetic syndromes that have been associated with risk for developing pancreatic cancer **(Chu et al., 2010; Donghui Li et al., 2004; Iovanna et al., 2012)**.

1.2.2 Precursor Lesions

Pancreatic cancer, like others epithelial cancers, do not arise *de novo* but undergo a stepwise progression through histologically well-defined non-invasive precursor lesions, culminating in frank invasive neoplasia **(Iovanna et al., 2012; Koorstra et al., 2008)**.

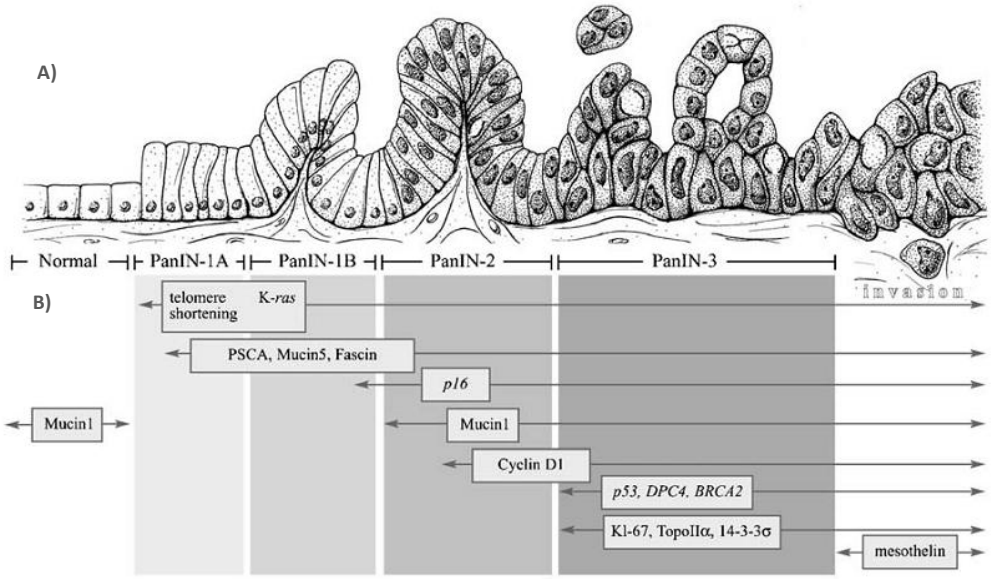


Figure 1: A "PanINgram" illustrating some of the molecular alterations that occur during the multi-step progression of pancreatic adenocarcinomas – PanIN-to-PDAC progression. As pancreatic ductal cells acquire successive molecular alterations, they develop from low-grade PanINs to high-grade PanINs. When cells invade beyond the basement membrane, they form invasive PDAC. A) Schematic drawing of the histopathological features of PanIN-to-PDAC progression. B) Known genetic alterations in PanIN-to-PDAC progression. Adapted from Koorstra et al., 2008.

Pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm (MCN), and intraductal papillary mucinous neoplasm (IPMN) are three PDAC precursor lesions **(Hezel et al., 2006; Mihaljevic et al., 2010; Koorstra et al., 2008)**.

Mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs) are less common precursor lesions. They are called "macrospcopic"

precursors lesions because they are presented typically as radiologically detectable cysts in the pancreas **(Koorstra et al., 2008)**. MCNs are large mucin-producing epithelial cystic lesions that harbor a distinctive ovarian-type stroma with a variable degree of epithelial dysplasia and focal regions of invasion. IPMNs resemble PanINs at the cellular level but grow into larger cystic structures **(Hezel et al., 2006)**.

PanINs are the most common precursor lesions. PanINs are microscopic lesions (less than 5 mm) in the smaller pancreatic ducts. PanINs can be papillary or flat, and are composed of columnar to cuboidal cells with varying amounts of mucin **(Koorstra et al., 2008; Iovanna et al., 2012)**. This lesion shows a spectrum of divergent morphological alterations relative to normal ducts (Figure 1) that seems to represent graded stages of increasingly dysplastic growth. PanINs are classified from stages I to III: PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3, reflecting the progression from non-invasive lesions to invasive PDAC. PanIN-1 lesions are characterized by nuclear atypia's absence and retained nuclear polarity. This is the lowest grade and can be flat (PanIN-1A) or papillary (PanIN-1B). The second stage of the disease, PanIN-2, includes loss of nuclear polarity, nuclear crowding, pleomorphism, nuclear hyperchromasia and nuclear pseudostratification, however in this phase mitoses are rarely seen. PanIN-3 lesions are also known as "carcinoma-*in-situ*". It is characterized by widespread loss of polarity, nuclear atypia and frequent mitoses **(Iovanna et al., 2012; Koorstra et al., 2008; Hezel et al., 2006)**.

1.2.3 Genetics of the Pancreatic Cancer

The genetics of pancreatic cancer makes it one of the most complex malignant diseases, with more mutations than any other common tumor type **(Mackenzie, 2004)**. Multiple subsets of genes were found to be activated or inactivated during the development and progression of pancreatic cancer and precursors lesions.The activation of oncogenes and the inactivation of tumor suppressor genes are in part responsible for the initiation and progression of pancreatic cancers. Moreover, the deregulation of molecules in several signaling pathways, such as EGFR (epidermal growth factor receptor), Akt, NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), Hedgehog, etc., and their molecular crosstalk also play important roles in the molecular pathogenesis of pancreatic cancer (see Figure 1 and Figure 2) **(Iovanna et al., 2012; Hezel et al., 2006; Donghui Li et al., 2004)**.

Oncogenes can be activated through a variety of mechanisms including point mutations within the gene and amplification of the gene itself **(Iovanna et al., 2012; Koorstra et al., 2008)**. Mutations of the K-ras gene are one of the earliest genetic abnormalities observed in the progression model of pancreatic cancer (Figure 1) **(Koorstra et al., 2008)**. The activation of the ras oncogene has been observed in more than 90% of pancreatic cancers. Patients with mutated K-ras have a shorter survival than patients with wild-type K-ras, suggesting that the K-ras mutation participates in the initiation and progression of pancreatic cancer. K-RAS is a member of the RAS family. It is a GTP-binding protein that mediates a wide variety of important cellular functions including proliferation, differentiation, cell survival and motility. The constitutively activated ras, resulting from the point mutations, binds to GTP and gives uncontrolled stimulation signals to downstream signaling cascades promoting uncontrolled cell growth **(Iovanna et al., 2012; Hezel et al., 2006; Kranenburg, 2005)**.

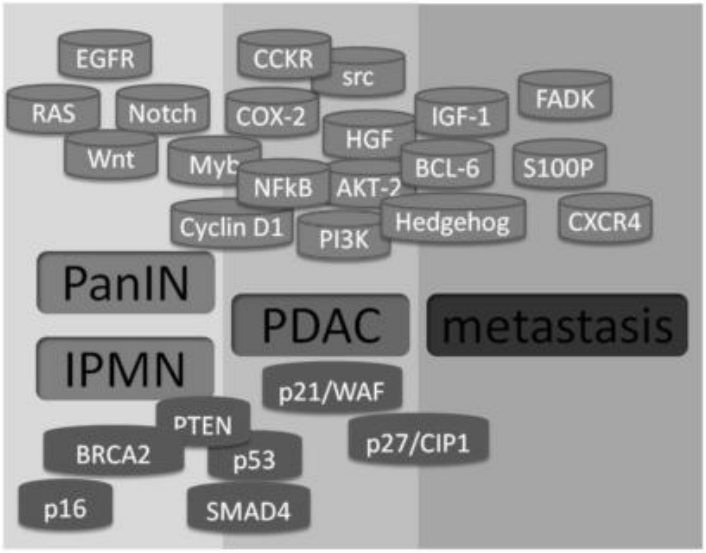


Figure 2 Altered genes in pancreatic cancer and precursors lesions. At the upper part, are shown the gene alterations with gain-of-function, and at the lower part, are shown the genes with lost-of-function that promote pre-cancerous lesions PanIN and IPMN, PDAC, and metastasis. Adapted from Iovanna et al., 2012.

Other example is Scr pathway that is activated in a number of malignancies including in 70% of pancreatic cancers. Src is one of the nine members of the Src family of non-receptor protein tyrosine kinases. In normal conditions Scr is maintained in a phosphorylated and inactive form. Src has diverse roles in cell proliferation, survival, motility, invasiveness, resistance to chemotherapy and angiogenesis **(Iovanna et al., 2012)**.

Pathways like: the cyclooxygenase pathway (affects about 50% of human pancreatic cancers); the MET oncogene that encodes the receptor for hepatocyte growth factor (HGFR) (overexpressed in 78% of pancreatic cancers); the insulin-like growth factor I (IGF-I) receptor that is overexpressed in 64% of pancreatic cancers; the Akt-2 gene and Myb gene that are amplified in 15% and 10% of pancreatic cancers, respectively, between others; are also molecular events involved in pancreatic cancer pathogenicity **(Iovanna et al., 2012)**.

Besides the oncogenes overexpression, inactivation of tumor suppressor genes also plays an important role in the initiation of pancreatic cancer. Tumor suppressor genes can be activated by homozygous deletion, intragenic mutations coupled with the loss of second allele and promoter hypermethylation. The tumor suppressor genes targeted in pancreatic cancer include p16, p53, SMAD4, and PTEN **(Iovanna et al., 2012)**.

Approximately 95% of pancreatic cancer patients have inactivated p16/INK4a in their tumors. p16/INK4a inhibits not only the activity of the cyclin D but also the activity of the cyclin-dependent kinase 4/6 (CDK4/6) complex. This complex interacts with cyclin D to phosphorylate the retinoblastoma (Rb) protein allowing activation of genes required for DNA synthesis along the cell cycle. In this way, p16/INK4a controls cell cycle progression through G1/S transition by inhibiting cyclin D and CDK4/6 mediated phosphorylation of Rb and therefore inhibiting cell growth **(Iovanna et al., 2012; Hruban et al., 2008)**.

The p53 tumor suppressor gene codifies the p53 protein. P53 has a number of important functions in the cells including regulation of the G1/S cell-cycle checkpoint, maintenance of G2/M arrest inhibiting cell growth and the induction of apoptosis. This gene is inactivated in 50-75% of pancreatic cancers and it is present in high-grade precursor lesions (PanIN-3) and adenocarcinomas. Inactivation of p53 during carcinogenesis can lead to uncontrolled cell growth and increased cell survival **(Iovanna et al., 2012; Hruban et al., 2008; Koorstra et al., 2008)**.

The inactivation of DPC4 (Deleted in Pancreatic Cancer locus 4, Smad4) tumor suppressor gene is another common genetic alteration. This mutation is identified in approximately 50% of the patients in pancreatic cancer. The DPC4 gene encodes a 64-kDa protein, Smad 4, which plays roles in the inhibition of cell growth and angiogenesis. Smad4 is very important in the transmission of signals from transforming growth factor type beta (TGFβ) and others related ligands. Loss of Smad4 interferes

with activin and TGFβ signaling cascades resulting in a decreased of growth inhibition via loss of pro-apoptotic signaling or inappropriate G1/S transition **(Iovanna et al., 2012;Hruban et al., 2008)**.

Others important tumor suppressor genes are: p21/WAF1, p27/CIP1 and BRCA2. p21/WAF1 is an inhibitor of CDK (cyclin dependent kinases). It forms complexes with cyclinA/CDK2 or cyclinD1/CDK4 and inhibits their activity causing cell cycle arrest in G1 phase. So, it is responsible for the regulation of the cell cycle arrest in G1 phase and its activity is absent in approximately 50% of pancreatic cancers. Loss of function of p27/CIP1, another CDK inhibitor which regulates cell cycle progression from G1 to S phases, is also common in PDAC patients. BRCA2 is involved in DNA damage repair. Mutations in BRCA2 are linked to a significantly increased risk of pancreatic cancer **(Iovanna et al., 2012)**.

The deregulation of EGFR, Atk, NFκB, Hedgehog signaling and theirs downstream signaling pathway are also important events for development and progression of pancreatic cancer. Telomerase abnormalities, present in more than 90% of the cases, are another promoting factor of pancreatic cancer malignancy. Centrosome abnormalities, chromosomal amplifications and deletions, inflammation, angiogenesis also occur in PDAC cells. Epigenetic alterations are another important factor, controlling gene function. DNA methylation, histone acetylation and deacetylation between others events are also correlated with pancreatic cancer malignancy **(Mihaljevic et al., 2010; Iovanna et al., 2012)**.

Cancer stem cells possess important properties associated with their normal counterparts, namely the ability for self-renewal and differentiation. This type of cells forms a small subset in the heterogeneous tumor population, contributing to neoplastic progression, metastasis, and resistance to chemotherapy and radiotherapy. Dysregulation of various signaling cascades, including the PTEN, Shh, Notch, and Wnt pathways, are frequently observed in cancer stem cells, turning these pathways good therapeutic targets **(Iovanna et al., 2012; Balic et al., 2012)**.

The idea that tumor development and progression is exclusive to cellular processes and molecular pathways existents in tumor cells themselves was abandoned. It has been demonstrated that the effects of the stroma on tumor cells may be pro- and anti-tumorigenic depending on the context. The microenvironment, also known as stroma, of pancreatic cancer comprises several different cell types including stellate cells, endothelial cells, nerve cells, immune cells such as macrophages, lymphocytes,

dendritic cells and the extracellular matrix. It is becoming clear that the desmoplastic microenvironment of pancreatic cancer – which is approximately eighty percent of the tumor mass – is not a passive scaffold for the tumor cells but an active player in carcinogenesis. Therefore, targeting the activated stroma in order to uncouple epithelial-stromal interactions may interrupt multiple aberrant autocrine and paracrine pathways that promote pancreatic cancer cells growth, invasion, metastasis, and angiogenesis **(Erkan et al., 2010; Mihaljevic et al., 2010; Christofori, 2006)**.

Recent studies indicate that microRNA (new class of small non-coding RNA molecules which play a crucial role in the regulation of gene expression by repression at translation level) are overexpressed in pancreatic cancer and present its own signature on PDAC **(Lee et al., 2007; Blenkiron and Miska 2007)**.

1.2.4 Invasion and Metastasis

Approximately 90% of all cancer deaths arise from the metastatic spread of primary tumors. Of all the processes involved in carcinogenesis, local invasion and the formation of metastasis are clinically the most relevant, but they are the least well understood at the molecular level. There is an embarrassing lack of therapies that can efficiently prevent metastasis **(Mihaljevic et al., 2010)**.

Multiple signal-transduction pathways, changes in the adhesive and migratory capabilities of tumor cells and the tumor microenvironment have critical roles in malignant tumor progression. Malignant tumor cells have the ability to migrate and to invade the surrounding tissue. This phenomenon can be done either as single cells or in collective clusters, thereby forming an invasive front **(Mihaljevic et al., 2010; Christofori, 2006)**.

Dissociation of cells from the epithelial layer requires deregulation of cell-to-cell contacts and the acquisition of migratory capabilities. In PDAC, these changes are associated to epithelial-mesenchymal transition. So, this can be considered as the initial step of the metastatic spread. Growth factors including TGF-β, HGF, EGF, IGF, and FGF are trigger to this program. For example, pancreatic stellate cells can be activated by a number of cytokines that are secreted by tumor cells. These cells will produce dense desmoplastic stroma, which may constitute a tumor-supportive microenvironment. Factors influencing growth, angiogenesis and invasion are present in the tumor stroma **(Mihaljevic et al., 2010; Christofori, 2006)**.

Angiogenesis and lymphangiogenesis are very important processes to tumor growth and progression. Angiogenesis is frequently induced by transforming signals that promote tumor progression and directly upregulate the expression of angiogenic factors such as Ras-Raf-MAPK pathway and hypoxia. Overexpression of these factors and the subsequent increase of the micro-vessel density together with the presence of inflammatory sites, enable invasive tumor cells to intravasate and disseminate through the bloodstream. Lymphangiogenesis, the outgrowth of new lymphatic vessels, can directly promote the formation of lymph node metastases, mainly at the draining regional lymph nodes of the tumor. Lymphangiogenesis is induced by the lymphangiogenic members of the VEGF family. Both angiogenesis and lymphangiogenesis contribute not only to primary tumor growth but also to the metastatic dissemination of tumor cells and, together, offer attractive targets for development of anti-metastatic therapies **(Christofori, 2006)**.

1.2.5 Symptoms and Diagnosis of PDAC

The symptoms of pancreatic cancer are generally ambiguous and can easily be attributed to other less serious and more common conditions. Symptoms of PDAC can include pain in the abdomen, back pain and jaundice (yellowing of the skin and the whites of the eyes, itchy skin, dark yellow urine, and pale bowel motions). These symptoms can also include weight loss, associated with loss of appetite (anorexia), bloating, diarrhea or steatorrhea (fat bowel movements that float in water). When a pancreatic-head tumor is quite small, painless jaundice might be the only sign of the disease **(Stathis and Moore, 2010; Donghui Li et al., 2004)**. This lack of specific symptoms explains the high number of people with advanced stages of the disease when pancreatic cancer is diagnosed. Furthermore, pancreas is placed in an inaccessible location within the abdomen, making the diagnosis of pancreatic cancer more difficult than others digestive tract cancers **(Lowenfels and Maisonneuve, 2004)**.

Pancreatic cancer is relatively rare, turning the screening of the entire population inappropriate when compared to other types of cancer, namely breast, colon and prostate cancers. It would cost a lot of money to screen everyone for a disease that only a few people get. Besides, at the present time there is no screening test reliable enough to use for pancreatic cancer in people at average risk. However, there are screening programs for people who may be at high risk of developing the disease. This screening

is more fit for people over 40 years old who have hereditary pancreatitis or a high incidence of pancreatic cancer in their family **(Lowenfels and Maisonneuve, 2004)**.

When some suspicions about pancreatic cancer exist, computed tomography is regularly the first test performed **(Donghui Li et al., 2004)**. Endoscopic ultrasound is another technique used. To obtain maximum yield from Endoscopic ultrasound it should be made after contrast computed tomography and before endoscopic retrograde cholangiopancreatography – technique that combines the use of endoscopy and fluoroscopy to diagnose and treat certain problems of the biliary or pancreatic ductal systems **(Tadić et al., 2010)**.

1.2.6 Current Treatments Strategies

Despite the advances in cancer therapy, the treatment of pancreatic cancer patients remains one of the major challenges of medical oncology. The conventional treatment approaches for PDAC are surgery (highly invasive), radiation, chemotherapy (usually gemcitabine that inhibits DNA synthesis promoting apoptosis) and/or combinations of these. For early stages of pancreatic cancer, radical surgery (Whipple's operation) is the standard and the only curative option nowadays. However, just 10 to 20% of the patients benefit from it. The majority of patients have locally advanced unresectable disease at diagnosis, due to local vascular invasion or metastatic disease. In late stages of the disease the palliative chemotherapy with purine analogue gemcitabine and/or fluoropyrimidine or a platinum agent is the standard approach **(Marco et al., 2010; Stathis and Moore, 2010; Strimpakos et al., 2010)**. Another alternative is radiotherapy. Radiotherapy offers an improving of local tumor in specific cases: patients with advanced non metastatic disease when surgery is either not feasible or incomplete **(Klautke and Brunner, 2008)**.

Being surgery a highly invasive technique and having in mind that the majority of patients eventually evolve advanced metastatic disease, development of new therapeutic approaches and detection techniques are essential.

1.2.7 Emerging Therapies and Molecular Targets

The poor prognosis of pancreatic cancer reflects both the difficulty of early diagnosis and the generally poor response to current therapies. Although the use of

chemotherapeutic drugs is the conventional therapeutic approach, the advances in understanding molecular abnormalities implicated in pancreatic cancer opened doors to create novel diagnostic tools and new molecular approaches. These novel approaches could also be combined with chemotherapeutic drugs in order to improve clinical outcomes of pancreatic cancer **(Shi et al., 2012)**.

Molecular targets allowing diagnose of PDAC are essential. Glycoproteins are the most common target. Carbohydrate antigen 19-9 (CA19-9) is one of that, having a sensitivity and specificity for pancreatic tumors around the 85% and 90%, respectively. However, it is not a specific marker and can be found in others conditions like liver-biliary cirrhosis, biliary obstruction and ascites. Mucins (MUC) are the second most known glycoproteins studied in pancreatic tumors. The most important are MUC1 and MUC4. MUC1 is highly expressed in invasive ductal carcinoma and MUC4 is overexpressed in pancreatic cancer but not in begin conditions. MUC4 is also associated with advanced stages and aggressiveness of pancreatic cancer having not only a diagnose potential but also a therapeutic potential **(Strimpakos et al., 2010)**.

Eph receptors are another molecule with a great potential for both, diagnose and therapy **(Iiizumi et al., 2006; Giaginis et al., 2010)**. Ephrin (Eph) receptors constitute the largest sub-family of receptor tyrosine kinases (RTKs). They interact with cell surface-bound ligands that are also part of a family of related proteins. Eph receptors and ephrins are grouped into class A and class B based on their ligand-binding-affinity and structure of the extracellular domain. Eph receptors and eph ligands have been shown to form a vital cell communication system capable of bi-directional signaling. They are implicated in a wide spectrum of biological activities: guidance and migration of neural crest cells in the nervous system; regulation of cellular adhesion; migration or chemo-repulsion; tissue/cell boundary formation; and are critical regulators of vascular remodeling during embryogenesis **(Arvanitis and Davy, 2008; Iiizumi et al., 2006; Giaginis et al., 2010)**.

Nakamura et al. (2004) identify 260 genes overexpressed to at least fivefold greater extent in cancer cells than in normal pancreatic epithelial cells. Eph receptors and their ligands are one of those. They are frequently overexpressed and/or functionally altered not only in pancreatic cancer but also in others types of cancers **(Nakamura, 2004; Giaginis et al., 2010)**. These receptors are involved in a broad range of processes directly related with tumorigenesis and metastasis, including cell attachment and shape, migration and angiogenesis **(Giaginis et al., 2010)**.

The Eph-A1 receptor seems to be associated with tumor size and tumor histopathological stage. Eph-A2 receptors expression, on the other hand, is significantly associated with patients' age and seems to enhance PDAC invasiveness, while Eph-A4 and Eph-A5 receptors are related with tumor proliferative capacity **(Giaginis et al., 2010)**.

An advantage of EphA4 receptors is the very restricted expression pattern in adult tissues. EphA4 receptors are essentiality expressed in central nervous system and in a subset of protest cancer and soft tissue sarcomas. In this way, EphA4 receptors can be used as target molecules for therapeutic interventions or as molecules for targeted therapies to pancreatic cancer cells. This possibility is due to the fact of the expression pattern of ephrin receptors in normal pancreatic epithelium be almost absent and because of the blood-brain barrier that is a natural barrier against the delivery systems **(Iizumi et al., 2006; Giaginis et al., 2010)**.

So, we are now in a time of therapeutic strategies that are based on mechanism that target specific biologic pathways of tumors triggering specific responses. This strategy is known as "molecularly targeted therapy". The traditional cytotoxic drugs also "target" specific cellular processes. However, these agents used in this new approach allow the targeting of a pathway or molecule that drives the growth, spread, survival, or maintenance of tumor cells specifically and preferentially **(Ko, 2007)**. Due to their importance in tumor development and progression VEFG (vascular endothelial growth factor) pathway, EGF (epidermal growth factor) pathway, cancer stem cells (CSC), Matrix metalloproteinases (MMPs), farnesyl transferase inhibitors (FTIs), NFkB inhibitors, mTOR inhibitors, are good targets for the "molecularly targeted therapy" **(Marco et al., 2010; Strimpakos et al., 2010)**.

Targeted therapies can be done by small-molecule inhibitors, monoclonal antibodies, short-hairpin RNAs (shRNAs), oncolytic viruses, gene therapy, and immunotherapy **(Ko, 2007; Shi et al., 2012; Andresen et al., 2005)**. For example small molecules inhibitors are commonly orally bioavailable agents, designed to disrupt a particular signaling pathway, enzyme, or cellular component playing some defined role in cell growth and tumorigenesis. Monoclonal antibodies are another example. They can be applied stand-alone or conjugated with radioisotopes or cytotoxic drugs. They have the advantage of being more specific than small molecules inhibitors **(Ko, 2007)**.

Although all efforts made to find more effective therapies for the treatment of pancreatic cancer, significant results have not yet been achieved **(Marco et al., 2010; Shi et al., 2012)** and there are an all new range of possibilities to explore.

1.2.8 microRNA Profile in Pancreatic Cancer

Small regulatory RNAs are essential and ubiquitous riboregulators, discovered by Andy Fire and Craig Mello in 1998. They are the key mediators of interference RNA (iRNA). They include microRNAs (miRNAs) and short interfering RNAs (siRNAs) **(Liu et al., 2008; Blenkiron and Miska, 2007)**.

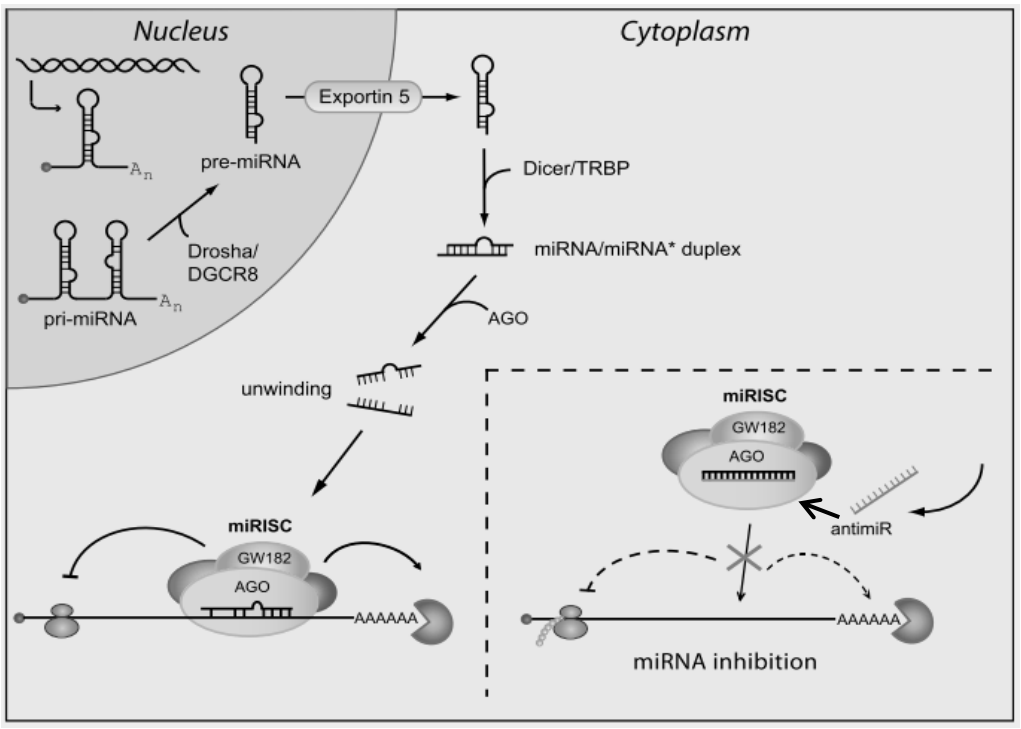


Figure 3 miRNA biogenesis and inhibition of miRNA function by antimiR oligonucleotides. miRNA genes are transcribed by RNA polymerase II and processed by nuclear Microprocessor complex in the nucleus to ~70 nt pre-miRNAs. Pre-miRNAs are exported to the cytoplasm by Exportin-5 and processed further by Dicer to ~22 nt double-stranded miRNA. The mature miRNA is retained in the miRISC, whereas the complementary strand, known as the miRNA star (miR*) is released. Mature miRNAs bond to 3' UTRs of target mRNAs promoting their translational repression or deadenylation and degradation. Chemically modified antimiR oligonucleotides sequester the mature miRNA in competition with cellular target mRNAs leading to functional inhibition of the miRNA and derepression of the direct targets. Adapted from Stenvang et al., 2012.

MicroRNAs (miRNAs), an evolutionary conserved class of small non-coding RNAs, are transcribed from endogenous genes as long RNA transcripts and are processed to their mature form of single-stranded RNA with approximately 22 nucleotides **(Nelson et al., 2003; Blenkiron and Miska, 2007)**. The primary transcripts of miRNA genes, known as pri-miRNAs, are usually several kilobases long and possess

a 5' CAP and a poly(A) tail. The nuclear Microprocessor complex, consisting of Microprocessor complex subunit DGCR8 and the RNase III enzyme Drosha, is responsible by the processing of pri-miRNAs into the nucleus to approximately 70 nucleotides hairpin-structures, termed pre-miRNAs. Pre-miRNAs are exported to the cytoplasm by Exportin-5 and processed further by Dicer to approximately 22 nucleotides double-stranded miRNA duplexes. The miRNA duplexes are loaded into an Argonaute protein in the miRNA-induced silencing complex (miRISC) and rapidly unwound. During this process the mature miRNA is retained in the miRISC, whereas the complementary strand, known as the miRNA star (miR*), is released (Figure 3). At this time, the microRNAs are in their mature form of 19 to 25 nucleotides **(Liu et al., 2008; Carthew and Sontheimer, 2009; Stenvang et al., 2012)**. The miRNAs biogenesis process is temporally and spatially regulated **(Bloomston et al., 2007; Nelson et al., 2003)**. At this point, the single-stranded miRNAs are able to bind messenger RNAs of hundreds of genes with perfect or near-perfect complementarity, resulting in degradation or inhibition of the target messenger RNA, respectively. Notice that the key specificity determinant for miRNA target recognition is based on Watson-Crick pairing of the so-called seed region (nucleotides 2 to 8) in the mature miRNA to the seed match site in the target 3' UTR, which nucleates the miRNA:target mRNA interaction **(Bartel, 2009; Stenvang et al., 2012; Nelson et al., 2003)**.

Many miRNAs have been discovered in the past few years in humans. The total number of miRNAs in each organism is unknown but is estimated to represent 1% of all genes **(Nelson et al., 2003)**. miRNAs have been implicated in regulation of a diverse number of cellular processes, including differentiation of adipocytes, maturation of oocytes, maintenance of the pluripotent cell state, regulation of insulin secretion, between others **(Lee et al., 2007)**. However, there are a growing number of direct and indirect evidences suggesting a relationship between altered miRNA expression and cancer, namely in pancreatic cancer **(Farazi et al., 2010; Lee et al., 2007)**, making them promising biomarkers and/or therapeutic targets to novel therapeutic approaches.

In humans, aberrant expression of miRNAs can contribute to carcinogenesis by promoting the expression of proto-oncogenes or by inhibiting the expression of tumor suppressor genes **(Bloomston et al., 2007; Farazi et al., 2010)**. Development of miRNA microarrays, qRT–PCR (quantitative real-time polymerase chain reaction) platforms and deep sequencing methodologies, has resulting in an exponential acquisition of miRNA profiles **(Farazi et al., 2010)**. In pancreatic cancer a unique

miRNA signature was identified distinguishing pancreatic cancer from normal and benign pancreas **(Lee et al., 2007)** and also differentiates from pancreatitis with 95% of accuracy **(Bloomston et al., 2007).** The expression profile of miRNAs in PDAC has a large number of miRNAs aberrantly expressed being the most significant represented in Table 1.

Table 1 Top 20 aberrantly expressed miRNAs precursors in pancreatic adenocarcinoma. Adapted from Lee et al., 2007.

Rank	Name	P-value (*t*-test)	Fold change	Chromossome location
1	miR-221	$5.66E^{-05}$	26.2	Xp11.3
2	miR- 424	$3.62E^{-08}$	56.3	Xq26.2
3	miR-301	$1.11E^{-05}$	34.2	17q23.2
4	miR-100	$4.40E^{-06}$	36.9	11q24.1
5	miR-376a	$7.00E^{-04}$	7.79	14q32.31
6	miR-125b-1	$1.00E^{-04}$	23.2	11q24.1
7	miR-021	$2.00E^{-04}$	15.7	17q23.2
8	miR-345	$1.44E^{-15}$	-14.5	14q32.2
9	miR-016-1	$3.73E^{-04}$	14.3	13q14.2
10	miR-181a,c	$8.31E^{-04}$	18.6	9q33.3, 19p13.13
11	miR-092-1	$3.40E^{-03}$	19.6	13q31.3
12	miR-015b	$4.00E^{-04}$	8.55	3q25.33
13	miR-142-P	$3.63E^{-07}$	-15.4	17q25.33
14	miR-155	$1.51E^{-03}$	14.0	21q21
15	Let-7F-1	$4.00E^{-04}$	10.9	9q22.32
16	miR-212	$2.00E^{-04}$	22.2	17p13.3
17	miR-107	$3.86E^{-05}$	8.20	10q23.31
18	miR-024-1,2	$9.12E^{-08}$	8.17	9q22.32, 19p13
19	Let-7d	$7.06E^{-04}$	8.38	9q22.32
20	miR-139	$6.79E^{-11}$	-7.91	11q13.4

miR-216 and miR-217 were identified to be specific for normal pancreas. They are down-regulated more than 200-fold in PDAC samples **(Mardin and Mees, 2009)**, making them promising biomarker candidates. On the other hand, miR-21, miR-221, miR-222, miR-181a, miR-181b, miR-181d and miR-155 are all overexpressed in tumor samples relative to benign pancreatic tissue **(Lee et al., 2007; Bloomston et al., 2007)**. Some of the overexpressed miRNAs in pancreatic cancer are also aberrantly expressed in others types of cancers, such as miR-155, miR-222 and miR-221**(Lee et al., 2007)**.

Greither et al. (2010) has shown by qRT-PCR significant correlations between elevated microRNA expression and overall survival. The miRNAs implied were miR-

155, miR-203, miR-210 and miR-222. Tumors from patients demonstrating elevated expression levels of all 4 microRNAs possessed a 6.2-fold increased risk of tumor-related death compared to patients whose tumors showed a lower expression of these microRNAs **(Greither et al., 2010)**. This study provides evidences of the miRNA importance and potential as future therapeutic targets.

miR-155 is a functional miRNA. miR-155 is responsible for the inhibition of TP53INP1 (tumor protein 53-induced nuclear protein 1) and it is upregulated 14 times in PDAC **(Gironella et al., 2007; Lee et al., 2007)**. TP53INP1 is linked to the regulation of the cell cycle progression through its anti-proliferative and pro-apoptotic activities. A reduction of this protein might be an indicator of pancreatic malignancy being the miR-155 a possible target for PDCA therapies **(Gironella et al., 2007; Hruban et al., 2008)**.

miR-221 is located 700 bp from miR-222 and both are part of a gene cluster located on chromosome X. In fact, the 5' "seed" regions of these two miRNAs are identical and theoretically target the same sites **(Galardi et al., 2007)**. miR-222 is overexpressed in pancreatic cancer at levels that are similar to miR-221 (Table 1) **(Lee et al., 2007)**. Monitored alterations of miRNA expression in pancreatic cancer observed a 26-fold increase in miR-221 expression **(Lee et al., 2007; Mardin and Mees, 2009)**. One of the targets sequence for the miR-221&222 is the 3'UTR of p27 mRNA **(Galardi et al., 2007)** and these microRNAs function as oncogenes by controlling cell cycle progression through inhibition of $p27^{kip1}$ **(le Sage et al., 2007)**. The $p27^{Kip1}$ gene is a member of the Cip/Kip family of CDK inhibitors that function to negatively control cell cycle progression. It binds to CDK2 and cyclin E complexes to prevent cell cycle progression from G1 to S phase. $p27^{Kip1}$ acts as a tumor suppressor **(Koff, 2006; le Sage et al., 2007)**. The requirement of miR-221&222 for tumor survival may suggest that it might be possible to use antagomiR-221&222 as a form of cancer treatment **(Koff, 2006; le Sage et al., 2007)**.

Looking to miRNAs as target molecules and giving special attention to the overexpressed miRNAs, there are three approaches used in miRNAs loss-of-function studies: genetic knockouts, miRNA sponges and antisense oligonucleotides. The antisense oligonucleotide approach is widely used allowing a transient to long-time inhibition ideally to cancer treatments. In this approach, chemically modified antisense oligonucleotides, termed antimiRs, sequester the mature miRNA in competition with

cellular target mRNAs leading to functional inhibition of the miRNA and de-repression of direct targets as illustrated in Figure 3 **(Stenvang et al., 2012)**.

There are different kinds of chemically modification on antisense oligonucleotides. The locked nucleic acids (LNAs) are an outstanding technology. They are more resistant to nucleases activity and possess a highest affinity towards complementary RNA. The LNAs nucleotides contain a methylene bridge that connects the 2'-oxygen of the ribose with the 4'-carbon. The bridge results in a locked 3'*endo* conformation reducing the conformational flexibility of the ribose and increasing the local organization of the phosphate backbone **(Braasch and Corey, 2001)**. However, it is necessary to keep in mind that the use of antimiR oligonucleotides as tools in miRNA loss-of-function studies or as therapeutic modalities carries the inherent risk of affecting others RNA species instead of the intended miRNA target – off target effects **(Stenvang et al., 2012)**.

So, in conclusion, although there are some challenges to overcome (like the *in vivo* delivery) the use of antimiR oligonucleotides to target disease-associated miRNAs shows great promise in the development of novel miRNA-based therapeutics.

1.3 Gene therapy

1.3.1 Origin and Different Approaches of Gene Therapy

The concepts of gene therapy (GT) arose initially during the 1960s and early 1970s whilst the development of genetically marked cells lines and the clarification of mechanisms of cell transformation by the papovaviruses polyoma and simian virus 40 were in progress. In 1972 Friedmann and Roblin authored a paper in Science titled "Gene therapy for human genetic disease?". They cite Rogers S for proposing "that exogenous "good" DNA could be used to replace the defective DNA in those who suffer from genetic defects". In this way, the initial concept of gene therapy was to introduce into the cells of a patient a therapeutic gene in an approach called gene replacement. This intervention simply added an additional gene to a specific population of cells without directly perturbing the structure of an endogenous nonfunctioning or pathogenic gene **(Friedmann and Roblin, 1972)**.

In the case of inherited monogenic diseases the aim of gene therapy is to transfer and express the defective gene **(Touchefeu et al., 2010)**. Gene therapy can be applied to the treatment of acquired diseases like cancer (almost 70% of the studies in GT are in the cancer area) **(El-Aneed, 2004)**. However, the situation is more complex in cancer gene therapy because cancer often results from sequential genetic and epigenetic alterations, affecting oncogenes, tumor-suppressor genes and microRNAs **(Touchefeu et al., 2010)**. In this way, there are four different strategies in cancer gene therapy: immunotherapy, oncolytic virotherapy, gene transfer and therapeutic RNA interfering **(Touchefeu et al., 2010; Cross and Burmester, 2006)**.

Immunotherapy or the concept of improving the immune system to target and destroy cancer cells is being used to create recombinant cancer vaccines. These vaccines are not meant to prevent disease, but to stimulate immune system responses against tumor by using highly antigenic and immunostimulatory cellular debris **(Touchefeu et al., 2010; Cross and Burmester, 2006)**.

Oncolytic gene therapy uses vectors, generally viruses, designed to infect cancer cells and induce cell death through the propagation of the virus, expression of cytotoxic proteins and cell lysis, while viruses remaining innocuous to the rest of the body **(Cross and Burmester, 2006)**. The two main characteristics of this approach are: replication selectively in cancer cells and self-amplification properties; and the cancer-

cell-specific toxicity; making this an exciting avenue for lowering the number of cancer deaths **(Touchefeu et al., 2010)**.

Gene transfer therapy involves the introduction of a foreign gene into the cancer cells or surrounding tissue. Gene transfer can use genes to correct gene mutations, restoring the expression of a tumor-suppressor gene. It can also use suicide genes to kill the cancer cells, antiangiogenesis genes and cellular stasis genes **(Touchefeu et al., 2010; Cross and Burmester, 2006)**.

RNA interfering therapies are the most recent, emerging from the relationship between miRNAs and cancer. As refereed before, miRNAs can have oncogenic activities when they are upregulated and inhibit the tumor suppressor genes translation. Nevertheless, they can also present a tumor suppressor potential. Additionally, miRNAs can modify the response to therapeutic agents. Having this in mind, RNA interference pathway has been exploited to develop other RNA interference molecules: synthetic, exogenous, double-stranded, short, interfering RNA between others. In this way, there are multiple opportunities for therapeutic approaches using interfering RNA or having miRNAs as the target molecules **(Touchefeu et al., 2010)**.

1.3.2 Delivery Systems

The effectiveness of gene therapy is highly dependent on the efficacy of gene transfer. Although the delivery of genetic material into the cells can be done without a delivery system, the delivery of naked nucleic acids is not enough to reach efficient gene transfer **(Nuno Penacho, 2009)**. So, the development of delivery systems became a need in order to overcome this limitation. Still nowadays, the reduced delivery of functional therapeutic genes into target cells and the use of efficient and safe gene delivery systems remain the primaries challenges of gene therapy **(Gao et al., 2007; El-Aneed, 2004)**. Requirements for successful gene delivery systems include safety (biocompatibility and biodegradability), resistance to degradation, avoid recognition by the immune cells (unless immune response is desired), capability of entering the appropriate cellular compartment, specificity towards the target sites and ability to selectively modulate the expression of the target gene or to express the therapeutic protein during the desired period of time **(Nuno Penacho, 2009; Elsabahy et al., 2011; Duzgunes et al., 2003)**. These vehicles are categorized into two groups: biological and non-biological systems.

Biological carries are viruses. They represent the most evolved shuttle system for nucleic acids **(Nuno Penacho, 2009)**. Viruses are naturally evolved to infect and transfer their genetic materials into the host cells **(El-Aneed, 2004)**. Adenovirus, retrovirus, adenoassociated virus and herpes simplex virus are some examples of viruses used in GT. These vectors are modified in laboratory eliminating pathogenicity and retaining their high gene transfer efficiency. Although the advantages of biological systems (demonstrated to be the most efficient vectors), they have some setbacks. Toxicity, severe inflammation and immunological problems, the risk of a new gene be inserted in the wrong location in DNA causing mutations or even cancer, and its difficult and expensive production are some of their disadvantages **(Elsabahy et al., 2011; El-Aneed, 2004; Nuno Penacho, 2009)**. The number of clinical trials using viral vectors as delivery systems is high, still many of them were interrupted. This happen because the application of these vectors had induced unexpected adverse effects such as immunogenicity and oncogenicity **(He et al., 2010)**.

Non-biological systems, also known as non-viral vectors, represent a simple and, most importantly, a safe alternative to viral vectors **(Nuno Penacho, 2009)**. However, the low transfection activity and transient nature of transgene expression continues to be the most severe bottlenecks in the clinical use of non-viral vectors. So, it is important and required the improvement of theirs transfection activity **(He et al., 2010)**. Non-viral delivery approaches have been developed and they can be categorized in two different groups: naked nucleic acid delivery using physical methods; and nucleic acid delivery mediated by chemical carriers **(Nuno Penacho, 2009)**. Physical approaches include needle injection, electroporation, gene gun, ultrasound, and hydrodynamic delivery. They are techniques that employ a physical force able to permeate the cell membrane facilitating intracellular gene transfer. The chemical approaches use synthetic or naturally occurring compounds as carriers to deliver the transgene into cells **(Gao et al., 2007)**. The advantages and limitations of these techniques are summarized in Table 2.

Table 2 Advantages and limitations of current non-viral gene delivery systems. Adapted from Gao et al., 2007.

Method	Route of Gene Delivery	Advantages	Limitations
Needle injection	Intratissue	Simplicity and safety	Low efficency
Gene Gun	Topical	Good efficiency	Tissue damage in some applications
Electroporation	Topical Intratissue	High efficiency	Limited working range; need for surgical procedure for nontopical applications
Hydrodynamic delivery	Systemic Intravascular	High efficiency, simplicity, effectiveness for liver gene delivery	Extremely effective in small animals; surgical procedure may be needed for localized gene delivery
Ultrasound	Topical Systemic	Good potential for site-specific gene delivery	Low efficiency in vivo
Cationic lipids	Topical Systemic Airway	High efficiency in vitro; low to medium high for local and systemic gene delivery	Acute immune responses; limited activity in vivo
Cationic polymers	Topical Intratissue Systemic Airway	Highly effective in vitro; low to medium high for local and systemic gene delivery	Toxicity to cells; acute immune responses
Lipid/polymer hybrids	Intratissue Systemic Airway	Low to medium-high efficiency in vitro and in vivo low toxicity	Low activity in vivo.

1.3.2.1 Non-viral Carriers: Cationic liposomes

Non-viral systems are generally cationic in nature **(El-Aneed, 2004)**. The most frequently used strategy for non-viral gene delivery is the formulation of nucleic acid with cationic lipids or cationic polymers, termed lipoplexes and polyplexes, respectively. Lipoplexes and polyplexes are usually formed through the electrostatic complexation between the negatively charged nucleic acid materials and positively charged lipids and/or polymers, respectively. An excess of positive charges for these complexes are required in order to facilitate interaction with the cell membrane **(Elsabahy et al., 2011)**. Internalization can occur via endocytosis, macropinocytosis, or phagocytosis in the form of intracellular vesicles, from which a small fraction of the NA are released into the cytoplasm and in case of DNA migrates into the nucleus, where transgene expression takes place **(Gao et al., 2007; El-Aneed, 2004; Nuno Penacho, 2009)**. In the presence of serum the complexes can be rapidly inactivated. This inactivation is probably due to interactions with negatively charged serum proteins,

which shield the positive charges on the surface of lipoplexes and polyplexes. In addition, due to their positive charge, they interact, non-specifically, with all kinds of cells membranes inducing toxicity **(Elsabahy et al., 2011)**. Nevertheless, it is important to note that non-viral vectors (cationic polymers, cationic peptides and cationic lipids) are less toxic, present a decreased immunogenicity and are more versatile than viral vectors despite its lower transfection efficient **(Duzgunes et al., 2003)**.

Cationic lipids are used mainly in the form of liposomes. Liposomes appear as carriers to transport drugs into the cell but rapidly became indispensable for GT. Cationic liposomes have been the most extensively studied delivery system since their introduction as gene carriers in 1987 by Felgner **(Gao et al., 2007; Nuno Penacho, 2009)**. Liposomes are lipid vesicles composed of amphiphilic molecules (lipids) surrounding an aqueous interior. They are produced by a self-assembly process and can have different structures like multilamellar vesicles (MLV), large unilamellar vesicles (LUV) or small lamellar vesicles (SUV) **(Nuno Penacho, 2009)**. The principal cationic liposomes characteristics: 1) opposite charge to DNA allowing condensation of plasmid DNA; 2) the positive net charge of these liposomes enabling the binding to negative plasma cell membranes; 3) their fusogenic capacity promoting fusion or destabilization of the plasma membrane facilitating the intracellular release of DNA; make them promising delivery vectors not only to plasmid DNA but also to interfering RNA, proteins and small molecules **(Pedroso de Lima et al., 2001; Spagnou et al., 2004)**. Considering that cationic liposomes transfection activity decreases with increasing of alkyl chain length and saturation, and having in mind that lipids with labile ester linkages are less toxic, phosphatidylcholine (PC) derivate seems to be a good option for liposomes formation **(Pedroso de Lima et al., 2001)**. For example, 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC), that is a synthetic cationic analogue of phosphatidylcholine, made of normal cellular metabolites linked with ester bonds, has been used in liposomes synthesis due to their low toxicity and high transfection efficiency **(Faneca et al., 2008)**. Although the efficient transfection activity that some of the cationic liposomes alone have, their activity can be improved by helper lipids **(He et al., 2010)**. The introduction of a co-lipid improves the ability of cationic liposome to transfect cells. Cholesterol has proven to be the most efficient helper-lipid for *in vivo* tests **(Pedroso de Lima et al., 2001; El-Aneed, 2004)**. Thus, cationic liposomes are generally composed by cationic and neutral helper lipids, which can interact with

plasmid DNA or iRNA condensing these molecules into small quasi-stable particles **(He et al., 2010; Faneca et al., 2008)**.

The efficiency of gene delivery is dependent of gene carriers and transfection systems. Physico-chemical properties of lipoplexes are important for biological activity regulation. Multiple factors influence the lipoplexes structure. These factors include the charge ratio, the concentration of individual lipids and NA, the structure of the cationic lipids and the helper lipids, the physical aggregation state of the lipids (multilamellar or unilamellar liposomes, or micelles), the salt concentration and the method of preparation **(Gao et al., 2007; El-Aneed, 2004)**. Lipoplexes have the ability to protect nucleic acids from nuclease degradation and are able to triggering cellular uptake and facilitate the release of nucleic acids from the intracellular vesicles before reaching the destructive lysosomal compartments **(El-Aneed, 2004)**. Charge ratio of lipid/DNA complexes is also a crucial factor in the lipofection process, since it determines the surface charge and the mean diameter of the complexes. High charge ratios are related with strong DNA condensation and with nuclear resistance increased. The lipoplex structure is also important. Lipid-DNA complexes are highly ordered structures, and inverted hexagonal phases of the complexes strongly promotes transfection efficiency. By contrast, a lamellar phase of the complexes correlates with stable particles, displaying substantially lower transfection potency, since lipids and fusogenic peptides usually induce the endosomal destabilization via bilayer-to-micelle or lamellar-to-inverted hexagonal (HII) transition **(Elsabahy et al., 2011; Shi et al., 2002)**.

1.3.3 Improvements of the Activity of Lipoplexes

The inclusion of ligands into DNA complexes to convert a simple DNA complex into a more sophisticated multicomponent gene carrier appears to be a reasonable approach to equip the complexes with more function and to reduce toxicity and lack of specificity problems. Human serum albumin (HSA), for example, enhance the expression of transgene by promoting internalization via endocytosis and escape from endocytotic pathway **(Nuno Penacho, 2009; Faneca et al., 2008)**. The use of flexible hydrophilic polymers like poly(ethylene glycol) (PEG) which is able to form a stabilizing interface between the cationic complexes and the external environment partially circumvented the toxicity of the delivery vectors. In addition, pegylation could prolong the circulation time of the vectors allowing their accumulation into pathological

sites with leaky vasculature (e.g. tumors and inflammations) **(Elsabahy et al., 2011; Duzgunes et al., 2003)**. PEG advantages will be discussed in more detail in the next section. Association of cell penetrating peptides (group of peptides able to efficiently accumulate inside cells) is also used in gene delivery. The ability of these peptides to accumulate inside the nucleus of cells renders them the suited particularity to act as gene delivery vectors *per se* or in association with other non-viral systems. Cell penetrating peptides are not only efficient for delivery of peptides and proteins to the cytoplasm, but also to mediate the intracellular delivery of plasmid DNA **(Trabulo et al., 2008)**.

Other attempts have been made in order to enhance cell internalization. The cationic lipid-based systems targeted to the tumor cells are one of them. Active targeting can be achieved via conjugation of targeted molecules to vectors, favoring their recognition by cell membrane-bound receptor proteins. A variety of molecules including antibodies, peptides, aptamers, vitamins and sugar molecules have been used to achieve cell targeting. The use of antibodies as the targeting moiety presents the advantage of selectivity, high affinity, and minimal competition for the receptor, contrary to what is observed with endogenous molecules such as folic acid or transferrin **(Elsabahy et al., 2011; Nuno Penacho, 2009)**. Antibody coated liposomes (immunoliposomes) are extensively used in cancer strategies. They can be targeted to surface molecules expressed either in the vascular system or in the extravascular system on tumor cell membranes **(Andresen et al., 2005; Iyer et al., 2006)**. However, antibodies might induce immunogenicity, could be difficult to produce/handle and present a large size putting strain on micelle self-assembly. Thus, the use of small molecules, such as sugars, could be advantageous, especially when their receptors are available and overexpressed on the target cells **(Elsabahy et al., 2011)**.

Although the importance of targeting and triggering strategies to improve delivery and bioavailability of the therapeutic molecule, they tend to accumulate more in tumor cells than normal cells when administrated to patients **(Iyer et al., 2006)**. What is the reason to this phenomenon? The answer to this question resides in tumor structure and it is known as enhanced permeability and retention (EPR) effect. When tumor cells multiply, angiogenesis is promoted to suppress oxygen and metabolic needs. However, their neovasculature differ significantly from normal tissue. Tumor blood vessels have irregular shape, are dilated, leaky or defective and endothelial cells are poorly aligned or disorganized with large fenestrations. They also have a wide lumen and a poor

lymphatic drainage. All these anatomical defectiveness allied to functional abnormalities result in extensive leakage of blood plasma components into the tumor. In this way, macromolecules, nanoparticles and lipid particles tend to be retained in the tumor. Polymer conjugates, micellar or liposomal drugs of anticancer agents take advantage of these characteristics to improve drugs therapy efficiency **(Andresen et al., 2005; Iyer et al., 2006)**.

1.3.3.1 PEG-lipoplexes

The incorporation of poly(ethylene glycol) (PEG) components in GT vectors is a very common improvement. Positively charged particles are especially prone to undesired interactions with plasma proteins, which can lead to destabilization and rapid clearance of the lipoplexes by macrophages before they reach the diseased tissue. So, ability of the vectors to circulate in the bloodstream for a prolonged period of time is an important requirement. Due to their chemical inertness, non-ionic character, high water solubility and low cost (easy synthesizes in large amounts at high purity) poly(ethylene glycol) is still the most used material for achieving steric stabilization. This so-called steric stabilization effect happens because the PEG molecules form a protective hydrophilic layer on the surface of the nanoparticle that opposes interaction with blood components. As a result, the PEG coating reduces uptake by macrophages of the mononuclear phagocyte system, provides relatively long plasma residence times and also enable extravasation at site with increased vascular permeability such as tumors and inflamed sites (EPR effect) **(Romberg et al., 2008; Peeters et al., 2007; Rejman et al., 2004)**. On the other hand, pegylation lowers the cellular interaction, internalization and endosomal escape of the lipoplexes. It occurs because PEG-lipid analogues strongly interfere with structural features of the complexes, causing a stabilization of the lamellar phase and preventing an intimate interaction with the endosomal membrane, thereby impeding cytosolic release of nucleic acids **(Gao et al., 2007; Rejman et al., 2004; Shi et al., 2002)**. In spite of the presence of pegylated lipids, such as distearoylphosphatidylethanolamine (DSPE)–PEG or ceramide–PEG (common bilayer anchor), lipoplexes are (at least partly) internalized by cells implying that the presence of PEG does not necessarily prevent the lipoplex-cell-membrane (receptor) interaction that is necessary for endocytic internalization **(Shi et al., 2002)**.

Incorporation of pegylated lipids in gene delivery systems can be done by two different strategies. One involves prior incorporation of lipid-PEG into liposomes – pre-pegylation. In other words, the complex formed results from the complexation between pegylated liposomes and nucleic acids. The second, post-pegylated lipoplexes are made by mixing the PEG-containing lipids and lipoplexes that are not pegylated (Figure 4) **(Rejman et al., 2004; Shi et al., 2002)**. It is important to notice that transfection efficiency of pegylated lipoplexes varies with different parameters, such as lipoplexes composition, the cell type used, the amount of PEG, the length of hydrocarbons in PEG, the lipid anchor used, the PEG insertion method (pre-pegylation *versus* post-pegylation). For example, the length of hydrocarbons in PEG needs to be adjusted in other to prevent huge lipoplexes formation that will cause dose-dependent inhibition in transfection activity **(Gao et al., 2007)**.

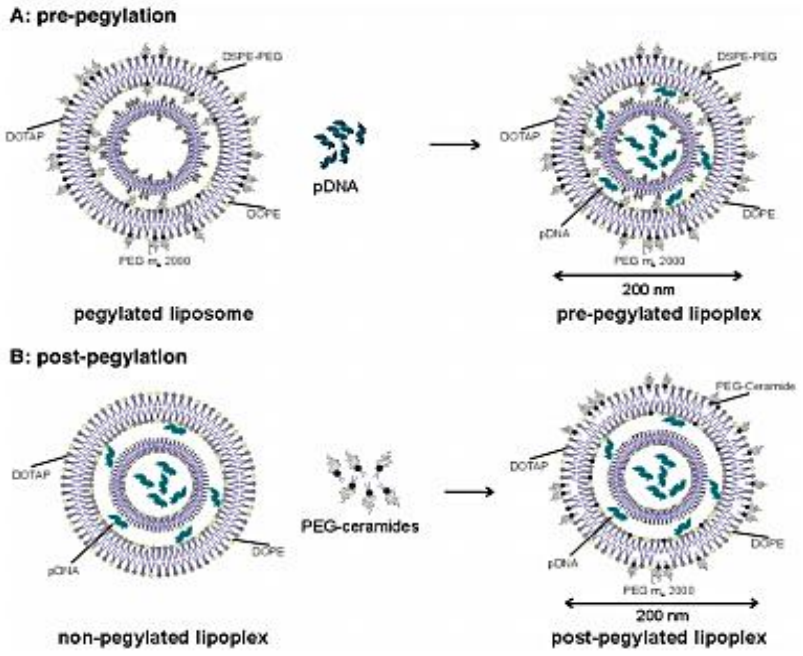


Figure 4 Schematic representation of different Pegylated lipoplexes preparation. A: illustration of a pre-pegylation strategy where the pegylated liposomes are complexed with pDNA; B: formation of post-pegylated lipoplexes resulting from the mixing of PEG-containing lipids (i.e. PEG-ceramides) with preformed non-pegylated lipoplexes. Adapted fromPeeters et al., 2007.

Some reports are already shown that PEG-ceramides present higher transfection efficiency compared to DSPE-PEG **(Peeters et al., 2007; Rejman et al., 2004)**. Ceramides are a family of lipid molecules known to promote cell suicide or apoptosis **(Khazanov et al., 2008)**. Ceramides are spontaneously adsorbed to the lipoplexes and their "de-pegylation" after lipoplexes endocytosis is easier, allowing the escape of lipoplexes from endosomal pathway by non-pegylated mechanism. The PEG-ceramide

lipoplexes proprieties are governed by the length of the acyl chain of the ceramide molecules **(Peeters et al., 2007; Rejman et al., 2004; Shi et al., 2002)**. Moreover, **Peeters et al. (2007)** demonstrated that post-pegylation with cerC_8-PEG (where C_8 denotes the fatty acyl chain length) spontaneously adsorb to the lipoplexes allowing the escape from endosomal pathway similar to non-pegylated lipoplexes *in vitro*. They verified that post-pegylation of the lipoplexes strongly improved the transfection efficiency compared with pre-pegylated lipoplexes.

Thus, post-pegylated lipoplexes, using PEG-ceramides containing small acyl chains, seems to be an efficient *in vitro* strategy. PEG molecules are used as a cover for lipoplexes before they reach the target cells. Once in the cells, PEG-lipids fall off, revealing highly active lipoplexes. The benefits of pegylation are well described and with the development of cleavable linker chemistries, a rational selection may result in the construction of stabilized lipoplexes with optimal structural features **(Peeters et al., 2007; Rejman et al., 2004)**.

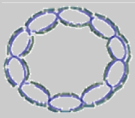
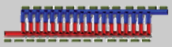
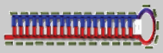
1.3.4 miRNAs in Gene Therapy Strategies for PDAC

Antisense gene therapy aims to prevent the transcription or translation of cancer-associated genes. Gene therapy using miRNAs or anti-miRNAs (miRNAs with the complementary sequence of miR) has emerge as a possible and advantageous therapeutic strategy **(Mackenzie, 2004)**. It will associate the power of gene therapy combined with specificity of the miRNAs, since the PDAC cancer presents a unique miRNAs expression pattern.

This type of therapeutic approach presents some advantages when compared with plasmid DNA (Table 3). iRNAs do not interact with chromosomal DNA reducing the possibility of gene alteration that might be occurring from DNA-based gene therapy. miRNAs interact with mRNA controlling the translation process which can be useful to prevent the expression of harmful protein. Few molecules of miRNAs are capable to promote gene silencing, so little amounts of miRNAs and anti-sense nucleotides are needed for therapeutic approaches. The delivery methods are easier because they have a cytosolic action. Nevertheless, the use of miRNAs has some limitations. miRNAs delivery is one of them but can be overpast using cationic liposomes, for example. The non-specific silencing of mRNAs, since one miRNA can bind different mRNAs, is another setback. miRNA lack of specificity could lead to inhibition of a gene with

partial homology to miRNA which is not supposed to be target – off target effects. The degradation of miRNAs by nucleases can also be a problem. However, the use of chemical modified molecules as LNAs can minimized this problem **(Elsabahy et al., 2011; Caplen, 2004)**.

Table 3 Characteristics of plasmid DNA and small nucleic acids. Adapted from Elsabahy et al., 2011.

Character	pDNA	AON	siRNA	shRNA	miRNA
Shape					
Molecular Weight*	~0.6-5Kbp	18-21 nucleotides	19-22 bp	Stem: 25-29 bp Loop: 4-23 nucleotides	21-24 nucleotides
Mechanism of action	Express particular gene Replace faulty gene Induce immunity by the unmethylated CpG motifs	Induce RNaseH enzyme Translational arrest by steric hidrance of ribosomes Interfere with mRNA maturation or incorrect spicing in the nucleus	RNA interference		
Immune response	TLR9	TRL9 (if there are CpG motifs in the AON sequence)	RNAs are generally recognized by three main types of immunoreceptors: TLR (3, 7 and 8), protein Kinase R and helicases		
Advantages	Can be used to express a missing gene Adjuvant therapy in vaccination	Lower amount of carrier (i.e. lower cost and toxicity) The vector design is easier No nuclear barrier Insertional mutagenesis can be circumvented			
Disadvantages	Nuclear barrier Possibility of insertional mutagenesis Difficult to construct and formulate	Nuclear barrier can exist for some therapeutic application of AON (i.e. modulating splicing) Can not be used to express a gene of interest			
Approved/clinical trials (examples)	LifeTide* SW 5 (VGX Animal Health): approved	Vitravene (Isis Pharmaceuticals): Approved	Bevasiranib (Opko Health): Phase III	NUC B1000** (Nucleonics Inc., PA): Phase I	SPC3649*** (Santaris Pharma): Phase I

*These are the approximate molecular weights of the common working range
**Gene construct (i.e. vector-expressing shRNA) for the treatment of chronic hepatitis B infection
***Targets and inhibits the liver specific miRNA-122 which is required for the hepatitis C virus replication

KRAS is the most commonly mutated oncogene in pancreatic cancer. *In vitro* studies have confirmed that transfection with plasmids that contain KRAS2 antisense inhibits the growth of pancreatic-cancer cell lines. An antisense inhibitor strategy against KRAS (ISIS 2503) has already in phase II trial despite some toxic effects like mild thrombocytopenia and asthenia appeared in certain patients. A combination of ISIS 2503 with gemcitabine was also performed and no clinical improving was obtained

when compared with gemcitabine alone. Many other strategies using the huge potential of these small molecules are being tested **(Mackenzie, 2004)**.

Thus, site-specific targeting and site-specific triggering are two principles that can be combined improving gene delivery for treatment of several diseases, namely cancer. It is also interesting to note that in the past 15 years, more than 400 clinical studies in gene therapy have been evaluated and almost 70% of these studies are in the cancer gene therapy area.

1.4 Objectives

The main goal of this work was the development of a new lipoplex formulation, based on EPOPC:Chol/NA lipoplexes, that had the ability to specifically deliver genetic material (namely plasmid (p)DNA encoding a therapeutic gene and/or LNAs against miRNAs overexpressed in PDAC) to pancreatic cancer using an intravenous administration pathway.

In order to achieve the main goal of the project several purposes were defined:

- Evaluation of the most efficient charge ratio (+/-) formulation of the EPOPC:Chol/DNA lipoplexes formulation for the used cell line;
- Assessment of the transfection activity of a combined strategy using several chemotherapeutic drugs and lipoplexes containing pDNA;
- Evaluation of the efficiency a combined strategy using LNAs and pDNA complexed with cationic liposomes;
- Development of a post-pegylation strategy using cerC_8-PEG molecules in order to obtain small stable lipoplexes with high biological activity;
- Development of a targeting strategy to the EphA4 receptor using a peptide attached covalently to the maleimide groups of the DSPE-PEG-MAL molecules;
- Evaluation of the cytotoxicity and the biological activity of the developed formulations.

These aims were designed in order to obtain an efficient system to deliver therapeutic plasmid DNA and anti-sense oligonucleotides against microRNAs overexpressed in the pancreatic cancer. The possibility of achieving a competent therapeutic strategy together with an efficient non-viral lipid-based vector, specific to the pancreatic tumor cells, would be an enormous advance in the fight against pancreatic cancer.

Chapter 2: Material and Methods

This chapter describes the methodologies used repeatedly in the experiments described in each result and discussion section. Any modification to the protocols here described will be further detailed.

2.1 Cell culture

Panc-1 and Mia Paca cells, immortalized pancreatic cell lines, were obtained from a human epithelial pancreatic carcinoma. The cell lines were maintained as subconfluent cultures in DMEM-HG medium (Sigma, St Louis, USA). Cells were grown at 37ºC in a humidified incubator with 5% CO_2. To maintain a continuous proliferation, cells were subcultivated two times per week. For this purpose, media was removed by aspiration. Cells were then washed with phosphate buffered saline (PBS) and harvested with 0.25% (w/v) Trypsin- EDTA solution for a few minutes. Cells were detached and suspended in the media to obtain the desired dilution (subcultivation ratio: between 1:3-1:5 for Panc-1 cells and between 1:4 - 1:8 for Mia PaCa cells). For the *in vitro* studies, the two cell lines were prepared 24 hours before the experiments. Cells were submitted to the same process of trypsinization described above. However, in this case, the detached cells were counted, in a hemacytometer, using trypan blue and were diluted in the culture medium to obtain the desired cellular density. Cells were seeded on different multi-well plates at different densities, according to the experiment. All cell work was carried out in aseptic conditions, using a laminar flow cabinet and only sterile material. Material was sterilized through autoclave and solutions were filtered through 0.22µm pore-diameter filters.

2.2 Lipoplexes Formation

2.2.1 Preparation of Cationic Liposomes

Small unilamellar cationic liposomes were prepared by extrusion of multilamellar liposomes composed of 1:1 (molar ratio) mixtures of (2) 1- palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC) and cholesterol (Chol) **(Faneca et al., 2008)**. EPOPC:Chol (1:1) liposomes were labeled with 1% of rhodamine-dioleoylphosphatidylethanolamine (Rh-PE), for binding and uptake spectrofluorimetry

and flow cytometry studies. Briefly, lipids (Avanti Polar Lipids, Alabaster, AL) dissolved in chloroform were mixed at the 1:1 molar ratio and dried in a rotatory evaporator under a nitrogen flux. The dried lipid films were hydrated with 1 mL of deionized water. The resulting MLV were then sonicated for 3 min and extruded 21 times through two stacked polycarbonate filters of 50 nm pore diameter using a LiposoFast mini extruder (Avestin, Toronto, Canada). The resulting liposomes (SUV) were then diluted three times with deionized water and filter-sterilized utilizing 0.22 μm pore-diameter filters. The suspension was stored at 4 °C until use.

2.2.1.1 Cholesterol Quantification Method

Liposomal concentration was obtained through the quantification of the cholesterol content and according to cholesterol/total lipid ratio. The technique is based on the liebermann-burchard method. Briefly, 50μL of the lipoplexes were incubated with 1.5mL of the cholesterol reagent (35% Glacial Acid Acetic, 55% Acetic Anhydrid, 10% Concentrated Sulfuric Acid and 1mg/mL sodium sulfate) for 20min. at 37ºC. Lieberman-Burchard method is a colorimetric reaction to detect cholesterol, which consists in adding acetic anhydride and sulfuric acid to the cholesterol. The hydroxyl group (-OH) of cholesterol react with the reagent and increase the conjugation of the unsaturation in the adjacent fused ring conferring a blue-green color **(Huang et al., 1961)**. This reaction can be followed spectrophotometrically (625nm) and is directly proportional to the cholesterol concentration in the sample. A calibration curve, built from a set of standard cholesterol samples with known concentrations (0-1mg/mL), was used to assess the cholesterol concentration in the liposomes sample.

2.2.2 Preparation of Peptide-Coupled PEG_{2000}-DSPE and $CerC_8$-PEG_{750} Micelles

The DSPE-PEG_{2000}-MAL (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000], Avanti Polar Lipids, Alabaster, AL) reacts with the thiol groups on the terminal cysteine side chains of modified APY or KYL peptides, respectively (APYCVYRGSWSCGSGSC; KYLPYWPVLSSLPKKKRKVC, GeneCust, Dudelange, LU), generating a stable 3-thiosuccinimidyl ether linkage. Three types of micelles: PEG_{2000}-DSPE-MAL, PEG_{750}-$CerC_8$ and PEG_{2000}-DSPE-MAL with

PEG_{750}-$CerC_8$ (N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)750]}, Avanti Polar Lipids, Alabaster, AL) were performed. A lipid film was prepared by solvent evaporation under a mild stream of nitrogen. This dried lipid film was then hydrated with 100µL of MES buffer (20mM HEPES, 20mM MES, pH 6.5), at a concentration above the critical micellar concentration of the lipid (2.3mM) **(Ishida et al., 1999)**. Micelles were formed by 3 cycles of 30s of strong vortex followed by 30s heating in a water bath at 37ºC. Then, the modified APY or KYL peptides were coupled to the freshly prepared DSPE-PEG_{2000}-MAL micelles at the molar ratio of 1:1. The coupling reaction was performed overnight, in the dark, at room temperature **(Takasaki et al., 2006; Mendonça et al., 2010)**.

The percentage of the peptide binding the DSPE-PEG_{2000}- MAL micelles was quantified by the Ellman's assay. Briefly, the free thiol groups of the peptide react with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, Sigma, St. Louis, MO) cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB^-), which ionizes to the NTB^{2-} dianion in water at neutral and alkaline pH. This rapid and stoichiometric reaction is quantified in a spectrophotometer by measuring the absorbance of visible light at 412 nm. A calibration curve, built from a set of standard L-glutathione reduced (Sigma, St. Louis, MO) samples with known concentrations (0-30µM), was used to assess the peptide concentration.

2.2.3 Preparation of Cationic Complexes

Lipoplexes were prepared by sequentially mixing the HEPES-buffered saline solution (HBS) (100 mM NaCl, 20 mM HEPES, pH 7.4), with liposomes (the volume necessary to obtain the desired 4:1 (+/−) lipid/DNA charge ratio) and with the HBS solution containing 1 µg of pCMVluc encoding luciferase, or 25nM of the antisense oligonucleotide 221 (Exiqon, Vedbaek, Dk). The mixtures were further incubated for 15 min at room temperature. For lipoplexes containing HSA (Sigma, St. Louis, MO), liposomes were pre-incubated with this protein (32 µg HSA/µg of DNA or LNA) for 15 min, followed by a further 15 min incubation with plasmid DNA or LNA 221 solutions at room temperature. Lipoplexes were used immediately after being prepared **(Faneca et al., 2008)**. For post-pegylated lipoplexes, the cationic liposomes/nucleic acids complexes were incubated with micelles for 20 minutes at 50ºC **(Gao et al., 2011; Peeters et al., 2007)**. The molar percentage of pegylated lipids were between 1% and

4% mol of the total lipid composition. Subsequently, the lipoplexes were cooled down to room temperature and immediately used for the experiments.

2.3 Transfection Activity Studies

2.3.1 Luciferase Assay

For transfection studies with complexes containing the plasmids pCMVluc, 7.5×10^4 Mia PaCa cells or 7×10^4 Panc-1 cells were seeded in 1 mL of medium in 48-well culture plates 24 h before transfection, in order to obtain 50–70% confluence. Cells were covered with 0.3 mL of DMEM-HG (without serum, unless indicated otherwise) before lipoplexes were gently added, containing 1 µg of DNA per well. In some experiments different amounts (0.1µM, 0.5µM and 1 µM) of Vinblastine, Gemcitabine, Vinorelbine and Docetaxel (Sigma, St. Louis, MO) were added. After 4 h incubation (5% CO_2 at 37°C) the medium was replaced with DMEM-HG and the cells were further incubated for 48 h. Cells were then washed twice with phosphate-buffered saline solution (PBS) and 100µL of lysis buffer (1mM DTT; 1mM EDTA; 25mM Tris-phosphate (pH=7.8); 8 mM $MgCl_2$; 15% glycerol; 1% (v/v) Triton X-100) were added to each well. After cell lysis at -80ºC, 50µL of each lysate were incubated with luciferin and ATP and light production was determined in a luminometer (LMax II 384; Molecular Devices, San Jose, CA). The protein content of the lysates was measured by the Dc Protein Assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. The data were expressed as RLU of luciferase per mg of total cell protein.

2.3.2 Extraction of Total RNA and cDNA Synthesis

For transfection studies with complexes containing LNAs (with or without plasmid DNA – pCMVluc) 2.6×10^5 Panc-1 cells were seeded in 1mL of medium in 12-well culture plates 24h before transfection, in order to obtain 80% confluence. Cells were covered with 0.4 mL of DMEM-HG before lipoplexes were gently added containing 25nM of LNAs per well. After 4 h incubation (5% CO_2 at 37°C) the medium was replaced with DMEM-HG and the cells were further incubated for 48 h. A LNA scramble was used as control.

Total RNA, including small RNA species, was extracted from Panc-1 cells using the miRCURY^{TM} Isolation Kit-Cells (Exiqon, Vedbaek, Dk), according to the

manufacturer's recommendations for cultured cells. Briefly, after cell lysis, the total RNA was adsorbed to a matrix, washed with the recommended buffers and eluted with 50µL of elution buffer by centrifugation. After RNA quantification, cDNA conversion for miRNA quantification was performed using the Universal cDNA Synthesis Kit (Exiqon, Vedbaek, Dk). For each sample, cDNA for miRNA detection was produced from 25 ng total RNA according to the following protocol: 60 min at 42ºC followed by heat-inactivation of the reverse transcriptase for 5 min at 95ºC. The cDNA was diluted 60 or 40× with RNase-free water before quantification by qRT-PCR.

2.3.3 Quantitative Real-Time PCR

Quantitative RT-PCR was performed in an iQ5 thermocycler (Bio-Rad) using 96-well microtitre plate. For miRNA quantification the miRCURY LNA^{TM} Universal RT microRNA PCR system (Exiqon, Vedbaek, Dk) was used in combination with pre-designed primers (Exiqon, Vedbaek, Dk) for miR-221 and sRNA U6 (reference gene). A master mix was designed for each primer set, according to the recommendations for the RT-PCR setup of individual assays suggested in this kit. For each reaction, 6µL master mix was added to 4 µL template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 10µL per well, using the iQ5 Optical System Software (Bio-Rad). The reaction conditions consisted of polymerase activation/denaturation and well-factor determination at 95ºC for 10 min, followed by 40 amplification cycles at 95ºC for 10 s and 65ºC for 1 min. For miR relative quantification a melting curve protocol was started immediately after amplification and consisted of 1 min heating at 55ºC followed by 80 steps of 10s, with a 0.5ºC increase at each step. Threshold values for threshold cycle determination (C_T) were generated automatically by the IQ5 OPTICAL SYSTEM software. The miRNA fold decrease with respect to control samples was determined by the $\Delta\Delta C_T$ (Livak) taking into consideration equals amplification efficiencies of all genes and miRNAs in all experiments. The amplification efficiency for target or reference RNA was determined according to the formula: $E=10^{(-1/S)}-1$, where S is the slope of the obtained standard curve.

2.4 Cell viability assay

Following transfection under the different experimental conditions, cell viability was assessed by a modified Alamar Blue assay **(O'Brien et al., 2000)**. The assay measures the redox capacity of the cells due to the production of metabolites as a result of cell growth and allows determination of viability over the period culture without the detachment of adherent cells. Briefly, the culture medium was replaced with 10% (v/v) resazurin dye in DMEM-HG, which was added to each well 48h following the initial transfection period. After the dye incubation at 37 °C, 200 µl of the supernatant were collected from each well and transferred to 96-well plates. The absorbance at 570 and 600 nm was measured in a SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices, Union City, CA). Cell viability (as a percentage of control cells) was calculated according to the formula: $cell\ viability\ (\%\ of\ control) = \frac{A570-A600\ of\ treated\ cells}{A570-A600\ of\ control\ cells} * 100.$

2.5 Binding and Uptake of the Lipoplexes

EPOPC:Chol (1:1) liposomes, labeled with 1% Rh-PE, were complexed in the presence or absence of HSA, with 1 µg of pCMVluc at a 4/1 (+/−) cationic lipid/DNA charge ratio. Some of lipoplexes were post-pegylated containing or not APY peptide (1-4% mol). Twenty-four hours before transfection, 2×10^5 Mia PaCa cells were seeded in 1 mL of medium in 48-well culture plates. After covering the cells with 0.3 mL of DMEM-HG medium without serum, lipoplexes were added. Following a 4 h incubation at 4 °C or 37 °C, cells were washed twice with PBS and lysed with 100 µL/well of 1% Triton X-100. Binding (4 °C) and uptake (37 °C) of lipoplexes were monitored in a SPECTRAmax GEMINI EM fluorometer (Molecular Devices, Union City, CA) by measuring the fluorescence at excitation and emission wavelengths of 545 and 587 nm, respectively.

2.6 Flow Cytometry Studies

To evaluate the extent of cell association of the EPOPC:Chol 4/1 (+/-) lipoplexes containing PEG molecules, in presence or absence of the APY peptide, Mia PaCa cells were transfected with lipoplexes labeled with 1% mol of Rh-PE. Cells were seeded in

DMEM-HG culture medium (4×10^5 cells/well) in 12-well culture plates 36h before the treatment. The different formulations under study were gently added to 0.4mL of DMEM-HG medium without serum and incubated for 4h at 37ºC. After transfection the cells were washed twice with PBS and detached with 300µL of trypsin (5 min at 37 ºC) and then 700µL of DMEM-HG medium was added to inactive the trypsin. Subsequently, cells were collected in conic tubes, washed twice with 1mL of cold PBS, resuspended in 400 µL of cold PBS and analyzed immediately. Flow cytometry analysis is performed in live cells and can be performed using a Becton Dickinson FACSCalibur flow cytometer. Live cells were gated by forward/side scattering from a total of a minimum of 20.000 events. Data were analyzed using Cell Quest software (BD), to determine the amount of cells that internalized the lipoplexes.

2.7 Statistical analysis

All data are presented as mean ± standard deviation (SD). Data were analyzed using the standard statistical software (GRAPHPADPRISM5, GraphPad Software, La Jolla, USA). Statistical significance of differences between data was evaluated by One-way ANOVA using the Tukey test or the Dunnett test. Statistical differences are presented at probability levels of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

Chapter 3: Results and Discussion

3.1 Biological Activity and Cytotoxicity of Lipoplexes

Panc-1 cells, an adherent cell line, were derived from *Homo sapiens* (56 years old Caucasian male). They are epithelial cells from epithelioid carcinoma with a doubling-time of 52 hours **(ATCC – CRL-1469™)**. There are some miRNAs (such as miR-196a, miR-190, miR- 186, miR-221, miR-222, miR-200b, miR-15b, and miR-95) that are highly expressed (more than 3.3-fold) both in pancreatic cancer tissue samples and cell lines (Panc-1 and Mia PaCa cell lines) when compared to normal pancreatic tissues **(Zhang et al., 2009)**.

The successful application of gene therapy depends on available carriers to efficiently deliver genetic material into target cells. The efficacy of such systems is strongly dependent on their physicochemical properties. There are several parameters affecting the final physicochemical properties of lipoplexes and consequently the transfection levels. The nature of the cationic and helper lipids and the relative proportion of cationic lipid and DNA are two crucial parameters that should be considered **(Pedroso de Lima et al., 2001)**.

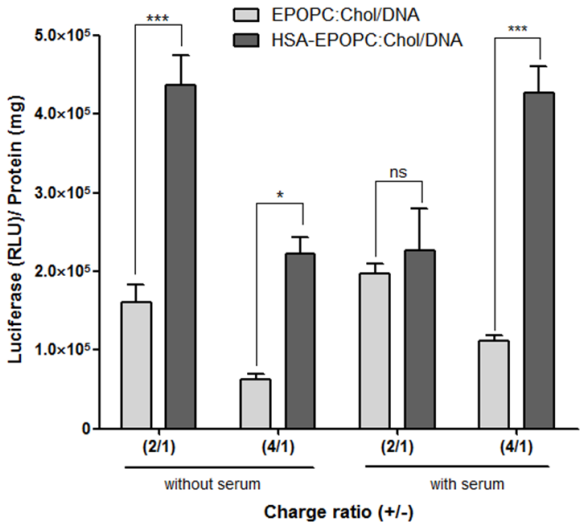


Figure 5 Effect of present of serum and lipoplex charge ratio (+/-) on luciferase gene expression in Panc-1 cells. Cells were covered with 0.3mL of DMEM-HG with 10% of FBS or without FBS before cationic liposome/DNA complexes were added. The liposomes (EPOPC:Chol) pre-incubated or not with HSA (32µg/µg of DNA), were complexed with 1µg of pCMVluc at the indicate theoretical lipid/DNA Charge ratios. After a 4h incubation, the medium was replaced with DMEM-HG containing 10% serum and the cells were further incubated for 48h. The level of gene expression was evaluated as described in "Materials and methods". The data are expressed as RLU of luciferase per mg of total cell protein (mean ± standard deviation obtained from triplicates) and are representative of at least two independent experiments. *p<0.5; ** p< 0.01; *** p<0.001.

In the present work, cationic lipome/DNA complexes (EPOPC:Chol (1/1) (mol/mol) cationic liposomes and pCMVluc – luciferase DNA plasmid), containing or not HSA, prepared at different charge ratios (+/−) were evaluated in terms of their biological activity, in the presence or absence of 10% of serum in Panc-1 cells. Serum is known to decrease the transfection activity of lipoplexes through interaction of their proteins with the complexes **(Lewis et al., 1996)**.

As shown in Figure 5, the biological activity mediated by lipoplexes in Panc-1 cells was dependent on their charge ratio (+/−). Association of albumin to lipoplexes proved to be highly effective in enhancing their transfection activity even in the presence of serum. As can be observed, for albumin-associated lipoplexes a great enhancement of transgene expression occurred for both charge ratios being particularly interesting at the 4/1 (+/-) in the presence of serum ($p<0.001$).

The biological activity of the complexes can be affected by their cytotoxicity. Regarding this, cell viability studies were also performed for the formulations described above. As illustrated in Figure 6, in presence of serum the viability of Panc-1 cells was not substantially affected by the lipoplexes. Nevertheless, the incubation of the Panc-1 cells with the complexes in the absence of serum showed a significant cytotoxicity. This is a common result in serum-free transfections suggesting that serum is able to protect cells from the aggression associated to the transfection process **(Faneca et al., 2004; Faneca et al., 2007; Faneca et al., 2008)**.

The enhancing effect caused by albumin in the biological activity of lipoplexes is due to two different facts. Although albumin was thought not to interact with human cells through a specific cell surface receptor, HSA-lipoplexes are able to bind non-specifically to receptors analogous to scavenger receptors, which in turn mediate their endocytosis, resulting in a transfection enhancement. Moreover, albumin can facilitate the escape of DNA from the endocytotic pathway. Albumin has been described as being able to undergo a low pH-induced conformational change, thereby acquiring fusogenic properties. HSA associated with lipoplexes expose hydrophobic domains under acidic conditions that could result in dissociation of the complexes and destabilization of the endosomal membrane **(Simões et al., 2000; Faneca et al., 2004)**. It is important to notice that the increase in biological activity caused by HSA depends on the lipid nature. The interaction between the two molecules determines the final structure and properties of the lipoplexes and consequently their transfection capacity **(Faneca et al., 2004; Schenkman et al., 1981)**.

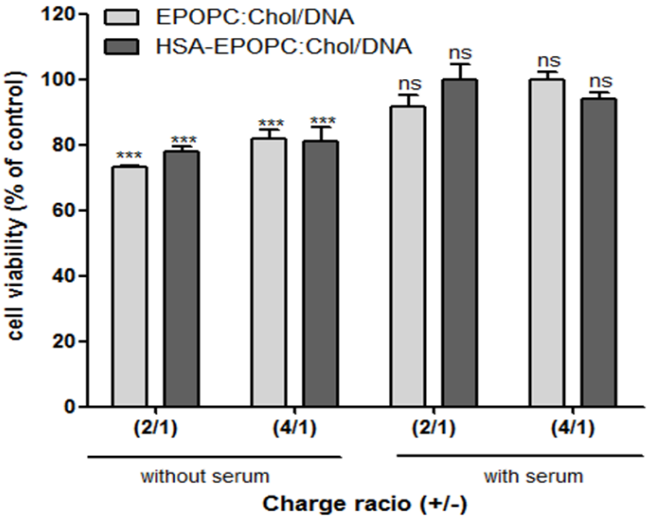


Figure 6 Effect of cationic liposome/DNA complexes on the viability of Panc-1 cells. Cells were covered with 0.3mL of DMEM-HG with 10% of serum or without serum before cationic liposome/DNA complexes were added. After a 4h incubation, the medium was replaced with DMEM-HG containing 10% serum and the cells were further incubated for 48h. Cell viability was measured by the Alamar Blue assay as described in "Materials and Methods". The data are expressed as the percentage of the untreated control cells (mean ± standard deviation obtained from triplicates) and are representative of at least two independent experiments. *p<0.5; ** p< 0.01; *** p<0.001.

HSA-EPOPC:Chol/DNA 4/1 (+/-) lipoplexes proved to be the best formulation tested in Panc-1 cell line. This formulation led to high levels of transfection, even in the presence of serum, and show low toxicity in these conditions. Moreover, the presence of albumin could reduce the problems associated with the use of highly positively charged complexes *in vivo*, such as avid complexation with serum proteins **(Faneca et al., 2004)**. However, the presence of albumin increases the mean diameter of the lipoplexes, most probably due to the neutralizing effect of the positive charges of the complexes (conferred by the cationic lipids) by albumin (which exhibits a net negative charge) **(Simões et al., 2000)**. Nevertheless, HSA-EPOPC:Chol/DNA 4/1 (+/-) lipoplexes were used due to its high biological activity.

3.2 Effect of Chemotherapeutic Drugs on Transfection Activity of HSA-EPOPC:Chol/DNA Lipoplexes

Previous reports have demonstrated that the presence of chemotherapeutic drugs can improve the biological activity of lipoplexes **(Chowdhury et al., 1996; Faneca et al., 2008)**. Chemotherapy is commonly the only treatment for PDAC patients. So, in this part of the project the aim was to evaluate the transfection efficiency of HSA-

EPOPC:Chol/pCMVluc 4/1 (+/-) lipoplexes in the presence of different anti-cancer agents (gemcitabine, vinorelbine, vinblastine and docetaxel) at different concentration (0.1µM, 0.5 µM and 1 µM) in Panc-1 cells. The most common chemotherapeutic drug used for PDAC is Gemcitabine. Gemcitabine is a nucleoside analog and its cytotoxic activity results from several actions on DNA synthesis. In this way, it impairs the cell cycle resulting at last in cell apoptosis **(Mini et al., 2006)**. On the other hand, Vinorelbine, vinblastine and docetaxel are chemotherapeutic drugs that interfere with microtubule dynamics. Microtubule dynamics is important for successful mitosis, particularly for the proper function of the mitotic spindle. Vinorelbine, vinblastine are microtubule-destabilizing agents while docetaxel has a stabilizing effect **(Perez, 2009)**.

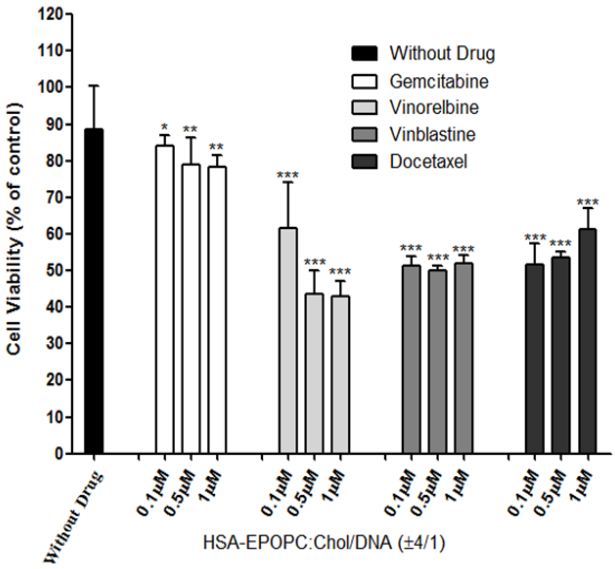


Figure 7 Effect of the combination of cationic liposome/DNA complexes with different drugs concentrations on the viability of Panc-1 cells. Cells were covered with 0.3mL of DMEM-HG with 10% of serum or without serum before cationic liposome/DNA complexes and chemotherapeutic drugs (0,1µM; 0,5µM; 1µM) were added. After 4h incubation, the medium was replaced with DMEM-HG containing 10% serum and the cells were further incubated for 48h. Cell viability was measured by the Alamar Blue assay as described in 'Materials and Methods'. The data are expressed as the percentage of the untreated control cells (mean ± standard deviation obtained from triplicates) and are representative of two independent experiments. *p<0.5; ** p< 0.01; *** p<0.001.

The correlation between cell proliferation rate and transfection activity is a common feature of cationic lipid-mediated transfection systems. Mitosis is important facilitating the entry of plasmid into the nucleus. The lipid-mediated transfection is dependent on cell proliferation rate. Consequently, they have important implications on the efficacy of these systems in gene therapy protocols. Tissues consisting of non-proliferating cells, such as muscle and neural tissue, would theoretically not be amenable to efficient lipid-mediated transfection. On the other hand, neoplastic tissue

and targets that are composed of rapidly proliferating cells will theoretically be more amenable to transfection **(Mortimer et al., 1999)**.

First, the toxicity of the lipoplexes alone and in the presence of the drugs was analyzed (Figure 7). According with the results presented above, a non-significant cytotoxicity caused by lipoplexes alone was obtained. The presence of chemotherapeutic drugs, as expected, showed a significant cytotoxic effect. However, no differences of toxicity were found with different concentrations of the same drug. When cells were incubated with lipoplexes and gemcitabine the cell death was about 20% of the control (non-treated cells). Transfection in the presence of vinorelbine, vinblastine or docetaxel resulted in approximately 50% of cytotoxicity.

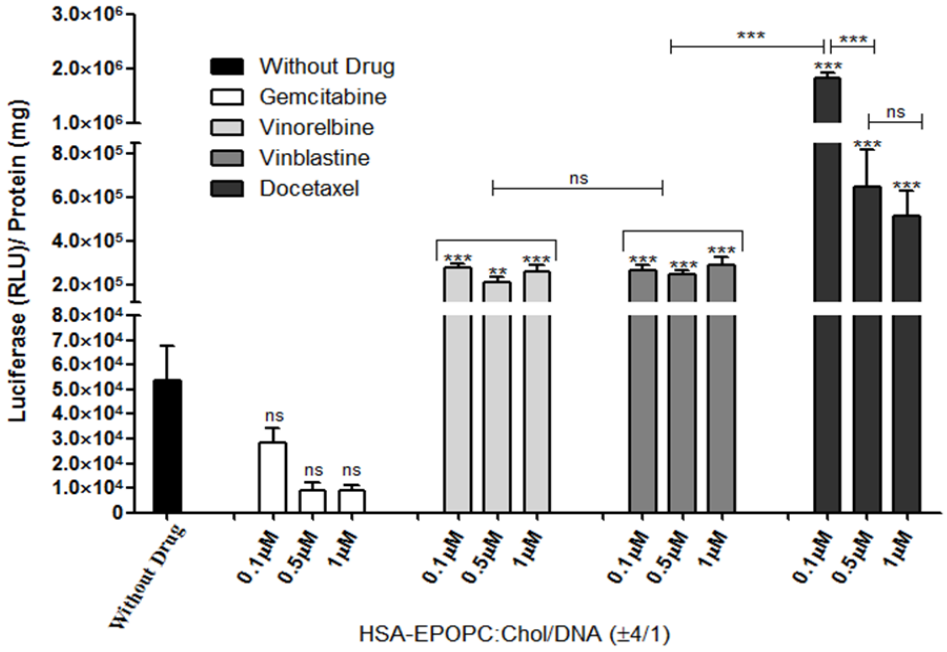


Figure 8 Effect of gemcitabine, vinorelbine, vinblastine and docetaxel on the transfection activity mediated by HSA-EPOPC:Chol/DNA lipoplexes in Panc-1 cells. Cells were simultaneously incubated with lipoplexes and drugs (0.1; 0.5; 1 µM). Levels of luciferase gene expression are presented as RLU of luciferase per mg of total cell protein (mean ± standard deviation obtained from triplicates) and are representative of two independent experiments. ** p< 0.01; *** p<0.001.

Transfection activity of lipoplexes was evaluated using the luciferase assay. As illustrated in Figure 8, the transfection activity of lipoplexes was significantly higher in the presence of vinorelbine, vinblastine and docetaxel than in its absence. This enhancing effect was only concentration-dependent for docetaxel being maximal at 0.1µM. Vinorelbine and vinblastine improved the biological activity in 5-fold while docetaxel led to an 8 to 20-fold increase.

Transfection efficiency is mitosis dependent and microtubules dynamics is essential for this biological process. However, these chemotherapeutic agents were able to enhance the transgene expression. This phenomenon could be attributed to the decrease in the intracellular traffic of lipoplexes to lysosomes allowing an increase in the amount of pDNA that has the ability to reach the nucleus. Other possible explanation could be the activation of the transcription factor NF-kB **(Faneca et al., 2008)**. Drugs interfering with microtubule dynamics are able to activate the sequence-specific transcription factor NF-kB and induce NF-kB-dependent gene expression. NF-kB is kwon to assist importation of pDNA and to enhance transcription and expression of transgenes **(Rosette and Karin, 1995; Kuramoto et al., 2006)**.

On the other hand, gemcitabine did not significantly alter the transfection activity of the complexes, although it had induced a slight decrease in lipofection. These results can be most probably due to the fact that this drug impairs the DNA synthesis and the cell cycle progression and it not interfere with the microtubule dynamics, like the others used drugs.

These studies were performed in the presence of 10% of serum. So, it is even more remarkably the inducing of a 5-fold to 20-fold increase in the transfection activity. Regarding this, from the obtained results, it was possible to conclude that the use of HSA-EPOPC:Chol/DNA 4/1 (+/-) lipoplexes in the present of some chemotherapeutic agents is a proficient strategy to promote high levels of expression in Panc-1 cell line. This strategy could be used in a development of an efficient antitumoral approach, since it combines the enhancement of transgene expression with the chemotherapeutic activity.

3.3 Effect of a DNA Cargo in the Inhibition Effect of LNAs Using HSA-EPOPC:Chol/NA 4/1 (+/-) Complexes

Pancreatic cancer has a high genetic component, including a substantial change in the microRNA expression pattern conferring to PDAC a unique miRNA signature. Gene therapy can contribute to the attempt of fight against this malignity by introducing key genes that are mutated in PDAC or by inhibiting the microRNAs overexpressed in this pathology, using antisense oligonucleotides (single strand).

In 2009, Rhinn et al. demonstrated that transfection using lipoplexes with small interfering RNA (siRNA – double strand) could be enhanced by the addition of pDNA

(cargo). They reveal that cargo addition has several advantages. The developed formulations containing DNA cargo needed lower amounts of siRNA to obtain the same inhibitory effect than formulations prepared without DNA cargo. It was also less toxic and had less off-target effects. The improvement in RNA interference efficiency for these formulations did not result from a better protection of the siRNAs but from a better availability and probably a facilitated release of siRNAs inside the cytoplasm **(Rhinn et al., 2009)**.

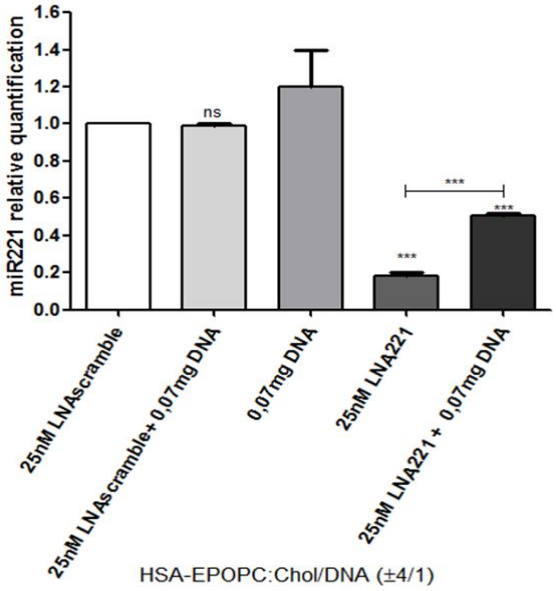


Figure 9 Quantification of miR221 expression levels following lipoplexes transfection. Panc-1cells were covered with 0.4mL of DMEM-HG with 10% of serum. Panc-1 cells were incubated with HAS-EPOPC:Chol/NA containing 25nM of LNAs against miR221 in presence or absence of plasmid DNA (pVMC-luc) (1/1 (-/-) charge ratio) for 4h. After the 4h incubation, the medium was replaced with DMEM-HG containing 10% of serum and the cells were further incubated for 48h. Then RNA was extracted as described in "Material and Methods" and miR-221 levels were determined by qRT-PCR using specific LNA probes for the mature form of this miRNA. Results are presented as miR-221 fold change with respect to control (lipoplexes with LNA scramble). Results are representative of two independent experiments performed in duplicates. *p<0.5; ** p< 0.01; *** p<0.001.

Plasmid DNA and siRNAs are different molecules. However, both are double strand molecules with anionic phosphodiester backbones with the same negative charge for nucleotide ratio and both can interact electrostatically with cationic agents **(Gary et al., 2007)**. On the other hand, LNAs are single strand molecules and, despite their similarity with siRNAs in terms of chain size, they could have a different behavior when complexed with cationic liposomes. So, at this point the objective was to understand if the presence of pDNA cargo in lipoplexes containing LNAs at 4/1 (+/-) charge ratio promoted an increase in the LNAs biological effect. For that HSA-EPOPC:Chol/NA 4/1 (+/-) formulation was used. LNAs were pre-associated with the

plasmid DNA in the charge ratio 1/1 (-/-) before mixing with cationic liposomes. Antisense oligonucleotides against miR-221 and pCMVluc plasmid that codify luciferase were used in the formulation. The levels of miR-221 in Panc-1 cells were evaluated by quantitative RT-PCR using LNAs scramble as control.

As illustrated in Figure 9, the administration of the lipoplex formulation containing 25nM of LNAs against miR-221 without the pDNA led to an 80% inhibition of the miR-221. On the other hand, application of 25nM of Lipopoplex containing LNAs and pDNA resulted in a decreased of the LNA inhibition efficacy from 80% to 50%. Moreover, the addition of more pDNA decreased in a higher extent the inhibitory efficacy of the complexes (data not shown). These results indicate that HSA-EPOPC:Chol/LNA221 4/1 (+/-) lipoplexes were efficient carriers to deliver LNAs molecules into the cells, since it was only necessary 25nM of LNAs to achieve a huge inhibitory effect (80%). Besides, these results also demonstrated that the simultaneous use of pDNA decrease the biological activity of the LNAs, suggesting a lower availability of the LNAs molecules inside the cells when this strategy is used. This may result from the interaction between the two nucleic acid molecules. LNAs are single strand molecules and could interact with the pDNA forming triple-helix structures **(Mishra and Tinevez, 1996; Mills et al., 1999)**. Other possibility may be a poor entrapment of LNAs molecules in the lipoplexes due to the presence of pDNA, consequently leading to a decreased transportation of LNAs into the cells. The pDNA feature ensures that this molecule is entirely encapsulated/encased by the used amount of cationic liposomes. However, the electrostatic interactions between LNAs, which are a small sub-nanometric nucleic acid, and a cationic liposome/lipid system can lead to lipoplexes with less stability and poor encase **(Spagnou et al., 2004; Rhinn et al., 2009)**.

Thus, HSA-EPOPC:Chol/LNA221 4/1 (+/-) proved to be able to silence specifically the miR-221 *per see* using reduced LNAs concentration. The simultaneous use of the pDNA and LNAs in lipoplexes led to less biological activity.

3.4 Development of a New Lipoplex Formulation Based on EPOPC:Chol Liposomes Targeted for Pancreatic Cancer

Mia PaCa is an adherent epithelial cell line derived from a pancreatic carcinoma of a 65 years-old Caucasian male, with a doubling time of 42 hours **(ATCC - CRL-**

1420™). These cells also present a microRNA signature like Panc-1 cells **(Zhang et al., 2009)**. Comparisons of EphA4 receptors expression patterns in PDAC cells and normal tissues, by Northern blot analysis, revealed a higher and specific expression of EphA4 receptors in PDAC cells, when compared with normal tissues, being the Mia PaCa cells the ones with a more extensive expression pattern **(Iiizumi et al., 2006)**.

EphA4 receptors are important because, as mentioned above, besides the brain expression, these receptors are only expressed in pancreatic cancer and others soft carcinomas. In this regard, they can be used as a target to specific deliver genetic material into the target cells using lipoplexes **(Iiizumi et al., 2006)**.

EphA4 receptors are associated with tumor growth, invasion, metastasis and angiogenesis. Ephrins are ligands that control ephrin receptors activity. For example, EphA4 receptor is rapidly internalized by endocytosis upon engagement of ephrin-A5. However, their size makes the ephrins difficult to produce in large quantities. The identification of short peptides that can mimic the binding properties of the ephrins and selectively target individual receptors is a beneficial alternative to target these molecules **(Shin et al., 2008; Giaginis et al., 2010; Murai et al., 2003)**. **Murai et al., 2003** identified and characterized three 12-amino acid peptides (APY, KYL and VTM) that preferentially bind to EphA4 receptors. They antagonize with ephrin binding to EphA4 receptors and block ephrin-induced EphA4 receptor activation and biological function. APY and KYL peptides have conserved motifs to bind EphA receptors but VTM peptide has not this motifs. APY peptide has also two cysteine residues that allow this peptide to form an intramolecular disulphide bond which is important to determine the high binding affinity and receptor-binding selectivity of the peptide **(Murai et al., 2003)**.

Complexes size, composition and surface charge play a relevant role in transfection activity. Lipoplexes with a positive zeta potential, like EPOPC:Chol/DNA 4/1 (+/-), have a higher biological activity than complexes with a negative zeta potential (1/1 (+/−)). This phenomenon can be explained by the extensive electrostatic interactions of the positively charged complexes with the negatively charged cell membrane **(Faneca et al., 2002)**. Complexes with higher positive charge ratio (4/1 (+/-)) have the smaller sizes due to the establishment of repulsive forces that prevent their aggregation. Nevertheless, the association of HSA leads to an increase of the lipoplexes mean diameter. HSA has negative net charge at physiological pH. When complexes are

associated with HSA the positive charges (from cationic liposomes) are neutralized by albumin **(Simões et al., 2000; Pedroso de Lima et al., 2001; Faneca et al., 2004)**. PEG is a recognized strategy to improve lipoplexes blood lifetime without promoting a high increase in size of the complexes. Besides this, PEG molecules allow the insertion of reactive groups (like maleimide), in the end of the molecule, that have the ability to react with the thiol groups of the cysteine side chains in proteins and peptides generating a stable ether linkage **(Takasaki et al., 2006)**. **Shi et al., 2002** reported that *in vitro* the lipoplexes pre-pegylated with PEG-ceramide remained entrapped in the lysosomes and, consequently, did not transfect cells. They also shown that post-pegylation with PEG-ceramides with long acyl chains or with DSPE–PEG resulted in lipoplexes with very low transfection efficiency. **Peeters et al., 2007** in concordance with Shi et al. showed that post-pegylation with cerC_8-PEG seems preferable to post-pegylation with cerC_{16}-PEG because the shorter the acyl chain length of the PEG-lipid, the easier the escape of the lipoplexes from the endosome **(Shi et al., 2002; Peeters et al., 2007)**.

Thus, DSPE–PEG_{2000}-MAL was used to bind the APY peptide and then added to the non-pegylated lipoplexes. CerC_8-PEG_{750} molecules were also used in the lipoplexes in order to test the best amount of PEG. Micelles of CerC_8-PEG_{750} and/or DSPE-PEG_{2000}-MAL with or without APY peptide were added to EPOPC:Chol/DNA 4/1 (+/-) lipoplexes by a post-pegylation process (incubation of lipoplexes with micelles for 20 minutes at 50ºC). Different amounts of PEG and/or APY peptide were added to lipoplexes, which were tested in Mia PaCa cell line using the luciferase assay to evaluate their biological activity. The alamar blue assay was also used to assess lipoplexes cytotoxicity.

3.4.1 Covalent Binding of APY Peptide to DSPE-PEG_{2000}-MAL or DSPE-PEG_{2000}-MAL with CerC_8-PEG_{750} Micelles

The first step to accomplish this goal was the binding between maleimide group of the PEG molecule and the terminal cysteine residue from APY peptide. Micelles of the DSPE-PEG_{2000}-MAL or micelles of the DSPE-PEG_{2000}-MAL with CerC_8-PEG_{750} were prepared as described in chapter 2. The peptide was then added to micelles in a molar ratio of 1:1. Binding efficiency of APY peptide to the maleimide groups was evaluated by Ellman´s assay and the results are presented in Figure 10. In both cases the

efficacy of the attachment to the maleimide groups was approximately 90% and no significant differences were found between the two types of micelles.

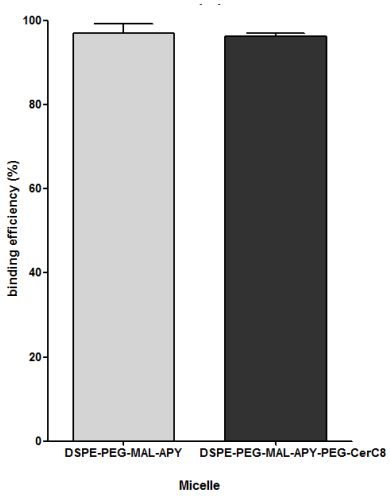


Figure 10 Characterization of the efficiency of the covalent attachment of the APY peptide to the maleimide groups of the DSPE-PEG moieties. The APY peptide was added to the micelles of DSPE-PEG-MAL or DSPE-PEG-MAL with $CerC_8$-PEG, in a ratio of the 1/1 (mol/mol), at acidic pH (6,5). The percentage of the binding efficiency was evaluated by Ellman's assay using a glutathione standard curve as described in "Material and Methods".

3.4.2 Biological Activity and Cytotoxicity of Different Lipoplexes Formulations

After optimization of the peptide binding protocol, different amounts of PEG were tested. The cytotoxicity of the different lipoplexes formulations as well as their biological activity were measured using the alamar blue assay and the luciferase assay, respectively. Figure 11a illustrates the cytotoxicity resulting from the different complexes formulations. The obtained results revealed that no significant differences were observed between the new formulations and the control lipoplexes (HSA-EPOPC:Chol/DNA 4/1 (+/-)) in terms of cytotoxicity. The Figure 11b presents the biological activity of the complexes. It was observed a small difference in luciferase activity between EPOPC:Chol/DNA 4/1 (+/-) lipoplexes and HSA-EPOPC:Chol/DNA 4/1 (+/-) lipoplexes. The obtained results also showed a significant decrease in the biological activity ($p<0.001$) in the majority of the tested conditions comparing with the control formulation. Post-insertion of $CerC_8$-PEG_{750} micelles to lipoplexes (condition with 2 mol% relative to the total lipid of lipoplexes) was the only formulation that did not exhibited a significant difference, in terms of transfection activity, when compared to control lipoplexes.

These results demonstrated that association of albumin does not enhance, at least in high levels, the biological activity of the lipoplexes in Mia PaCa cells. However, the transfection was performed without serum what could explain in part the results. Additionally, it was possible to infer that presence of DSPE-PEG_{2000} molecules in the lipoplexes lead to an extensive decrease of the biological activity. In fact, all formulations containing DSPE-PEG_{2000} molecules presented a reduced biological activity. This happened even in the formulations with 2 mol% of DSPE-PEG_{2000}. Nevertheless, the biological activity of the formulation with 2 mol% of $CerC_8$-PEG_{750} did not differ significantly from the control lipoplexes. These observations were in accordance with the results of **shi et al., 2002** that observed that lipoplexes post-pegylated with DSPE-PEG molecules present low transfection activity.

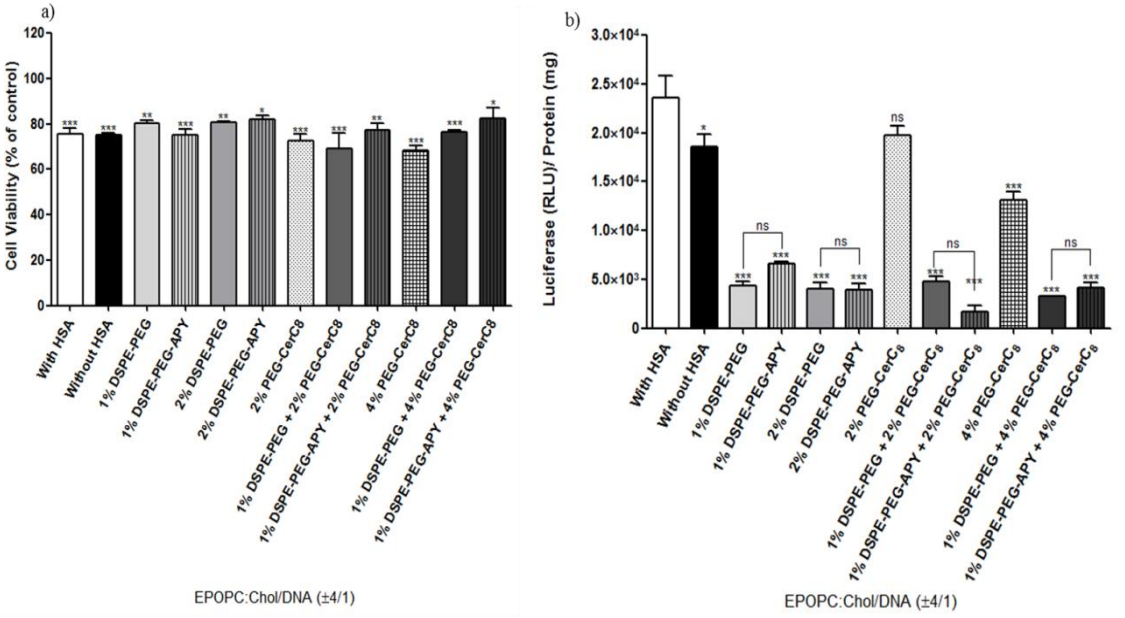


Figure 11 Effect of post-pegylation with different amounts (mol %) of DSPE-PEG and/or $CerC_8$-PEG with or without APY peptide on viability of a) and luciferase gene expression b) in Mia PaCa cells. Cells were covered with 0.3mL of DMEM-HG without serum before the different formulations of the complexes were added. After 4h incubation, the medium was replaced with DMEM-HG containing 10% serum and the cells were further incubated for 48h. The cytotoxicity and the level of gene expression were evaluated as described in "Materials and Methods". The data are expressed a) as the percentage of the untreated control cells and b) expressed as RLU of luciferase per mg of total cell protein. Both are presented as mean ± standard deviation obtained from triplicates, and are representative of at least two independent experiments. *p<0.5; ** p< 0.01; *** p<0.001.

APY peptide incorporation in lipoplexes should enhance the biological activity of the complexes by the interaction of the peptide with EphA4 receptors existing on cell surface. The interaction should lead to receptor mediated endocytosis conferring specificity to the formulation. This specificity would allow a higher biological activity as a consequence of the higher internalization of lipoplexes into the cells. However, as shown in Figure 11b the presence of the APY peptide did not alter the transfection

efficiency of the complexes. There are some possible explanations to this outcome. APY peptide could bind to the EphA4 receptor but it may not allow the internalization by receptor-mediated endocytosis. The APY peptide could have lost its secondary structure and, consequently not be able to bind to the EphA4 receptors. Alternately, there might be a lack of exposure of the peptide to the receptors due to the low size of the peptide and/or to the PEG conformation when attached to lipoplexes. When PEG molecules assume a mushroom regime there is less exposure of the peptide to the receptors **(Allen et al., 2002)**.

The ability of PEG to increase the circulation lifetime of the liposomes has been found to depend on both, the amount of PEG incorporated and the length or molecular weight of the polymer. Generally, the longer chain PEGs allowed the greatest improvements in blood residence time **(Allen et al., 2002)**. The presence of PEG_{2000} is described to increase the amount of lipid remaining in the plasma by two-fold when compared to formulations containing PEG_{750} **(Allen et al., 1991)**. So, it is a better strategy the use of PEG_{2000} instead of PEG_{750} for *in vivo* studies. However, for *in vitro* studies PEG_{750} molecules can be used instead of the PEG_{2000} maintaining the same activity of the lipoplexes.

The ability for PEG to extend the circulation lifetime of liposomes is also dependent on the lipid anchor attached to the PEG **(Allen et al., 2002)**. **Shi** and **Peeters** reports proved that using the $CerC_8$-PEG molecules they achieved the best results in *in vitro* studies, due to the easier escape of the lipoplexes from the endosome **(Shi et al., 2002; Peeters et al., 2007)**. The results shown in Figure 11b are in agreement with those reports.

However, *in vivo* data showed that formulations containing Cer-PEG with short acyl chains (C8 and C14) are submitted to an identical clearance, after intravenous administration, than that observed for liposomes lacking Cer-PEG reflecting the rapid exchange of Cer-PEG from lipid bilayers. Nevertheless, formulations containing Cer-PEG with long acyl chains (like C20 and C24) are submitted to a slower elimination rate, after intravenous administration. The long chain Cer-PEG remained in the carriers after administration and confers increased circulation life-times to the liposomes **(Webb et al., 1998)**. This strategy also has an important advantage relatively to DSPE-PEG molecules. PE-PEG derivatives are sensitive to both the cationic lipid content, via electrostatic attraction, and the lipid acyl chain length via hydrophobic interactions,

while PEG-ceramides are simply controlled by the strength of the hydrophobic interactions that can be plainly regulated via the alterations in acyl chain length **(Webb et al., 1998)**. In this way, Cer-PEG with long acyl chains is a good strategy to effectively increase the circulation lifetimes of lipoplexes *in vivo*, however, for evaluation of this strategy *in vitro* the use of $CerC_8$-PEG seems to be a best option.

Overall, it is important to emphasize that the attachment of the APY peptide to the micelles resulted in a 90% efficiency. Moreover, complexes *per see* did not present a high cytotoxicity and post-pegylation with 2% of $CerC_8$-PEG did not reduce the biological activity of the complexes *in vitro*.

3.4.3 Binding and Cell Interaction Studies of Lipoplexes

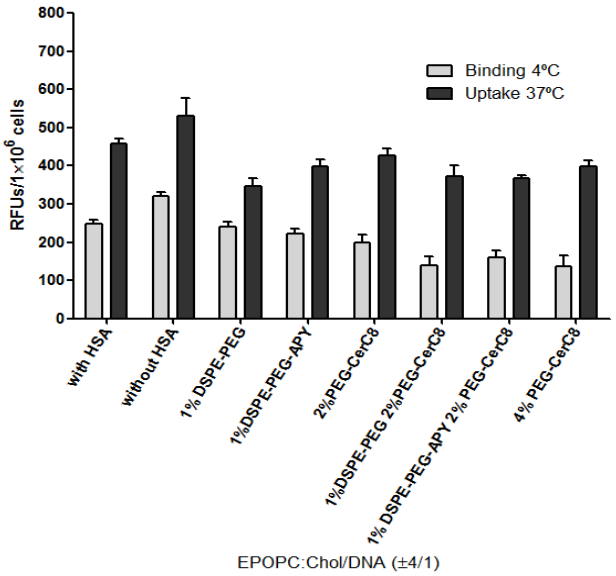


Figure 12 Effect of post-pegylation with different amounts of $CerC_8$-PEG_{750} and/or DSPE-PEG_{2000}-MAL with or without APY peptide on the extent of binding and uptake of lipoplexes in Mia PaCa cells. Liposomes were labeled with 1% of Rh-PE. Cells were incubated for 4h with the lipoplexes at 4 °C (binding) or 37 °C (uptake). The data are expressed as RFU per 10^6 cells (mean ± S.D. obtained from triplicates) and are representative of three independent experiments.

In order to understand the lack of biological activity of the developed lipoplex formulations lipoplexes-cell interaction studies were performed by spectrofluorimetry and flow cytometry. These experiences were done using cationic liposomes labeled with 1 mol% of Rh-PE. For binding and uptake studies the Mia PaCa cells were incubated 4h with the lipoplexes formulations at 4ºC and 37ºC, respectively. At 4ºC the metabolic activity of the cells is inhibited and it is possible to evaluate the extent of lipoplexes bind to the cell surface. At 37ºC the cells present their normal activity and it is possible to measure the extent of lipoplexes that are associated to the cells (binding and uptake).

After the 4h incubation, the cells were lysed and the fluorescence was evaluated. In the flow cytometry studies cells were also incubated for 4h with the labeled complexes. Then, the internalization of the lipoplexes was evaluated by the Rh-PE fluorescence of the cells.

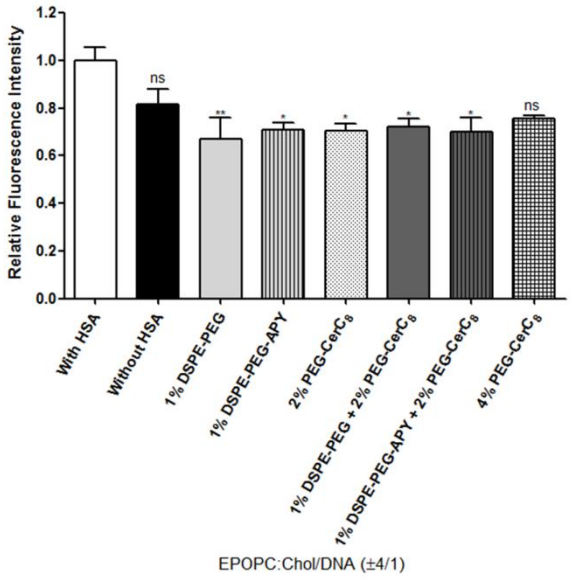


Figure 13 Flow cytometry of Mia PaCa cells treated with different amounts (mol %) of $CerC_8$-PEG_{750} and/or DSPE-PEG_{2000}-MAL with or without APY peptide. Liposomes were labeled with 1% of Rh-PE. Cells were incubated for 4h with the lipoplexes at 37 °C as described in "Material and Methods". The data are expressed as relative fluorescence intensity of Rh-PE being the HAS-EPOPC:Chol/DNA 4/1 (+/-) the control complexes. Results are presented as mean ± standard deviation obtained from duplicates, and are representative of two independent. *p<0.5; ** p< 0.01.

The binding and uptake values of the tested formulations did not show significant differences from that obtained with the control complexes – HSA-EPOPC:Chol/DNA 4/1 (+/-) (Figure 12). On the other hand, the flow cytometry studies (Figure 13) showed that the control lipoplexes (HSA-EPOPC:Chol/DNA 4/1 (+/-)) were significantly more internalized by Mia PaCa cells than the developed lipoplexes formulations containing DSPE-PEG, with or without APY peptide, and/or Cer-PEG. However, these differences are not enough to explain the high lack of biological activity observed for the developed lipoplexes (Figure 11b), particularly containing DSPE-PEG_{2000} molecules. The obtained results in the spectrofluorimetry and flow cytometry studies indicate that the developed lipoplexes were internalized by the cells in similar amounts. Nevertheless, the EPOPC:Chol/DNA 4/1 (+/-) lipoplex formulations containing 1 or 2 mol% of DSPE-PEG_{2000}-MAL, with or without APY peptide were not able to efficiently deliver the pDNA into the cell, consequently resulting in a reduced biological activity. The intracellular traffic of cationic liposome/DNA complexes has

been the subject of several studies aiming at identifying the cellular barriers that limit efficient gene delivery to target cells. To this regard, the endolysosomal entrapment of lipoplexes has been identified as one of the major limiting factors of the transfection efficiency **(Wasungu and Hoekstra, 2006)**. All these data led to the belief that lipoplexes with DSPE-PEG were not able to escape from the endosome being degraded in lysosomes. In this way, there was less pDNA achieving the nucleus and consequently less transgene expression. These observations were in agreement with **Peeters (2007)** and **Shi (2002)** that shown that DSPE-PEG did not allow transgene expression *in vitro*. Looking for cell association studies on EPOPC:Chol/DNA 4/1 (+/-) lipoplexes with APY peptide and without APY peptide no alterations were observed. With these results we can conclude that APY peptide was not interacting with EphA4 receptors.

Based on these findings, we can conclude that despite all lipoplexes formulations were able to interact and be internalized by cells, only the control and plain lipoplexes and the formulation prepared with 2% of Cer-PEG_{750} presented biological activity. This suggests that the others formulations were not able to escape the endolysosomal pathway. Moreover, it is also possible to conclude that APY peptide did not confer the desired and expected specificity to the formulations.

3.5 KYL peptide – an Alternative Ligand to the Targeting Strategy

KYL peptide, like the APY peptide, is an antagonist of the EphA4 receptors. This peptide also presents a high affinity for EphA4 receptors. On contrary to the APY peptide, KYL peptide does not present a secondary structure what may facilitate its binding to the EphA4 receptors.

As described above, the binding efficiency of the KYL peptide to the DSPE-PEG_{2000}-MAL or DSPE-PEG_{2000}-MAL with $CerC_8$-PEG_{750} (1/1, mol/mol) micelles was evaluated by the Ellman's assay. Then, the formulations EPOPC:Chol/DNA 4/1 (+/-), EPOPC:Chol/DNA 4/1 (+/-) with 2 mol% of $CerC_8$-PEG_{750}, EPOPC:Chol/DNA 4/1 (+/-) with 1 mol% of DSPE-PEG_{2000}-MAL, in present or absence of the KYL peptide, and EPOPC:Chol/DNA 4/1 (+/-) with 2 mol% $CerC_8$-PEG_{750} and 1 mol% of DSPE-PEG_{2000}-MAL, in the presence or absence of the KYL peptide, were evaluated for their cytotoxicity and biological activity.

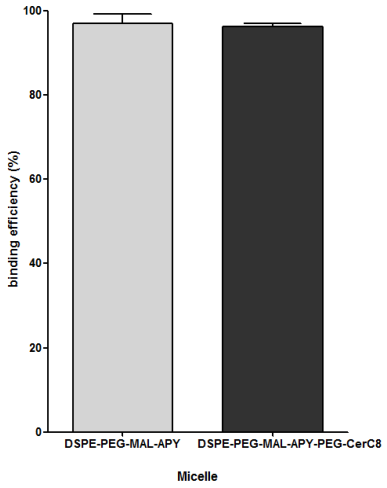


Figure 14 Characterization of the efficiency of the covalent attachment of the KYL peptide to the maleimide groups of the DSPE-PEG moieties. A ratio of the 1/1 (mol/mol) of the KYL peptide were added to the micelles of DSPE-PEG-MAL or DSPE-PEG-MAL with $CerC_8$-PEG at acidic pH (6,5). The percentage of the binding efficiency was evaluated by Ellman's assay using a glutathione standard curve as described in "Material and Methods".

Figure 14 reveals the results corresponding to the covalent binding of the PEG-maleimide groups to the terminal cysteine residue of the KYL peptide present in the micelles. The obtained results showed a binding efficiency of approximately 95% for both types of micelles demonstrating the high efficiency of the process.

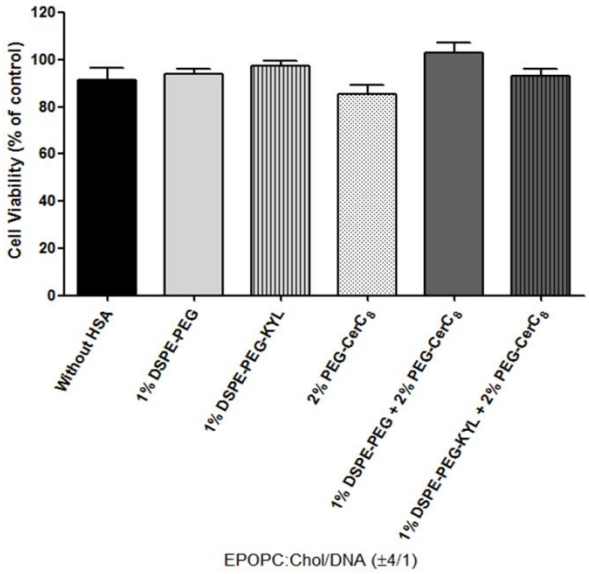


Figure 15 Effect of post-pegylation with $CerC_8$-PEG_{750} and/or DSPE-PEG_{2000}-MAL with or without KYL peptide on viability of Mia PaCa cells. Cells were covered with 0.3mL of DMEM-HG without serum before the different formulations of the complexes were added. After 4h incubation, the medium was replaced with DMEM-HG containing 10% serum and the cells were further incubated for 48h. The cytotoxicity was evaluated as described in "Materials and Methods". The data are expressed as the percentage of the untreated control cells and the results are presented as mean ± standard deviation obtained from triplicates and are representative of two independent experiments.

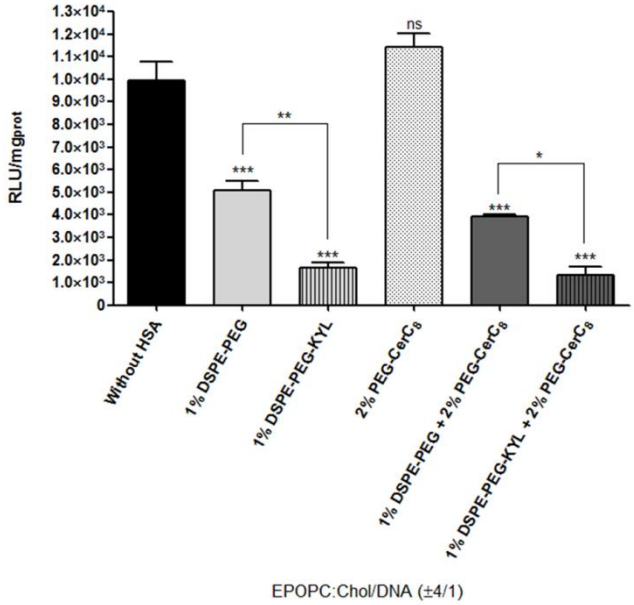


Figure 16 Effect of post-pegylation with $CerC_8$-PEG_{750} and/or DSPE-PEG_{2000}-MAL with or without KYL peptide on luciferase gene expression in Mia PaCa cells. Cells were covered with 0.3mL of DMEM-HG without serum before the different formulations of the complexes were added. After 4h incubation, the medium was replaced with DMEM-HG containing 10% of serum and the cells were further incubated for 48h. The levels of gene expression were evaluated as described in "Materials and Methods". The data are expressed as RLU of luciferase per mg of total cell protein. The results are presented as mean ± standard deviation obtained from triplicates and are representative of two independent experiments. *p<0.5; ** p< 0.01; *** p<0.001.

Figure 15 shows the toxicity levels, obtained after incubation of Mia PaCa cells with lipoplexes, evaluated by the alamar blue assay. No significant toxicity was observed for all tested formulations. After, the biological activity was evaluated by the luciferase assay. The data illustrated in Figure 16 revealed, in accordance with the results obtained with the APY peptide, that the presence of 2 mol% of $CerC_8$-PEG_{750} did not significantly change the biological activity of the complexes. On the other hand, the presence of DSPE-PEG_{2000} significantly decreased the luciferase gene expression. These results were also in agreement with the observations by **Shi et al. (2002)** and **Peeters et al. (2007)** and with the results presented for the APY peptide. Nevertheless, in this case, contrary to what was observed with the APY peptide, it was possible to visualize significant differences between the formulations containing and not containing the KYL peptide. Surprisingly, the presence of KYL peptide in the lipoplexes resulted in a lower biological activity. The KYL peptide (KYLPYWPVLSSLPKKKRKVC) had a nuclear localization sequence with a high positive charge density. The high positive charge of the peptide was probably establishing strong interactions with the negative charge of the genetic material which could promote a change in the structure of the lipoplexes, a high level of superficial hydration and a decrease in the DNA release

inside the cells. These facts could result in a lower lipoplex-cell interaction, in a reduced cell internalization and in a lower DNA translocation to the nucleus, consequently resulting in a decreased biological activity.

From these results it was possible to infer that the covalent binding between maleimide and cysteine residue is a highly efficient strategy for different types of molecules. The reduction in the biological activity caused by DSPE-PEG molecules was once more confirmed, suggesting the entrapment of the lipoplexes in the endolysomal pathway. On the other hand, the efficacy of post-pegylation strategy with $CerC_8$-PEG_{750} (low amounts – 2 mol%) *in vitro* was also confirmed. KYL peptide, like APY peptide, did not confer the desired specificity to the lipoplexes formulations.

Chapter 4: Conclusion and Future Perspectives

During the past decade thousands of patients with advanced pancreatic carcinoma were treated in several phase II and phase III clinical trials. Until now, the majority of the developed treatments failed on improving survival of the patients with pancreatic cancer. It is a tragedy that 10 years of trials had added little to the overall knowledge of the disease. So, the development of new therapeutic approaches is urgent and should join all the advantages of the different used strategies to fight the disease. A multiple targeting strategy seems the best possibility to get effectiveness on pancreatic cancer treatment.

The main goal of this project was the development of a new non-viral lipid-based vector for specific delivery of genetic material into pancreatic cancer cells. This vector was designed in order to obtain small and stable lipoplexes resistant to serum (able to be used in intravenous administrations) with high biological activity and specificity for the pancreatic tumors cells (targeting strategy). Beside this, it was also planned to evaluate the efficiency of combined therapeutic strategies in order to act against this malignancy in different fronts (interfering in diverse pathways of the tumor cells). In this project it was not possible to accomplish all the defined objectives as it is noticeable from the results described above. However, it is important to mention some relevant conclusions from this work.

One of the conclusions was that association of albumin to EPOPC:Chol/DNA 4/1 (+/-) lipoplexes promotes not only the enhancement of their biological activity but also the resistance to the inhibitory effect of serum on transfection of Panc-1 cell line. The combination of therapeutic approaches was an important part of the work. From the obtained results it is possible to conclude that the presence of chemotherapeutic drugs that interfere with microtubules dynamics of the cells enhance more than 5-fold the lipoplexes biological activity. This is a promising strategy because it is possible to have the benefits from chemotherapy and at the same time potentiate the efficacy of a gene therapy approach. HSA-EPOPC:Chol/LNAs 4/1 (+/-) lipoplexes also showed to be highly efficient in the inhibition of microRNAs even at low LNAs concentrations. So, it is possible to conclude that EPOPC:Chol liposomes, when associated to albumin, are an efficient vector to deliver both plasmid DNA and antisense oligonucleotides in the Panc-1 cells.

The high deliver efficacy of the non-viral vectors as well as their high stability and specificity remains a challenge in the gene therapy approaches. The specificity can be achieved using different strategies. In this work, the incorporation of a peptide in the delivery vector to target EphA4 receptors was the selected strategy. A covalent binding strategy was successfully optimized in order to obtain a high attachment level of the ligands to the reactive groups of the PEG molecules. Then, the formulations developed, using a post-pegylation strategy, were evaluated in their cytotoxicity and biological activity. The lipoplexes EPOPC:Chol/pDNA 4/1 (+/-) containing 2 mol% $CerC_8$-PEG_{750} demonstrated to be an effective formulation for *in vitro* studies. However, it was also possible to conclude that the incorporation of DSPE-PEG_{2000} in the formulations causes a reduction in their biological activity proving to be an inadequate strategy. Another important point was the formation of stable lipoplexes with targeting ability. The incorporation, into lipoplexes, of the two peptides (APY and KYL) chosen to confer specificity to the pancreatic tumor cells were not successful. Thus, the aim of develop a stable and specific formulation was not achieved.

Having in mind the results obtained in this work it would be interesting the improvement of some used strategies as well as the fulfillment of additional experiments. It would be interesting to improve the combined strategy using pDNA and LNAs. It could be a viable way to reach different altered pathways in pancreatic tumor cells. Another interesting study would be the evaluation of the chemotherapeutic drugs (vinblastine, vinorelbine and/or docetaxel) in the biological activity of the lipoplexes with LNAs. The combination of the three strategies, inhibition of overexpressed miRNAs using LNAs, insertion of a pDNA encoding a tumor suppressor gene, such as p53, and the application of chemotherapeutic agents would be a very interesting approach.

Regarding the development of the delivery system initially proposed, it would be interesting to investigate some alternatives. The ligand used for targeting could be, for instance, EphrinA5 which is a protein that allows the internalization of EphA4 receptor **(Shin et al., 2008)**. The use of an anti-body or the Fab' fragment of the antibody against the EphA4 receptor would be another interesting ligand **(Gao et al., 2011)**. After the optimization of the formulation, the physicochemical characterization of the lipoplexes would be essential. Parameters such as size, charge density, serum resistance and genetic material protection would be important issues to validate the quality of the

developed formulations. Obviously after all the *in vitro* optimization and physicochemical characterization the application of this new formulation in a mouse model would be essential.

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