THE FEASIBILITY OF COATING CATIONIC LIPOSOMES WITH MALARIA CIRCUMSPOROZOITE (CS) REGION II+ PEPTIDE FOR HEPATOCYTE SELECTIVE TARGETING

ANAS EL-ANEED

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THE FEASIBILITY OF COATING CATIONIC LIPOSOMES WITH MALARIA CIRCUMSPOROZOITE (CS) REGION II+ PEPTIDE FOR HEPATOCYTE SELECTIVE TARGETING

by

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Abstract

PURPOSE: Cationic liposomes are non-viral vectors studied for cancer gene therapy. However, liposomal-mediated transfection levels in the liver are significantly lower than those observed in other organs. In this study, we evaluated the feasibility of coating liposomal preparations with a liver targeting ligand derived from malaria circumsporozoite (CS) region II+ peptide.

METHODS: A novel derivative of region II+ peptide was designed and synthesized. It was studied for its liver targeting potential for liposomal-mediated gene delivery. **Liposomes-peptide association:** the targeting ligand was either added in the hydration step of liposomes preparation or in the final stage after extrusion. The technique of density gradient airfuge was used to evaluate liposome-peptide association. **Liposomes-HepG2 interactions:** cells were incubated with liposomes labeled with ^{3}H cholesterol. Cells were then lysed and radioactivity was measured. **Transfection Experiment:** PCMV53 plasmid containing the tumor suppressor gene p53 was used for the preparation of the liposomal complexes. The transfection experiments were executed using liver cancer cell lines. Western blotting analysis was performed to determine p53 expression.

RESULTS: more than 70 % of the targeting peptide was associated with the cationic liposomes when the former was added in the hydration step. The percentage however dropped to less than 40 % if peptide addition was performed in the final stage. No difference was observed between liposomal and liposomal-peptide preparations after incubation with HepG2 cells. Similar results were obtained by western blotting analysis.

CONCLUSION: the peptide was successfully incorporated with the liposomal formula. Its binding specificity however was not achieved by our methodology.

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Table of Contents

List of Figures

List of Tables

List of abbreviations and symbols

%	percent
α	alpha, a Greek letter
β	beta, a Greek letter
^{o} C	degrees Celsius
µl	microliter, 10^{-6} liter, unit of volume
µM	micromole, 10^{-6} M, unit of concentration
µ	mu, a Greek letter, used to indicate micro in metric units
µg	micrograms 10^{-6} g, unit of mass
^{3}H	tritium
^{3}H-CHE	Cholesterol [1,2-^{3}H –(*N*)]-hexadecyl ether
^{3}H-Chol	^{3}H radio-labeled cholesterol
^{99m}Tc	^{99m}technetium
AA	amino acids
AAV	adeno- associated virus
ADA	adenosine deaminase
ADA-SCID	adenosine deaminase- severe combined immuno-deficiency
AF	asialofetuin
AFP	α-fetoprotien
APC	antigen presenting cells
ASGP-R	asialoglycoprotein receptors
B_{max}	maximum receptor number/cell
CCS	combined consensus scale
CD	cytosine deaminase
CD	circular dichroism
Chol	cholesterol
Ci	Curie, unit of radioactivity
CMDA	carboxypeptidase/ 4-[2-chloroethyl 2-mesyloxyethel-0-amino] bensoyl-L-glutamic acid
CNS	central nervous system
cpm	count per minute, unit for radioactivity
CS	circumsporozoite
CTL	cytotoxic T lymphocytes
Cx32	connexine32
D5W %	dextrose 5%
Da	Dalton, atomic mass unit
DC	dendritic cells
DC-Chol	3β [N-(N′, N′ -dimethylaminoethane) carbamoyl] cholesterol
DMF	dimethyl sulfoxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOPE	dioleylphosphatidylethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	N [1-(2,3-dioleyloxy) propyl] –N, N, N-trimethylammonium chloride
ECL	enhanced chemiluninescence

EDTA	EthylenediamineTetraacetic Acid
EGFR	epidermal growth factor receptor
EM	electron microscopy
ES	electrospray
Fab	fragment antigen binding
Fc	fragment crystallizable
GCV	ganciclovir
G	gravity unit
hAAT	human alpha antitrypsin
HCC	hepatocellular carcinoma
HIV	human immunodeficiency virus
HSV	herps simplex virus
HSV-tk	herpes simplex virus thymidine kinase
i.e.	id est (that is)
IL-12	interleukin-12
Inc.	incorporated
iv	intravenously
K_d	dissociation equilibrium constant
kDa	kilo Daltons.
LDL	low density lipoprotein
LPD	liposomes/ Protamine/ DNA
LS	liquid scintillation
mAb	monoclonal antibody
MALDI-TOF	Matrix-assisted laser desorption ionization – time of flight
MDR-1	multiple drug resistance gene-1
mg	milligram (s), 10^{-3} g, unit of mass
MHC	major histocompatibility complex
min	minute(s)
ml	milliliter (s), 10^{-3} liter, unit of volume
mmol	milimole
mRNA	messenger ribonucleic acid
NBD-Chol	7-nitrobenz-2-oxa-1,3-diazol-4-yl-labeled. Cholesterol
ng	nanograms, 10^{-9} g, unit of mass
NK	natural killer
nm	nanometer, 10^{-9} m, unit for distance
NSCLC	non-small cell lung cancer
P. faciparum	*Plasmodium faciparum*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PEI	poly(ethylenimine)
pI	isoelectric point
PLL	poly-L-lysine
PMSF	phenylmethylsulfonyle fluoride
RBC	red blood cells
RPC	reducible polycations

SA	sinapic acid
SDS	sodium dodecyl sulfate
TBS-T	Tris-buffered saline-Tween
TEMED	N,N,N',N',- tetramethylethylenediamine
TF	transferrin
TFA	tri-flouro acetic acid
ULV	unilamellar vesicles
VEGF	vascular endothelial growth factor
wt p53	wild type *p53*

1. Introduction

1.1 Gene therapy:

Gene therapy includes the treatment of both genetically based and infectious diseases by introducing genetic materials which have therapeutic effects (Anderson FW 1998; Crystal RG 1995; Miller AD 1992). In its simplest terms, a wild type gene (which is non-functional in the cell leading to disease development) is introduced into the somatic cell lacking this gene to restore the normal gene function in this cell. Many gene therapy strategies, however, utilize genes to destroy specific cells. Such strategy is widely encountered in cancer gene therapy (Fillat C *et al.*, 2003; Zeh HJ, and Bartlett DL 2002). Another gene therapy strategy is found in the diseases of the nervous system where the genetic basis is very complicated or not well understood. Therapeutic genes in this case encode for a protein which is missing in the neuro-cells. The loss of dopaminergic neurons, for example, plays a major role in the development of Parkinson's disease (Lindvall O *et al.*, 1990). Therefore, genes that can enhance dopamine production will have therapeutic effects (Latchman DC and Coffin RS, 2000). Genes can also boost the body's defense system against foreign infectious microorganisms. Gene therapy for human immunodeficiency virus (HIV), which relies on boosting T-cell immunity, for instance, has entered phase I clinical trails (Clayton J, 2002).

During the past 15 years, intensive research in the area of gene therapy has embarked worldwide with the first approved gene therapy clinical trial in 1990 in which the adenosine deaminase (ADA) gene was transferred into T-cells of two children with severe combined immuno-deficiency (ADA-SCID) (Blaese RM et al., 1995). After a

decade, there are more than 400 clinical studies in gene therapy. Almost 70% of these studies are in the area of cancer gene therapy (Breyer B *et al.*, 2001).

1.2. Cancer gene therapy:

Gene therapy for cancer is one of the most studied applications of gene therapy. Over 2,500,000 cancer patients died in the United States alone between the years of 1976-1996 (Ries LAG *et al.*, 1999). The transformation of normal cells into neoplastic ones is associated with multi-mutational alterations at the genetic level in these cells (Bertram JS 2000). Therefore, gene therapy can provide a new treatment which may reduce the mortality rate among cancer patients, especially if combined with conventional therapy. Due to the complex nature of cancer, cancer gene therapy includes many therapeutic strategies. These strategies can be categorized into two main avenues: immunologic and molecular (Heo DS 2002; Brand K 2000).

1.2.1. Immunologic approaches in cancer gene therapy:

There are two arms for the immune system to encounter foreign antigens. One arm includes antibodies which are secreted by B cells after being activated through membrane immunoglobulin (B cell receptors) -antigen binding. Antibodies are soluble proteins that circulate in the blood to reach their targeted soluble antigens. On the other hand, T cells, the second arm of the immune system, do not secrete antibodies and interact directly with the antigen which can be a synthesized antigen presented at the cell surface through major histocompatibility complex (MHC) within a certain cell population

(e.g. cancer cells). T cells can then mediate multiple immune reactions including cytotoxic effects (Benjamini E *et al.*, 2000).

Cancer cells are immunogenic in nature with cancer antigens being intracellular molecules (Oettgen HF, and Old LJ 1999). Therefore, cellular immunity (T-cell mediated) is more prominent than humoral immunity (B-cell mediated) (Rosenberg SO *et al.*, 1999). The regular immune response, however, is not enough to eradicate tumor cells. The ability of cancer cells to escape the immune system is related to the secretion of immunosuppressive factors (Cochran AJ *et al.*, 2001), down-regulation of antigen expression (Kurnick JT *et al.*, 2001; Uyttenhove C *et al.*, 1983) or MHC molecules (Cabrera CM *et al.*, 2003; Hui K *et al.*, 1984), and the lack of co-stimulation (Pardoll DM 1998; Galea-Lauri J *et al.*, 1996). In fact, the antigen is presented by the tumor cell itself rather than the antigen presenting cells (APC) capable of co-stimulants secretion. Genetic immunotherapy can be utilized mainly to boost T-cell mediated immune response against cancer.

One of the most frequently encountered genetic immunotherapy strategies involves the transfer of the genes of the immune-stimulant molecules such as cytokines. Intensive research has focused on the transfection with Interleukin-12 (IL-12) gene. Complete tumor regression in rat animal models was observed in hepatocellular carcinoma (HCC) and adenocarcinoma after successful IL-12 gene transfection into the cancer cells (Shi F *et al.*, 2002; Barajas M *et al.*, 1996). The production of IL-12 by tumor cells mediates the immune response by the activation of many components in the immune system, in particular cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (Saudemont A *et al.*, 2002; Caruso M *et al.*, 1996).

Another genetic immunotherapy approach includes the *in vitro* manipulation of APC cells to enable them of active tumor antigen presentation. Dendritic cells (DC) are the most powerful APC cells. Engineered DC, for example, expressing α-fetoprotien (AFP), HCC antigen, was able to provoke a strong immune response against the cancerous cells (Vollmer CM Jr *et al.*, 1999). Acute leukemic cells can also boost body immunity after being modified *ex vivo* into functional APC cells (Stripecke R *et al.*, 2002). These strategies of *in vitro* manipulation are very efficient in treating minimal disease status observed after conventional chemo- and radio- therapies (i.e. cell vaccines).

Direct genetic vaccination by the antigen-encoding genes can also induce the desired immune reaction against cancer cells. When injected by subcutaneous or intramuscular routes, DNA enters local cells (fibroblasts or myocytes) which can then produce and secret the antigen. APC will capture the new antigen and migrate to the lymphoid organs initiating the desired immune response (Ribas A *et al.*, 2000). AFP-expressing tumors, for instance, were rejected by at least 60% of tested mice after being vaccinated with AFP-expressing gene. The life span in the treated animals was also significantly prolonged (Hanke P *et al.*, 2002; Grimm CF *et al.*, 2000). Similarly, immunization of monkeys by carcino-embryonic antigen gene resulted in both humoral and lympho- proliferative immune responses (Conry RM *et al.*, 1999).

1.2.2. Molecular approaches in cancer gene therapy:

Up-regulation or down-regulation of some genes is the basis of tumor initiation and progression. The underlying mechanism of gene dysfunction includes many mutations on the genetic level. Many genes are involved in the transformation from

normal cell state to neoplastic state (Bertram JS 2000). The two gene groups believed to be mainly involved in cancer development are oncogenes and tumor suppressor genes. Oncogenes are growth promoting while tumor suppressor genes are growth inhibiting. Therefore, the later group and antioncogenes can be used in cancer therapy. In either case, the aim is to induce cell cycle arrest or better apoptosis (programmed cell death) in cancer cells. In addition to antioncogenes and tumor suppressor genes, suicide genes which also target cancer cells on the molecular levels is another molecular approaches in cancer gene therapy.

1.2.2.1 Suicide genes:

This strategy relies on the conversion of non-toxic substances (prodrugs) into physiologically active agents by means of non-mammalians enzymes. These enzymes were over-expressed in the neoplastic cells as a result of a successful transfection with their genes (Kirn D *et al.*, 2002; Mullen CA 1994).

One of the most investigated suicide gene/prodrug systems is the herpes simplex virus thymidine kinase (HSV-tk)/ ganciclovir (GCV) system. HSV-tk is a herpetic enzyme that catalyzes the phosphorylation of nucleoside analogs such as the antiviral drug GCV (Fillat C *et al.*, 2003; Dubowchik GM, and Walker MA 1999). The phosphorylated GCV mediates the killing of cancer cells via apoptotic (Wei SJ *et al.*, 1998; Hamel W *et al.*, 1996) and non apoptotic mechanisms (Kwon GY et al., 2003; Link CJ Jr *et al.*, 1997).

One of the powerful features in these systems is the bystander effect. It is the mechanism by which the toxic metabolites are transferred from transduced cells to

neighboring cancerous cells via gap junctions and/ or apoptotic vesicles described below (Tanaka T *et al.*, 2001; Freeman SM *et al.*, 1993). It has been shown that the treatment with GCV for cancer cells with as few as 10% of the cells expressing the HSV-tk gene *in vitro* and 50% *in vivo* will lead to the same degree of cell death and tumor regression as that obtained with 100% cell transfection (Freeman SM *et al.*, 1993; Takamiya Y *et al.*, 1993; Culver KW *et al.*, 1992).

Gap junctions or metabolic cooperation are cylindrical structures in the cellular membrane which link the cytoplasm of two adjunct cells. The main structure protein of gap junctions is Connexine32 (Cx32) (Ladish H *et al.*, 2000). These cellular communicating units enable cells to transfer ionic and low molecular weight substances (i.e. < 2000 Da) between each other (Ladish H *et al.*, 2000). The relationship between gap junctions and HSV-tk/ CGV system is well established (Asklund T *et al.*, 2003; Nicholas TW *et al.*, 2003; Marconi P *et al.*, 2000; Touraine RL et al., 1998).

On the other hand, apoptosis is characterized by chromatin condensation, cell shrinkage, and the formation of apoptotic vesicles (apoptotic bodies) (Gschwind M and Huber G 1997) which are phagocytosed by adjunct cells. Apoptotic bodies were detected, for example, in HSV-tk negative colon cancer cells which were co-cultured with HSV-tk positive cells after GCV treatment (Freeman SM *et al.*, 1993).

This two step approach in cancer gene therapy, however, may affect the surrounding non-cancerous cells. Normal tissue damage was reported when rat hepatoma was treated with the HSV-tk/GCV system (Bustos M *et al.*, 2000).

In addition to HSV-tk/ GCV system, there are many other systems which are under investigation. These systems include, but not limited to, cytosine deaminase

(CD)/5-fluorocytosine (Yoshimura I *et* al., 2001; Li Z *et al.*, 1997), cytochrome P450/ cyclophosphamide (Chen L *et al.*, 1996; Wei MX *et al.*, 1995), and carboxypeptidase/ 4-[2-chloroethyl 2-mesyloxyethel-0-amino] bensoyl-L-glutamic acid (CMDA) (Marais R *et al.*, 1996).

1.2.2.2 Antioncogenes:

The biological activity of oncogenes can be modulated and suppressed either on the RNA or DNA levels. Oligonucleotides are short nucleic acid segments that can bind to a specific sequence of the RNA (antisense oligonucleotides) or the DNA (antigene oligonucleotides) (Zhang WW, and Roth JA, 1994; Helene C, 1994).

1.2.2.2.1 Antisense oligonucleotides:

Antisense oligonucleotides bind to mRNA through Waston- Crick base pairing inhibiting the translation step of protein synthesis (Kibler-Herzog L *et al.*, 1990). One of the most prominent oncogenes is *bcl-2* gene, a prototypical inhibitor of apoptosis (Gross A *et al.*, 1999). Over-expression of *bcl-2* also increases resistance to chemo- and radio-therapies in cancer cells (Reed J 1999). Expression of *bcl-2 in vitro* was significantly reduced after treatment with the liposomal solutions of the antisense oligonucleotide G3139 (Hu Q *et al.*, 2002; Duggan BJ *et al.*, 2001). The intravenous infusion of the short oligonucleotide G3139 in patients with solid tumors, however, did not show antitumor effects (Morris MJ *et al.*, 2001). In contrast, the combination with chemotherapy revealed encouraging therapeutic results for leukemic patients (Marcucci G *et al.*, 2003).

Other important targets for antisense therapy are *c-myc* (Potter M, and Marcu KB 1997) and *ras* family oncogenes (Scharovsky OG *et al.*, 2000). For example, retardation in cell growth rate was observed in melanoma cells treated with antisense oligonucleotide targeting the *c-myc* gene (Chana JS *et al.*, 2002). *In vivo* treatment showed significant reduction in tumor growth in adenocarcinoma-implanted rats only when combined with the chemotherapeutic agent Carboplatin which was not effective when used alone (Walker TL *et al.*, 2002). Oncogenes seem to play a central role in cancer resistance against chemotherapy.

It is also possible to include cleavage capable fragment in the oligonucleotides (i.e. ribozymes) (James HA 1999). This strategy will lead to the destruction of the targeted RNA. Point mutation in codon 12 of the *K-ras* oncogene was utilized successfully to design a site-specific antisense ribozyme (Kijima H, and Scanlon KJ 2000). Apoptosis and tumor growth suppression were observed both *in vitro* and *in vivo* when colon cancer cells were treated with the *K-ras* antisense (Tokunaga T *et al.*, 2000).

1.2.2.2.2 Antigene oligonucleotieds:

Antigene oligonucleotieds bind to the DNA through Hoogsteen hydrogen bonding forming a non-functional triple helical structure (Helene C *et al.*, 1992). In this strategy, gene expression is blocked at the transcription stage. The main benefit of this approach over the antisense strategy is the limited targets for the therapeutic oligonucleotieds (two targets per cell versus hundreds to thousands of mRNA for the antisense oligonucleotieds). For example, the combined radiotherapy, via ^{99m}technetium (^{99m}Tc), and *bcl-2* antigene treatment was illustrated *in vitro* through the use of ^{99m}Tc-conjugated

bcl-2 antigene resulting in successful transcriptional cessation of the *bcl-2* gene (Shen C *et al.*, 2003).

In addition, the combination of more than one antigen can result in synergistic effects. Dual treatment with *c-myc* and *c-erbB2* (also known as *HER/neu*) antigenes, for example, increased cell growth inhibition in ovarian cancer cells by almost 20 % more than individual antigene treatments (about 60% cell growth inhibition) (Fei R, and Shaoyang L 2002).

1.2.2.3. Tumor suppressor genes:

Tumor suppressor genes constrain unusual cell proliferation (Weinberg RA, 1991). These genes are capable of apoptosis and cell cycle arrest induction in the malignant cells (Opalka B *et al.*, 2002). The main representative gene of this family is the *p53* gene which is responsible for the detection of DNA damage followed by repair initiation or apoptosis induction (Sager R, 1989). Mutational alterations in the *p53* gene occur in almost 40% of all tumors (Greenblatt MS *et al.*, 1994). Despite that wild type *p53 (wt p53)* belongs to tumor suppressor gene family, some of its mutant forms can act as oncogenes (Marshall CJ, 1991; Lane DP and Benchimol S, 1990). Therefore, successful transfection of the *wt p53* into cancerous cells will have therapeutic outcomes. It is well documented that *p53* gene induces apoptosis and cell cycle arrest in cultured cells (Sauter ER *et al.*, 2002; Roy I *et al.*, 2002; Mitry RR *et al.*, 1997). Similarly, tumor growth inhibition and tumor regression in animal models were observed after *p53* transfection (Dolivet G *et al.*, 2002; Anderson SC *et al.*, 1998; Hsiao M *et al.*, 1997).

p53 treatment has found its way into clinical stages. Intratumoral injections of adenovirus mediated transfection of *p53* (Ad-*p53*) for 28 patients diagnosed with non-small cell lung cancer (NSCLC) resulted in disease stabilisation in 16 patients (64%) and more than 50% tumour size reduction in two patients (Swisher SG *et al.*, 1999). More promising outcomes were reported when Ad-*p53* treatment was combined with chemotherapy (Cisplatin) (Nemunaitis J *et al.*, 2000) or radiation (Swisher SG *et al.*, 2003). In the later combination, no viable tumor was observed in 63% of patients after three month of the completion of the therapy.

Another tumor suppresser gene which has been also tested for clinical trials is the *E1A* gene (Hortobagyi GN *et al.*, 2001). *E1A* gene is efficient in treating cancer cells that over-express the *HER/neu* oncogene such as ovarian and breast cancers (Ueno NT *et al.*, 2001; Wang SC and Hung MC 2001). Many other apoptotic genes such as p16, p21, p27, E2F-1, FHIT, E4 and PTEN are currently investigated in animal experimental settings (Opalka B *et al.*, 2002).

1.2.2.4. Other molecular approaches in cancer gene therapy:

Cancer tissue proliferation is associated with vasculature growth from existing blood vessels (i.e. angiogenesis) (Folkman J 1990). Angiogenesis provides cancerous cells with the necessary nutrients; therefore, interfering with this process in the tumor can produce therapeutic effects. The main targets for anti-angiogenesis cancer therapy includes inhibiting angiogenic inducers (namely vascular endothelial growth factor (VEGF) and angiopoietine) or introducing angiogenic inhibitors such as angiostatine, endostatine, IL-12, and p53 (Chen QR *et al.*, 2001). For example, tumor growth of

implanted HCC cells in athymic mice was proportional to the percentage of cells transfected with angiostatin gene. No tumor was detected when 90-100% of cells were transfected with the antiangiogenesis gene (Ishikawa H *et al.*, 2003). It was also illustrated that significant tumor regression was achieved when only 5% of human breast cancer cells were transduced with the apoptotic gene *p53* (Xu M *et al.*, 1997). This tumor regression was, however, associated with 60% reduction in the number of the blood vessels (i.e. anti-angiogenesis effect). The main advantages of this cancer therapy strategy are the easy accessibility to the endothelial cells of the blood vessels and its applicability to different cancer types.

Another cancer gene therapy approach includes the prevention of toxic side effects, mainly myelosuppression, of antineoplastic agents. This can be achieved by transfering the drug resistance genes such as multiple drug resistance gene-1 (MDR-1) into the hematopoietic progenitors (Licht T and Peschel C 2002; Koc ON *et al.*, 1999). MDR-1 gene encodes for P-glycoprotein, a cell membran transporter which effluxes many hydrophobic and amphipathic substances (Gottesman MM *et al.*, 1995). The main advantage of this strategy is overcoming the dose-limiting toxicity of traditional chemotherapy.

1.3. Gene delivery systems:

The main objective in gene therapy is successful *in vivo* transfer of the genetic materials to the targeted tissues. The aim from the delivery system varies according to the application. For example, prolonged and sustained expression is needed for treating diseases related to one gene dysfunction like hypercholesterolemia while short period of

gene expression is sufficient for most cancer gene therapy strategies. Despite that naked DNA was used successfully when injected directly into the tumor (Shi F *et al.*, 2002; Walther W *et al.*, 2002) or as DNA vaccines (Hanke P *et al.*, 2002; Conry RM *et al.*, 1998), it is highly prone to tissue clearance and totally inefficient for systematic delivery (Kawabata K *et al.*, 1995).

Gene therapy vehicles can be categorized into two groups: biological and non-biological systems. Each group has its own advantages and limitations. Biological carriers are viruses which were naturally evolved to infect cells and transfer their genetic materials into the host cells. Viral vectors used in gene therapy were modified to eliminate their pathogenicity and retain their high efficiency in gene transfer. They are, however, difficult to produce and toxic (in particular immunogenic), as well as having a limitation in terms of the size of the inserted gene (Cusack JC Jr and Tanabe KK 2002). These limitations are not encountered in the less efficient non-viral gene carriers such as liposomes. In either case, selected modifications that can produce safe, efficient and targetable gene carriers are desirable.

1.3.1. Biological gene delivery systems (viral vectors):

1.3.1.1 Retrovirus:

Retrovirus carriers are developed by replacing the vital viral genes with therapeutic ones. The ability of retroviral vectors to successfully deliver foreign genetic materials was first described in 1981 (Wei CM *et al.*, 1981; Shimotohno K and Temin HM 1981). Retroviruses are small RNA viruses with DNA intermediates, that integrates into the host genome producing the viral proteins (*gag*, *pol*, and *env*) which are removed

when developing the gene delivery carrier. Most of these viruses infect actively dividing cells during mitosis (Lewis PF and Emerman M 1994; Miller DG *et al.*, 1990). Despite that this feature might protect the normal tissues and provide natural targeting to the tumor, all tumors contain non-dividing cells in the resting phase G_0. These cells will not be affected by retrieval-mediated gene transfer. Lentiviruses such as HIV and their vectors can, however, infect non-proliferating cells (Buchschacher GL Jr and Wong-Staal F 2000; Lewis PF and Emerman M 1994). Transfection efficiency was 10 times higher in ovarian cancer cells when lentiviruses were used in comparison to retroviral vectors (Indraccolo S *et al.*, 2002). The usage of lentiviruses, however, has a major drawback because of the original serious clinical consequences of these viruses.

In this context, the new retroviral vectors, namely replication-competent retroviruses, were developed and engineered to replicate specifically in the targeted neoplastic tissues; thus, increasing the vectors' non-toxic transduction ability (Solly SK *et al.*, 2003; Logg CR *et al.*, 2002).

1.3.1.2 Adenovirus:

Adenoviruses are double-stranded DNA viruses that can infect both dividing and non-dividing cells (Li Q *et al.*, 1993; Quantin B *et al.*, 1992). The wild type viruses can cause benign respiratory infections in humans (Doerfler W 2000). The defective-competent adenoviral vectors were first generated by substituting the viral *E1* gene with a therapeutic gene. More efficient gene carriers were obtained by altering more genes in the viral genome such as the *E2* gene (Engelhardt JF *et al.*, 1994). The removal of the whole coding sequence of the viral genome resulted in better gene carriers in terms of

their capacity (Harrington KJ *et* al., 2001). Transfection with adenoviruses is transient since the DNA genome does not permanently integrate into the host cell genetic material (Kelly TJ Jr 1984). Therefore, repetitive administration of the adenoviral vectors is needed to obtain the desired therapeutic outcomes.

Adenoviral vectors have been widely used for cancer therapy applications (Swisher SG *et al.*, 2003; Roy I *et al.*, 2002; Anderson SC *et al.*, 1998). It has been shown that adenovirus-mediated gene transfer is more efficient in immuno-deficient animals (Ragot T *et al.*, 1993). Both cellular and humoral immune responses limit the *in vivo* efficiency of these gene carriers (Mack CA *et al.*, 1997; Yang Y *et al.*, 1994). Therefore, co-administration of immunosuppressive agents may increase the adenoviral transduction ability. Such strategy may not be attractive for cancer therapy since the immune response can be utilized for tumor destruction. On the other hand, it was hypothesized that systemic immunity can reduce the toxic effects of these vectors (Bramson JL *et al.*, 1997) which accounts for the first reported death in clinical gene therapy trials (Raper SE *et al.*, 2002). Surprisingly, the pre-immunization of mice bearing cancerous tissues with the null vector increased the mortality rate between high-dose treated animals in comparison with non-immunized animals. Positive results (less toxic effects), however, were observed for moderate dosing (Vlachaki MT *et al.*, 2002). It was suggested that adenovirus/ antibody immune complex at high doses will induce complement activation which may lead substantially to systemic lethal inflammatory reactions (which does not occur in moderate dosing).

Similar to retrovirus vectors, conditionally replicative adenoviruses were also successfully developed for selective cancer gene therapy (Heise C *et al.*, 2000; Rodriguez

R *et al.*, 1997). In one study, adenoviruses were developed to replicate selectively in *wt p53*- deficient tumor cells. This was achieved by gene deletion of the *E1B* viral protein which binds naturally to *wt p53* allowing viral propagation in *wt p53* cells (Bischoff JR *et al.*, 1996).

1.3.1.3. Herpes simplex virus (HSV):

HSV family naturally infects humans in the eye or the oral and vaginal mucosa causing lytic curable effects. During their life cycle, they infect the sensory nerve ending and migrate to the neuronal cells resulting in a latent infection (Corey L, and Spear PG 1986). This feature was utilized to deliver genes effectively to brain tumors (Parker JN *et al.*, 2000). HSV vectors are produced by eliminating the sequence of some of the viral proteins expressed early in the infection such as *ICP0*, *ICP4*, *ICP27* and *ICP22* (Wu N *et al.*, 1996; Marconi P *et al.*, 1996). These proteins can trigger the production of other essential viral components.

The large linear double strand genome of HSV virus (about 150 kb), which is almost 15 and 4 times bigger than that of lentiviruses and adenoviruses respectively, can be replaced by almost 40 kb of foreign genes, ranking at the top of viral vectors' capacity (Latchman DS 2001). This capacity was utilized successfully to simultaneously deliver multiple genes using one vector. Despite that both HSV-tk and CD suicide genes, for example, were delivered successfully to malignant glioma *in vivo* using single HSV vector, their single treatments were superior to the combined delivery (Moriuchi S *et al.*, 2002).

On the other hand, both the original pathologic and latent infectious nature of these viruses can limit their therapeutic applications. In the case of cancer gene therapy, latency is not a major concern since temporary active gene expression can produce the desired destruction effects on cancer cells.

1.3.1.4. Adeno- associated virus (AAV):

AAVs are single stranded DNA viruses. They encode for two viral proteins namely *Rep* and *Cap* which are removed in the defective vectors used in gene therapy (Jain KK 1998). Similar to adenoviruses, AAVs can infect both dividing and non-dividing cells. Their DNA, however, integrates into the host cell genome in a similar way to the retroviruses. AAV vectors cause little toxicity since their wild type version does not cause any pathologic effects in humans and they integrate specifically into chromosome 19 of the human genome (Samulski RJ *et al.*, 1991). Such specificity will reduce the risks of insertional mutagenesis encountered in retroviral mediated gene transfer. In addition, the site of integration does not encode any important gene.

The main drawback in this system, however, is the need for helper viruses (adenoviruses or HSV) for AAV production (Buller RM *et al.*, 1981; Janik JE *et al.*, 1989). This may result in contaminated AAV vectors during preparation. This disadvantage was overcome by inducing viral replication through genotoxic stimuli such as heat shock, chemicals or irradiation (Yakinoglu AO *et al.*, 1988). In fact, AAV mediated gene transduction was significantly enhanced when combined with UV and gamma radiation treatments (Alexander IE *et al.*, 1996; Alexander IE *et al* 1994). In

addition to replication difficulties, the capacity of these vectors is very limited (less than 5 kb of DNA) (Cusack JC Jr, and Tanabe KK 2002).

Despite that AAV vectors were used in cancer gene therapy (Ponnazhagan S *et al.*, 2001), it has been shown that other viral systems such as adenoviruses possess better transfection ability (Vermeij J *et al.*, 2001).

1.3.1.5. Pox virus (Vaccina Virus):

These viruses were used as vaccines which eradicated smallpox virus worldwide. They are double stranded DNA viruses that can infect both dividing and non-dividing cells. Similar to HSV, they have a large genome (about 186 kb) such that they can accommodate up to 25 kb transgenic sequence (Smith GL and Moss B 1983).

Because of the landmark success in recombinant vaccination via poxviruses, which can induce T-cell mediated immune reaction against infectious and malignant diseases (Gomella LG *et al.*, 2001; Paoletti E 1996), they were successfully tested for cytokine gene delivery against cancer in preclinical studies (Qin H *et al.*, 2001; Peplinski GR *et al.*, 1995). Such strategy can produce synergistic immunological effects. Moreover, the anti-tumor effects and survival rates between tumor bearing mice were significantly enhanced when *IL-2* and *IL-12* were delivered simultaneously using single vaccina viral vector along with the tumor antigen (Kaufman HL *et al.*, 2002). The previous study also demonstrates the feasibility of delivering multiple genes using single vaccina viral carrier. This feature in viral-mediated gene delivery systems is only possible with vaccina and HSV vectors.

The long history in the usage of these vectors in vaccination, their low toxicity, and high capacity for foreign DNA make them excellent carriers for gene delivery.

1.3.2. Non- biological gene delivery systems (non-viral vectors):

All non-viral systems are cationic in nature. They interact with negatively charged DNA through electrostatic interactions. The total charge, however, should maintain a net positive value. This will enable the carrier of efficiently interacting with the negatively charged cell membranes and internalizes into the cell which occurs mainly through the endocytosis pathway (behr JP 1994).

1.3.2.1. Cationic polymers:

This group of gene carriers includes any synthetic cationic polymer (at physiological pH) that can be combined with DNA to form a particulate complex, polyplex, capable of gene transfer into the targeted cells. Since they are synthetic compounds, many modifications such as molecular weight and ligand attachment can be easily achieved. The most widely studied polymers for gene therapy include poly-L-lysine (PLL) and poly(ethylenimine) (PEI).

1.3.2.1.1. Poly(ethylenimine) (PEI):

PEI was used in gene delivery more recently than PLL. It is usually branched with every third amino nitrogen atom being protonated such that PLL has a buffer capacity virtually at any pH value (*proton sponge*) (Boussif O *et al.*, 1995). This feature and PEI's ability to destabilize Lysosomal membranes enables PEI polyplexes to

efficiently escape the degradation within the acidic endosomal environment (Kichler A *et al.*, 2001; Klemm AR *et al.*, 1998).

Many factors affect the efficiency/ cytotoxicity profile of PEI polyplexes (and almost any non-viral vector) such as molecular weight, degree of branching, ionic strength of the solution, zeta potential and particle size (Kunath K *et al.*, 2003; Kircheis R *et al.*, 1999). One study, for instance, showed that low molecular weight (10 kDa), moderately branched polymer resulted in efficient delivery with low toxicity in comparison with commercial high molecular weight PEI (Fischer D *et al.*, 1999). Another study demonstrated that linear PEI (22 kDa) was more efficient in both salt and salt-free buffers than branched polymers (25 and 800 kDa) (Wightman L *et al.*, 2001). Lethal side effects in mice, however, were observed when the linear PEI (22 kDa) polyplex was injected intravenously within its therapeutic window (Chollet P *et al.*, 2002). Therefore, more studies are needed to produce optimum PEI carriers with respect to efficiency/ toxicity behavior.

The nature of these polymers enables the researchers to successfully introduce targeting ligands and/ or polyethylene glycol (PEG) (that produces sterically stabilized gene carriers) to their surfaces. Pegylated PEI polyplexes, for instance, were linked to tumor specific ligand transferrin, an asialoglycoprotein, and then applied intravenously, resulting in five fold increase in the transfection efficiency with lower toxicity in comparison with pegylated (transferrin-free) PEI polyplexes (Kircheis R *et al.*, 1999).

1.3.2.1.2. Poly-L-lysine (PLL):

PLL is one of the first cationic polymers employed for gene transfer (Wu GY, and Wu CH 1987). They are linear polypeptides with the amino acid lysine as the repeat unit; thus, they posses a biodegradable nature. This property is very useful for *in vivo* applications. PLL polyplexes are, however, rapidly bound to plasma proteins and cleared from the circulation (Ward CM *et al.*, 2001; Dash PR *et al.*, 1999). In addition, successful transfection requires co-application of chloroquine, a lysosomotropic agent, which reduces the lysosomal degradation of lipoplexes (Pouton CW *et al.*, 1998; Shewring L *et al.*, 1997). The exact mechanism in which chloroquine acts is not well understood. Chloroquine can be substituted with fusogenic peptides which undergo pH-related conformational changes perturbing the lysosomal/ endosomal membranes, thus the DNA is successfully delivered into the cell cytoplasm (Lee H *et al.*, 2002; Wagner E *et al.*, 1992). In fact, PLL has poor transfection ability when applied alone or without modifications (Brown MD *et al.*, 2000; Pouton CW *et al.*, 1998). One popular modification that can increase both the transfection ability and the circulation half-life of these vectors is coating with PEG (Ward CM *et al.*, 2002; Lee H *et al.*, 2002). Targeting ligands were also linked to the polymer chain (even in early studies) resulting in enhanced transfection (Suh W *et al.*, 2001; Wu GY, and Wu CH 1987). A common efficient strategy implies the addition of both PEG and a targeting ligand to PLL polymer to optimize transfection (Faraasen S *et al.*, 2003; Nah JW *et al.*, 2002). Another approach can create the desirable '*proton sponge*' effect similar to that of PEI polyplexes by introducing histidine residues to PLL backbone (Pichon C *et al.*, 2001). Histidylated PLL

showed better transfection efficiency than PLL/ chloroquine mixture (Midoux P and Monsigny M 1999).

Many PLL polymers with different molecular weights were tested and evaluated for gene transfer (Mannisto M *et al.*, 2002; Ward CM *et al.*, 2001; Nishikawa M *et al.*, 1998). It has been shown that DNA condensation and transfection efficiency increased with high molecular weight PLL, which was also associated with undesirable high toxicity (Wolfert MA et al., 1999). The creation of amphiphilic PLL, by linking both PEG and palmitoyl groups to the polymer, reduced toxicity without compromising the gene delivery efficiency (Brown MD *et al.*, 2000).

1.3.2.1.3. Other polymeric delivery systems:

Many other cationic polymers such as chitosans (a biodegradable linear aminopolysacharides) (Koping-Hoggard M *et al.*, 2003; Lee M *et al.*, 2001) and dendimers (highly branched polyamidoamine) (Vincent L *et al.*, 2003; Bielinska AU *et al.*, 2000) were tested for gene transfer. A novel polycations known as reducible polycations (RPC) prepared by oxidative polycondensation of the peptide Cys-Lys_{10}-Cys resulted in enhanced transfection in comparison with PLL (Read ML *et al.*, 2003). It is believed that cellular reduction of disulfide bonds of these vectors will facilitate gene delivery and reduce cytotoxicity. Despite the low number of clinical trials which utilize polymer-mediated gene transfer in gene therapy, it is expected that this area will continue to grow and expand in the near future.

1.3.2.2. Cationic peptides:

Cationic peptides employed for gene transfer are amphiphilic peptides which can undergo conformational changes in acidic environments escaping the endosomal /lysosomal pathways. They contain the positively charged amino acids (i.e. histdine, lysine, and/ or arginine) such that they can effectively condense DNA. The α-helical KALA peptide (derived from the influenza HA-2 subunit which enables the virus to infuse into the cell membrane) is one of the early cationic peptides used successfully for gene delivery in cultured cells (Wyman TB *et al.*, 1997). Despite that 7 positively charged amino acids (lysine) exist in the (30 AA) KALA peptide, it was demonstrated that only 4 cationic AA (arginine) in other α-helical peptide (total 16 AA) were sufficient to condense DNA and deliver it to the cytoplasm (Niidome T *et al.*, 1999). The efficiency of the peptide vector will also depend on the hydrophobic portion that plays a major role in aggregation and endosomal escape (Haines AM *et al.*, 2001; Ohmori N *et al.*, 1998). The relationship between peptide aggregation and efficient gene delivery is not well understood. DNA release into the cytoplasm can also be enhanced by the introduction of cysteine moieties into the peptide backbone, resulting in the formation of reducible disulfide bonds within the DNA/peptide complex (McKenzie DL *et al.*, 2000). The reduction occurs after the internalization of the delivery complex.

Similar to other vectors, receptor-mediated gene transfer can be achieved through ligand attachment (Niidome T *et al.*, 2000).

Peptide gene carriers have been mainly explored *in vitro* (Kim HH *et al.*, 2003; Niidome T *et al.*, 1999; Plank C *et al.*, 1999; Wyman TB *et al.*, 1997). Their *in vivo* behavior is still under investigation. In a recent study, transfection with peptide vectors

by intravenous administration in mice was reported and occurred mainly in the lungs. It was, however, 10 to 40 folds less efficient than liposomes and PEI vectors (Rittner K *et al.*, 2002).

1.3.2.3. Cationic Lipids (liposomes):

Since their introduction as gene carriers in 1987 (Felgner PL *et al.*, 1987), liposomes have become one of the most studied non-viral vectors. They include a group of positively charged lipids at the physiological pH. As with other non-viral vectors, they interact with the negatively charged DNA through electrostatic attractions. Cationic lipids were used mainly in the form of liposomes. More recently, however, cationic lipid emulsions have been described and evaluated as possible non-viral gene carriers (Yi SW et al., 2000; Choi BY *et al.*, 2002).

1.3.2.3.1. Liposoms and liposomes/ DNA complex structures:

Cationic lipids are amphiphilic molecules composed of one or two fatty acid side chains (acyl) or alkyl, a linker, and hydrophilic amino group. Figure 1 shows an example of cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) which consists of two unsaturated diacyl side chains (oleoyl), ester linker and propyl ammonium group (Lasic DD 1997). Other lipids may contain different linkers such as the more stable ether linkages e.g. DOTMA (N [1-(2,3-dioleyloxy) propyl] –N, N, N-trimethylammonium chloride) (Felgner PL *et al.*, 1987). The hydrophobic part can also be cholesterol-derived moieties (Gao H, and Hui KM 2001). Multivalent cationic lipids (which can condense DNA more efficiently than monovalent lipids) were synthesized and successfully

evaluated for liposomal-mediated gene transfer (Ewert K *et al.*, 2002; Behr JP *et al.*, 1989).

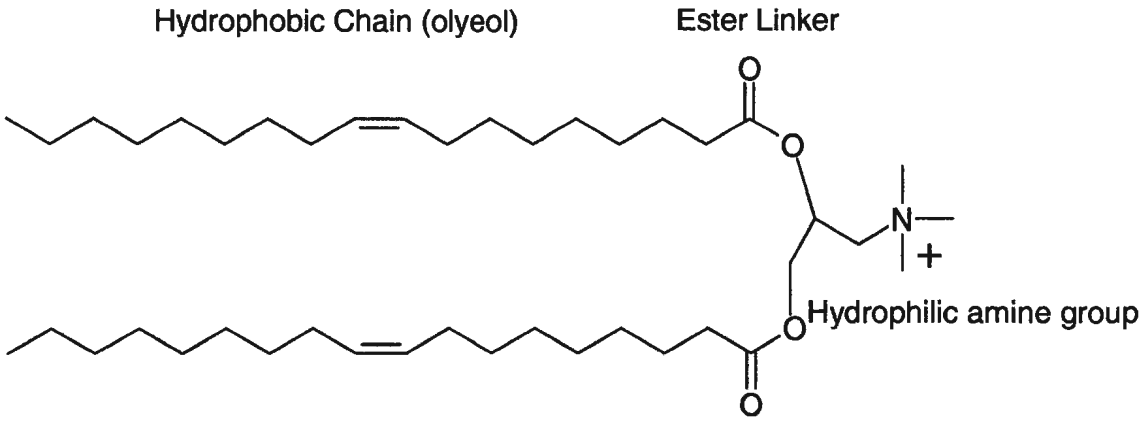


Figure 1: Schematic representation of the cationic lipid DOTAP. The hydrophobic part (olyel), the ester linker, and the hydrophilic portion are presented. Most other cationic lipids share the same general structure.

In aqueous media, cationic lipids are assembled into a bilayer vesicular-like structure (liposomes). Liposomes are first arranged into multilamellar vesicles. Unilamellar vesicles can then be obtained by sonication (Huang CH 1969), detergent removal (Jiskoot W *et al.*, 1986) or extrusion through porous membranes (Hope MJ *et al.*, 1985; Mayer LD *et al.*, 1986). The liposomes/DNA complex is usually termed a lipoplex. Based on freeze-fracture electron micrographs and x-ray diffraction studies, it was suggested that DNA is sandwiched between many liposomal particles (Radler JO *et al.*, 1997; Sternberg B *et al.*, 1994). This structure is in agreement with the increase of particle size after the addition of DNA to cationic liposomes (Almofti MR *et al.*, 2003b). Negatively-charged DNA will neutralize cationic liposomes resulting in aggregation and

continuous fusion with time while DNA being entrapped during this process. Because of poor stability (i.e. continuous aggregation), lipoplexes are usually administered directly after their formation. Many physical factors influence stability, complex formation and transfection efficiency of lipoplexes such as particle size, zeta potential, DNA/ liposomes ratio and ionic strength of the medium (Almofti MR *et al.*, 2003b; Pedroso de Lima MC *et al.*, 2001; Ross PC *et al.*, 1999). Producing the favorable, stable, small lipoplex particles was obtained with the development of the novel liposomal formulation LPD (liposomes/ Protamine/ DNA) which will be discussed later.

1.3.2.3.2. Structure/ activity relationship:

Despite the numerous studies that focus on the possible relationship between cationic lipid structure/ composition and lipoplex transfection activity, solid conclusions are rarely obtained. This is mainly due to the complex factors affecting gene transfer, even different cell lines, for example, will produce different behavior with the same lipoplex formulations (Zou Y *et al.*, 2000; Balasubramaniam RP *et al.*, 1996). More important, the correlation between *in vitro* and *in vivo* experiments is not always obtained (Gorman CM *et al.*, 1997; Lee ER *et al*, 1996; Solodin I et al., 1995). Therefore, it is expected that empirical findings will remain the main source for structure/ activity relationships.

It has, however, been proven that the addition of neutral lipids (colipids) will increase the transfection ability of lipoplexes both *in vitro* and *in vivo*. The most common colipids are cholesterol (Chol) (Liu Y *et al.*, 1997; Bennett MJ *et al.*, 1995) and dioleylphosphatidylethanolamine (DOPE) (Hui SW *et al.*, 1996; Farhood H *et al.*, 1995).

While the former is more efficient *in vivo*, the later enhances *in vitro* lipoplex transfection (Simberg D et al., 2001; Li S *et al.*, 1998; Wang J *et al.*, 1998; Hong K *et al.*, 1997). It was demonstrated that lipoplexes enter cells through endocytosis and fusion pathways (Almofti MR *et al.*, 2003b; Farhood H *et al.*, 1995). Neutral lipids facilitate conformational changes from a bilayer structure into hexagonal arrangements at the endosomal level (Hafez IM et al., 2001; Litzinger DC, and Huang L 1992). This change will trigger the release of the encapsulated DNA into the cytoplasm before reaching the destructive lysosomal environment.

1.3.2.3.3. Liposomes/ Protamine/ DNA (LPD):

Protamine is an arginine-rich peptide which can condense negatively-charged DNA before being complexed with cationic lipids. The polycation PLL (which is less efficient) was also tested for LPD preparation in early studies (Gao X and Huang L 1996). Liposomes will interact with condensed DNA resulting in lipid rearrangement and the formation of compact liposomes/ DNA complex (LPD) (Li S and Huang L 1999). Figure 2 compares proposed complex structures between LPD and conventional lipoplexes.

DOTAP and DC-Chol (3β [N-(N′, N′ -dimethylaminoethane) carbamoyl] cholesterol) were used for the preparation of LPD with the most common co-lipids: cholesterol and DOPE respectively (Tan Y *et al.*, 2002). Particle size distribution of LPD ranged from 100-250 nm which is almost three to five times less than conventional lipoplexes (Ueno NT *et al.*, 2002; Sorgi FL *et al.*, 1997; Gao X and Huang L 1996).

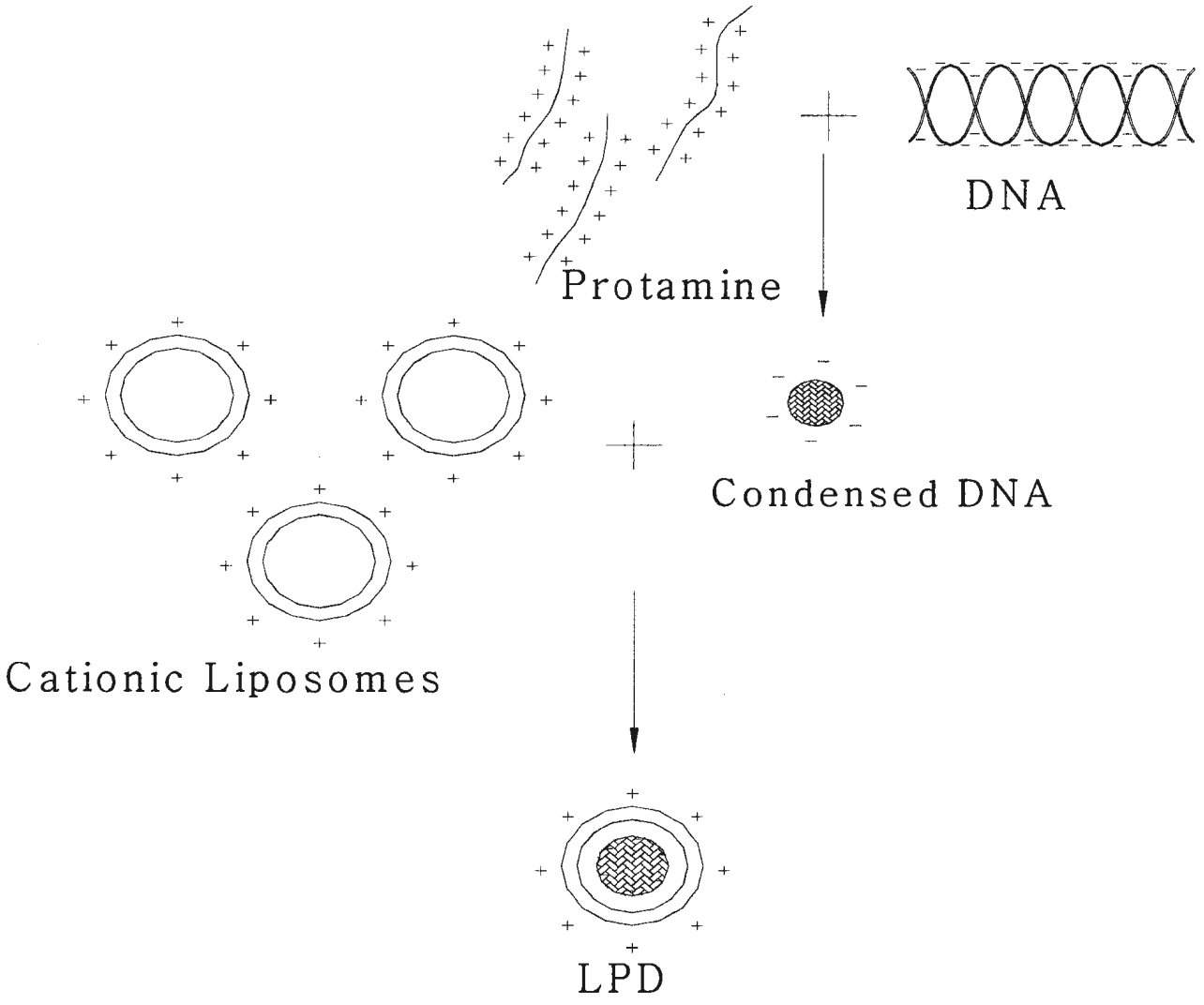


Fig.2A

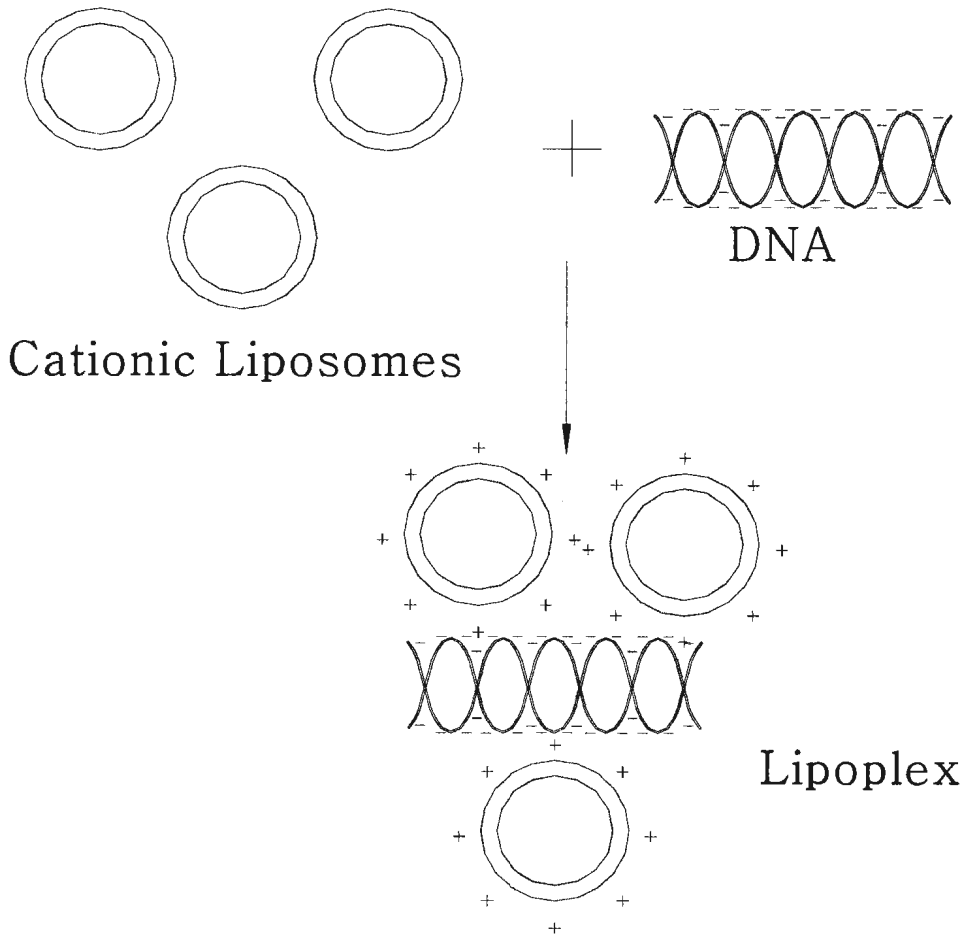


Fig.2B

Figure 2: Schematic comparison between LPD and lipoplex particles. In Fig.2A: DNA is nutrlaized and condensed with the protamine before the formation of the complex LPD. In Fig.2B: DNA is sandwiched between liposomal particles forming the 'conventional' lipoplex.

Stability with no compromise in the transfection ability was maintained for 4 months when LPD was stored at 4°C or at room temperature after lyophylization (freeze-drying or spray-drying) (Seville PC *et al.*, 2002; Li B *et al.*, 2000). Both *in vitro* and *in vivo* studies showed superiority of LPD mediated gene transfer over conventional liposomes (Li S *et al.*, 1998; Sorgi FL *et al.*, 1997). It is believed that the small size of LPD will facilitate endocytosis and increase the in vivo circulating half life. Like other non-modified liposomes, LPD tends to accumulate preferably in the lungs after injection from the tail vein of a mouse (Li S *et al.*, 1998; Li S and Huang L 1997). In addition to the lungs, reporter gene expression, however, was also detected in other organs such as the kidney, the spleen and the liver (Li S *et al.*, 1998; Li S and Huang L 1997). Reporter genes encode usually for readily detected proteins such that the transfection ability can be easily monitored and evaluated.

For therapeutic applications, iv administration of DOTAP: Chol LPD carrying tumor suppressor genes *Rb* or *E1A* in cancer animal models resulted in apoptosis induction, tumor size reduction, and life span increase in the treated animals (Ueno NT *et al.*, 2002; Nikitin AY *et al.*, 1999). As expected, antitumoral synergistic effects were obtained when *E1A* LPD treatment was combined with the chemotherapeutic agent paclitaxel (Ueno NT *et al.*, 2002). These studies demonstrate the possibility of effective IV gene carrier administration, whereas intratumoral application (or as close as possible to the tumor) are more effective in most studies of various gene delivery systems. LPD composed of DC-Chol/ DOPE was also tested in clinical settings when two children with Canavan disease (a fatal CNS disease characterised by spongy degradation of cerebral

white matter) were treated with *ASPA* gene via intracerebral application; both subjects showed some clinical improvements (Leone P *et al.*, 2000)

1.3.2.3.4. Targeted liposomes:

The same strategies applied at anionic liposomes to develop tissue-specific formulations are also encountered in targeted cationic liposomes. More emphasis on tumor-specific liposomes and liposomes/ ligand attachment techniques are discussed below. The two main strategies in developing targeted liposomes are achieved by attaching monoclonal antibody (mAb) (i.e. immunoliposomes) or tissue specific ligand to the surface of the liposomes.

1.3.2.3.4.1. Immunoliposomes:

Antibodies are soluble proteins produced by B cells of the immune system to bind specifically to the antigens mediating its destruction directly or with the help of other immune system components. They consists of two major parts, Fab (fragment antigen binding) fragments responsible for antigen recognition and Fc (fragment crystallizable) fragments which play a role in the biological activity (Benjamini E *et al.*, 2000).

Immunoliposomes are widely studied because of their relative ease of preparation and high specificity. Antibodies or Fab fragments were first linked to liposomes in early eighties by attaching them directly to the lipids (Leserman LD *et al.*, 1980; Heath TD *et al.*, 1980). Because of their short half lives, immunoliposomes are mostly used in long circulating peglated liposomes (Bendas G 2001). There are two methods in attaching antibodies to the surface of the peglated vesicles, either through the terminal end of PEG

chain (Shi N *et al.*, 2001; Allen TM *et al.*, 1995) or directly to the lipids (Klibanov AL *et al.*, 1991). The former attachment methodology is extensively used and favored since the ligand will be away from the liposomal surface providing easy access to the antibodies. PEG chains will cause steric barriers when the mAb or Fab is attached to the lipids. It has been shown, for instance, that PEG 2000 will mask the lipid-linked antibody less than the longer PEG 5000 (Mori A *et al.*, 1991). In addition, a comparison between PEG-linked and lipid-linked antibodies showed that efficient coupling was better achieved with the PEG chains (Hansen CB *et al.*, 1995). While the coupling reaction to PEG is usually proceeded after the preparation of the liposomes, anchor lipid molecules are generally attached first to the antibody before being assembled into the liposoaml structure during preparation (in case of direct linkage of the antibody to the lipids). On the other hand, a novel simple preparation method for immunoliposomes were developed by transferring the lipid conjugated mAb or Fab micelles to preformed, drug loaded liposomes under specified conditions of temperature and pH (Allen TM *et al.*, 2002; Iden DL, and Allen TM 2001; Ishida T *et al.*, 1999). This method is referred to as post insertion technique.

In cancer gene therapy, gene expression in tumors was significantly enhanced with immunoliposomes technology compared to conventional liposomes (Lee CH *et al.*, 2003; Kao GY *et al.*, 1996). Life span of mice bearing aggressive brain tumor was, for instance, increased by 100% after treatment with epidermal growth factor receptor (EGFR) antisense mRNA delivered by intravenous injection of immunoliposomes (Zhang Y *et al.*, 2002).

In all the above studies, antibodies were covalently linked to the liposomal surface. Non covalent linkages were, however, used by simple mixing of the antibody with the liposomal vesicles resulting in two to four fold increases in the transfection efficiency of the reporter gene in glioma cell line (Yoshida J and Mizuno M 1995; Mizuno M, and Yoshida J 1996). More efficient non-covalent linkages were obtained through avidin-biotin binding. Biotinated lipids were bound to strepavidin (contains four biotin binding sites) which was then attached to biotinated mAb by simple incubation (Hansen CB *et al.*, 1995; Loughrey H *et al.*, 1987).

Immunogenicity is the main concern associated with immunoliposomes applications. This drawback was minimized by the usage of Fab subunits (instead of the whole antibodies) or the fully humanized mAb produced first in the mid eighties (Verhoeyen M *et al.*, 1988; Jones PT *et al.*, 1986). The linkage techniques of Fab fragments are identical to those applied on the complete mAb, covalently (Lee CH *et al.*, 2003; Heath TD *et al.*, 1980) or non-covalently (Mizuno M, and Yoshida J 1996; Loughrey H *et al.*, 1987). These liposomes/ ligand attachment methods are also applicable on all other peptides and proteins ligands.

1.3.2.3.4.2. Ligand targeted liposomes:

This group of targeted liposomes has the advantage of low immunogenicity in comparison with immunoliposomes. Ligands vary according to the targeted tissues. One popular target is the liver associated with many genetically based diseases such as hemophilia, lipoprotein receptor deficiency and α 1-antitrypsin deficiency as well as liver cancer. Many receptors are expressed specifically on the surface of this organ namely

low density lipoprotein (LDL) and asialoglycoprotein receptors. Therefore, the discussion in this section will focus on liver targeted liposomes. The main challenge for successful liver targeting is to reach the parenchymal cells rather than being captured by kupffer cells.

Asialoglycoprotein receptors (ASGP-R) are very specifically abundant on the mammalian parenchymal liver cells. Their major role is to clear glycoproteins and lipoproteins from the circulation. The receptor contains carbohydrate recognition domain which can bind to galactose derivatives (Wu J *et al.*, 2002).

Asialofetuin (AF) is a natural ligand for ASGP-R. It is a glycoprotein with several terminal galactose sugar chains (Spiro RG 1973). It was effectively incorporated into the liposomal surface by covalently attaching a hydrophobic moiety (palmitic acid) as an anchor among the lipids of the liposomal vesicles (AF-liposomes) (Hara T *et al.*, 1988; Tsuchiya S et al., 1986). AF-liposomal uptake by the liver in mice was increased 11 times in comparison with non modified liposomes (Wu J *et al.*, 1998). Similarly, AF-liposomal mediated transfection of the human alpha antitrypsin (hAAT) gene was significantly enhanced in comparison with regular liposomes. After one year of the treatment, hAAT mRNA in the liver was detected in all animals transfected with AF-liposomes versus only 25 % with those treated with regular liposomes with more than 4000 fold increase in case of AF-liposomes condition (Dasi F *et al.*, 2001). In this study, AF was covalently linked to an anchor lipid on the surface of preformed liposomes. AF, however, can induce immunogenic reaction. Therefore, simpler glycosylated liposomes were developed and evaluated for liver targeting (Kawakami S *et al.*, 2002). Glycosylated cholesterol, for example, was synthesized and incorporated into the cationic

liposomal vesicles resulting in 10-fold increase of gene expression in the liver (Kawakami S *et al.*, 2000).

Liver cancer is another important target for gene delivery. HCC (hepatocellular carcinoma) is a leading cause for cancer related deaths worldwide (Bosch FX 1997). Gene therapy can provide a new approach to treat this fatal disease. In addition to the different specific receptors on the hepatocyte cells, there are some receptors which are over-expressed in hepatoma cells. One example is transferrin (TF) receptors which are also elevated in other malignant cells (Keer HN *et al.*, 1990). TF-liposomes will not only target the cancerous cells but it will also reduce non-desired transfection levels in the surrounding normal tissues. This feature can be exaggerated by hepatic arterial injection of TF-liposomes mediating DNA delivery (Seol JG *et al.*, 2000). TF-liposomes complexes are usually prepared through charge-charge interactions by simple mixing and incubation (Li X *et al.*, 2003; Seki M *et al.*, 2002; Seol JG *et al.*, 2000). Tumor growth was inhibited up to 70% in liver tumor xenografts after treatment with TF-liposomes containing the antiangiogenesis gene, endostatin (Li X *et al.*, 2003). Linkages to the PEG terminal end of pegylated liposomes were also used for TF-liposome preparations (Derycke AS, and De Witte PA 2002; Iinuma H *et al.*, 2002).

1.4. Rational, research hypothesis, and objectives:

Despite the many advantages of cationic liposomes as gene carriers over the viral vectors in terms of safety and unlimited DNA size, they have natural affinity towards the lungs (McLean JW *et al.*, 1997; Solodin I *et al.*, 1995). In this study, we investigated the possibility of coating cationic liposomes with a liver targeting ligand. We aimed to

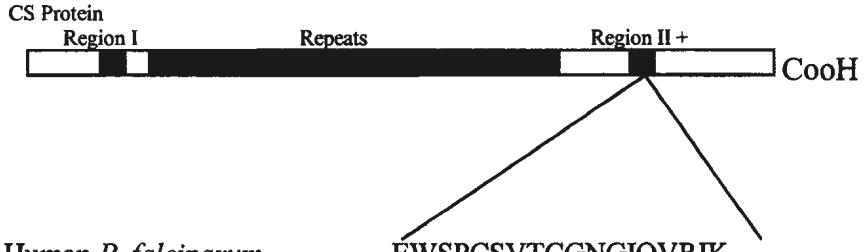
deliver the apoptotic gene *p53* specifically to liver cancer cells. Traditional therapies such as surgery, radiotherapy, chemotherapy, and/or liver transplantation have shown limited success on the survival rates of HCC patients (Okuda K *et al.*, 1984). Tumor suppressor gene *p53* can induce apoptosis and/or cell cycle arrest in cancerous cells lacking the *wt p53* (Zou Y *et al.*, 1998; Anderson SC *et al.*, 1998). In liver cancer, mutational alterations occur in about 30-50% of the *p53* gene (Greenblatt MS *et al.*, 1994; Tabor E 1994), thus successful transfection of the *p53* gene to hepatic cells may have therapeutic effects.

Our targeting ligand was derived from the malaria circumsporozoite (CS) surface protein. It has been illustrated that CS protein is responsible for directing the malaria parasite within minutes towards the liver after mosquito bite. The injection of CS protein into mice, for instance, resulted in direct homing of the protein into the liver (Cerami C *et al.*, 1994). The protein also plays important role in the development of the sporozoite inside the cells (Frevert U *et al.*, 1998).

CS protein of the human *Plasmodium falciparum* (*P. falciparum*), for example, consists basically of three segments, the N-terminus region (containing region I peptide), repeat domain (37 NANP and 4 NVDP) and C-terminus region (containing region II+ peptide) (Dame JB *et al.*, 1984) (Fig. 3). It has been proven that the two conserved peptide sequences (region I and region II +) in the CS protein play a critical role in receptor recognition on the surface of the hepatic cells (Ying P *et al.*, 1997; Chatterjee S *et al.*, 1995; Aley SB *et al.*, 1986). In our study, we explored the possibility of utilizing region II + peptide for developing liver specific liposomal formulation.

1.4.1. Malaria region II + peptide:

Region II+ peptide is located on the carboxyl terminus region of CS protein. It consists of 18 amino acids conserved among rodent, monkey and human malaria parasites (see fig. 3). Our ligand was derived from the human *P. falciparum* CS protein. Region II+ initiates the binding of CS protein by interacting with heparan sulfate proteoglycans on the cell surface (Frevert U *et al.*, 1993; Ying P *et al.*, 1997). It has been shown that region II+ can totally inhibit CS protein from selective binding to hepatic cells (Sinnis P *et al.*, 1994; Cerami C *et al.*, 1992). The peptide has hydrophobic motif at its N-terminus EWSPCSVTCG with two cysteins. The sequence CSVTCG mediates cell adhesion and it is identical to adhesion domain of thrombospondin, a modular adhesive glycoprotein (Prater CA *et al.*, 1991; Lawler J and Hynes RO 1987).



Human *P. falciparum*	EWSPCSVTCGNGIQVRIK
Human *P. vivax*	EWTPCSVTCGVGVRVRRR
Monkey *P. brasilianum*	EWSPCSVTCGSGIRARRK
Monkey *P. knowwlesi*	EWTPCSVTCGNGVRIRRK
Monkey *P. malariae*	EWSPCSVTCGSGIRARRK
Monkey *P. cynomolgi*	EWSPCSVTCGKRVRMRRK
Rodent *P. yoelii*	EWSQCSVTCGSGVRVRKR
Rodent *P. berghei*	EWSQCNVTCGSGIRVRKR
Avian *P. gallinaceum*	EWTNCNVTCGKGIQAKIK

Figure 3: Schematic representation of CS protein and region II +peptide between different malaria species. Amino acids that varies from the human *P. Falciparum* used in this study are bolded.

The amino acid sequences were obtained from the following sources:
P. falciparum (Caspers P *et al.*, 1989; de la Cruz VF *et al.*, 1987); *P. vivax* (McCutchan TF *et al.*, 1985); *P. brasilianum* (Lal AA *et al.*, 1988b); *P. knowwlesi* (Godson GN *et al.*, 1983); *P. malariae* (Lal AA *et al.*, 1988a); *P. yoelii* (Lal AA *et al.*, 1987); *P. berghei* (Eichinger DJ *et al.*, 1986); *P. cynomolgi* (Galinski MR *et al.*, 1987); *P. gallinaceum* (McCutchan TF *et al.*, 1996).

1.4.2. Modifications on malaria region II+ peptide:

Fatty acid chains namely palmitate and myristate were linked to the N-terminus of region II + peptide. These fatty acids will serve as an anchor between the fatty acyl chains of the liposomes. This strategy was adopted for protein and glycoprotein ligand incorporation into the liposomal surface for selective targeting (Sliedregt LA *et al.*, 1999; Wu J *et al.*, 1998; Hughes BJ *et al.*, 1989; Huang A *et al.*, 1980).

Two modified fatty acid linked region II+ peptides were used in this study to be incorporated into the lipid membranes of the liposomes. These peptides will be refered to as peptide I and peptide II (See fig. 4). Peptide I was linked to palmitic acid at its N-terminus, while peptide II was linked to myrstic acid. Peptide I has an extension of 5 amino acids derived from the original CS protein sequence at its C-terminus. We used the segment described by Cerami C *et al.*, (1992) for specific targeting. It was shown later that this extension is not essential for the binding specificity of region II+ peptide (Sinnis P *et al.*, 1994). Therefore, we tested the extension at the N-terminus for peptide II. The extension at the N-terminus was also derived from CS protein original sequence (de la Cruz VF *et al.*, 1987). The later strategy may provide a spacer between the lipid layers and the ligand such that the ligand will be more accessible for binding. Tyrosine was added at the N-terminus of peptide I versus C-terminal of peptide II. Tyrosine will enable the radiolabeling of the peptide with ^{125}I for detection and quantification purposes.

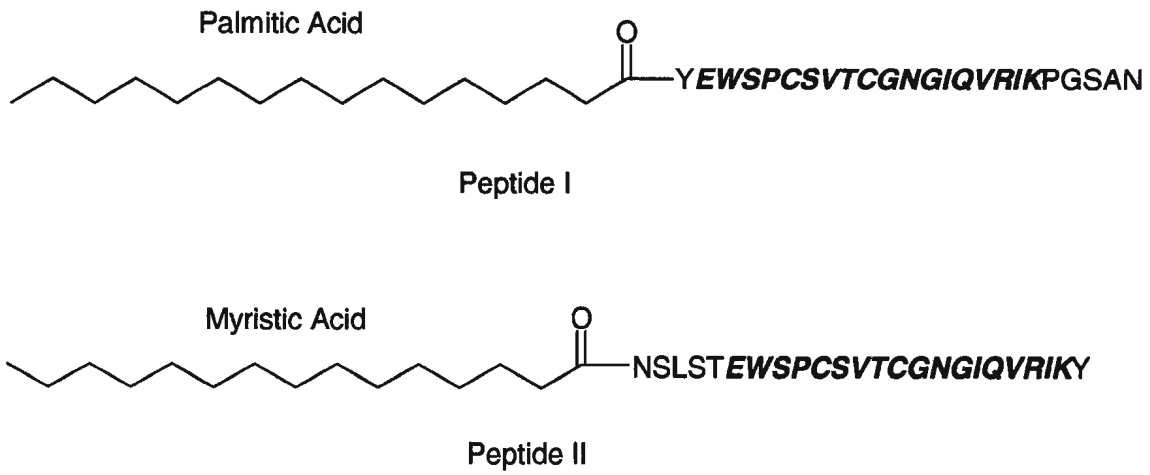


Figure 4: Structures of peptides I and II of malaria CS region II+ peptide used in this study.

1.4.3. Statement of research objective:

The aim of this study is to coat the novel cationic liposomal formulation LPD (bearing the *p53* gene for liver cancer treatment) with the malaria region II+ peptide for hepatocyte selective targeting. We investigated the feasibility of *in vitro* liver targeting as prestudy for any possible future *in vivo* investigations.

2. Materials and methods

2.1. Chemicals:

All chemicals were purchased from Sigma-Aldrish (St. Louis, MO, USA) unless otherwise indicated.

2.2. Tissue culture and the choice of the cell lines:

Hepatoma cell lines Hep G2, McA RH7777 and Hep 3B were used in this study. Both human HepG2 and rat McA RH7777 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% (penicillin /streptomycin), 4 mM L-glutamine, 0.11 g/L sodium pyruvate and 4.5 g/L glucose. Human Hep 3B with integrated hepatitis B virus (Aden *et al.*, 1979) were cultured in minimum essential medium Eagle (EMEM) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 10% fetal bovine serum, and 1% (penicillin/streptomycin). Cells were incubated in humidified incubator at 37° C in 5% CO_2. All cell lines were obtained from American Type Culture Collection (ATCC), Manassas, VA, USA.

The Hep G2 cell line was used in this study since binding affinity of malaria CS protein and region II+ peptide to this hepatoma cell line is well-documented (Rathore D *et al.*, 2002; Suarez JE *et al.*, 2001; Sinnis P *et al.*, 1994; Cerami C *et al.*, 1992). However, the Hep G2 cell line naturally expresses the human *wt p53* protein, and therefore is not a suitable model for *p53* transfection studies. Detection of *p53* in Hep G2 cells after successful transfection with *wt p53* gene will not differ from non-treated cells

since the levels of the protein is controlled within the cells. In contrast, both McA RH 7777 and Hep 3B cell lines do not contain the human *wt p53* in their genome. McA RH 7777 cell line contains mutated rodent *p53* that can not be detected by human monoclonal antibody used in this study (Anderson SC *et al.*, 1998). Human Hep 3B has deletion in *p53* gene (Puisieux A *et al.*, 1993; Bressac B *et al.*, 1990). Therefore, both cell lines were used as model for the *in vitro* transfection experiments.

2.3. Targeting peptide solution:

The two modified forms of region II+ peptide (as discussed earlier) were synthesized according to our suggestion (Genemed Synthesis Inc, San Francisco, CA, USA) with a purity of 80%. Peptides were dissolved in a minimum volume of dimethyl sulfoxide (DMSO) and then diluted with deionized water or dextrose 5% (D5W %) solution such that the final percentage of DMSO did not exceed 2% of the total volume. Under these conditions, no aggregates were observed under microscopic examination (Wilovert®; 100x magnification).

2.4. Preparation of ^{125}I peptide:

Tyrosine was added to the N terminus of peptide I and the C terminus of peptide II to enable us to radiolabel the peptides with ^{125}I for subsequent qualitative analysis of peptide/liposomes complexation and peptide/ HepG2 cells interactions. IODO-BEADS® iodination reagent (Pierce Inc., pockford, IL, USA) was used to perform the labeling as instructed by the manufacturer. The bead was first washed three times with 300 µl of

D5W % solution. It was then dried on filter paper and placed in Eppendorf® tubes (Fisher Scientific, Pittsburg, PA, USA) containing 100 µl (D5W %). Ten to fifteen µl of 65.5 Ci/mmol $Na^{125}I$ (NEN products, Boston, MA, USA) were added to the tube and allowed to react for about 5 min. Peptide solution was then added to the tube and the reaction was allowed to proceed for 5 min. The reaction was stopped by removing the solution from the tube. The beads were washed two time with 200 µl (D5W %). To remove the free iodine, ^{125}I peptide solution was dialyzed against 2 liters of D5W% solution at 4° C using molecular porous dialysis tubes (Spectra/Por mwCo: 2000, Los Angeles, CA, USA). Dialysis solution was changed several times till the radioactivity of 1 ml of the dialysis solution was less than 1000 cpm measured with Gamma counter (1272 Clinigamma LKB Wallac, Turku, Finland). ^{125}I-labeled peptide was stored at 4° C for future use. The specific radio-activity of the peptide is specified for each individual experiment. Peptide concentration was measured by Bio-Rad DC protein assay kit (Hercules, CA, USA), using micro-plate assay.

2.5. Protein assay:

Protein assays were performed by Lowry method (Lowry et al., 1951) using the DC protein assay kit (Bio-Rad, Hercules, CA, USA). Briefly, working reagent A' was formed by combining twenty microliter of surfactant solution (reagent S) with 1 ml of alkaline copper tartarate solution (reagent A). Within the range of 0.2 mg/ml to 1.37 mg/ml, six dilutions of the protein standard (bovine serum albumin BSA 1.37 mg/ml; Bio-Rad) were prepared. Five microliters of standards and samples were pipeted in 96-well microliter plate. Twenty-five microliters of reagent A' were then added to each well

followed by 200 µl of dilute folin reagent (reagent B). Mixtures were allowed to stand at room temperature for almost 15 min. Absorbencies were then measured spectrophotometrically at a wavelength of 630 nm by Microplate reader (Bio-Rad Model 550, Japan). The sensitivity of this assay is 0.2 mg/ml.

For micro-microplate assay (suggested by the manufacturer) with higher sensitivity (5µg/ml), volumes of sample (or standard), reagent A' and reagent B were 20, 10, 80 µl respectively. Reagent S was not used when samples did not contain surface-active ingredient.

2.6 Liposomes preparation:

At a molar ratio of 1:1, DOTAP (Avanti Polar Lipids, Inc., Albaster, AL, USA), a positively charged phospholipid, and cholesterol (Chol) were dissolved in chloroform (HPLC grade). The organic solvent was then evaporated in a round-bottomed flask under vacuum for at least 30 minutes or until a dry lipid film was formed at the bottom of the flask. The lipid film was then hydrated by deionized water or by an aqueous solution of the targeting peptide. In either case, the final concentration of DOTAP was 10 mg/ml in the lipid mixture. The hydration process was then continued by rotating first for 45 minutes at 50° C followed by 10 minutes at 30° C. The mixture was allowed to stand at room temperature overnight under argon atmosphere. Unilamellar vesicles (ULV) were prepared by the extrusion method using a mini extruder (Avanti polar lipids, Inc., Albaster, AL, USA) as described by others (MacDonald RC *et al.*, 1991; Templeton NS *et al.*, 1997). The lipid solution was first sonicated at low frequency for 5 minutes at 50°

C, kept in hot water bath (50° C) for 10 minutes and then sequentially extruded through polycarbonate membrane with the following pore sizes 1, 0.6, 0.2, 0.1 μm. Liposomes were used within 24 hours.

For radioactive experiments, ^{3}H radio-labeled cholesterol (^{3}H-Chol) (NEN products, Boston, MA, USA) was included in a percentage of 0.5 or 1% molar of the total cholesterol used in liposomal preparations.

2.7. Liposomal/ peptide complexes preparation:

Two methods were adapted for the inclusion of the targeting peptide into the liposomal formulas.

a) Liposomes/ peptide complex I: the peptide was added to the preparation after extrusion (the final stage).

b) Liposomes/ peptide complex II: the peptide was incorporated during the hydration step of liposomal preparation. The final concentration of the peptide in liposome solutions was 0.5 or 1 μg/μl.

2.8. Assessment of liposomes/ peptide association:

The technique of sucrose gradient airfuge was applied to test liposomes/ peptide complex formation. Airfugation was performed as described (Rivnay B and Metzger H, 1983) with some modifications. Stock solutions of sucrose (ultracentrifugation grade, Fisher, FairLawn, NJ, USA) 5, 10, 20, 25, 30, 60 % (w/w) were prepared and checked by

refractive index measurements using Abbe-type refractometer (American Optical Inc., Buffalo, NY, USA). Airfuge tubes (ultra-clear 5 x 20 mm, Beckman Instruments Inc., Palo Alto, CA, USA) were weighted before the initiation of the centrifugation such that each pair matched and were within 1-2 mg difference to reduce rotor wobbling during the acceleration and deceleration. Four non-continuous gradients containing 45 µl 25%, 40 µl 20%, 25 µl 10%, and 30 µl 5% of sucrose solutions were prepared unless otherwise indicated. In order to prevent turbulence and solution mixing during gradient preparation, tubes were placed on dry ice then sucrose solutions were sequentially added. This methodology allowed us to prepare six tubes in less than 15 minutes. Gradient was thawed at room temperature and 50 µl of the sample was applied at the bottom with hamilton syringe. Twenty five µl of the samples were first diluted with the same volume of 60% of sucrose solution.

The following Samples were produced: liposomes/ no peptide, peptide/ no liposomes, liposomes/ peptide complex I, and liposomes/ peptide complex II (1µg/µl). The fixed angle rotor A-100/30 designed for Beckman Airfuge used in this study (Beckman ®, Palo Alo, CA, USA).

Since the centrifuge was not designed to run the experiment at 4° C, centrifugation was carried out at room temperature at top speed (approximately 167,000 g) for 60 minutes. Each tube was fractioned 25 µl from the top to the bottom of the tube with minimum insertion of the pipettor tip in the gradient. The last fraction was always less than 25 µl.

One-mole percent of ^{3}H-Chol and 0.4 % (w/w) of ^{125}I peptide (specific activity 30 cpm/ ng) were added to detected liposomes and ligand respectively. Counting was

performed in a Gamma counter (1272 Clinigamma LKB Wallac). ^{3}H was counted in liquid scintillation (LS) counter (Beckman LS 5000 TD, fullerton, CA, USA) with ^{3}H channel set to 0-400 KeV. ^{125}I interference with the ^{3}H count in the LS machine was less than 1% in this experimental design. The only exception was liposomes/ peptide complex II in which suitable correction was established via a standard curve of ^{125} I count in LS vs. the counts in gamma counter to exclude ^{125}I contribution in the sample when LS is used.

Sucrose gradient was run with no samples to check the density gradient pattern.

2.9. Liposomes/ protamine/ DNA (LPD) preparation:

PCMV53 plasmid containing the human wild type *p53* gene (wt *p53*) was used for the preparation of LPD complexes. *p53* plasmid was a gift from Dr. Pater lab, Basic Science, faculty of Medicine, Memorial University. In a ratio that was optimized by others (Li S *et al.*, 1998): 1μg DNA: 0.6 μg protamine sulfate: 12 nmol DOTAP-Chol liposomes., DOTAP-Chol liposomes were first mixed with protamine before adding the plasmid. The mixture was allowed to stand at room temperature for at least 20 minutes before either starting the transfection or adding the targeting peptide.

2.10. LPD/ Peptide complex preparations:

For liposomes/ peptide complexes, two methods were used for the preparation of the LPD/ peptide complexes:

a) The peptide was included in the formulation at the final stage. In this method, various

amounts of the peptide ranging from 0.1-10 μg were added to LPD formulation. The LPD/ peptide complexes were incubated at room temperature for 20 minutes before the initiation of transfection. This complex will be referred to as LPD/ peptide complex I.

b) The peptide was incorporated during the hydration step of liposomal preparation such that peptide concentration was 1 μg/μl. This method resulted in the following ratio of the formulation components: 1 μg DNA: 0.6 μg protamine: 12 nmol DOTAP: Chol (1:1) liposomes: 0.8 μg peptide. This complex will be referred to as LPD/ peptide complex II.

Similar procedure and methodology were used to prepare lipoplex/ peptide complexes (i.e. no protamine).

2.11. Cell transfection:

The MCA RH 7777 rat hepatoma and human hepatoma Hep 3B cell lines were used as a model since human wt *p53* is not expressed in these cell lines. About 3 x 10^3 cells were seeded per well in 24-well plates. After about 48 hours, 80-90% cell confluence was reached. Cells were washed with phosphate buffered saline (PBS) (KCl 2.67 mM, KH_2PO_4 1.47 mM, NaCl 138 mM and $Na_2HPO_4.7H_2O$ 8.10 mM) and then incubated with 200 μl of serum free medium containing LPD or LPD/ peptide complexes. The transfection was terminated after six hours by replacing the transfecting medium with the full serum medium. All transfections were performed at 37° C in the presence of 5 % CO_2.

Protein purification was performed after 48 hours from the initiation of the experiment. Cells were washed twice with ice-cold PBS then lysed with 75 μl extraction

buffer [50 mM Tris.HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% Igepal CA-630, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.1 mg/ml phenylmethylsulfonyle fluoride (PMSF) and 0.01 mg/ml aprotinin] for 30 minutes and centrifuged at 12,000 g at 4° C for 10 min. The supernatant was then transferred to another micro-centrifuge tube and stored at -80° C. Protein concentration in each sample was determined by Bio-Rad DC Lowry protein assay kit.

The expression of *p53* protein was evaluated by western blotting analysis. Equal amounts of lysate (usually 10 µg of proteins) were boiled for 5 min in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (200 mM Tris'HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Samples were then loaded on SDS-PAGE gel and run at 200 Volts using mini-protein II electrophoresis cell (Bio-Rad) until bromophenol blue reach the bottom of the gel (about 45 min). The running gel was composed of 8-12 % acrylamide mix, 375 mM Tris'HCl (pH 8.8), 0.1 SDS, 0.1% ammonium persulfate, and 6 µl of N,N,N',N',- tetramethylethylenediamine (TEMED) (Bio-Rad, Richmond, CA, USA). The staking gel was prepared with 5% acrylamide mix, 125 mM Tris'HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulfate and 5 µl of TEMED.

After electrophoresis, the gel was incubated in Towbin gel transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) for 15-30 min at room temperature. The proteins were transferred onto hybond enhanced-chemiluminescence nitrocelluse membrane (Pharmacia Biotech., Uppsala, Sweden) using electrophoretic transfer cell (mini trans-Blot®, Bio Rad, Hercules, CA, USA). Transfer was performed at 4° C for two hours at voltage of 100 volt. Following transfer, the non-specific binding sites on the

membrane were blocked with 5% fat free milk in Tris-buffered saline-Tween: TBS-T (20 mM Tris base pH 7.6, 137 mM NaCl, 1 M hydrochloric acid, and 0.1 % Tween) for 1 hr at room temperature with gentle shaking. The human *wt p53* was probed by incubating monoclonal anti *p53* antibody (NCL-*p53*-D07) (Novocustra Laboratories Ltd, Newcastle, UK) diluted in the blocking buffer (i.e. 5% fat free milk in TBS-T) overnight at 4° C with shaking. Membrane was then washed twice with TBS-T, incubated with horse-radish peroxidase conjugated secondary antibody diluted with blocking buffer for 1 hr at room temperature with agitation, and finally rinsed three times with TBS-T. The immunoblots were developed by the use of the enhanced chemiluminescence (ECL) detection system (Amersham Life Science, Inc., Buckinghamshire, UK) by exposing the membrane to ECL film as instructed by the manufacturer.

β-Actin expression served as a control since its expression is not affected by *p53* treatment. Membranes were incubated at 50° C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris.HCl pH 6.7) with gentle agitation. After washing with TBS-T at room temperature, membranes were reprobed with anti-β-actin antibody (Sigma- Aldrish, Steinhelim, Germany) as described for *p53* detection.

2.12. Hemolytic activity:

Hemolytic assay of the peptide were evaluated by incubating different concentrations of the peptide with freshly isolated rat erythrocytes such that the final concentration of the rat red blood cells (RBC) was 10^8 cells /ml. RBCs containing EDTA as anti-coagulant were first centrifuged at 1000 rpm for 10 minutes to remove the buffy

coat. Cells were then washed three times with D5W % solution and finally suspended in the same solution with a density of 2 x 10^8 cells/ ml.

Prior to the assay, the peptide was dissolved first in DMSO then diluted with D5W % solution. Half milliliter of cell suspension was incubated with different concentrations of the peptide at 37° C for 30 minutes with gentle mixing. The mixture was then centrifuged for five minutes at 500 G. The supernatants were measured spectrophotometrically at a wavelength of 570 nm by Microplate reader (Bio-Rad Model 550). One hundred percent hemolysis was obtained with 1% triton X 100. RBC exposed to D5W % solution were used as blank. To evaluate if DMSO interferes with the assay, hemolysis with D5W % solutions containing DMSO served as a control.

2.13. Particle size measurement:

Cationic liposomes were mixed with different amounts of the targeting peptide. After 20 min incubation at room temperature, samples were diluted 50 times with de-ionized water. Particle size was measured by Delsa 440 Zeta sizer (Beckman Coulter, Miami, FL, USA)

Similarly, liposomes /no peptide stored at 4° C under argon atmosphere were tested for any possible aggregation during the storage period.

2.14. Negative stain electron microscopy (EM):

Copper grids were floated on a droplet of liposomal formula for five minutes. The grid was dried for 10 min then it was stained with 10 % phosphotungstic acid for 5

minutes. Samples were viewed on JEOL 1200 EX electron microscopy, Japan. Photographs were taken at 20,000 magnification.

2.15.HepG2-liposomes interaction:

Cells were plated in 24-well plates in a density of 3×10^5 cells/ well. After 48 hours, 90% cell confluence was obtained. Growth medium was then removed and cells were washed two times with PBS. One hundred nmoles of radiolabeled liposomes (^3H liposomes) were incubated with the cells in D5 W % solution. Incubation was performed at 4° C to minimize internalization. At 1, 2.5, 4, and 5 hours, cells were washed three times with ice cold PBS and then lysed with 250 µl NaOH 0.1 M. Lysate (200 µl) were then measured for radioactivity by liquid scintillation counter (Beckman LS 5000TD, Fullertan, CA, USA). Protein concentration was measured as described above.

2.16. Binding of the targeting ligand to HepG2 cells:

Hep G2 cells were seeded in 24-well plates in a density of 3×10^5 cells/well. After 90% confluence was reached, cells were washed three times with PBS, and fixed for 10 min with 4 % paraformaldehyde as described previously for CS and region II+ peptide/ Hep G2 binding studies (Sinnis P *et al.*, 1996; Sinnis P *et al.*, 1994; Cerami C *et al.*, 1992). After washing with PBS three times, cells were blocked for 2 hrs with 1 % BSA in D5W % solution at 37° C. Cells were then rinsed twice with D5W % solution, incubated with different concentrations of peptide II for 1 hr at 37° C (concentration ranging from 0-1 mg/ml), washed three times with D5W % solution, incubated with 4 µg

of ^{125}I peptide II (specific activity 5.5 cpm/ng) for 1 hr at 37° C. Finally cells were rinsed three times with D5W % solution and lysed with 0.1 M NaOH solution.

Two hundred µl of lysate were then measured for radioactivity to assess the inhibition effects of the pre-incubation with cold region II+ peptide on the binding of ^{125}I peptide using a gamma counter (Beckman Gamma 5500, Irvine, CA, USA).

2.17 Mass spectroscopy:

2.17.1. Electrospray (ES) Ionization:

Quadropole tandem mass spectroscopy (Micromass Quadro II Triple Quade MS/MS, Fisons, Manchester, UK) was used. Traces of the peptides were dissolved in 50/50 methanol/ dichloromethane. Ten µl of the solution were introduced into the ES ion source. Peptide II was also tested in its aqueous solution (1µg/µl).

2.17.2. Matrix-assisted laser desorption ionization – time of flight (MALDI-TOF):

The dried droplet method was used in this technique (Beavis RC and Chait BT 1991). The matrix was sinapic acid (SA) dissolved in 30% acetonitrile, 0.1% TFA (Tri-flouroacetic acid) (10 mg/ml). SA solution was then purified from the undissolved particles through mild centrifugation. Peptide II (0.5 mg/ml) was diluted with the matrix and the dilution varied from 1/1 to1/4 (sample/ matrix). One micro-liter of this working solution was placed on the mass spectrometer's probe and air dried. The linear, time of flight MALDI-TOF system (Voyager-DE PRO, Applied Bio Systems, Foster City, CA, USA) was used to ionize the sample with N_2 laser beam (wavelength 337 nm).

2.18. Prediction of theoretical mass, isoelectric point (pI), and mean hydrophobicity:

Theoretical mass and pI values for peptide segments were predicted using PeptideMass program available on line: http://www.expasy.org/tools/peptide-mass.html (Wilkins MR et al., 1997).

Mean hydrophobicity was calculated via hydropathical scales: Kyte-Doolittle (Kyte J and Doolittle RF 1982), Eisenberg (Eisenberg D *et al.*, 1982), and combined consensus scale (CCS) (Tossi A *et al.*, 2002). These values did not consider the fatty acid chains linked to the modified region II+ peptides.

2.19. Statistical analysis:

Student t-test was performed to evaluate the significant difference between various conditions. Significant level was set at $P<0.05$. Sigma plot 2001 version 7.00 (SPSS Inc., Chicago, IL, USA) was used for the analysis.

3. Results

3.1. Assessment of liposomes/ peptide association:

The technique of density gradient ultracentrifugation is routinely employed for the separation of liposomes from associated or encapsulated substances (Nguyen XT *et al.*, 2002; Dipali SR *et al.*, 1996; Kurrle A *et al.*, 1990). Sample volume is usually in the range of milliliter for conventional ultracentrifugation. In our study, however, sample volume was in some cases less than 100 µl. Therefore, an air-driven ultracentrifuge (Airfuge) method was adopted as described previously (Rivnay B and Metzger H, 1983) with some modifications (rotor type, speed, time and temperature).

3.1.1. Gradient maintenance:

To evaluate if our methodology would maintain the gradient pattern, sucrose gradients were prepared and run at the experimental conditions. As seen in Figure 5, the gradient was maintained in monotonic pattern whether the run time was 30 or 60 min. In 30 min run, the recovered sucrose concentrations varied from 12% at the top fraction to 23% at the bottom (Figure 5A) compared to 14% to 22% for one hour run (Figure 5B). The original gradient falls in the range of 5-30%. In addition, the average slope of the recovered gradient was only about one-half the original for ½ hr run and one-third for 1 hr run (slope values: original gradient 1.612×10^{-1}, ½ hr run 7.731×10^{-2}, 1 hr run 5.146×10^{-2}). The reproducibility between duplicate tubes was excellent. In our study, we adopted the 1 hr protocol since sedimentation equilibrium is better attained with longer centrifugation times (Eikenberry EF, 1982).

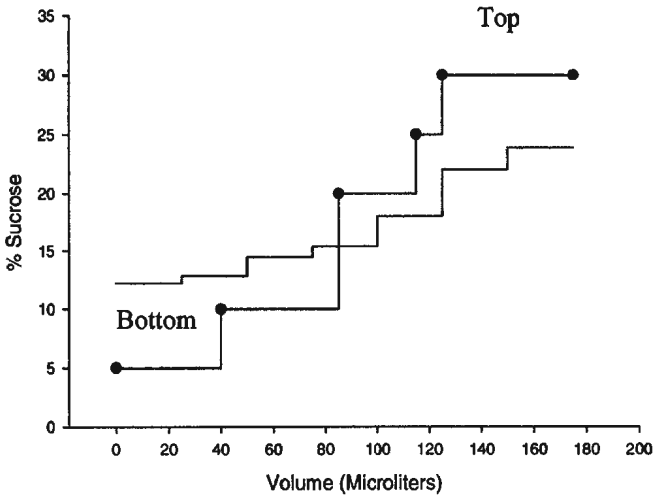


Fig. 5 (A)

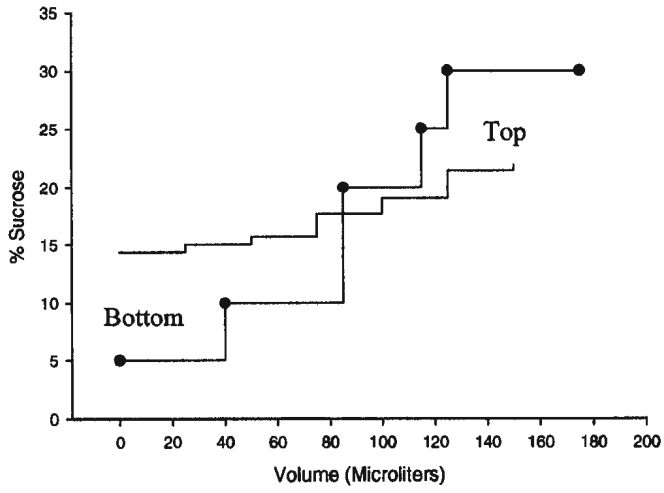


Fig. 5 (B)

Figure 5: Recovered gradients after centrifugation compared to the original sucrose gradient containing 50 µl 30%, 10 µl 25%, 30 µl 20%, 45 µl 10%, and 40 µl 5%. (A) Gradient was run for ½ hr at room temperature at top speed. 25 µl fractions were collected from top to the bottom and translated to concentrations by measuring the refractive index. (B) Gradient was run for 1 hr at room temperature at top speed. Fractions were collected as for ½ hr run. (•——•) refers to the original gradient.

3.1.2. Liposomes/ peptide complex II airfugation:

It is expected that unilamellar liposomes will float to the top of the tube after ultracentrifugation (liposomes flotation) while the free peptide (ligand) will migrate to the bottom (Dipali SR *et al.*, 1996; Rivnay B and Metzger H, 1983; Huang A *et al.*, 1980). The first four fractions will be considered as the top of the tube while the last four as the bottom. In our study, almost 50% of the peptide adsorbed to the surface of the tube when peptide/ no liposomes was centrifuged. Thirty percent of the peptide was detected in the final four fractions (the bottom of the tube), leaving just 20% for the top fractions. In contrast, the addition of the peptide to the liposomes in the hydration step (i.e. liposomes/ peptide complex II) reduced the peptide adsorption to the surface of the tube to only 5%. Seventy five percent of the peptide floated to the top of the tubes suggesting successful association between the liposomes and the peptide. Figure 6 shows the patterns of the recovered peptide in both free peptide and liposomes/ peptide complex II.

On the other hand, since cholesterol is one of the lipid components of liposomal formulations (50%), tracers of ^{3}H-Chol was used to quantify and detect the phospholipids after centrifugation. It was found that ^{3}H-Chol detection for liposomes/ peptide complex II revealed very close pattern to that observed with ^{125}I detection (i.e. the incorporated peptide) (see Figure 7). These findings suggest that liposomes/ ligand association was almost complete when the peptide was added in the hydration step of liposomal preparation.

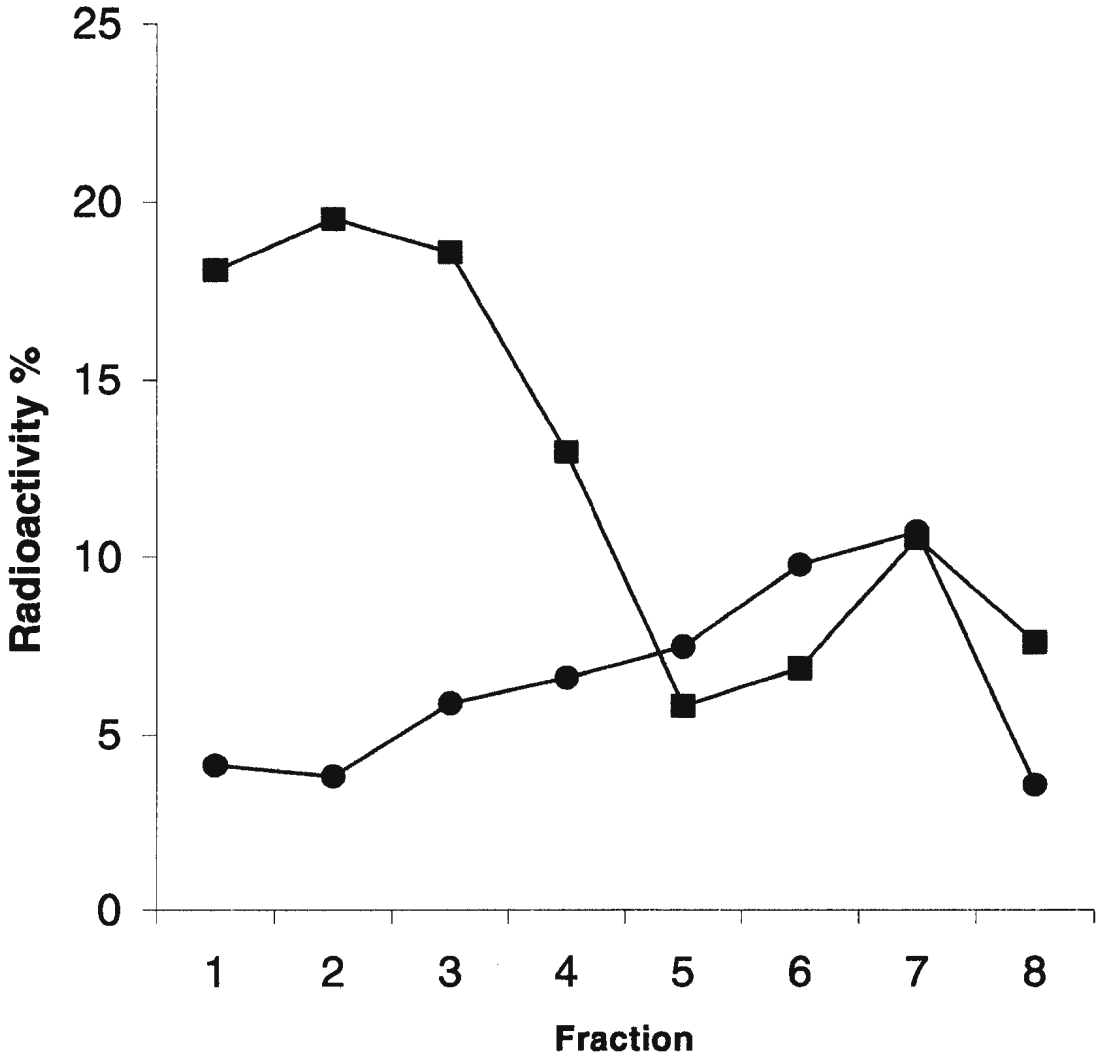


Figure 6: Airfuge flotation for liposomes-peptide complex II. ^{3}H detection (■) representing liposomes in comparison to ^{125}I detection (●) representing the peptide. Values were normalized as a percentage of the total radioactivity. Less than 1 % of ^{3}H was detected in the empty tube compared to 5 % of the peptide. Each data point represents the average between duplicated tubes.

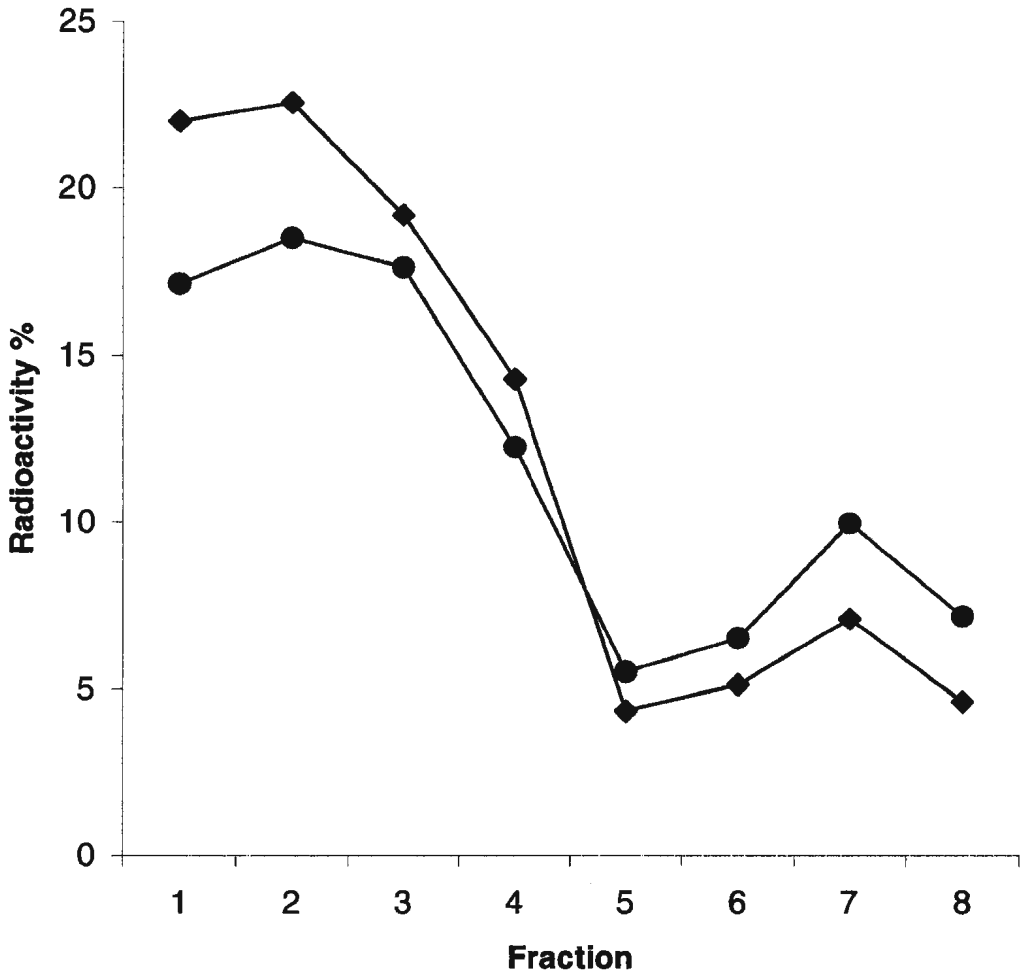


Figure 7: Airfuge flotation for liposomes-peptide complex II. ^{3}H detection (◆) representing liposomes in comparison to ^{125}I detection (●) representing the peptide. Values were normalized as a percentage of the total radioactivity. Less than 1 % of ^{3}H was detected in the empty tube compared to 5 % of the peptide. Each data point represents the average between duplicated tubes.

3.1.3. Liposomes/ peptide complex I airfugation:

The addition of the peptide in the final stage (i.e. after extrusion) resulted in very different patterns between the peptide and the associated liposomes after centrifugation. Figure 8 compares the patterns of ^{125}I and ^{3}H for liposomes/ peptide complex I. Twenty five percent of the peptide adhered to the tube surface while the remaining amount was equally divided between the top and the bottom fractions. It is important to notice that almost 20 % of the peptide was recovered in fraction 7. These results revealed that more than 60% of the peptide was not associated with the liposomes. ^{3}H detection for liposomes/ peptide complex I, however, revealed a pattern that is very close to both free liposomes and liposomes/ peptide complex II (refer to Figure 9). In the three conditions, more than 70% of liposomes floated to the top fractions.

3.2. Cell Transfection:

Transfection was executed on both rat McA RH 7777 and human hep 3B cell lines. Both cell lines does not express the human *wt p53* (Anderson SC *et al.*, 1998; Bressac B *et al.*, 1990; Puisieux A *et al.*, 1993). We used the newly developed lipospmal formula LPD that contains a polycationic peptide protamine (Li S *et al.*, 1998; Sorgi FL *et al.*, 1997; Gao X and Huang L, 1996).

3.2.1. LPD/ peptide complex I:

As seen in Figure 10, transfection efficiency on McA RH7777 cell line of LPD/ peptide complex I did not differ from that observed with LPD/ no peptide formula. Peptide amounts varied from 0.1 to 10 μg (e.g. 1 μg DNA: 0.6 μg protamine: 12 nmol

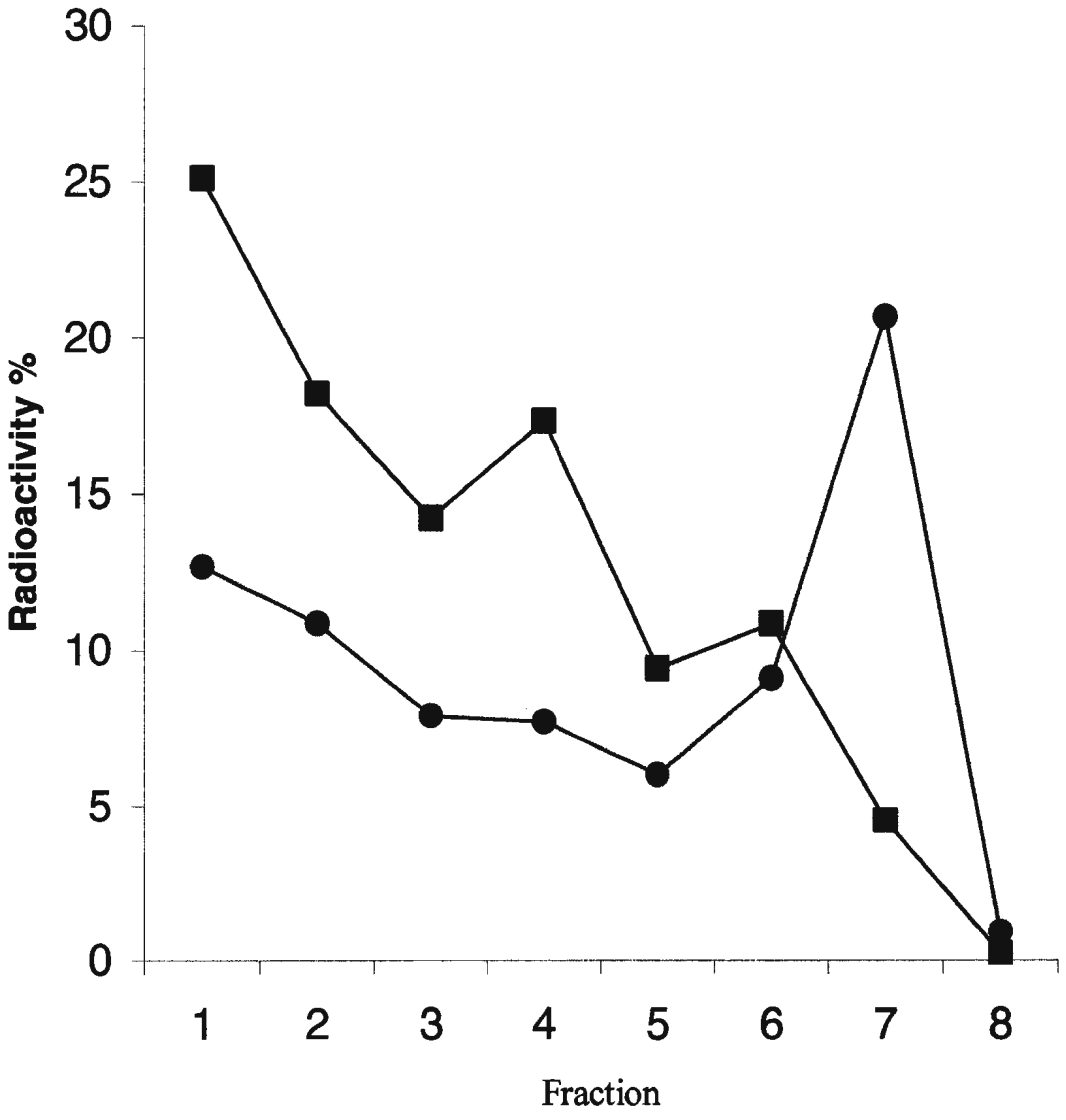


Figure 8: Airfuge flotation for liposomes-peptide complex I. ^{3}H detection (■) representing liposomes in comparison to ^{125}I detection (●) representing the peptide. Values were normalized as a percentage of the total radioactivity. Less than 1 % of ^{3}H was detected in the empty tube compared to 25 % of the peptide. Each data point represents the average between duplicated tubes.

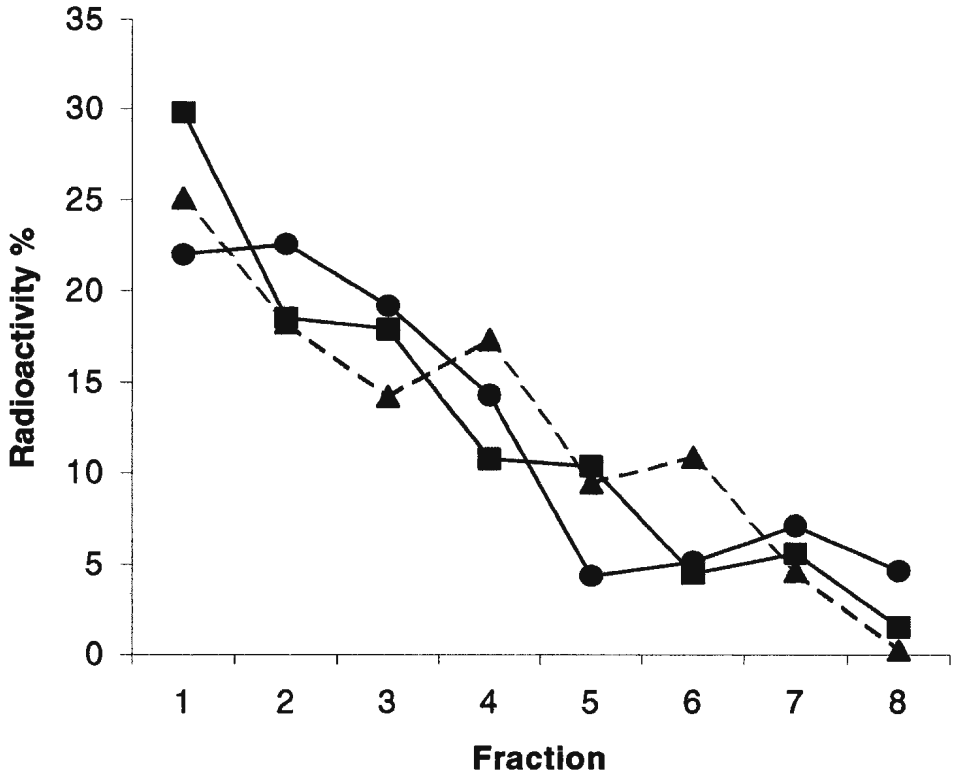


Figure 9: A comparison between liposomes flotation patterns (^3H detection) for different liposomal preparations: Liposomes no peptide (■), liposomes-peptide complex I (▲), and liposomes-peptide complex II (●). Values were normalized as a percentage of the total radioactivity. Very close percentages of the recovered radioactivity were detected at the top and bottom fractions between various preparations. Each data point represents the average between duplicated tubes.

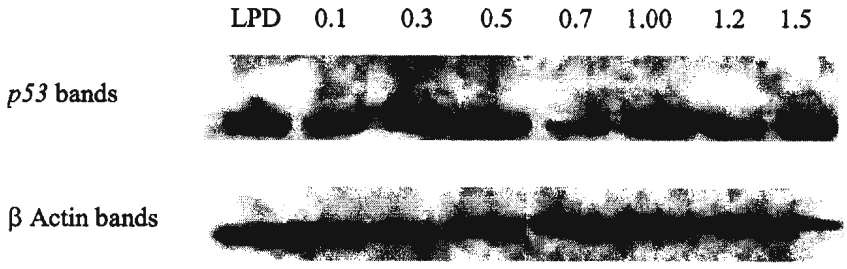


Fig 10 A

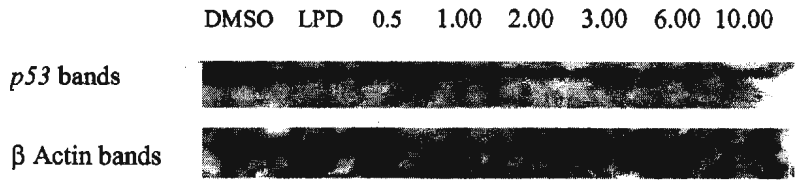


Fig 10 B

Figure 10: Transfection with LPD-peptide complex I on rat cell line McA RH7777. Numbers refer to the amount of the included peptide in LPD formula. LPD labeling refers to no peptide treatment and DMSO labeling is the control (LPD+ DMSO). Fig. 10 A and B correlates to two different experiments. Fig. 10 A: peptide amounts ranged from 0.1 to 1.5 μg. Fig. 10 B: peptide amounts ranged from 0.5 to 10 μg. β Actin control bands were used to detect gel loading accuracy from lane to lane

Dotap: 0.1 μg peptide). It was also illustrated that the addition of DMSO in its highest concentration (i.e. 0.1 % V/V) that corresponds to the addition of 10 μg of the peptide did not influence the transfection levels of the original LPD preparation. Despite that no increase in *p53* expression was observed with LPD/ peptide complex I compared to LPD/ no peptide, a remarkable reduction in transfection efficiency was observed when the peptide ratio in the formula exceeded 6 μg (refer to Figure 10). These results showed that no targetability was observed when the peptide was added after the LPD preparation.

These finding are in agreement with the minimal liposomes/ peptide association obtained when the peptide was incorporated into the liposomes after extrusion.

3.2.2. LPD/ peptide complex II:

The inclusion of the peptide in the hydration step of liposomal preparations (i.e. LPD/ peptide complex II) did not result in higher *p53* expressions than those observed with LPD/ no peptide treatments. Figure 11 showed the results of experiments conducted on both human Hep 3B and rat McA RH 7777 cell lines. In addition, the exclusion of protamine sulfate (considered as another variable in the formulation) did not affect the outcomes of the transfection. No increase in *p53* expression was observed. DMSO volume in this experimental setting was 0.016% of the total volume of the transfecting medium.

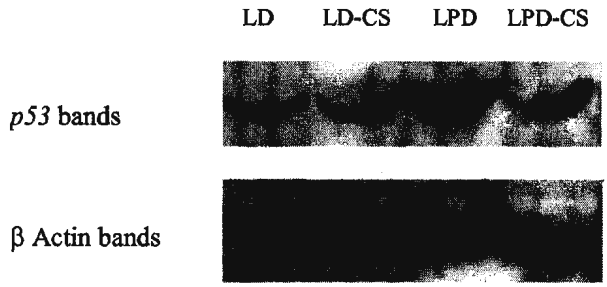


Fig. 11A

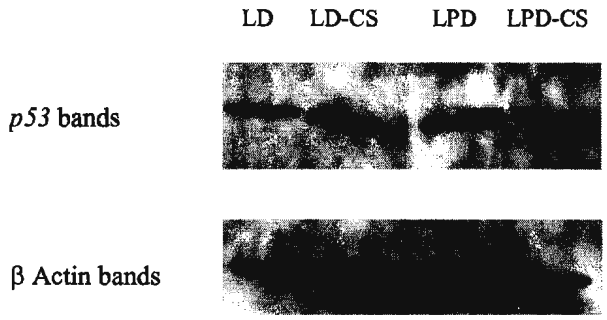


Fig. 11B

Figure 11: Transfection with LPD-peptide complex II on human Hep 3B (Fig. 11 A) and rat McA RH7777 (Fig. 11 B) cell lines. Labels: Lipoplex (LD); Lipoplex-peptide (LD-CS); Liposomes-Protamine-DNA (LPD); LPD-peptide (LPD-CS). β Actin control bands were used to detect gel-loading accuracy from lane to lane.

3.3. Hemolytic activity:

Hemolytic activity is an *in vitro* test used to evaluate the toxicity of the tested substance (Kobayashi S *et al.*, 2001; Pape WJ, and Hoppe U 1987; Anderson RJ *et al.*, 1984). It relies on the ability of this material to destabilize the red blood cell membrane releasing its containment. Liposomes have been used as artificial biological membranes since their discovery in the early sixties (Bangham AD et al., 1965). Therefore, the hemolytic activity of the peptide on rat erythrocytes can provide some assessment of the potential destructive effects of the targeting ligand on liposomal membranous structures.

Figure 12 shows the dose-response curve of peptide hemolytic activity. The peptide possesses mild hemolytic activity which increases in linear fashion with the increase of the peptide concentration. However, a peptide concentration higher or equal to 300 μg/ml (i.e. 107 μM) resulted in very close hemolytic activity values. The maximum hemolysis was in the range of 30%. The best fit line of the data points showed a sigmoid pattern. There was, however, a strong linearity for concentrations <300 μg/ml (R2=0.9871) (by excluding the data point at the concentration of 400 μg/ml). The contribution of DMSO in the hemolytic assay was minimal (less than1.8 % in its highest contributions).

In this study, the ratio between the lipids and the targeting ligand which resulted in lower transfection levels (Fig. 10 B) was 12nmol (DOTAP or Chol): 10 μg (peptide) or 0.13×10^{-4} g (total lipids): 10 μg (peptide). On the other hand, lipids are the main component of the cell membrane. It has been shown that total lipids are 3.15×10^{-13} g / rat erythrocyte and it is composed of almost 70 % phospholipids, 25 % neutral lipids

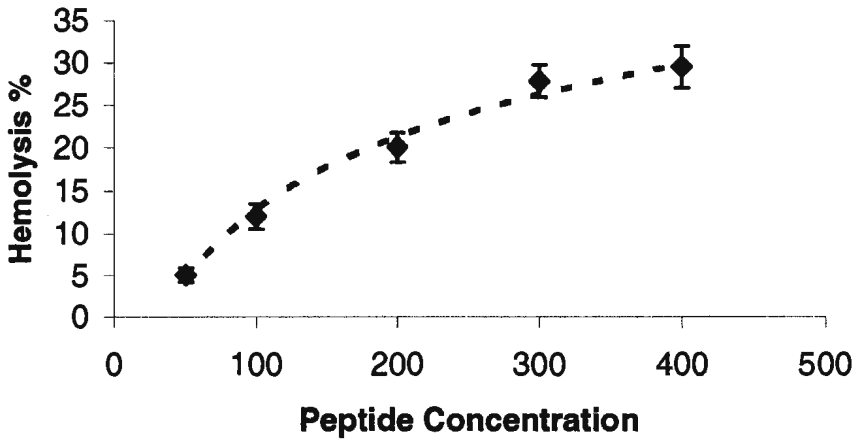


Figure 12: Percent hemolysis versus peptide I concentrations. Dotted line represents the best fit line. Each data point is a mean of three measurements.

(mainly cholesterol) with the rest being glycolipids and ganglioside (Nelson GJ 1967). Therefore, the (total lipid: peptide) ratio was 3.15×10^{-4} g: 300- 400 μg (when the hemolytic activity reached its plateau) or 0.0787×10^{-4} g: 10 μg (corresponding to 400 μg peptide). This ratio is almost two times lower (in terms of the total lipids) than that used in liposomes/ peptide complex I preparations. It is thus expected that lipid bilayer destruction in liposomes might occur more efficiently resulting in liposomal fusion and subsequently lowering transfection efficiency.

3.4. Particle size measurements:

Particle size of liposomes can be indication of stability and transfection efficiency (Regelin AE *et al.*, 2000; Birchall JC *et al.*, 2000; Ross PC and Hui SW 1999). The addition of the peptide after extrusion and the incubation for 20 min at room temperature resulted in significant increase ($P<0.05$) in the particle size only when the peptide amount was 10 μg. Liposomes: peptide ratios were consistent with tissue culture transfection experiment. Liposomal size increased by almost 35% from the original size. Figure 13 shows the sizes of the liposomal preparations after the incubation with different amounts of peptide I. Usually the size of the liposomes falls in the range of 150-220 nm.

In contrast, the addition of the peptide in the hydration step in a concentration of 1 μg/μl did not affect the size of different liposomal preparations. Figure 14 illustrates the size of Liposomes, LPD, and lipoplex (i.e. no protamine) with and without the targeting ligand. In addition, the presence of 4% of DMSO in the hydration step does not affect the size of the liposomes (refer to Figure 14). Some LPD preparations were bigger than

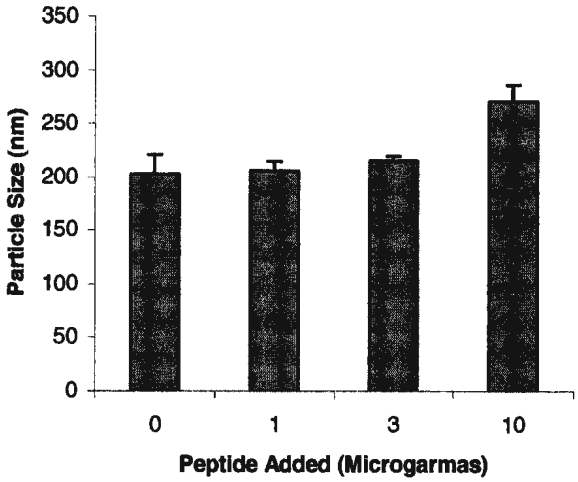


Figure 13: Particle size measurements of liposomal preparations incubated with different amounts of peptide I. X-axis represents the amount of the peptide. Y-axis is the particle size of the preparation after 20-min incubation with the peptide at room temperature. Each condition is a mean of five measurements.

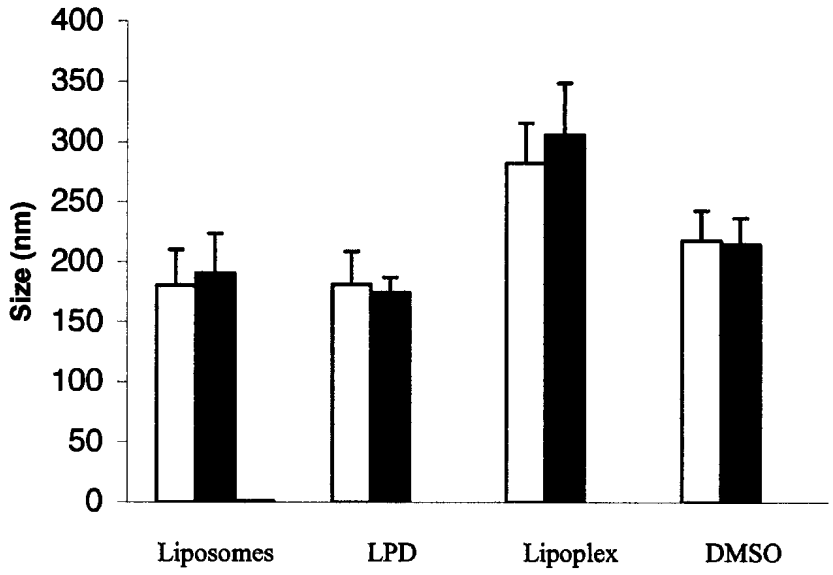


Figure 14: Particle size measurements of different liposomal preparations in two conditions: no peptide ligand (empty bars) and with peptide ligand added in the hydration step of the preparation procedure (filled bars). DMSO labeling refers to the size of the original liposomes (empty bars) in comparison with liposomes hydrated with 4% DMSO solution. Each condition is a mean of five measurements.

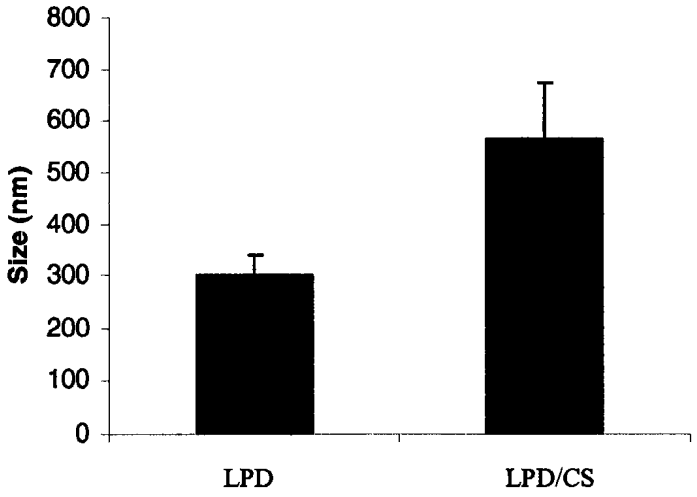


Figure 15: LPD size before and after incubation with 10 μg peptide. Each condition is a mean of five measurements.

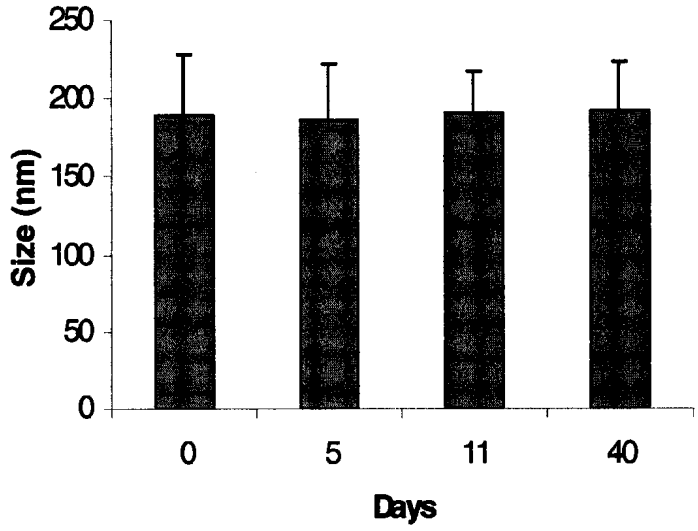


Figure 16: Particle size of liposomes stored at 4° C under argon atmosphere. No aggregates were observed within a month period. Each condition is a mean of five measurements.

expected (as large as lipoplex). The incubation of these LPD particles with 10 μg ligand resulted in almost 100% increase in the size of the particles (see Figure 15).

Since transfection experiments were conducted using freshly prepared liposomes, there was no need to check the effect of storage conditions (4°C under argon atmosphere) on the sizes of the liposomes. In other experiments, however, liposomes were used within a month period from their preparation. No aggregates were observed during this period under the specified storage conditions (see Figure 16).

3.5. Negative stain electron microscopy (EM):

EM pictures showed similar liposomes and LPD particles to those reported by others (Li B *et al.*, 2000; Birchall JC *et al.*, 1999; Thierry AR *et al.*, 1997; Gao X *et al.*, 1996). Membranous structures with stained core region were very clear in all pictures. Spherical shapes were much more abundant than the tubular ones. In this method, the addition of the ligand did not result in any observed morphological changes. In addition to membranous structures, LPD pictures showed some condensed particles and bigger aggregates. More aggregation was observed in liposomal pictures. Figure 17 shows the different Electron micrographs for liposomes and LPD preparations.

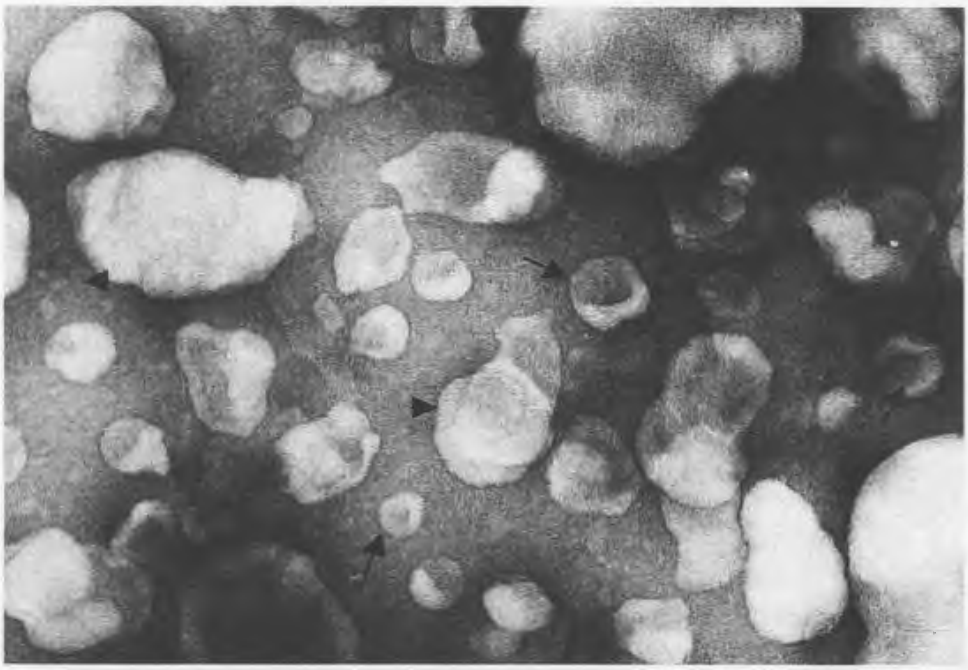


Figure 17 A

Figure 17 B

Figure 17 C

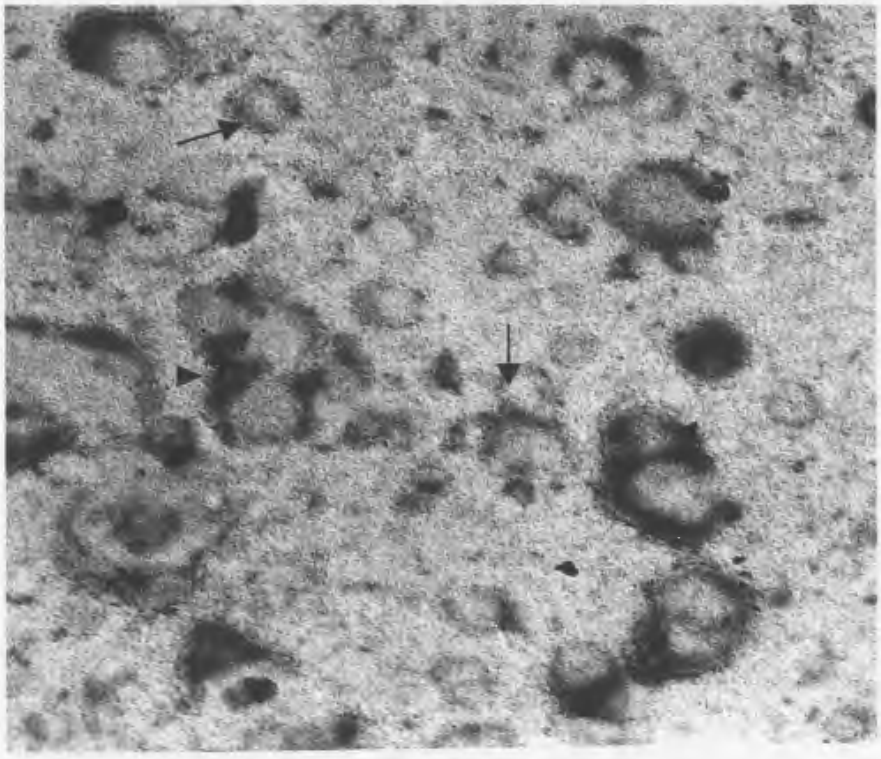


Figure 17 D

Figure 17: Electron micrographs of ULV (A), ULV-peptide complex II (B), LPD (C), and LPD-peptide complex I (D). Arrows show particles with membranous structures. Head arrows indicate the larger aggregates. Peptide I was used for LPD-peptide and ULV-peptide complexes preparations. LPD-peptide complex I: 1 μg DNA: 0.6 μg Protamine: 12 nmol Dotap: 1 μg Peptide.

3.6. HepG2-liposomes interaction:

One percent of ^{3}H cholesterol was added to both liposomes and liposomes/ peptide complex II to assess the complexes' binding affinity to HepG2 cell membranes at 4° C. Two different concentrations of the peptide were tested (0.5 μg/μl and 1 μg/μl). As seen in Figure 18, there was no increase in the binding affinity when the peptide was incorporated into the liposomes. HepG2-(liposomes/ peptide complex II) interaction profiles resembled that obtained with liposomes/ no peptide preparation

3.7. Binding of the targeting ligand to HepG2 cells:

It was reported that CS region II+ peptide inhibits the binding of CS protein to HepG2 cells by almost 90% (Cerami C *et al.*, 1992; Sinnis P *et al.*, 1994). In our study, the pretreatment with the cold peptide inhibited the binding of the ^{125}I peptide by less than 35% with the highest concentrations. A T-test, however, showed that there was no significant difference between different conditions ($P<0.05$). Figure 19 summarizes the inhibition effects of different concentrations of the cold peptide on ^{125} I peptide binding. These results suggest that our peptide design and experimental conditions may have resulted in the loss of the original high binding affinity of region II+ peptide to hepatoma cells.

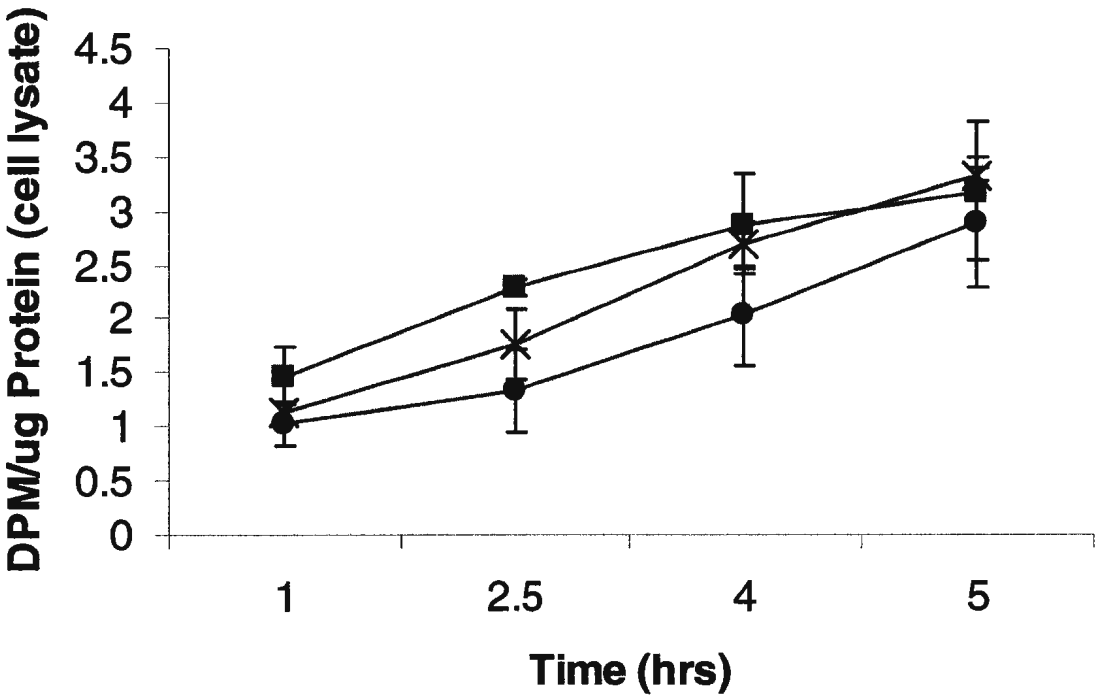


Figure 18: Comparison of the binding between liposomes and liposomes-peptide complex II with Hep G2 cells. 100 nmol of ^{3}H Liposomes were incubated at 4° C with HepG2 cells (♦) liposomes-no peptide (×) liposomes-peptide complex II (0.5 μg/μl) (•) liposomes-peptide complex II (1 μg/μl). Each data point represents the mean of three measurements.

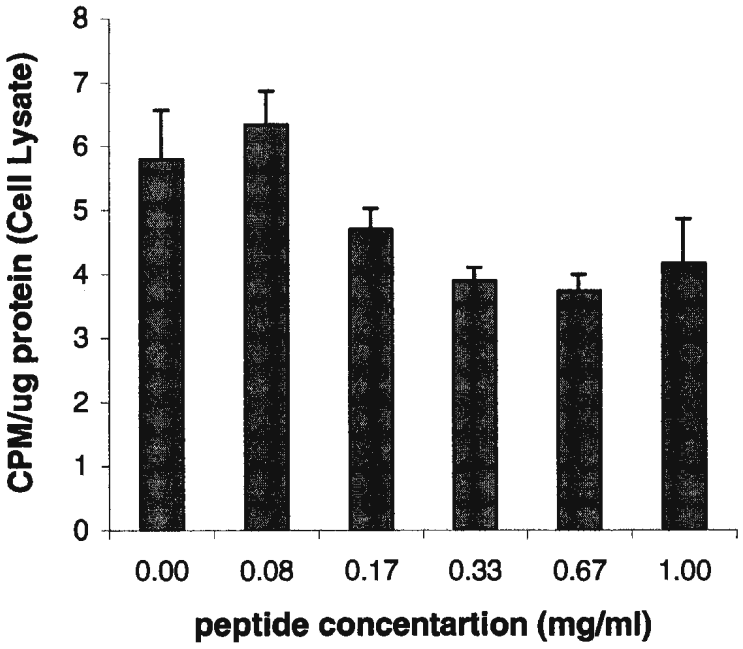


Figure 19: Binding of ^{125}I peptide (4μg) to HepG2 cells after preincuabation with different concentrations of cold peptide. Radioactivity was normalized to protein concentration in cell lysate. Peptide II was tested.

3.8. Mass spectroscopy:

3.8.1. Electrospray (ES) Ionization:

The ES ionization technique did not show the protonated molecular ion of the two peptides (expected by PeptideMass program (Wilkins MR et al., 1997): $[M+H]^+$ at m/z 2804.4474 for peptide I and 2852.4685 for peptide II). However, we noticed the formation of both $[M+2H]^{+2}$ and $[M+3H]^{+3}$.

$[M+2H]^{+2}$ (m/z 1401.83) and $[M+3H]^{+3}$ (m/z 935.37) of peptide I are shown in Figure 20. Similarly, Peptide II ionization with $[M+2H]^{+2}$ (m/z 1425) and $[M+3H]^{+3}$ (951) are shown in Figure 21. Ions were better formed when the sample was dissolved in 50/50 methanol/ dichloromethane rather than the aqueous solution containing 2% DMSO (see Figure 21). The strongest peak, however, for peptide II appeared at m/z 835.97. Tandem mass spectroscopy showed that the parent ion of 835.97 ion is $[M+3H]^{+3}$ ion (refer to Figure 22).

3.8.2. MALDI-TOF:

This analysis was applied on peptide II. As seen in Figure 23, the protonated molecular ion $[M+H]^+$ appeared as expected at m/z 2853.4 which was not the major peak. The later peak appeared at m/z 2984.9, which differ by almost 131 Da from the expected protonated molecule of the peptide. This difference comes from TFA which has a molecular weight of 113 absorbing one H_2O (i.e. 114+18= 132). Other peaks including a major peak appeared at 1680 does not relate to the original peptide and may represent impurities or degradation products.

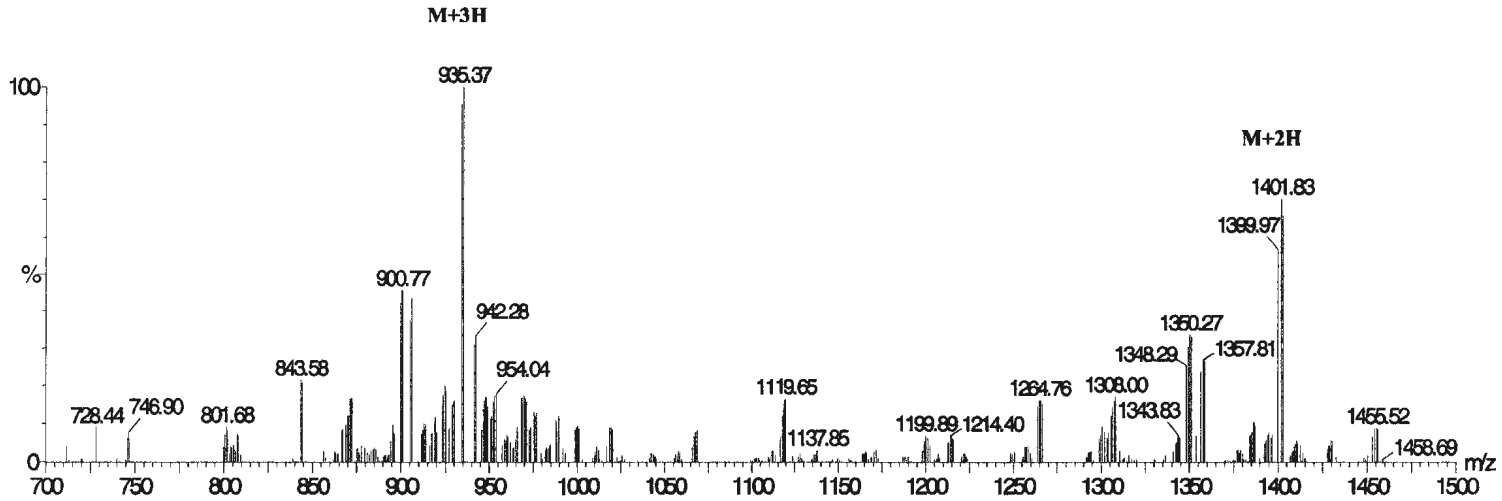


Figure 20: Fragmentation pattern of peptide I using electrospray ionization method. M+2H and M+3H are shown on the spectra. The peptide was dissolved in 50/50 methanol/ dichloromethan.

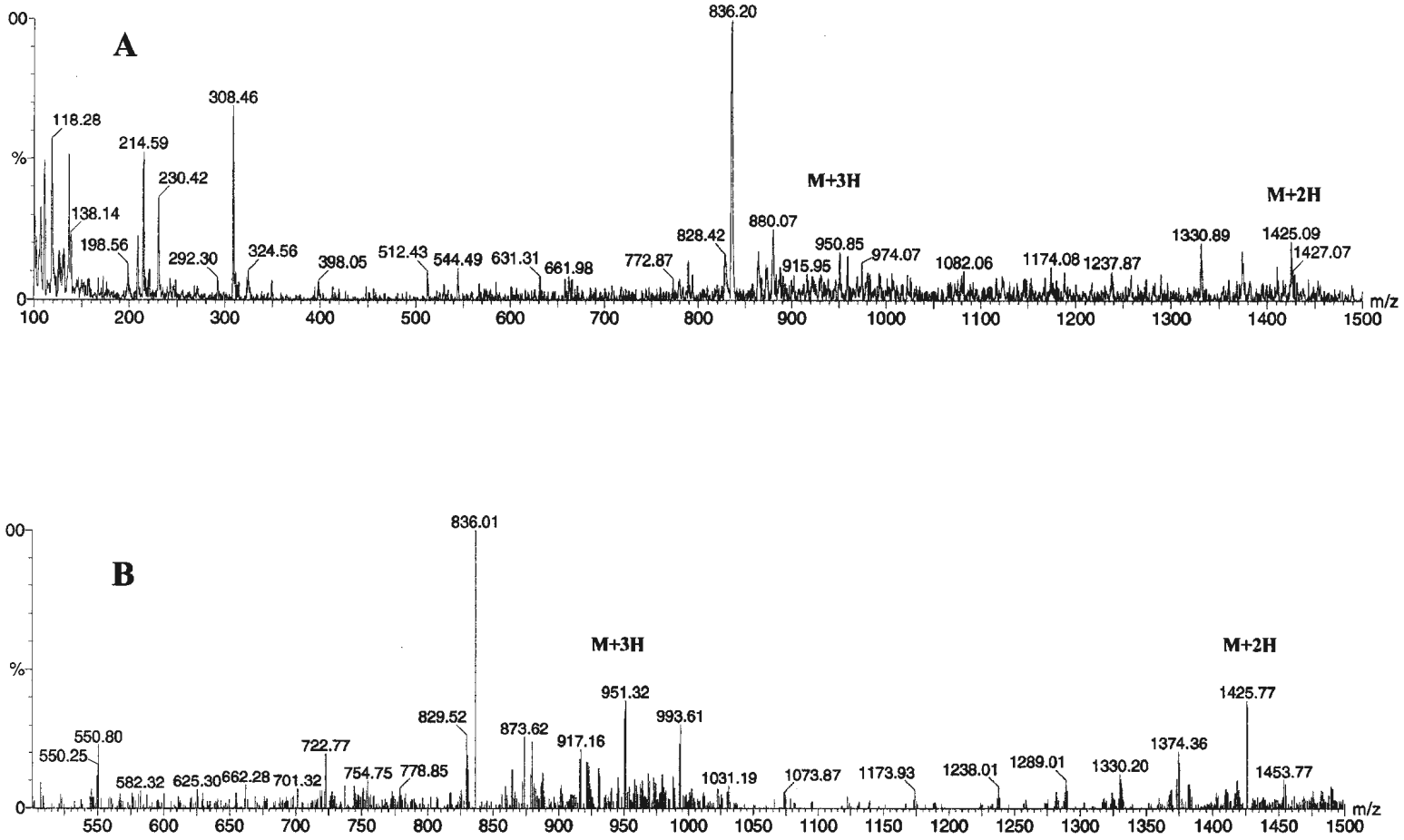


Figure 21: Fragmentation pattern of peptide II using electrospray ionization method. M+2H and M+3H are shown on the spectra. The peptide was dissolved either in dH_2O containing 2% DMSO (A) or 50/50 methanol/ dichloromethan (B).

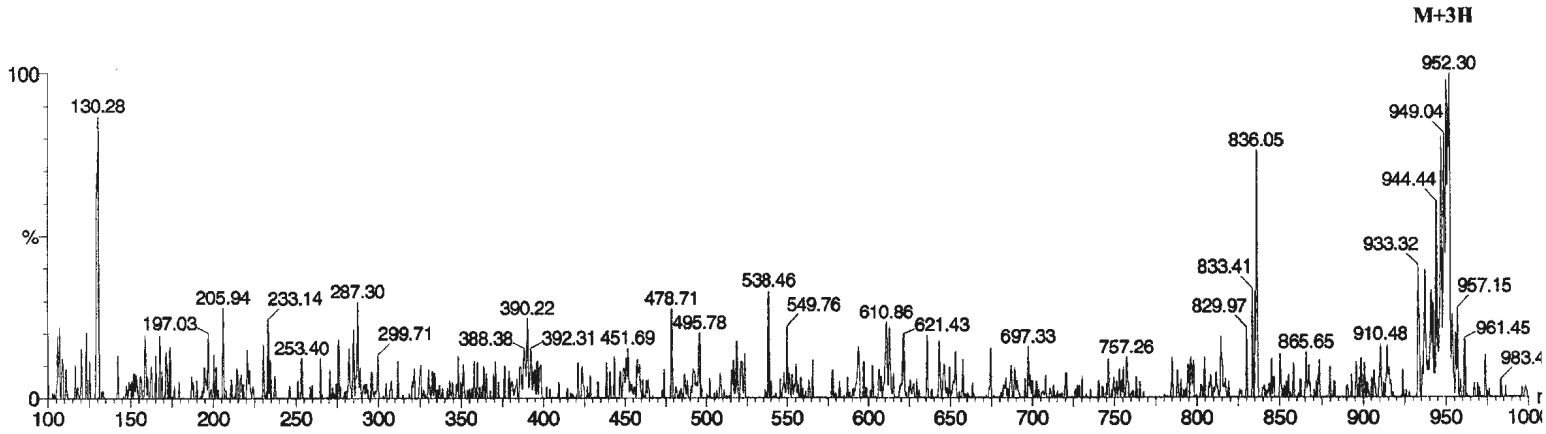


Figure 22: Tandem mass spectra for M+3H appeared at m/z 951.

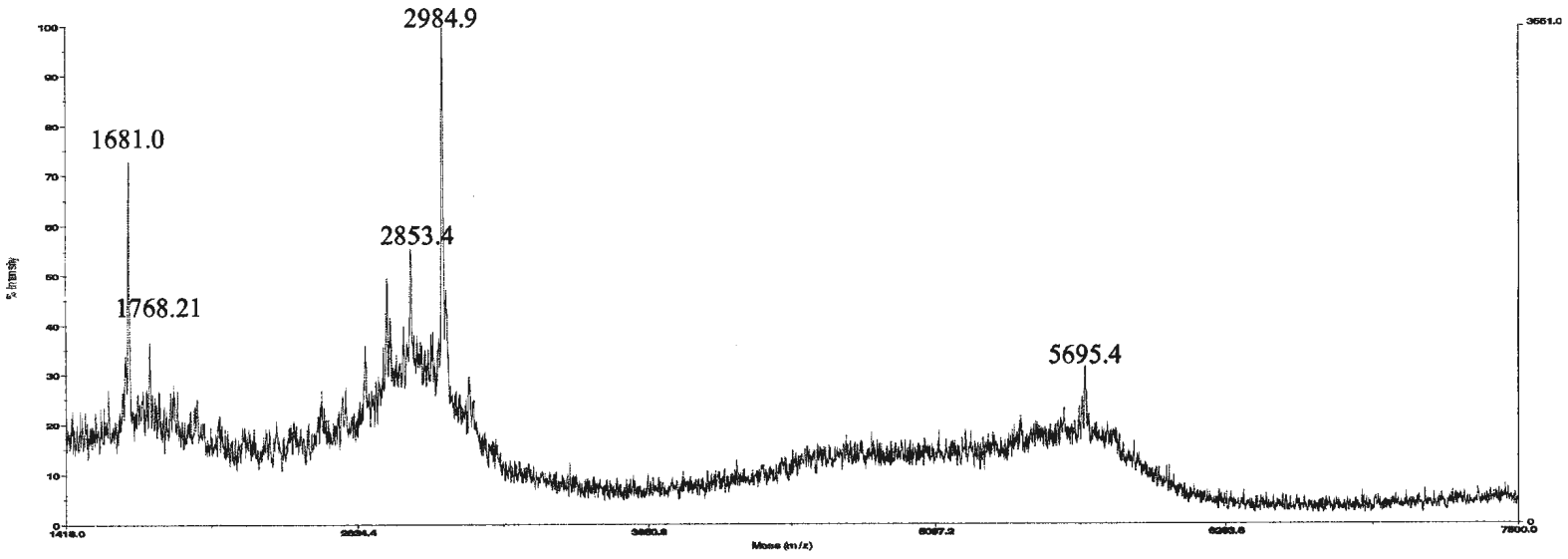


Figure 23: MALDI-TOF spectrum of peptide II. M+H ion appeared at m/z 2853.4.

3.9. Theoretical hydrophobicity:

Table 1 summarizes the hydrophobicity values of peptide segments:

Region II+: EWSPCSVTCGNGIQVRIK

Peptide I: YEWSPCSVTCGNGIQVRIKPGSAN

Peptide II: NSLSTEWSPCSVTCGNGIQVRIKY

Peptide\ hydrophobicity	CCS	Kyte-Doolittle	Eisenberg
Region II+	- 1.555	-0.116	-0.126
Peptide I	-1.691	-0.329	-0.117
Peptide II	-1.470	-0.225	-0.127

Table 1: Theoretical hydrophobicity of the peptide segments. Scales used were CCS, Kyte-Doolittle, and Eisenberg hydropathical scales.

4. Discussion

The principle goal of this study was to develop a liver specific liposomal gene carrier that can deliver the apoptotic gene *p53* efficiently to hepatic cells. Liver cancer treatment with *p53* gene was chosen because it can eradicate hepatic cancerous cells effectively (Anderson SC *et al.*, 1998), as well as enhance the efficacy of traditional therapies (Nishizaki M *et al.*, 2001). Non viral gene carriers are less efficient than the viral vectors. This limitation was, however, counterbalanced with safety (especially low immunogenicity), simplicity of preparation, and high gene encapsulation capability in comparison with viral vectors (Cusack JC Jr, and Tanabe KK 2002). Modifications to improve the efficiency of non-viral vectors including liposomal preparations are currently under intense research.

A good liposomal formulation for cancer gene therapy should encapsulate and protect the nucleic acid materials, escape the endosomal degradation and reach specifically to the tumor site. The last goal can be achieved by incorporating a tumor specific ligand which can deliver DNA specifically to the cancerous tissue. In the case of hepatocyte cells, the main challenge is to divert the liposomes from the lung "trap". Conventional liposomes (Iyer M *et al.*, 2002; McLean JW *et al.*, 1997; Osaka G *et al.*, 1996) and LPD (Ma Z et al., 2002; Li B *et al.*, 2000; Li S and Huang L 1997) tend to be trapped by capillary embolism in the lungs where transfection occurred mainly. Liver accumulation of lipoplexes can be enhanced through manipulations of the particle size (Hwang SH *et al.*, 2001; Alino SF *et al.*, 1996; Wu J, and Zern MA 1996) or lowering the complex surface charge (Mahato RI *et al.*, 1998). More recently, transfection occurred mainly in the liver with the development of the serum resistance poly(cationic lipid) (Liu

L *et al.*, 2003). It is also shown that significant amount of LPD accumulates in the liver (Li S *et al.*, 1998; Li S and Huang L 1997).

In liver uptake, it is essential to ensure that liposomes have actually reached the parenchymal liver cells rather than the phagocytic kupffer cells. Some studies have shown that kupffer cells were actually the main destination for liposomes in the liver (Litzinger DC *et al.*, 1996; Roerdink F *et al.*, 1981). This is less of a problem when receptor specific ligand is attached to the surface of the liposomes. The ligand will bind to its receptors on the parenchymal cells before internalization occurs.

Malaria region II+ peptide of CS protein is an attractive liver ligand since its specific binding to heparan sulfate proteoglycans receptors is essential to successful CS protein binding to the cell membranes and substantial malaria parasite invasion into the liver. It has been illustrated that CS-poly (L-lysine) conjugate successfully targeted recombinant DNA to liver cultures (Ding Z et al., 1995). In this study, we explored the possibility of utilizing region II+ peptide for cationic liposomal liver targeting. A lipid moiety was introduced to the N-terminus of the peptide to be anchored within the lipid bilayers of the liposomes. This strategy for developing liver-targeted liposomes was successfully used for antibodies (Harsch M *et al.*, 1981; Huang A *et al.*, 1980), glycoproteins (Hara T *et al.*, 1995; Tsuchiya S *et al.*, 1986) and glycopeptides (Kallinteri P *et al.*, 2001). The main advantage in this strategy is the ease of ligand incorporation (simple incubation) and the reduction of materials used. Two forms of the modified peptide were used as discussed in the introduction. Peptide I was linked to a palmitic acid chain while peptide II was linked to a myristic acid chain. Both peptides have extension of five amino acids derived from the original sequence of malaria CS protein.

The extension was, however, in the C-terminus of peptide I versus the N-terminus of peptide II. Based on the dates when the modified region II+ peptides were synthesized, all the experiments except the binding studies were performed on peptide I. Since both peptide I and II contain region II+ peptide motif and were modified in a very similar fashion, it is expected that the general behavior of these peptides will be similar.

4.1. Peptide solubility:

The peptides (with no modifications) exhibit a hydrophilic nature as calculated by Kyte-Doolittle (Kyte J and Doolittle RF 1982), Eisenberg (Eisenberg D *et al.*, 1982), and CVS (Tossi A *et al.*, 2002) hydropathical scales. The mean theoretical hydrophobicity for peptide segments were shown in table 1. All the predicted measurements exhibited slightly negative values (i.e. more hydrophilic). These results are in agreement with previous studies on this malaria peptide in which aqueous media were used to dissolve the peptide and other motifs from its sequence (Chatterjee S et al., 1995; Sinnis P et al., 1994; Cerami C et al., 1992). Peptides I and II (without the fatty acid linkage) were slightly more hydrophilic than the original region II+ segment. Our aim was that the attachment of the fatty acid chain will enable us to dissolve the peptide in an organic solvent that can be added to lipid mixture such as methanol or chlorophorm.

Our modified forms of the peptide, however, were only soluble in DMSO. DMSO brought the peptides into aqueous solutions such that the final concentration of DMSO did not exceed 2%. All other common organic solvents including methanol, ethanol, chloroform, acetonitrile, and dimethyl sulfoxide (DMF) did not dissolve the

peptides. Particulates were observed with the naked eye. The only exception was DMF in which aggregates were observed after microscopic examination.

4.2. Liposomes/ peptide complex formation:

Both size exclusion chromatography and ultracentrifugation can be used to separate liposomes, free ligand and liposomes/ligand complex from each other. The main obstacle to apply either of these techniques is the small volume of the samples in our study (in the range of microliters). Therefore, an air driven ultracentrifuge (Airfuge) was used. This technique was utilized before for liposome-related separation evaluation (Rivnay B and Metzger H, 1983).

Free liposomes float to the top of the tube (Fig. 9), while free peptide was concentrated at the bottom of the tube, as well as adsorb to its surface (Fig. 6). We have shown here, through liposome flotation experiments that complete liposomes/ ligand association was achieved when the peptide was added during the hydration step of liposomal preparations (liposomes-peptide complex II). In this complex, the peptide floats to the top in a similar manner to that obtained with the associated liposomes (Fig. 7). On the other hand, the association was less than 40% when the peptide was incubated with unilamellar liposomes after extrusion (Fig. 8). This association is minimal since 20 % of the free peptide (i.e. no liposomes) was normally detected at the top fractions after ultracentrifugation (refer to Fig 6).

4.3. LPD preparation:

We choose to use the novel LPD preparation because of its high efficiency compared to conventional liposomes (Li S *et al.*, 1998; Sorgi FL *et al.*, 1997; Gao X and Huang L 1996). LPD utilizes protamine to condense nucleic acid before being complexed with the lipid vesicles. For liver targeting purposes, LPD was recently coated with the targeting ligand asialofetuin through charge-charge interactions, which significantly increases HepG2 cells uptake of the encapsulated DNA (Arangoa MA *et al.*, 2003).

LPD mean particle size is usually in the range of 100-250 nm as reported by others (Ueno NT *et al.*, 2002; Sorgi FL *et al.*, 1997). Our LPD particle size was, however, in the range of 100-350 nm and varied from batch to batch. Some LPD formulations have a larger particle size than expected, almost similar to that obtained when protamine is excluded (i.e. lipoplexes). This inconsistency in the particle size measurements is due to the existence of salts as our DNA was stored in TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0) as provided by collaborators. Salts can interfere with the charge-charge interactions between LPD ingredients causing the observed inconsistent LPD size measurements (Tan Y *et al.*, 2002). Therefore, it is essential to exclude any salts from the formulation in any future work. LPD is also naturally composed of heterogeneous particles of free liposomes, lipoplex and LPD (Li S *et al.*, 1998). This heterogeneous mixture in terms of different sizes was observed in our preparations by EM picture (Figs. 17C and 17 D). Sample preparation for EM analysis, however, included air drying which might lead to liposomal aggregation. These aggregates were observed in ULV preparations (see Figs. 17 A and 17 B).

4.4. LPD transfection and liposomes/ HepG2 binding:

Based on liposomes/ ligand association experiments, it is expected that LPD or lipoplex mediated *p53* delivery to hepatoma cells will be better achieved with liposomes-peptide complex II. In our study, *p53* expressions were at the same levels for all the tested formulations. Western blotting analysis is illustrated in Figs 10 and 11. It was, however, observed that the transfection level was significantly reduced when more that 6 μg peptide was incubated with LPD preparation as shown in Fig 10 B. Monitoring liposomal stability through particle size measurements and assessing the hemolytic activity of the peptide provided some explanation for this observation.

Size increases in both liposomes and LPD were observed when liposomal formulations were incubated with high concentration of the peptide. The size of the free liposomes was significantly increased more than one third of the original formulation (Fig 13) while the LPD size was doubled (Fig 15). Particle size is a critical factor in liposomal transfection efficiency (Almofti MR *et al.*, 2003a; Ross PC, and Hui SW 1999). It has been demonstrated that the size increase of the LPD formulation will reduce the ability of the complex to deliver the encapsulated DNA to cultured cells (Li B et al., 2000). Since our LPD size was increased by almost 100%, this may explain the observed reduction in LPD mediated gene transfer when more than 6 μg peptide were used for LPD-peptide complex I formation. This conclusion is in agreement with the mild hemolytic activity possessed by the peptide (Fig. 12). On the other hand, the addition of the peptide in the hydration step (which was 0.8 peptide: 12 nmol DOTAP) did not influence liposomes stability as monitored by particle size analysis illustrated in Fig. 14.

It seems that the peptide can destabilize the membrane lipid bilayers in both the

liposomes and red blood cell membranes. This will facilitate fusion among the liposomal particles resulting in aggregation and consequent reduction of transfection.

To mimic region II+ peptide in the malaria parasite, the peptide should, however, exist in close proximity on the surface of the liposomes. It has been shown that region II+/ receptor interactions involves more than one peptide segment (Sinnis P et al., 1994; Cerami C et al., 1992). It is possible that the limitation we encountered in terms of the total peptide added (because of both DMSO and the destruction ability of region II + on the lipid bilayers) may have prevented sufficient coating on the liposomal surface.

In addition to the non-targetability observed with the transfection experiments, liposomes-peptide complex II/ HepG2 interactions resulted in no increase in liposomal binding to the cell surface as measured by ^{3}H-Chol detection in comparison with liposomes/ no peptide preparation (refer to Fig. 18).

These findings suggest that the targeting ligand might have been oriented inwards from the liposomal surface or buried within the lipid bilayers. It has been shown, however, that different ligands, linked to a hydrophobic moiety, were oriented to a large extent to the outer side of the liposomes (Kallinteri P et al., 2001; Zalipsky S *et al.*, 1997; Tsuchiya S *et al.*, 1986; Huang A *et al.*, 1980). Therefore, it is expected that our ligand will be aligned in the same fashion within the lipid bilayers. This assumption needs to be explored in any future investigation.

On the other hand, it is also possible that our methodology resulted in the loss of the peptide binding specificity to its receptors on the liver cells. While in previous studies the pretreatment of fixed HepG2 cells with region II+ peptide resulted in almost complete inhibition of the binding of the CS protein to these cells (Sinnis P *et al.*, 1994;

Cerami C *et al.*, 1992), our modified region II+ peptide pretreatment to HepG2 cells inhibited the binding of ^{125}I peptide by only 30%. This inhibition was, however, statistically insignificant in comparison with no pretreatment control (Fig. 19). This finding confirmed our assumption for the loss of the peptide binding specificity. One possibility is that oxidation mediated by DMSO occurred during peptide solution storage. DMSO has destructive effects on many amino acids (Spencer RL, and Wold F, 1969) such as tryptophan and cysteine which exist in the peptide sequence. Oxidation by DMSO will alter the peptide properties including its liver-specificity. This may explain the non-targatability observed in our methodology. Despite the serious potential of the oxidation problem especially with cysteine moieties that are prone to air oxidation, MS analysis by both ES and MALDI-TOF techniques showed spectrum peaks related to the molecular ion (m/z 2804.4 peptide I and 2852.4 Peptide II). Non related peaks are mainly due to the impurities since peptide purity was 80%. MS spectrums as obtained from the working solution are illustrated in Figs. 21, 22, and 23.

On the other hand, it is possible that conformational changes have occurred in the peptide after modification and dissolving in 2% DMSO aqueous solution. Circular dichroism (CD) analysis can provide information on the secondary structure (2D structure) of peptides and proteins (Venyaminov SY and Yang JT 1996). Structural analysis of peptide segments of CS protein including region II + were previously analyzed by this technique (Roggero MA *et al.*, 1995; Verdini AS *et al.*, 1991). We were, however, unable to load the modified peptide into the CD cell (Jasco J-810, Easton, MD USA) without the formation of air bubbles. Bubbling prevented us from performing this essential analysis.

The nuclear magnetic resonance spectroscopy technique (NOESY NMR) can also provide some insight into the possible conformational changes (Skelton NJ and Chain WJ 2000). These tests should be considered for any future work with region II+ peptide designed for liver specific targeting.

4.5. Future directions:

Despite the well documented evidence for region II+ specificity towards liver cells (Ying P *et al.*, 1997; Chatterjee S *et al.*, 1995; Aley SB *et al.*, 1986), our chemically-modified version of the peptide, as well the liposome-peptide complexes lack this specificity. The main problem with our peptide was its poor solubility. DMSO was the only solvent that dissolved the peptide and brought it into aqueous media. DMSO is not the solvent of choice for peptides and proteins. In addition, it has toxic effects on living tissues whether cultured cells *in vitro* or animal models *in vivo*. Therefore, any future modifications should consider increasing the aqueous solubility of the peptide or at least have better range of solvent choice. One simple modification is the inclusion of some hydrophilic amino acids at the N-terminus of the peptide. A repeat of (Asn-Gln) $_3$(which will not contribute to the peptide net charge), for example, will elevate the theoretical hydrophobicity of the peptide segment almost two times as predicted by the hydrophobicity scales (*CSS* -2.59; *Kyte-Doolittle* -0.976; *Eisenberg* -0.2496). In addition, these peptides might serve as a spacer from the lipid layers.

More efficient modification might be the usage of PEG linkage between the lipid part and the ligand. This approach is widely used especially for mAb (Bendas G 2001). In a recent study, a peptide ligand (specific to restenosis-involved cells) was linked to the

lipid through PEG 3400. The lipid-PEG-ligand conjugate was added to the lipid mixture in the first stage of liposome preparation (Lestini BJ *et al.*, 2002). In a similar manner, we can link the region II+ peptide with palmotic acid for instance. One of the most widely used PEG as a linkage for targeting ligands is PEG 2000 (Iden DL, and Allen TM 2001; Shi N, and Pardridge WM 2000; Bendas G *et al.*, 1999; Huwyler J *et al.*, 1996). This linkage will increase the water solubility as PEG-linked compounds are more hydrophilic than the original-no PEG molecules (Katre NV *et al.*, 1987; Chen RH et al., 1981). In addition, a reasonably long spacer (in comparison with peptide linkages) will be provided through the PEG portion. It has been shown that longer spacers will result in better targeting than short spacers on the liposomal surface (Zhou W et al., 2002; Kawakami S et al., 1998). The peptide will contain tyrosine moiety at its C-terminus for radio-labeling. The possibility of disulfide linkages between two moieties of the modified peptide also need to be explored since such modification will create the necessary multi region II+ required for peptide receptor interactions (Sinnis P et al., 1994; Cerami C et al., 1992). Figure 24 shows the proposed modifications for region II+ peptide as a liver targeting ligand.

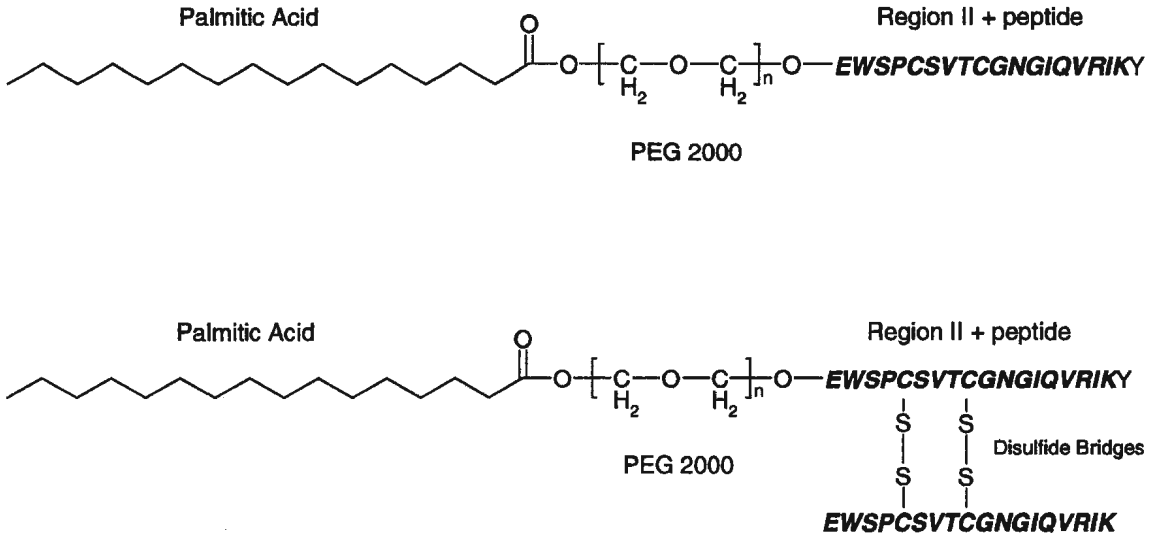


Figure 24: Schematic representation for region II+ peptide as liver targeting ligand to be anchored within the lipid membranes of cationic liposomes.

The sequence of the investigation will follow the following order:

4.5.1. The evaluation of the binding affinity of the peptide to hepatoma cells and generation of binding curve:

Binding dissociation equilibrium constant (K_d) and maximum receptor number/cell (B_{max}) can be calculated. This calculation was not possible in our study because of the existence of DMSO which can lead to cell death in high concentrations required for competition study. K_d Value will be compared with the non-modified peptide. It is possible that pegylated compounds will have lower affinity toward their receptors *in vitro* in comparison with the non-modified molecule. This relationship is reversed *in vivo* where the retarded renal clearance and the long half life in the blood

stream will increase the binding of the pegylated substances to their receptors (Bailon P and Berthold W 1998).

4.5.2. The formation of cationic liposomes/ ligand complex:

Peptides can be added at the hydration step or after extrusion. In spite that our study has shown that liposomes/ peptide complex was better formed if the addition was performed at the hydration step of liposomal preparation, the addition of the peptide after extrusion will ensure that the ligand is oriented outside (Zalipsky S *et al.*, 1997). It has been shown that this can be facilitated through mild heat (Zalipsky S *et al.*, 1997). In addition, Incubation time can play a role in the complex formation. The usage of low concentrations of sodium cholate or deoxycholate, mild detergents, was used to enhance the formation of liposomes/ ligand complexes (Kallinteri P *et al.*, 2001; Hara T *et al.*, 1995; Huang A *et al.*, 1980). These detergents can adsorb to and solubilize the lipid membrane forming a transient pores within the membranous structure (de la Maza A and Parra JL 1997; Schubert R *et al.*, 1986) such that the ligand is readily available to be incorporated within the lipid bilayers. It was reported that liposomes/ mAb (linked to palmitate) conjugate was not assembled when Na deoxycholate was excluded from the preparation (Shen DF *et al.*, 1982). Newly developed post insertion technique can also be evaluated. It was recently applied successfully for hexapeptide linked to DSPE (distearoylphatidyl ethanolamine) through PEG 2000 linkage (Moreira JN *et al.*, 2002). Free ligand can then be separated from the complex by ultracentrifugation, column chromatography or dialysis. All these factors need to be explored and optimized.

The evaluation of liposomes/ HepG2 interactions can be improved (over the methodology we adopted in this study) through the usage of ^{3}H-CHE (Cholesterol [1,2-

^{3}H –(*N*)]-hexadecyl ether) as a radiolabel tracer. ^{3}H CHE does not exchange with cell membranes after internalization. Therefore, valuable information about cell uptake can be obtained. In our study, binding (which is the first step for internalization) was evaluated at 4 C since ^{3}H-Chol can be exchanged with the cell membranes at 37 C (i.e. after internalization). Another membrane non-exchangeable lipid radioactive marker is ^{14}C Chol-oleate (Kirby C, and Gregoriadis G 1980). Fluorescent lipid markers are also available such as NBD-Chol (7-nitrobenz-2-oxa-1,3-diazol-4-yl-labeled. Cholesterol) (Mukherjee S. and Chattopadhyay A 1996).

In addition, competition experiment with free peptide (labled liposomes incubated with HepG2 cells in the presence of free peptide) will confirm the efficiency of this methodology of complex formation.

4.5.3. Transfection experiments:

Transfection experiments with LPD and lipoplexes bearing the *p53* gene can then be evaluated in cultured cells (Hep3B and McA RH 7777). *p53* therapeutic effects includes apoptosis induction and cell cycle arrest (Zou Y *et al.*, 1998).

4.5.4. In vivo evaluation:

In vivo evaluation can be applied on mice or rats bearing liver cancer lesions through tail vein or intra-arterial injection of the liposomal formulation. It has been shown the transfection efficacy in the liver can be elevated through hepatic artery delivery (Seol JG *et al.*, 2000; Anderson SC *et al.*, 1998). Therapeutic effects such as tumor size and animal life span can then be evaluated and compared with LPD- no peptide treatment.

4.6. Closing remarks:

CS protein derived liver targeted ligands is an attractive option for developing liver specific liposomal formulations. Highly hepatic-specific, revolutionary conserved region I and region II+ peptides of the malaria CS protein are possible candidates as targeting moieties. Despite that our liposomes/ region II+ complexes did not show the expected targeting effects, alternative ligand modifications and complex preparation methods (that may overcome the obstacles experienced with this study) are currently under experimental design and evaluation.

References

Aden DP, Fogel A, Plotkin S, Damjanov I, and Knowles BB (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* 282, 615-6.

Alexander IE, Russell DW, and Miller AD (1994). DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. *J. Virol.* 68, 8282-7.

Alexander IE, Russell DW, Spence AM, and Miller AD (1996). Effects of gamma irradiation on the transduction of dividing and nondividing cells in brain and muscle of rats by adeno-associated virus vectors. *Hum. Gene Ther.* 7, 841-50.

Aley SB, Bates MD, Tam JP, and Hollingdale MR (1986). Synthetic peptides from the circumsporozoite proteins of Plasmodium falciparum and Plasmodium knowlesi recognize the human hepatoma cell line HepG2-A16 in vitro. *J. Exp. Med.* 164, 1915-22.

Alino SF, Bobadilla M, Crespo J, and Lejarreta M (1996). Human alpha 1-antitrypsin gene transfer to in vivo mouse hepatocytes. *Hum. Gene Ther.* 7, 531-6..

Allen TM, Brandeis E, Hansen CB, Kao GY, and Zalipsky S (1995). A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. *Biochim. Biophys. Acta.* 1237, 99-108.

Allen TM, Sapra P, and Moase E (2002). Use of the post-insertion method for the formation of ligand-coupled liposomes. *Cell Mol. Bio.l Lett.* 7, 889-94.

Almofti MR, Harashima H, Shinohara Y, Almofti A, Li W, and Kiwada H (2003a). Lipoplex size determines lipofection efficiency with or without serum. *Mol. Membr. Biol.* 20, 35-43.

Almofti MR, Harashima H, Shinohara Y, Almofti A, Baba Y, and Kiwada H (2003b). Cationic liposome-mediated gene delivery: biophysical study and mechanism of internalization. *Arch. Biochem. Biophys.* 410, 246-53

Anderson FW (1998). Human gene therapy. *Nature* 392 (Suppl), 25-30.

Anderson RJ, Glasgow CE, and Dunham CB (1984). Hemolysis as a possible indicator of neurotoxicity induced by organic solvents. *Environ. Health Perspect.* 58, 393-6.

Anderson SC, Johnson DE, Engler H, Hancock W, Huang W, Wills KN, Gregory RJ, Sutjipto S, Wen SF, Lofgren S, Shepard HM, and Maneval DC (1998). *p53* gene therapy in a rat model of hepatocellular carcinoma: intra-arterial delivery of recombinant adenovirus. *Clin. Cancer. Res.* 4, 1649-59.

Arangoa MA, Duzgunes N, and Tros de Ilarduya C (2003). Increased receptor-mediated

gene delivery to the liver by protamine-enhanced-asialofetuin-lipoplexes. *Gene Ther.* 10, 5-14.

Asklund T, Appelskog IB, Ammerpohl O, Langmoen IA, Dilber MS, Aints A *et al.*, (2003). Gap junction-mediated bystander effect in primary cultures of human malignant gliomas with recombinant expression of the HSVtk gene. *Exp Cell Res.* 284, 185-95.

Bailon P and Berthold W (1998). Polyethylene glycol-conjugated pharmaceutical proteins. *Pharm. Sci. Tech. Today.* 1, 352-6.

Balasubramaniam RP, Bennett MJ, Aberle AM, Malone JG, Nantz MH, and Malone RW (1996). Structural and functional analysis of cationic transfection lipids: the hydrophobic domain. *Gene Ther.* 3, 163-72.

Bangham AD, Standish MM, and WatkinsJC (1965). The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. *J Mol Biol* 13, 138- 44.

Barajas M, Mazzolini G, Genove G, Bilbao R, Narvaiza I, Schmitz V *et al.*, (2001). Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. *Hepatology.* 33, 52-61

Beavis RC and Chait BT. (1991). Matrix assisted laser desorption/ ionization mass spectropmetry of biopolymers. *Anal. Chem.* 63, 1193A-1202A.

Behr JP (1994). Gene Transfer with Synthetic Cationic Amphiphiles: Prospects for Gene Therapy. *Bioconjugate Chem.* 5, 382-9.

Behr JP, Demeneix B, Leffler JP, and Perez-Mutul J (1989). Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. USA.* 86, 6982-6.

Bendas G (2001). Immunoliposomes: a promising approach to targeting cancer therapy. *Bio. Drugs.* 15, 215-24.

Bendas G, Krause A, Bakowsky U, Vogel J, and Rothe U (1999). Targetability of novel immunoliposomes prepared by a new antibody conjugation technique. *Int. J. Pharm.* 181, 79-93.

Benjamini E, Coico R, and Sunshine G (2000). *Immunology: A short course.* New York: Wiley- Liss.

Bennett MJ, Nantz MH, Balasubramaniam RP, Gruenert DC, and Malone RW (1995). holesterol enhances cationic liposome-mediated DNA transfection of human respiratory epithelial cells. *Biosci. Rep.* 15, 47-53.

Bertram JS (2000). The molecular biology of cancer. *Mol. Aspects Med.* 21, 167-223.

Bielinska AU, Yen A, Wu HL, Zahos KM, Sun R, Weiner ND, Baker JR Jr, and Roessler BJ (2000). Application of membrane-based dendrimer/DNA complexes for solid phase transfection in vitro and in vivo. *Biomaterials.* 21, 877-87.

Birchall JC, Kellaway IW, and Gumbleton M (2000). Physical stability and in-vitro gene expression efficiency of nebulised lipid-peptide-DNA complexes. *Int. J. Pharm.* 197, 221-31

Birchall JC, Kellaway IW, and Mills SN (1999). Physico-chemical characterisation and transfection efficiency of lipid-based gene delivery complexes. *Int. J. Pharm.* 183, 195-207.

Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M *et al.*, (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science.* 274, 373-6.

Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M *et al.*, (1995). lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science.* 270, 475-80.

Bosch FX (1997). Global epidemiology of hepatocellular carcinoma. In: (Okuda K and Tabor E, Eds). *Liver cancer* (pp. 13-28.). New York: Churchill Livingstone.

Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B *et al.*, (1995). Versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. USA.* 92, 7297-301.

Bramson JL, Hitt M, Gauldie J, and Graham FL (1997). Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination. *Gene Ther.* 4, 1069-76.

Brand K (2000). Gene therapy for cancer. In (Templeton NS and Lasic DD. Eds) *Gene therapy: Therapeutic mechanisms and strategies* (pp. 439-72). New York: Dekker.

Bressac B, Galvin KM, Liang TJ, Isselbacher KJ, Wands JR, and Ozturk M (1990). Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* 87, 1973-7.

Breyer B, Jiang W, Cheng H, Zhou L, Paul R, Feng T *et al.*, (2001). Adenoviral vector-mediated gene transfer for human gene therapy. *Curr. Gene Ther.* 1, 149-62

Brown MD, Schatzlein A, Brownlie A, Jack V, Wang W, Tetley L *et al.*, (2000). Preliminary characterization of novel amino acid based polymeric vesicles as gene and drug delivery agents. *Bioconjug. Chem.* 11, 880-91.

Brown MD, Schatzlein AG, and Uchegbu IF (2001). Gene delivery with synthetic (non viral) carriers. *Int. J. Pharm.* 229, 1-21.

Buchschacher GL Jr, and Wong-Staal F (2000). Development of lentiviral vectors for gene therapy for human diseases. *Blood.* 95, 2499-504.

Buller RM, Janik JE, Sebring ED, and Rose JA (1981). Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. *J. Virol.* 40, 241-7.

Bustos M, Sangro B, Alzuguren P, Gil AG, Ruiz J, Beraza N *et al.*, (2000). Liver damage using suicide genes. A model for oval cell activation. *Am. J. Pathol.* 157, 549-59.

Cabrera CM, Jimenez P, Cabrera T, Esparza C, Ruiz-Cabello F, and Garrido F (2003). Total loss of MHC class I in colorectal tumors can be explained by two molecular pathways: beta2-microglobulin inactivation in MSI-positive tumors and LMP7/TAP2 downregulation in MSI-negative tumors. *Tissue Antigens* 6, 211-9.

Caruso M, Pham-Nguyen K, Kwong YL, Xu B, Kosai KI, Finegold M *et al.*, (1996). Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. *Proc. Natl. Acad. Sci. USA.* 93, 11302-6.

Caspers P, Gentz R, Matile H, Pink JR, and Sinigaglia F (1989). The circumsporozoite protein gene from NF54, a Plasmodium falciparum isolate used in malaria vaccine trials. *Mol. Biochem. Parasitol.* 35, 185-90.

Cerami C, Fevert U, Sinnis P, Takacs B, Clavijo P, Santos MJ *et al.*, (1992). The bastolateral domain of the hepatocyte plasma membrane bears receptors for circumsporozoite protein of plasmodium flaciparum sporozoites. *Cell.* 70, 1021-33.

Cerami C, Frevert U, Sinnis P, Takacs B, and Nussenzweig V (1994). Rapid clearance of malaria circumsporozoite protein (CS) by hepatocytes. *J. Exp. Med.* 179, 695-701.

Chana JS, Grover R, Tulley P, Lohrer H, Sanders R, Grobbelaar AO *et al.*, (2002). The c-myc oncogene: use of a biological prognostic marker as a potential target for gene therapy in melanoma. *Br. J. Plast. Surg.* 55, 623-7.

Chatterjee S, Wery M, Sharma P, and Chauhan VS (1995). A conserved peptide sequence of the Plasmodium falciparum circumsporozoite protein and antipeptide antibodies inhibit Plasmodium berghei sporozoite invasion of Hep-G2 cells and protect immunized mice against P. berghei sporozoite challenge. *Infect. Immun.* 63, 4375-81.

Chen L, Pulsipher M, Chen D, Sieff C, Elias A, Fine HA *et al.*, (1996). Selective transgene expression for detection and elimination of contaminating carcinoma cells in hematopoietic stem cell sources. *J. Clin. Invest.* 98, 2539-48.

Chen QR, Zhang L, Gasper W, and Mixson AJ (2001). Targeting tumor angiogenesis

with gene therapy. *Mol. Genet. Metab.* 74, 120-7.

Chen RH, Abuchowski A, Van Es T, Palczuk NC, and Davis FF (1981). Properties of two urate oxidases modified by the covalent attachment of poly(ethylene glycol). *Biochim Biophys Acta.* 13; 660, 293-8.

Choi BY, Chung JW, Park JH, Kim KH, Kim YI, Koh YH *et al.*, (2002). Gene delivery to the rat liver using cationic lipid emulsion/DNA complex: comparison between intra-arterial, intraportal and intravenous administration. *Korean J. Radiol.* 3, 194-8.

Chollet P, Favrot MC, Hurbin A, and Coll JL (2002). Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. *J. Gene Med.* 4, 84-91.

Clayton J (2002). Gene therapy progress for HIV. *Drug Discovery Today.* 7, 481-2.

Cochran AJ, Morton DL, Stern S, Lana AM, Essner R, and Wen DR (2001). Sentinel lymph nodes show profound downregulation of antigen-presenting cells of the paracortex: implications for tumor biology and treatment. *Mod. Pathol.* 14, 604-8.

Conry RM, White SA, Fultz PN, Khazaeli MB, Strong TV, Allen KO *et al.*, (1998). Polynucleotide immunization of nonhuman primates against carcinoembryonic antigen. *Clin. Cancer Res.* 4, 2903-12

Corey L, and Spear PG (1986). Infections with herpes simplex viruses (2). *N. Engl. J. Med.* 314, 749-57.

Crystal RG (1995). Transfer of genes to humans: early lessons and obstacles to success. *Science.* 270, 404-10

Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, and Blaese RM (1992). In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science. 256, 1550-2.

Cusack JC Jr, and Tanabe KK (2002). Introduction to cancer gene therapy. *Surg. Oncol. Clin. N. Am.* 11, 497-519.

Dame JB, Williams JL, McCutchan TF, Weber JL, Wirtz RA, Hockmeyer WT *et al.*, (1984). Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite Plasmodium falciparum. *Science* 225, 593-9.

Dash PR, Read ML, Barrett LB, Wolfert MA, and Seymour LW (1999). Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. *Gene Ther.* 6, 643-50.

Dasi F, Benet M, Crespo J, Crespo A, and Alino SF (2001). Asialofetuin liposome-mediated human alpha1-antitrypsin gene transfer in vivo results in stationary long-term

gene expression. *J. Mol. Med.* 79, 205-12.

de la Cruz VF, Lal AA, and McCutchan TF (1987). Sequence variation in putative functional domains of the circumsporozoite protein of Plasmodium falciparum. Implications for vaccine development. *J. Biol. Chem.* 262, 11935-9.

de la Maza A and Parra JL (1997). Vesicle to micelle phase transitions involved in the interaction of sodium cholate with phosphatidylcholine liposomes. Colloids Surfaces. 127: 125- 34.

Derycke AS and De Witte PA (2002). Transferrin-mediated targeting of hypericin embedded in sterically stabilized PEG-liposomes. *Int. J. Oncol.* 20, 181-7..

Ding Z, Cristiano RJ, Roth JA, Takacs B and Kuo MT (1995). Malaria circumsporozoite protein is a novel gene delivery vehicle to primary hepatocyte cultures and cultured cells. *J. Biological. Chem.* 270, 3667-76.

Dipali SR, Kulkarni SB, and Betageri GV (1996). Comparative study of separation of non-encapsulated drug from unilamellar liposomes by various methods. *J. Pharm. Pharmacol.* 48, 1112-5.

Doerfler W (2000). *Foreign DNA in mammalian systems.* Weinheim, Germany: WILEY-VCH.

Dolivet G, Merlin JL, Barberi-Heyob M, Ramacci C, Erbacher P, Parache RM *et al*, (2002). In vivo growth inhibitory effect of iterative wild-type p53 gene transfer in human head and neck carcinoma xenografts using glucosylated polyethylenimine nonviral vector. *Cancer Gene Ther.* 9, 708-14.

Dubowchik GM, and Walker MA (1999). Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. *Pharmacol. Ther.* 83, 67-123.

Duggan BJ, Maxwell P, Kelly JD, Canning P, Anderson NH, Keane PF *et al.*, (2001). The effect of antisense Bcl-2 oligonucleotides on Bcl-2 protein expression and apoptosis in human bladder transitional cell carcinoma. *J. Urol.*166, 1098-105.

Eichinger DJ, Arnot DE, Tam JP, Nussenzweig V, and Enea V (1986). Circumsporozoite protein of Plasmodium berghei: gene cloning and identification of the immunodominant epitopes. *Mol. Cell Biol.* 6, 3965-72.

Eikenberry EF (1982). Analytical centrifugation in density gradients. In: (Price CA Ed) *Centrifugation in density gradients* (pp. 244- 63). New York: Academic Press Inc.

Eisenberg D, Weiss RM, Terwilliger TC and Wilcox (1982). *Faraday Symp. Chem. Soc.* 17, 109-20.

Engelhardt JF, Ye X, Doranz B, and Wilson JM (1994). Ablation of E2A in recombinant

adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc. Natl. Acad. Sci. USA.* 91, 6196-200.

Ewert K, Ahmad A, Evans HM, Schmidt HW, Safinya CR (2002). Efficient synthesis and cell-transfection properties of a new multivalent cationic lipid for nonviral gene delivery. *J. Med. Chem.* 45, 5023-9.

Faraasen S, Voros J, Csucs G, Textor M, Merkle HP, and Walter E (2003). Ligand-specific targeting of microspheres to phagocytes by surface modification with poly(L-lysine)-grafted poly(ethylene glycol) conjugate. *Pharm. Res.* 20, 237-46.

Farhood H, Serbina N, Huang L (1995). The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta.* 1235, 289-95.

Fei R and Shaoyang L (2002). Combination antigene therapy targeting c-myc and c-erbB(2) in the ovarian cancer COC(1) cell line . *Gynecol. Oncol.* 85, 40-4.

Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M *et al.*, (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA.* 84, 7413-7.

Fillat C, Carrio M, Cascante A, and Sangro B (2003). Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application. *Curr. Gene Ther.* 3, 13-26.

Fischer D, Bieber T, Li Y, Elsasser HP, and Kissel T (1999). A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm. Res.* 16, 1273-9.

Folkman J (1990). What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst.* 82, 4-6.

Freeman SM, Abboud CN, Whartenby KA, Packman CH, Koeplin DS, Moolten FL *et al.*, (1993). The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.* 53, 5274-83.

Frevert U, Galinski MR, Hugel FU, Allon N, Schreier H, Smulevitch S *et al.*, (1998). Malaria circumsporozoite protein inhibits protein synthesis in mammalian cells. *EMBO J.* 17, 3816-26.

Frevert U, Sinnis P, Cerami C, Shreffler W, Takacs B, and Nussenzweig V (1993). Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *J. Exp. Med.* 177, 1287-98.

Galea-Lauri J, Farzaneh F, andGaken J (1996). Novel costimulators in the immune gene therapy of cancer. *Cancer Gene Ther.* 3, 202-14.

Galinski MR, Arnot DE, Cochrane AH, Barnwell JW, Nussenzweig RS, and Enea V (1987). The circumsporozoite gene of the Plasmodium cynomolgi complex. *Cell.* 48, 311-9.

Gao H, and Hui KM (2001). Synthesis of a novel series of cationic lipids that can act as efficient gene delivery vehicles through systematic heterocyclic substitution of cholesterol derivatives. *Gene Ther.* 8, 855-63.

Gao X and Huang L (1996). Potentiation of cationic liposome-mediated gene delivery by polycations.*Biochemistry.*35, 1027-36.

Godson GN, Ellis J, Svec P, Schlesinger DH, and Nussenzweig V (1983). Identification and chemical synthesis of a tandemly repeated immunogenic region of Plasmodium knowlesi circumsporozoite protein. *Nature.* 305, 29-33.

Gomella LG, Mastrangelo MJ, McCue PA, Maguire HC JR, Mulholland SG, and Lattime EC (2001). Phase i study of intravesical vaccinia virus as a vector for gene therapy of bladder cancer. *J. Urol.* 166, 1291-5.

Gorman CM, Aikawa M, Fox B, Fox E, Lapuz C, Michaud B *et al.*, (1997). Efficient in vivo delivery of DNA to pulmonary cells using the novel lipid EDMPC. *Gene Ther.* 4, 983-92.

Gottesman MM, Hrycyna CA, Schoenlein PV, Germann UA, and Pastan I (1995). Genetic analysis of the multidrug transporter. *Annu. Rev. Genet.* 29, 607-49.

Greenblatt MS, Bennett WP, Hollstein M and Harris CC (1994). Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.* 54, 4855-78.

Grimm CF, Ortmann D, Mohr L, Michalak S, Krohne TU, Meckel S *et al.*, (2000). Mouse alpha-fetoprotein-specific DNA-based immunotherapy of hepatocellular carcinoma leads to tumor regression in mice. Gastroenterology 119, 1104-12.

Gross A, McDonnell JM, and Korsmeyer SJ (1999). BCL-2 family members and the mitochondria in apoptosis. *Genes Dev.* 13, 1899-911.

Gschwind M and Huber G. (1997). Detection of apoptotic or necrotic death in neuronal cells by morphological, biochemical and molecular analysis. In: (Poirier J ED). *Apoptosis techniques and protocols* (pp. 13-31). New Jersey. Human press Inc.

Hafez IM, Maurer N, and Cullis PR (2001). On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther.* 8, 1188-96

Haines AM, Irvine AS, Mountain A, Charlesworth J, Farrow NA, Husain RD *et al.*, (2001). CL22 - a novel cationic peptide for efficient transfection of mammalian cells.

Gene Ther. 8, 99-110.

Hamel W, Magnelli L, Chiarugi VP, and Israel MA (1996). Herpes simplex virus thymidine kinase/ganciclovir-mediated apoptotic death of bystander cells. *Cancer Res.* 56, 2697-702

Hanke P, Serwe M, Dombrowski F, Sauerbruch T, and Caselmann WH (2002). DNA vaccination with AFP-encoding plasmid DNA prevents growth of subcutaneous AFP-expressing tumors and does not interfere with liver regeneration in mice. *Cancer Gene Ther.* 9, 346-55.

Hansen CB, Kao GY, Moase EH, Zalipsky S, and Allen TM (1995). Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. *Biochim. Biophys. Acta.* 1239, 133-44.

Hara T, Aramaki Y, Takada S, Koike K, and Tsuchiya S (1995). Receptor-mediated transfer of pSV2CAT DNA to a human hepatoblastoma cell line HepG2 using asialofetuin-labeled cationic liposomes. *Gene.* 159, 167-74.

Hara T, Ishihara H, Aramaki Y, and Tsuchiya S (1988). Specific uptake of asialofetuin-labeled liposomes by isolated hepatocytes. *Int J Pharm* 42, 69-75.

Harrington KJ, Spitzweg C, Bateman AR, Morris JC, and Vile RG (2001). Gene therapy for prostate cancer: current status and future prospects. *J. Urol.* 166, 1220-33.

Harsch M, Walther P, and Weder HG (1981). Targeting of monoclonal antibody-coated liposomes to sheep red blood cells. *Biochem. Biophys. Res. Commun.* 103, 1069-76.

Heath TD, Fraley RT, and Papahdjopoulos D (1980). Antibody targeting of liposomes: cell specificity obtained by conjugation of F(ab')2 to vesicle surface. *Science.* 210, 539-41.

Heise C, Hermiston T, Johnson L, Brooks G, Sampson-Johannes A, Williams A *et al.*, (2000). An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat. Med.* 6: 1134-9.

Helene C (1994). Control of oncogene expression by antisense nucleic acids. *Eur. J. Cancer.* 30A, 1721-6.

Helene C, Thuong NT, and Harel-Bellan A (1992). Control of gene expression by triple helix-forming oligonucleotides. The antigene strategy. *Ann. N. Y. Acad. Sci.* 660, 27-36.

Heo DS (2002). Progress and limitations in cancer gene therapy. *Genet. Med.* 4 (6 Suppl), 52S-5S.

Hong K, Zheng W, Baker A, and Papahadjopoulos D (1997). Stabilization of cationic

liposome-plasmid DNA complexes by polyamines and poly (ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery. *FEBS Lett.* 400, 233-7.

Hope MJ, Bally MB, Webb G, and Cullis PR (1985). Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped violume and ability to maintain a membrane potential. *Biochim. Biophys. Acta.* 812, 55-65.

Hortobagyi GN, Ueno NT, Xia W, Zhang S, Wolf JK, Putnam JB *et al.*, (2001). Cationic liposome-mediated E1A gene transfer to human breast and ovarian cancer cells and its biologic effects: a phase I clinical trial. *J. Clin. Oncol.* 19, 3422-33.

Hsiao M, Tse V, Carmel J, Tsai Y, Felgner PL, Haas M *et al.*, (1997). Intracavitary liposome-mediated p53 gene transfer into glioblastoma with endogenous wild-type p53 in vivo results in tumor suppression and long-term survival. *Biochem. Biophys. Res. Commun.* 233, 359-64.

Hu Q, Shew CR, Bally MB, and Madden TD (2001). Programmable fusogenic vesicles for intracellular delivery of antisense oligodeoxynucleotides: enhanced cellular uptake and biological effects. *Biochim. Biophys. Acta.* 1514, 1-13.

Huang A, Huang L, and Kennel SJ (1980). Monoclonal antibody covalently coupled with fatty acid. A reagent for in vitro liposome targeting. *J. Biol. Chem.* 255, 8015-8.

Huang CH (1969). Studies on phosphatidylcholine vesicles. Formation and physical characterization. *Biochemistry.* 8, 344-51.

Hughes BJ, Kennel S, Lee R, and Huang L (1989). Monoclonal antibody targeting of liposomes to mouse lung in vivo. *Cancer Res.* 49, 6214-20.

Hui K, Grosveld F, and Festenstein H (1984). Rejection of transplantable AKR leukaemia cells following MHC DNA-mediated cell transformation. *Nature.* 311, 750-2.

Hui SW, Langner M, Zhao YL, Ross P, Hurley E, and Chan K (1996). The role of helper lipids in cationic liposome-mediated gene transfer. *Biophys. J.* 71, 590-9.

Huwyler J, Wu D, and Pardridge WM (1996). Brain drug delivery of small molecules using immunoliposomes. *Proc. Natl. Acad. Sci USA.* 93, 14164-9.

Hwang SH, Hayashi K, Takayama K, and Maitani Y (2001). Liver-targeted gene transfer into a human hepatoblastoma cell line and in vivo by sterylglucoside-containing cationic liposomes. *Gene Ther.* 8, 1276-80.

Iden DL, and Allen TM (2001). In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. *Biochim. Biophys. Acta.* 1513, 207-16.

Iinuma H, Maruyama K, Okinaga K, Sasaki K, Sekine T, Ishida O *et al.*, (2002). Intracellular targeting therapy of cisplatin-encapsulated transferrin-polyethylene glycol liposome on peritoneal dissemination of gastric cancer. *Int. J. Cancer.* 99, 130-7.

Indraccolo S, Habeler W, Tisato V, Stievano L, Piovan E, Tosello V *et al.*, (2002). Gene transfer in ovarian cancer cells: a comparison between retroviral and lentiviral vectors. *Cancer Res.* 62, 6099-107.

Ishida T, Iden DL, and Allen TM (1999). A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs. *FEBS Lett.* 460, 129-33.

Ishikawa H, Nakao K, Matsumoto K, Ichikawa T, Hamasaki K, Nakata K et al., (2003). Antiangiogenic gene therapy for hepatocellular carcinoma using angiostatin gene. *Hepatology.* 37, 696-704.

Iyer M, Berenji M, Templeton NS, and Gambhir SS (2002). Noninvasive imaging of cationic lipid-mediated delivery of optical and PET reporter genes in living mice. *Mol. Ther.* 6, 555-62.

Jain KK (1998). *Textbook of gene therapy.* Gottingen, Germany: Hogrefe & Huber Publishers.

James HA (1999). The potential application of ribozymes for the treatment of hematological disorders. *J. Leuk. Biol.* 14, 361-68.

Janik JE, Huston MM, Cho K, and Rose JA (1989). Efficient synthesis of adeno-associated virus structural proteins requires both adenovirus DNA binding protein and VA I RNA. *Virology.* 168, 320-9.

Jiskoot W, Teerlink T, Beuvery EC, and Crommelin DJ (1986). Preparation of liposomes via detergent removal from mixed micelles by dilution. The effect of bilayer composition and process parameters on liposome characteristics. *Pharm. Weekbl. Sci.* 8, 259-65.

Jones PT, Dear PH, Foote J, Neuberger MS, and Winter G (1986). Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature.* 321, 522-5.

Kallinteri P, Liao WY, Antimisiaris SG, and Hwang KH (2001). Characterization, stability and in-vivo distribution of asialofetuin glycopeptide incorporating DSPC/CHOL liposomes prepared by mild cholate incubation. *J. Drug. Target.* 9, 155-68.

Kao GY, Change LJ, and Allen TM (1996). Use of targeted cationic liposomes in enhanced DNA delivery to cancer cells. *Cancer Gene Ther.* 3, 250-6.

Katre NV, Knauf MJ, and Laird WJ (1987). Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma

model. *Proc Natl Acad Sci USA.* 84, 1487-91.

Kaufman HL, Flanagan K, Lee CS, Perretta DJ, and Horig H (2002). Insertion of interleukin-2 (IL-2) and interleukin-12 (IL-12) genes into vaccinia virus results in effective anti-tumor responses without toxicity. *Vaccine.* 20, 1862-9.

Kawabata K, Takakura Y, and Hashida M (1995). The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm. Res.* 12, 825-30.

Kawakami S, Fumoto S, Nishikawa M, Yamashita F, and Hashida M (2000). In vivo gene delivery to the liver using novel galactosylated cationic liposomes. *Pharm. Res.* 17, 306-13.

Kawakami S, Yamashita F, Nishida K, Nakamura J, and Hashida M (2002). Glycosylated cationic liposomes for cell-selective gene delivery. *Crit. Rev. Ther. Drug Carrier Syst.* 19, 171-90.

Kawakami S, Yamashita F, Nishikawa M, Takakura Y, and Hashida M (1998). Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes. *Biochem. Biophys. Res. Commun.* 252, 78-83.

Keer HN, Kozlowski JM, Tsai YC, Lee C, McEwan RN, and Grayhack JT (1990). Elevated transferrin receptor content in human prostate cancer cell lines assessed in vitro and in vivo. *J. Urol.* 143, 381-5.

Kelly TJ Jr (1984). Adenovirus DNA replication. In: (Ginsberg HS Ed) The Adenoviruses (pp. 271-308). New York: Plenum Press.

Kibler-Herzog L, Kell B, Zon G, Shinozuka K, Mizan S, and Wilson WD (1990). Sequence dependent effects in methylphosphonate deoxyribonucleotide double and triple helical complexes. *Nucleic Acids Res.* 18, 3545-55.

Kichler A, Leborgne C, Coeytaux E, and Danos O (2001). Polyethylenimine-mediated gene delivery: a mechanistic study. *J. Gene Med.* 3, 135-44.

Kijima H, and Scanlon KJ (2000). Ribozyme as an approach for growth suppression of human pancreatic cancer. *Mol. Biotechnol.* 14, 59-72.

Kim HH, Lee WS, Yang JM, and Shin S (2003). Basic peptide system for efficient delivery of foreign genes. *Biochim Biophys Acta.* 1640, 129-36.

Kirby C, and Gregoriadis G (1980). The effect of the cholesterol content of small unilamellar liposomes on the fate of their lipid components in vitro. *Life Sci.* 27, 2223-30.
Kircheis R, Schuller S, Brunner S, Ogris M, Heider KH, Zauner W *et al.*, (1999)Polycation-based DNA complexes for tumor-targeted gene delivery in vivo. *J.*

Gene Med. 1, 111-20.

Kirn D, Niculescu-Duvaz I, Hallden G, and Springer CJ (2002). The emerging fields of suicide gene therapy and virotherapy. *Trends Mol. Med.* 8(4 Suppl), S68-73.

Klemm AR, Young D, and Lloyd JB (1998). Effects of polyethyleneimine on endocytosis and lysosome stability. *Biochem. Pharmacol.* 56, 41-6.

Klibanov AL, Maruyama K, Beckerleg AM, Torchilin VP, and Huang L (1991). Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. *Biochim. Biophys. Acta.* 1062, 142-8.

Kobayashi S, Hirakura Y, and Matsuzaki K (2001). Bacteria-selective synergism between the antimicrobial peptides alpha-helical magainin 2 and cyclic beta-sheet tachyplesin I: toward cocktail therapy. *Biochemistry.* 40, 14330-5.

Koc ON, Davis BM, Reese JS, Friebert SE, and Gerson SL (1999). Transfer of drug-resistance genes into hematopoietic progentitors. In (Lattime EC and Gerson SL, Eds). *Gene therapy of cancer* (pp. 177-200). San Diego: Academic Press..

Koping-Hoggard M, Mel'nikova YS, Varum KM, Lindman B, Artursson P (2003). Relationship between the physical shape and the efficiency of oligomeric chitosan as a gene delivery system in vitro and in vivo. *J. Gene Med.* 5, 130-41.

Kunath K, von Harpe A, Fischer D, Petersen H, Bickel U, Voigt K *et al.*, (2003). Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *J. Control Release.* 89, 113-25.

Kurnick JT, Ramirez-Montagut T, Boyle LA, Andrews DM, Pandolfi F, Durda PJ *et al.*, (2001). A novel autocrine pathway of tumor escape from immune recognition: melanoma cell lines produce a soluble protein that diminishes expression of the gene encoding the melanocyte lineage melan-A/MART-1 antigen through down-modulation of its promoter. *J. Immunol.* 167, 1204-11.

Kurrle A, Rieber P, and Sackmann E (1990). Reconstitution of transferrin receptor in mixed lipid vesicles. An example of the role of elastic and electrostatic forces for protein/lipid assembly. *Biochemistry.* 29, 8274-82.

Kwon GY, Jeong J, Woo JK, Choi HY, Lee MJ, Ko JK *et al.*, (2003). Co-expression of bfl-1 enhances host response in the herpes simplex virus-thymidine kinase/ganciclovir gene therapy system. *Biochem. Biophys. Res. Commun.* 303, 756-63.
Kyte J and Doolittle RF (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157, 105-32.

Ladish H, Berk A, Zipusky SL, Matsudaira P, Baltimore D, and Darenell J. (2000). *Molecular cell biology*. New York: W.H. Freeman and Company.

Lal AA, de la Cruz VF, Campbell GH, Procell PM, Collins WE, and McCutchan TF (1988a). Structure of the circumsporozoite gene of Plasmodium malariae. *Mol. Biochem. Parasitol.* 30, 291-4.

Lal AA, de la Cruz VF, Collins WE, Campbell GH, Procell PM, and McCutchan TF (1988b). Circumsporozoite protein gene from Plasmodium brasilianum. Animal reservoirs for human malaria parasites? *J. Biol. Chem.* 263, 5495-8.

Lal AA, de la Cruz VF, Welsh JA, Charoenvit Y, Maloy WL, and McCutchan TF (1987). Structure of the gene encoding the circumsporozoite protein of Plasmodium yoelii. A rodent model for examining antimalarial sporozoite vaccines. J. Biol. Chem. 262, 2937-40.

Lane DP and Benchimol S (1990). p53: oncogene or anti-oncogene? *Genes Devel.* 4, 1-8.

Lasic DD (1997). *Liposomes in gene delivery*. Boca Raton, Florida : CRC Press.

Latchman DC, and Coffin RS (2000). Viral vectors in the treatment of Parkinson's disease. *Mov. Disord.* 15, 9-17.

Latchman DS (2001). Gene delivery and gene therapy with herpes simplex virus-based vectors. *Gene*. 264, 1-9.

Lawler J and Hynes RO (1987). Structural organization of the thrombospondin molecule. *Semin. Thromb. Hemost.* 13, 245-54.

Lee CH, Hsiao M, Tseng YL, and Chang FH (2003). Enhanced Gene Delivery to HER-2-Overexpressing Breast Cancer Cells by Modified Immunolipoplexes Conjugated with the Anti-HER-2 Antibody. *J. Biomed. Sci.*10, 337-344.

Lee ER, Marshall J, Siegel CS, Jiang C, Yew NS, Nichols MR *et al.*, (1996). Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum. Gene Ther.* 7, 1701-17.

Lee H, Jeong JH, and Park TG (2002). PEG grafted polylysine with fusogenic peptide for gene delivery: high transfection efficiency with low cytotoxicity. *J. Control Release.* 79, 283-91.

Lee M, Nah JW, Kwon Y, Koh JJ, Ko KS, and Kim SW (2001). Water-soluble and low molecular weight chitosan-based plasmid DNA delivery. *Pharm. Res.* 18, 427-31.

Leone P, Janson CG, Bilaniuk L, Wang Z, Sorgi F, Huang L *et al.*, (2000). Aspartoacylase gene transfer to the mammalian central nervous system with therapeutic

implications for Canavan disease. *Ann. Neurol.* 48, 27-38.

Leserman LD, Barbet J, Kourilsky F, and Weinstein JN (1980). Targeting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or protein A. *Nature.* 288, 602-4.

Lestini BJ, Sagnella SM, Xu Z, Shive MS, Richter NJ, Jayaseharan J *et al.*, (2002). Surface modification of liposomes for selective cell targeting in cardiovascular drug delivery. *J. Control Release* 78, 235-47.

Lewis PF, and Emerman M (1994). Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol.* 68, 510-6.

Li B, Li S, Tan Y, Stolz DB, Watkins SC, Block LH *et al.*, (2000). Lyophilization of cationic lipid-protamine-DNA (LPD) complexes. *J. Pharm. Sci.* 89, 355-64.

Li Q, Kay MA, Finegold M, Stratford-Perricaudet LD, and Woo SL (1993). Assessment of recombinant adenoviral vectors for hepatic gene therapy. *Hum. Gene Ther.* 4, 403-9.

Li S and Huang L (1997). In vivo gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. *Gene Ther.* 4, 891-900.

Li S and Huang L (1999). Novel lipidic vectors for gene transfer. In (Huang L, Hung MC and Wagner E Eds). *Nonviral vectors for gene therapy* (pp. 289-309.). San Diego: Academic Press.

Li S, Rizzo MA, Bhattacharya S and Huang L (1998). Characterization of cationic lipid-protamin-DNA (LPD) complexes for intravenous gene delivery. *Gene Ther.* 5, 930-7.

Li X, Fu GF, Fan YR, Shi CF, Liu XJ, Xu GX *et al.*, (2003). Potent inhibition of angiogenesis and liver tumor growth by administration of an aerosol containing a transferrin-liposome-endostatin complex. *World J. Gastroenterol.* 9, 262-6.

Li Z, Shanmugam N, Katayose D, Huber B, Srivastava S, Cowan K *et al.*, (1997). Enzyme/prodrug gene therapy approach for breast cancer using a recombinant adenovirus expressing Escherichia coli cytosine deaminase. *Cancer Gene Ther.* 4, 113-7.

Licht T, and Peschel C (2002). Restoration of transgene expression in hematopoietic cells with drug-selectable marker genes. *Curr. Gene Ther.* 2, 227-34.

Lindvall O, Brundin P, Widner H, Rehncrona S, Gustavii B, Frackowiak R *et al.*, (1990). Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science* 247, 574-7.

Link CJ Jr, Levy JP, McCann LZ, and Moorman DW (1997). Gene therapy for colon cancer with the herpes simplex thymidine kinase gene. *J. Surg. Oncol.* 64, 289-94.

Litzinger DC, and Huang L (1992). Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. *Biochim. Biophys. Acta.* 1113, 201-27.

Litzinger DC, Brown JM, Wala I, Kaufman SA, Van GY, Farrell CL, and Collins D (1996). Fate of cationic liposomes and their complex with oligonucleotide in vivo. *Biochim. Biophys. Acta.* 1281, 139-49.

Liu L, Zern MA, Lizarzaburu ME, Nantz MH, and Wu J (2003). Poly(cationic lipid)-mediated in vivo gene delivery to mouse liver. *Gene Ther.* 10, 180-7.

Liu Y, Mounkes LC, Liggitt HD, Brown CS, Solodin I, Heath TD, and Debs RJ (1997). Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat. Biotechnol.* 15, 167-73.

Logg CR, Logg A, Matusik RJ, Bochner BH, and Kasahara N (2002). Tissue-specific transcriptional targeting of a replication-competent retroviral vector. *J. Virol.* 76, 12783-91.

Loughrey H, Bally MB, and Cullis PR (1987). A non-covalent method of attaching antibodies to liposomes. *Biochim. Biophys. Acta.* 901, 157-60.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951). Protein measurement with the Folin Phenol reagent. *J. Bio. Chem.* 193, 265-75.

Ma Z, Zhang J, Alber S, Dileo J, Negishi Y, Stolz Dn *et al.*, (2002). Lipid-mediated delivery of oligonucleotide to pulmonary endothelium. *Am. J. Respir. Cell Mol. Biol.* 27, 151-9.

MacDonald RC, MacDonald RI, Menco BP, Takeshita K, Subbarao NK, and Hu LR (1991). Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta.* 106, 297-303.

Mack CA, Song WR, Carpenter H, Wickham TJ, Kovesdi I, Harvey BG *et al.*, (1997). Circumvention of anti-adenovirus neutralizing immunity by administration of an adenoviral vector of an alternate serotype. *Hum. Gene Ther.* 8, 99-109.

Mahato RI, Anwer K, Tagliaferri F, Meaney C, Leonard P, Wadhwa MS *et al.*, (1998). Biodistribution and gene expression of lipid/plasmid complexes after systemic administration. *Hum. Gene Ther.* 9, 2083-99.

Mannisto M, Vanderkerken S, Toncheva V, Elomaa M, Ruponen M, Schacht E *et al.*, (2002). Structure-activity relationships of poly(L-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. *J. Control Release.* 83, 169-82.

Marais R, Spooner RA, Light Y, Martin J, and Springer CJ (1996). Gene-directed enzyme prodrug therapy with a mustard prodrug/carboxypeptidase G2 combination. *Cancer Res.* 56, 4735-42.

Marconi P, Krisky D, Oligino T, Poliani PL, Ramakrishnan R, Goins WF *et al.*, (1996). Replication-defective herpes simplex virus vectors for gene transfer in vivo. *Proc. Natl. Acad. Sci. USA.* 93, 11319-20.

Marconi P, Tamura M, Moriuchi S, Krisky DM, Niranjan A, Goins WF et al., (2000). Connexin 43-enhanced suicide gene therapy using herpesviral vectors. *Mol Ther.* 1, :71-81.

Marcucci G, Byrd JC, Dai G, Klisovic MI, Kourlas PJ, Young DC *et al.*, (2003). Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. *Blood* 101, 425-32.

Marshall CJ (1991). Tumor suppressor genes. *Cell.* 89, 124-33.

Mayer LD, Hope MJ, and Cullis PR (1986). Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta.* 858, 161-8.

McCutchan TF, Kissinger JC, Touray MG, Rogers MJ, Li J, Sullivan M *et al.*, (1996). Comparison of circumsporozoite proteins from avian and mammalian malarias: biological and phylogenetic implications. *Proc. Natl. Acad. Sci. USA.* 93, 11889-94.

McCutchan TF, Lal AA, de la Cruz VF, Miller LH, Maloy WL, Charoenvit Y et al., (1985). Sequence of the immunodominant epitope for the surface protein on sporozoites of Plasmodium vivax. *Science.* 230, 1381-3.

McKenzie DL, Smiley E, Kwok KY, and Rice KG (2000). Low molecular weight disulfide cross-linking peptides as nonviral gene delivery carriers. *Bioconjug Chem.* 11, 901-9.

McLean JW, Fox EA, Baluk P, Bolton PB, Haskell A, Pearlman R *et al.*, (1997). Organ-specific endothelial cell uptake of cationic liposome-DNA complexes in mice. *Am. J. Physiol.* 273, 387-404.

Merdan T, Kopecek J, and Kissel T (2002). Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Adv. Drug Deliv. Rev.* 54, 715-58.

Midoux P and Monsigny M (1999). efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconjug. Chem.* 10, 406-11.

Miller AD (1992). Human gene therapy comes of age. *Nature* 357, 455-60.

Miller DG, Adam MA, and Miller AD (1990). Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol. *Cell Biol.* 10, 4239-42.

Mitry RR, Sarraf CE, Wu CG, Pignatelli M, and Habib NA (1997). Wild-type p53 induces apoptosis in Hep3B through up-regulation of bax expression. *Lab Invest.* 77, 369-78.

Mizuno M, and Yoshida J (1996). Repeated exposure to cationic immunoliposomes activates effective gene transfer to human glioma cells. *Neurol. Med. Chir.* 36, 141-4.

Moreira JN, Ishida T, Gaspar R, and Allen TM (2002). Use of the post-insertion technique to insert peptide ligands into pre-formed stealth liposomes with retention of binding activity and cytotoxicity. *Pharm. Res.* 19, 265-9.

Mori A, Klibanov AL, Torchilin VP, and Huang L (1991). Influence of the steric barrier activity of amphipathic poly(ethyleneglycol) and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo. *FEBS Lett.* 284, 263-6.

Moriuchi S, Wolfe D, Tamura M, Yoshimine T, Miura F, Cohen JB *et al.*, (2002). Double suicide gene therapy using a replication defective herpes simplex virus vector reveals reciprocal interference in a malignant glioma model. *Gene Ther.* 9, 584-91.

Morris MJ, Tong WP, Cordon-Cardo C, Drobnjak M, Kelly WK, Slovin SF *et al.*, (2002). Phase I trial of BCL-2 antisense oligonucleotide (G3139) administered by continuous intravenous infusion in patients with advanced cancer. *Clin. Cancer Res.* 8, 679-83.

Mukherjee S. and Chattopadhyay A (1996). Membrane organization at low cholesterol concentrations: a study using 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labeled. cholesterol. *Biochemistry.* 35, 1311-22.

Mullen CA (1994). Metabolic suicide genes in gene therapy. *Pharmacol. Ther.* 63, 199-207.

Nah JW, Yu L, Han SO, Ahn CH, and Kim SW (2002). Artery wall binding peptide-poly(ethylene glycol)-grafted-poly(L-lysine)-based gene delivery to artery wall cells. *J. Control Release.* 78, 273-84.

Nelson GJ (1967). Lipid composition of erythrocytes in various mammalian species. *Biochim Biophys Acta.* 144, 221-32.

Nemunaitis J, Swisher SG, Timmons T, Connors D, Mack M, Doerksen L *et al.*, (2000). Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J. Clin. Oncol.* 18, 609-22.

Nguyen XT, Pabarue HA, Geyer RR, Shroyer LA, Estey LA, Parilo MS *et al.*, (2002).

Biochemical and biophysical properties of purified phospholipid vesicles containing bovine heart cytochrome *c* oxidase. *Protein Expr. Purif.* 26, 122-30.

Nicholas TW, Read SB, Burrows FJ, and Kruse CA (2003). Suicide gene therapy with Herpes simplex virus thymidine kinase and ganciclovir is enhanced with connexins to improve gap junctions and bystander effects. *Histol Histopathol.* 18, 495-507.

Niidome T, Takaji K, Urakawa M, Ohmori N, Wada A, Hirayama T *et al.*, (1999). Chain length of cationic alpha-helical peptide sufficient for gene delivery into cells. *Bioconjug. Chem.* 10, 773-80.

Niidome T, Urakawa M, Sato H, Takahara Y, Anai T, Hatakayama T *et al.*, (2000). Gene transfer into hepatoma cells mediated by galactose-modified alpha-helical peptides. *Biomaterials*. 21, 1811-9.

Nikitin AY, Juarez-Perez MI, Li S, Huang L, and Lee WH (1999). RB-mediated suppression of spontaneous multiple neuroendocrine neoplasia and lung metastases in Rb+/- mice. *Proc. Natl. Acad. Sci. USA.* 96, 3916-21.

Nishikawa M, Takemura S, Takakura Y, and Hashida M (1998). Targeted delivery of plasmid DNA to hepatocytes in vivo: optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties. *J. Pharmacol. Exp. Ther.* 287, 408-15.

Nishizaki M, Meyn RE, Levy LB, Atkinson EN, White RA, Roth JA *et al.*, (2001). Synergistic inhibition of human lung cancer cell growth by adenovirus-mediated wild-type p53 gene transfer in combination with docetaxel and radiation therapeutics in vitro and in vivo. *Clin. Cancer Res.* 7, 2887-97.

Oettgen HF, and Old LJ (1991). The history of cancer immunotherapy. In: (DeVita VT, Hellmann S, and Rosenberg SA, Eds). *Biological therapy of cancer: principles and practice* (87-119). Philadelphia. JB Lppincott Co.

Ohmori N, Niidome T, Kiyota T, Lee S, Sugihara G, Wada A *et al.*, (1998). Importance of hydrophobic region in amphiphilic structures of alpha-helical peptides for their gene transfer-ability into cells. *Biochem. Biophys. Res. Commun.* 245, 259-65.

Okuda K, Obata H and Nakajima Y (1984). Prognosis of primary hepatocellular carcinoma. *Hepatology*. 4 (Suppl), 3-6.

Opalka B, Dickopp A, and Kirch HC (2002). Apoptotic genes in cancer therapy. *Cells Tissues Organs.* 172, 126-32

Osaka G, Carey K, Cuthbertson A, Godowski P, Patapoff T, Ryan A *et al.*, (1996). Pharmacokinetics, tissue distribution, and expression efficiency of plasmid [33P]DNA

following intravenous administration of DNA/cationic lipid complexes in mice: use of a novel radionuclide approach. *J. Pharm. Sci.* 85, 612-8.
Paoletti E (1996). Applications of pox virus vectors to vaccination: an update. *Proc. Natl. Acad. Sci. USA.* 93, 11349-53.

Pape WJ, and Hoppe U (1987). Validation of the red blood cell test system as in vitro assay for the rapid screening of irritation potential of surfactants. *Mol. Toxico.* 1, 525-36.

Pardoll DM (1998). Cancer vaccines. *Nature Med.* 4(suppl), 525-31.

Parker JN, Gillespie GY, Love CE, Randall S, Whitley RJ, and Markert JM (2000). Engineered herpes simplex virus expressing IL-12 in the treatment of experimental murine brain tumors. *Proc. Natl. Acad. Sci. USA.* 97, 2208-13.

Pedroso de Lima MC, Simoes S, Pires P, Faneca H, and Duzgunes N (2001). Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Adv. Drug Deliv. Rev.* 47, 277-94.

Peplinski GR, Tsung K, Meko JB, and Norton JA (1995) In vivo gene therapy of a murine pancreas tumor with recombinant vaccinia virus encoding human interleukin-1 beta.*Surgery*.118,185-91.

Pichon C, Goncalves C, and Midoux P (2001). Histidine-rich peptides and polymers for nucleic acids delivery. Adv. *Drug Deliv. Rev.* 53, 75-94.

Plank C, Tang MX, Wolfe AR, and Szoka FC Jr (1999). Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and in vitro activity of DNA polyplexes. *Hum Gene Ther.* 10, 319-32.

Ponnazhagan S, Curiel DT, Shaw DR, Alvarez RD, and Siegal GP (2001). Adeno-associated virus for cancer gene therapy. *Cancer Res.* 61, 6313-21.

Potter M, and Marcu KB (1997). The c-myc story: where we've been, where we seem to be going. *Curr. Top. Microbiol. Immunol.* 224, 1-17.

Pouton CW, Lucas P, Thomas BJ, Uduehi AN, Milroy DA, and Moss SH (1998). Polycation-DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. *J. Control. Release.* 53, 289-99.

Prater CA, Plotkin J, Jaye D, and Frazier WA (1991). The properdin-like type I repeats of human thrombospondin contain a cell attachment site. *J. Cell. Biol.* 112, 1031-40.

Puisieux A, Galvin K, Troalen F, Bressac B, Marcais C, Galun E *et al.*, (1993). Retinoblastoma and p53 tumor suppressor genes in human hepatoma cell lines. *FASEB J.* 7, 1407-13.

Qin H, Valentino J, Manna S, Tripathi PK, Bhattacharya-Chatterjee M, Foon KA et al., (2001). Gene therapy for head and neck cancer using vaccinia virus expressing IL-2 in a murine model, with evidence of immune suppression. *Mol. Ther.* 4, 551-8.

Quantin B, Perricaudet LD, Tajbakhsh S, and Mandel JL (1992). Adenovirus as an expression vector in muscle cells in vivo. *Proc. Natl. Acad. Sci. USA.* 89, 2581-4.

Radler JO, Koltover I, Salditt T, and Safinya CR (1997). Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science.* 275, 810-4.

Ragot T, Vincent N, Chafey P, Vigne E, Gilgenkrantz H, Couton D *et al.*, (1993). Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice. *Nature.*361, 647-50.

Raper SE, Yudkoff M, Chirmule N, Gao GP, Nunes F, Haskal ZJ, Furth EE, Propert KJ, Robinson MB, Magosin S, Simoes H, Speicher L, Hughes J, Tazelaar J, Wivel NA, Wilson JM, and Batshaw ML. A pilot study of in vivo liver-directed gene transfer with an adenoviral vector in partial ornithine transcarbamylase deficiency. Hum. Gene Ther. 13, (2002): 163-75.

Rathore D and McCutchan TF (2000). Role of cysteines in Plasmodium falciparum circumsporozoite protein: interactions with heparin can rejuvenate inactive protein mutants. *Proc. Nat.l Acad. Sci. USA.* 97, 8530-5.

Rathore D, Sacci JB, de la Vega P, and McCutchan TF (2002). Binding and invasion of liver cells by Plasmodium falciparum sporozoites. Essential involvement of the amino terminus of circumsporozoite protein. *J. Biol. Chem.* 277, 7092-8.

Read ML, Bremner KH, Oupicky D, Green NK, Searle PF, and Seymour LW (2003). Vectors based on reducible polycations facilitate intracellular release of nucleic acids. J. *Gene Med.* 5, 232-45.

Reed J (1999). Dysregulation of apoptosis in cancer. J. Clin. Oncol. 17, 2941-53.

Regelin AE, Fankhaenel S, Gurtesch L, Prinz C, von Kiedrowski G, and Massing U (2000). Biophysical and lipofection studies of DOTAP analogs. *Biochim. Biophys. Acta.* 1464, 151-64

Ribas A, Butterfield LH, and Economou JS (2000). Genetic immunotherapy for cancer. *Oncologist.* 5, 87-98.

Ries LAG, Kosary CL, Hankey BF, Miller BA, Clegg LX, and Edwards BK (Eds) (1999). SEER cancer statistics review, 1973-1996, National cancer institute. NIH Pub. No 99-2789. Bethesda, MD.

Rittner K, Benavente A, Bompard-Sorlet A, Heitz F, Divita G, Brasseur R et al., (2002). New basic membrane-destabilizing peptides for plasmid-based gene delivery in vitro and in vivo. *Mol. Ther.* 5, 104-14.

Rivnay B and Metzger H (1983). Use of the airfuge for analysis and preparation of receptors incorporated into liposomes: studies with the receptor for immunoglobulin E. *Anal Biochem.* 130, 514-20.

Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, and Henderson DR (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* 57, 2559-63.

Roerdink F, Dijkstra J, Hartman G, Bolscher B, and Scherphof G (1981). The involvement of parenchymal, Kupffer and endothelial liver cells in the hepatic uptake of intravenously injected liposomes. Effects of lanthanum and gadolinium salts. *Biochim. Biophys. Acta.* 677, 79-89.

Roggero MA, Filippi B, Church P, Hoffman SL, Blum-Tirouvanziam U, Lopez JA *et al.*, (1995). Synthesis and immunological characterization of 104-mer and 102-mer peptides corresponding to the N- and C-terminal regions of the Plasmodium falciparum CS protein. Mol Immunol. 32, 1301-9.

Rosenberg SO, Gunther VS, Armstrong TA, Pulaski BA, Pipeling MR, Clements VK, and Lamouse-Smith N (1999). Immunologic targets for the gene therapy of cancer. In (Lattime EC and Gerson SL, Eds). *Gene therapy of cancer* (pp. 33-48). San Diego: Academic Press.

Ross PC, and Hui SW (1999). Lipoplex size is a major determinant of in vitro lipofection efficiency. *Gene Ther.* 6, 651-9.

Roy I, Holle L, Song W, Holle E, Wagner T, and Yu X (2002). Efficient translocation and apoptosis induction by adenovirus encoded VP22-p53 fusion protein in human tumor cells in vitro. *Anticancer Res.* 22, 3185-9.

Sager R (1989). Tumor suppresser genes: the puzzle and the promise. *Science.* 246, 1406-12.

Samulski RJ, Zhu X, Xiao X, Brook JD, Housman DE, Epstein N *et al.*, (1991). Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J.* 10, 3941-50.

Saudemont A, Buffenoir G, Denys A, Desreumaux P, Jouy N, Hetuin D et al., (2002). Gene transfer of CD154 and IL12 cDNA induces an anti-leukemic immunity in a murine model of acute leukemia. *Leukemia.* 16, 1637-44.

Sauter ER, Takemoto R, Litwin S, and Herlyn M (2002). p53 alone or in combination with antisense cyclin D1 induces apoptosis and reduces tumor size in human melanoma. *Cancer Gene Ther.* 9, 807-12.

Scharovsky OG, Rozados VR, Gervasoni SI, and Matar P (2000). Inhibition of ras oncogene: a novel approach to antineoplastic therapy. *J. Biomed. Sci.* 7, 292-8.

Schubert R, Beyer K, Wolburg H, and Schmidt KH (1986). Structural changes in membranes of large unilamellar vesicles after binding of sodium cholate. Biochemistry. 25, 5263-9.

Seki M, Iwakawa J, Cheng H, and Cheng PW (2002). p53 and PTEN/MMAC1/TEP1 gene therapy of human prostate PC-3 carcinoma xenograft, using transferrin-facilitated lipofection gene delivery strategy. *Hum. Gene Ther.* 13, 761-73.

Seol JG, Heo DS, Kim HK, Yoon JH, Choi BI, Lee HS et al., (2000). Selective gene expression in hepatic tumor with trans-arterial delivery of DNA/liposome/transferrin complex. *In Vivo.* 14, 513-7.

Seville PC, Kellaway IW, and Birchall JC (2002). Preparation of dry powder dispersions for non-viral gene delivery by freeze-drying and spray-drying. *J. Gene Med.* 4, 428-37.

Shen C, Rattat D, Buck A, Mehrke G, Polat B, Ribbert H *et al.*, (2003). Targeting bcl-2 by Triplex-Forming Oligonucleotide-A Promising Carrier for Gene-Radiotherapy. *Cancer Biother. Radiopharm.* 18, 17-26.

Shen DF, Huang A, and Huang L (1982). An improved method for covalent attachment of antibody to liposomes. *Biochim. Biophys. Acta.* 689, 31-7.

Shewring L, Collins L, Lightman SL, Hart S, Gustafsson K, and Fabre JW (1997). A nonviral vector system for efficient gene transfer to corneal endothelial cells via membrane integrins. *Transplantation.* 64, 763-9.

Shi F, Rakhmilevich AL, Heise CP, Oshikawa K, Sondel PM, Yang NS *et al.*, (2002). Intratumoral injection of interleukin-12 plasmid DNA, either naked or in complex with cationic lipid, results in similar tumor regression in a murine model. Mol. *Cancer Ther.* 1. 949-57.

Shi N, and Pardridge WM (2000). Noninvasive gene targeting to the brain. *Proc. Natl. Acad. Sci. USA.* 97, 7567-72.

Shi N, Boado RJ, and Pardridge WM (2001). Receptor-mediated gene targeting to tissues in vivo following intravenous administration of pegylated immunoliposomes. *Pharm. Res.* 18, 1091-5.

Shimotohno K, and Temin HM (1981). Formation of infectious progeny virus after

insertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. *Cell.* 26, 67-77.
Simberg D, Danino D, Talmon Y, Minsky A, Ferrari ME, Wheeler CJ *et al.*, (2001). Phase behavior, DNA ordering, and size instability of cationic lipoplexes. Relevance to optimal transfection activity. *J. Biol. Chem.* 276, 47453-9.

Sinnis P, Clavijo P, Fenyo D, Chait BT, Cerami C, and Nussenzweig V (1994). Structural and functional properties of region II-plus of the malaria circumsporozoite protein. *J. Exp. Med.* 180, 297-306.

Sinnis P, Willnow TE, Briones MR, Herz J, and Nussenzweig V (1996). Remnant lipoproteins inhibit malaria sporozoite invasion of hepatocytes. *J. Exp. Med.* 184, 945-54.

Skelton NJ and Chain WJ (2000). Solution structure determination of proteins by nuclear magnetic resonance spectroscopy. In (Reid RE Ed). *Peptide and protein drug analysis* (pp. 683-726). New York: Marcel Dekker, Inc.

Sliedregt LA, Rensen PC, Rump ET, van Santbrink PJ, Bijsterbosch MK, Valentijn AR *et al.*, (1999). Design and synthesis of novel amphiphilic dendritic galactosides for selective targeting of liposomes to the hepatic asialoglycoprotein receptor. *J. Med. Chem.* 42, 609-18.

Smith GL and Moss B (1983). Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene.* 25, 21-8.

Solly SK, Trajcevski S, Frisen C, Holzer GW, Nelson E, Clerc B *et al.*, (2003). Replicative retroviral vectors for cancer gene therapy. *Cancer Gene Ther.* 10, 30-9.

Solodin I, Brown CS, Bruno MS, Chow CY, Jang EH, Debs RJ *et al.*, (1995). A novel series of amphiphilic imidazolinium compounds for in vitro and in vivo gene delivery. *Biochemistry.* 34, 13537-44.

Sorgi FL, Bhattacharya S, and Huang L (1997). Protamine sulfate enhances lipid-mediated gene transfer. *Gene Ther.* 4, 961-8.

Spencer RL, and Wold F (1969). A new convenient method for estimation of total cystine-cysteine in proteins. *Anal Biochem.* 32, 185-90.

Spiro RG (1973). Glycoproteins. *Adv. Protein Chem.* 27, 349-467.

Sternberg B, Sorgi FL, and Huang L (1994). New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. *FEBS Lett.* 356, 361-6.

Stripecke R, Levine AM, Pullarkat V, and Cardoso AA (2002). Immunotherapy with acute leukemia cells modified into antigen-presenting cells: ex vivo culture and gene

transfer methods. *Leukemia.* 16, 1974-83.

Suarez JE, Urquiza M, Puentes A, Garcia JE, Curtidor H, Ocampo M *et al.*, (2001). Plasmodium falciparum circumsporozoite (CS) protein peptides specifically bind to HepG2 cells. *Vaccine.* 19, 4487-95.

Suh W, Chung JK, Park SH, Kim SW (2001). Anti-JL1 antibody-conjugated poly (L-lysine) for targeted gene delivery to leukemia T cells. *J. Control Release.* 72, 171-8.

Swisher SG, Roth JA, Komaki R, Gu J, Lee JJ, Hicks M *et al.*, (2003). Induction of p53-regulated genes and tumor regression in lung cancer patients after intratumoral delivery of adenoviral p53 (INGN 201) and radiation therapy. *Clin. Cancer Res.* 9, 93-101.

Swisher SG, Roth JA, Nemunaitis J, Lawrence DD, Kemp BL, Carrasco CH *et al.*, (1999). Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer. *J. Natl. Cancer Inst.*91, 763-71.

Tabor E (1994). Tumor suppressor genes: growth factor genes, and oncogenes in hepatitis B virus-associated hepatocellular carcinoma. *J. Med. Virol.* 42, 357-65.

Takamiya Y, Short MP, Moolten FL, Fleet C, Mineta T, Breakefield XO *et al.*, (1993). An experimental model of retrovirus gene therapy for malignant brain tumors. *J. Neurosurg.* 79, 104-10

Tan Y, Whitmore M, Li S, Frederik P, and Huang L (2002). LPD nanoparticles--novel nonviral vector for efficient gene delivery. *Methods Mol. Med.* 69, 73-81.

Tanaka T, Yamasaki H, and Mesnil M (2001). Induction of a bystander effect in HeLa cells by using a bigenic vector carrying viral thymidine kinase and connexin32 genes. *Mol. Carcinog.* 30, 176-80.

Templeton NS, Lasic DD, Fredrik PM, Strey HH, Roberts DD and Pavlahis GN (1997). Improved DNA: Liposome complexes for increased systemic delivery and gene expression. *Nature Biotech.* 15, 647-52.

Thierry AR, Rabinovich P, Peng B, Mahan LC, Bryant JL, and Gallo RC (1997). Characterization of liposome-mediated gene delivery: expression, stability and pharmacokinetics of plasmid DNA. *Gene Ther.* 4, 226-37.

Tokunaga T, Tsuchida T, Kijima H, Okamoto K, Oshika Y, Sawa N *et al.*, (2000). Ribozyme-mediated inactivation of mutant K-ras oncogene in a colon cancer cell line. *Br. J. Cancer.* 83, 833-9.

Tossi A, Sanri L, and Giangaspero A. (2002) Synthetic antimicrobial peptides designed from sequence templates. In E. Benedetti and C. Pedone Eds. *Peptides 2002, Proceedings of the 27th European Peptide Symposium.* Edizioni Ziino, Naples, Italy, pp. 416-417.

Touraine RL, Vahanian N, Ramsey WJ, and Blaese RM (1998). Enhancement of the herpes simplex virus thymidine kinase/ganciclovir bystander effect and its antitumor efficacy in vivo by pharmacologic manipulation of gap junctions. *Hum Gene Ther.* 9, 2385-91.

Tsuchiya S, Aramaki Y, Hara T, Hosoi K, and Okada A (1986). Preparation and disposition of asialofetuin-labelled liposome. *Biopharm. Drug Dispos.* 7, 549-58.

Ueno NT, Bartholomeusz C, Xia W, Anklesaria P, Bruckheimer EM, Mebel E et al., (2002). Systemic gene therapy in human xenograft tumor models by liposomal delivery of the E1A gene. *Cancer Res.* 62, 6712-6.

Ueno NT, Yu D, and Hung MC (2001). E1A: Tumor suppressor or oncogene? Preclinical and clinical investigations of E1A gene therapy. *Breast Cancer.* 8, 285-93.

Uyttenhove C, Maryanski J, and Boon T (1983). Escape of mouse mastocytoma P815 after nearly complete rejection is due to antigen-loss variants rather than immunosuppression. *J. Exp. Med.* 157, 1040-52

Venyaminov SY and Yang JT (1996). Determination of protein secondary structure. In: (Fasman GD Ed). *Circular dichroism and the conformational analysis of biomolecules* (pp. 69- 107). New York: Plenum Press.

Verdini AS, Chiappinelli L, and Zanobi A (1991). Toward the elucidation of the mechanism of attachment and entry of malaria sporozoites into cells: synthetic polypeptides from the circumsporozoite protein of Plasmodium falciparum bind Ca2+ and interact with model phospholipid membranes. Biopolymers. 31, 587-94.

Verhoeyen M, Milstein C, and Winter G (1988). Reshaping human antibodies: grafting an antilysozyme activity. *Science.* 239, 1534-6.

Vermeij J, Zeinoun Z, Neyns B, Teugels E, Bourgain C, and De Greve J (2001). Transduction of ovarian cancer cells: a recombinant adeno-associated viral vector compared to an adenoviral vector. *Br. J. Cancer.* 85, 1592-9.

Vincent L, Varet J, Pille JY, Bompais H, Opolon P, Maksimenko A et al., (2003). Efficacy of dendrimer-mediated angiostatin and TIMP-2 gene delivery on inhibition of tumor growth and angiogenesis: In vitro and in vivo studies. *Int. J. Cancer.* 105, 419-29.

Vlachaki MT, Hernandez-Garcia A, Ittmann M, Chhikara M, Aguilar LK, Zhu X *et al.*, (2002). Impact of preimmunization on adenoviral vector expression and toxicity in a subcutaneous mouse cancer model. *Mol. Ther.* 6, 342-8.

Vollmer CM Jr, Eilber FC, Butterfield LH, Ribas A, Dissette VB, Koh A *et al.*, (1999). Alpha-fetoprotein-specific genetic immunotherapy for hepatocellular carcinoma. *Cancer*

Res. 59, 3064-7.

Wagner E, Plank C, Zatloukal K, Cotton M and Birnstiel ML (1992). Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferring-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. USA* 89, 7934–38.

Walker TL, Dass CR, and Burton MA (2002). Enhanced in vivo tumour response from combination of carboplatin and low-dose c-myc antisense oligonucleotides. *Anticancer Res.* 22, 2237-45.

Walther W, Stein U, Fichtner I, Voss C, Schmidt T, Schleef M et al., (2002). Intratumoral low-volume jet-injection for efficient nonviral gene transfer. *Mol. Biotechnol.* 21, 105-15.

Wang J, Guo X, Xu Y, Barron L, and Szoka FC Jr (1998). Synthesis and characterization of long chain alkyl acyl carnitine esters. Potentially biodegradable cationic lipids for use in gene delivery. *J. Med. Chem.* 41, 2207-15.

Wang SC and Hung MC (2001). HER2 overexpression and cancer targeting. *Semin. Oncol.* 5 (Suppl 16), 115-24.

Ward CM, Pechar M, Oupicky D, Ulbrich K, and Seymour LW (2002). Modification of pLL/DNA complexes with a multivalent hydrophilic polymer permits folate-mediated targeting in vitro and prolonged plasma circulation in vivo. *J. Gene Med.* 4, 536-47.

Ward CM, Read ML, and Seymour LW (2001). Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy. *Blood.* 97, 2221-9.

Wei CM, Gibson M, Spear PG, and Scolnick EM (1981). Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. *J. Virol.* 39, 935-44.

Wei MX, Tamiya T, Rhee RJ, Breakefield XO, and Chiocca EA (1995). Diffusible cytotoxic metabolites contribute to the in vitro bystander effect associated with the cyclophosphamide/cytochrome P450 2B1 cancer gene therapy paradigm. *Clin. Cancer Res.* 1, 1171-7.

Wei SJ, Chao Y, Hung YM, Lin WC, Yang DM, Shih YL *et al.*, (1998). S- and G2-phase cell cycle arrests and apoptosis induced by ganciclovir in murine melanoma cells transduced with herpes simplex virus thymidine kinase. *Exp. Cell Res.* 241, 66-75.

Weinberg RA (1991). Tumor suppressor genes. *Science.* 254, 1138-46.

Wightman L, Kircheis R, Rossler V, Carotta S, Ruzicka R, Kursa M *et al.*, (2001).

Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J. Gene Med.* 3, 362-72.
Wilkins MR, Lindskog I, Gasteiger E, Bairoch A, Sanchez JC, Hochstrasser DF et al., (1997). Detailed peptide characterisation using PEPTIDEMASS - a World-Wide Web accessible tool. *Electrophoresis* 18, 403-8.

Wolfert MA, Dash PR, Nazarova O, Oupicky D, Seymour LW, Smart S, Strohalm J, and Ulbrich K (1999). Polyelectrolyte vectors for gene delivery: influence of cationic polymer on biophysical properties of complexes formed with DNA. *Bioconjug. Chem.* 10, 993-1004.

Wu GY, and Wu CH (1987). Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J. Biol. Chem.* 262, 4429-32.

Wu J, and Zern MA (1996). Modification of liposomes for liver targeting. *J. Hepatol.* 24, 757-63.

Wu J, Liu P, Zhu JL, Maddukuri S, and Zern MA (1998). Increased liver uptake of liposomes and improved targeting efficacy by labeling with asialofetuin in rodents. *Hepatology*. 27, 772-8.

Wu J, Nantz MH, and Zern MA (2002). Targeting hepatocytes for drug and gene delivery: emerging novel approaches and applications. *Front. Biosci.* 7, d717-25.

Wu N, Watkins SC, Schaffer PA, and DeLuca NA (1996). Prolonged gene expression and cell survival after infection by a herpes simplex virus mutant defective in the immediate-early genes encoding ICP4, ICP27, and ICP22. *J. Virol.* 70, 6358-69.

Wyman TB, Nicol F, Zelphati O, Scaria PV, Plank C, and Szoka FC Jr (1997). Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. *Biochemistry*. 36, 3008-17.

Xu M, Kumar D, Srinivas S, Detolla LJ, Yu SF, Stass SA *et al.*, (1997). Parenteral gene therapy with p53 inhibits human breast tumors in vivo through a bystander mechanism without evidence of toxicity. *Hum. Gene Ther.* 8, 177-85.

Yakinoglu AO, Heilbronn R, Burkle A, Schlehofer JR, zur Hausen H (1988). DNA amplification of adeno-associated virus as a response to cellular genotoxic stress. *Cancer Res.* 48, 3123-9.

Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, and Wilson JM (1994). Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA*. 91, 4407-11.

Yates JL, Warren N, and Sugden B (1985). Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature*. 313, 812-5.

Yi SW, Yune TY, Kim TW, Chung H, Choi YW, Kwon IC et al., (2000). A cationic lipid emulsion/DNA complex as a physically stable and serum-resistant gene delivery system. *Pharm. Res.* 17, 314-20.

Ying P, Shakibaei M, Patankar MS, Clavijo P, Beavis RC, Clark GF et al., (1997). The malaria circumsporozoite protein: interaction of the conserved regions I and II-plus with heparin-like oligosaccharides in heparan sulfate. *Exp. Parasitol.* 85, 168-82.

Yoshida J and Mizuno M (1995). Simple preparation and characterization of cationic liposomes associated with monoclonal antibody against glioma- associated antigen (immunoliposomes). *J. Liposome Res.*5, 981-95.

Yoshimura I, Suzuki S, Tadakuma T, and Hayakawa M (2001). Suicide gene therapy on LNCaP human prostate cancer cells. *Int. J. Urol.* 8, S5-8.

Zalipsky S, Mullah N, Harding JA, Gittelman J, Guo L, and DeFrees SA (1997). Poly(ethylene glycol)-grafted liposomes with oligopeptide or oligosaccharide ligands appended to the termini of the polymer chains. *Bioconjug. Chem.* 8, 111-8.

Zeh HJ, and Bartlett DL (2002). Development of a replication-selective, oncolytic poxvirus for the treatment of human cancers. *Cancer Gene Ther.* 9, 1001-12.

Zhang WW, and Roth JA (1994). Anti-oncogene and tumor suppressor gene therapy examples from a lung cancer animal model. *In Vivo.* 8, 755-69

Zhang Y, Zhu C, and Pardridge WM (2002). Antisense gene therapy of brain cancer with an artificial virus gene delivery system. *Mol. Ther.* 6, 67-72.

Zhou W, Yuan X, Wilson A, Yang L, Mokotoff M, Pitt B *et al.*, (2002). Efficient intracellular delivery of oligonucleotides formulated in folate receptor-targeted lipid vesicles. *Bioconjug. Chem.* 13, 1220-5.

Zou Y, Zong G, Ling Y, Hao MM, Lozano G, Hong WK *et al.*, (1998). effective treatment of early endobranchial cancer with regional administration of liposome-*p53* complexes. *J. Natl. Cancer. Inst.* 90, 1130-7.

Zou Y, Zong G, Ling YH, and Perez-Soler R (2000). Development of cationic liposome formulations for intratracheal gene therapy of early lung cancer. *Cancer Gene Ther.* 7. 683-96