

**Lipoplex-mediated gene transfer: Influence of selected
chemotherapeutic agents on transgene expression**

by

Lynn Karen Nicholson
B.Sc. (Honours), Queen's University, 1993

A thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in
The Faculty of Graduate Studies
Department of Pathology
and Laboratory Medicine

We accept this thesis as conforming
to the required standard.

The University of British Columbia
April 1999

© Lynn Karen Nicholson, 1999

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Pathology & Lab Medicine

The University of British Columbia
Vancouver, Canada

Date Wed Apr 28

Abstract

The ultimate goal of anticancer therapies should be the elimination and/or suppression of tumour cells. Lipid-based gene transfer systems have been employed in promoting transgene expression of exogenous DNA whose products are capable of performing these roles. Since improvements in antitumour efficacy may be obtained by combining gene therapy with other therapeutic modalities such as chemotherapy, it is pertinent to characterize the influence of conventional cytotoxic drugs upon transgene expression, achieved following use of a lipoplex-mediated gene transfer system.

SKOV-3 human ovarian carcinoma and B16/BL6 murine melanoma cells were used as *in vitro* models to evaluate levels of transgene expression. Lipoplexes, [DODAC/DOPE (1:1 mol ratio) liposomes complexed with plasmid DNA containing the luciferase or chloramphenicol acetyltransferase reporter gene] were added to cells that had been pre-exposed for 24h to various cytotoxic drugs. Transgene expression levels were established 48h post-transfection. Two *in vivo* models were also established to evaluate whether chemotherapeutic agents would affect transgene expression following lipoplex-mediated gene transfer. C57BL/6 mice bearing B16/BL6 tumours were injected intraperitoneally with lipoplexes, three days after receiving an intravenous drug injection equivalent to the maximum therapeutic dose. The second model analyzed transgene expression in spleen and bone marrow samples from CD1 mice. Lipoplexes were intravenously administered to mice 3, 7, 14 or 21 days after they had received an intravenous drug injection equivalent to the maximum therapeutic dose. In both models, transgene expression was determined 24h post-transfection.

In vitro cytotoxicity data indicates that lipoplexes did not influence the sensitivity of SKOV-3 or B16/BL6 cells to cisplatin, doxorubicin, vincristine or bleomycin. Transgene expression levels increased, decreased or remained the same (relative to lipoplex only controls), depending upon the drug, its concentration and the cell type. Combining chemotherapy with an intraperitoneal model for lipoplex-mediated gene transfer did not affect transgene expression levels evaluated in B16/BL6 tumours. The *in vivo* intravenous model measuring spleen and bone marrow transfection levels showed that all selected drugs caused some inhibition of transgene expression. These results suggest that DNA damage is not a mechanism by which to increase gene transfer by lipoplexes, rather drug-induced alterations in cellular uptake and processing of lipoplexes are believed to be responsible for inducing changes (increases or decreases) in transgene expression.

Table of Contents

Table of Contents	iv
Abstract	iii
List of Figures	vii
List of Tables	ix
Abbreviations	x
Acknowledgments	xi
Dedication	xii

Chapter 1 Introduction

1.1	Combination gene therapy: past predictions/future prospects	1
1.2	Hypothesis statement	1
1.3	Advances in gene therapy	2
1.4	Methods of gene transfer	3
1.4.1	Viral vectors	4
1.4.1.1	Retroviruses	4
1.4.1.2	Adenoviruses	5
1.4.1.3	Herpesviruses	6
1.4.1.4	Other viral systems	6
1.4.2	Plasmid expression vectors	7
1.4.2.1	Polymer-based delivery systems	9
1.4.2.2	Lipid-based delivery systems	9
1.4.2.3	Composite “artificial” viral vectors	10
1.5	Lipid-based delivery systems	11
1.5.1	Lipids	11

1.5.2	Liposomes	15
1.5.3	Cationic liposomes	17
1.5.3.1	Lipoplexes	19
1.5.3.2	Lipoplex structures	20
1.5.4	Mechanism of gene transfer	22
1.5.4.1	Uptake of lipoplexes	22
1.5.4.2	Nuclear localization	24
1.6	Evaluating gene transfer efficiency	25
1.6.1	Chloramphenicol acetyltransferase	26
1.6.2	Luciferase	27
1.7	Problems with lipoplex-mediated gene therapy	27
1.8	Chemotherapeutic agents	28
1.8.1	Combination therapy strategies	29
1.8.2	Cisplatin	30
1.8.3	Doxorubicin	31
1.8.4	Vincristine	34
1.8.5	Bleomycin	35
1.9	Thesis objectives	37

Chapter 2 Material and Methods

2.1	Materials	38
2.2	Methods	39
2.2.1	Cell culture	39
2.2.2	Chemotherapeutic agents	40
2.2.3	Liposome preparations	40
2.2.4	Lipoplex preparations	41
2.2.5	<i>In vitro</i> analysis	42
2.2.5.1	MTT cytotoxicity assay	42
2.2.5.2	<i>In vitro</i> luciferase expression analysis	43
2.2.5.3	<i>In vitro</i> CAT expression analysis	44

2.2.6	<i>In vivo</i> analysis	46
2.2.6.1	Intraperitoneal model	46
2.2.6.2	Intravenous model	47
2.2.6.3	<i>In vivo</i> CAT expression analysis	47
2.2.7	Statistical analysis	48
Chapter 3	Results	
3.1	Examination of cytotoxicity	49
3.2	<i>In vitro</i> lipoplex-mediated transfection in the presence of selected chemotherapeutic agents	51
3.2.1	Luciferase transgene expression in SKOV-3 cells	51
3.2.2	Luciferase transgene expression in B16/BL6 cells	54
3.2.3	CAT transgene expression in B16/BL6 cells	56
3.3	<i>In vivo</i> lipoplex-mediated transfection in the presence of selected chemotherapeutic agents	57
3.3.1	Transfection of B16/BL6 tumors following intraperitoneal administration of lipoplexes	58
3.3.2	Transfection following intravenous administration of lipoplexes	60
Chapter 4	Discussion	65
Chapter 5	References	74

List of Figures

Figure 1A.	Schematic representation of linear, relaxed and supercoiled plasmid DNA.	8
Figure 1B.	Agarose gel electrophoresis for separation of plasmid DNA.	8
Figure 2A.	Structure of phosphatidylcholine, a common bilayer-forming phospholipid.	12
Figure 2B.	Common phospholipid headgroup components.	13
Figure 3.	Polymorphic phases and corresponding molecular shape of various lipids.	14
Figure 4.	Schematic representation of multilamellar, large unilamellar and small unilamellar vesicles.	16
Figure 5.	Chemical structures of cationic lipids used in lipid-based gene transfer delivery systems.	18
Figure 6.	Models proposed for the structures of lipoplexes formed following addition of DNA to cationic lipids.	21
Figure 7.	Lipoplex-mediated gene transfer.	23
Figure 8.	Structure of cisplatin and cisplatin-DNA adducts.	31
Figure 9.	Structure of doxorubicin.	32

Figure 10.	Schematic illustration of (A) Topo (II) involvement in DNA strand breakage and resealing; (B) doxorubicin interference with Topo II-DNA interactions; (C) enzymatic activation of doxorubicin and subsequent activation of superoxide anion, hydrogen peroxide and hydroxyl radicals.	33
Figure 11.	Structure of vincristine.	35
Figure 12.	Structure of bleomycin.	36
Figure 13.	SKOV-3 cytotoxicity of selected anticancer agents in the presence and absence of added lipoplexes.	50
Figure 14.	Luciferase transgene expression in SKOV-3 cells following lipoplex-mediated gene transfer.	53
Figure 15.	Luciferase transgene expression in B16/BL6 cells following lipoplex-mediated gene transfer.	55
Figure 16.	CAT transgene expression in intraperitoneal B16/BL6 tumours following intraperitoneal administration of lipoplexes.	59
Figure 17.	CAT transgene expression in various tissues following intravenous administration of lipoplexes.	61
Figure 18.	CAT transgene expression in spleen (A) and bone marrow (B) following intravenous administration of lipoplexes.	63

List of Tables

Table 1.	IC ₅₀ for selected drugs and cell lines in the presence and absence of lipoplexes.	51
Table 2.	CAT transgene expression in B16/BL6 cells following transfection with DODAC/DOPE lipoplexes prepared using pINEXCatv2.0, in the presence and absence of cisplatin.	57

Abbreviations

^{14}Cm	radiolabel [^{14}C] chloramphenicol
$^3\text{H-CHDE}$	[^3H]-cholesteryl hexadecyl ether
BCA	bicinchonic acid
BLEO	bleomycin sulfate
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CDDP	<i>cis</i> -diamminedichloroplatinum, cisplatin
ddH ₂ O	demineralized distilled water
DODAC	dioleoyldimethylammoniumchloride
DOPE	dioleoylphosphatidylethanolamine
DOX	doxorubicin hydrochloride
dpm	disintegrations per minute
FBS	fetal bovine serum
x g	relative centrifugal force
h	hours
HBSS	Hank's Balanced Salt Solution
H _{II}	hexagonal phase
HSV	herpes simplex virus
IC ₅₀	drug concentration at which 50% cell kill occurs
i.p	intraperitoneal
i.v	intravenous
LUVs	large unilamellar vesicles
min	minutes
MLVs	multilamellar vesicles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
p	probability
PBS	phosphate buffered saline
RLU	relative light units
RT	room temperature
SEM	standard error of the mean
SUVs	small unilamellar vesicles
Topo	topoisomerase
VINC	vincristine sulfate

Acknowledgments

As with most endeavors, the number of accredited people behind the ideas, the execution and the documentation does not reflect the actual depth of participation. My time at the BCCRC extended beyond the scope of this thesis and as such, there are numerous individuals whom deserve my thanks and appreciation. Likewise, a great number of people outside this scientific realm have been a tremendous support and must know how grateful I am to them.

First and foremost is my greatest thanks to my supervisor, Marcel Bally, for his enthusiasm, motivation and the opportunity to work within a fantastic environment. His patience, attention and above all, his wonderfully accepting humour made it a pleasure to work within his group. May all his future students realize this and play at least one good practical joke a semester on him! Thanks Marcel.

Included within this eclectic group of mad scientists were a number of people I'm pleased to call my friends and would like to thank for their scientific insight and in particular, their uncanny ability to pinpoint just exactly where I had gone wrong and how on earth I'd managed to kill my cells this time!! To Tash, for offering to scrape my unconscious body off the floor whenever we had to do the mousey bloodfest and for being my constant sidekick for gossip and gab. To Howie, for always being there to squirm at the gossip and gab. To my French globetrotting coterie – Pierrot, Frances and Howie for allowing me to practice my atrocious French. Pierrot, may your bountiful sense of optimism serve you well on the 'Bench of 1000 Curses' (and thank you once again for all your homogenization buffer, sterile Eppendorfs, CAT plasmid, incomplete buffer, marathon tips, hiking maps and stories, more homogenization buffer.....). And of course to Spencer, for his endless patience when faced with a hurricane of data, a frustrated grad student with a scientific mind block and a pathetic plea for "heeeeeelp" May all your cells live long and healthy and may you continue to make the world beautiful with your art. Thank you very much.

The downstairs gang is filled with terrific people whose company I have greatly enjoyed over the past couple of years. Whether it was a suggestion to help my experiments along, shared sympathy when Origin would indiscriminately gobble data or just a friendly chat at the Friday parties, this group was amazing. In particular I would like to acknowledge a few individuals who stand out in my mind for any of these reasons. Thank you very much to the furry creature experts Rebecca and Dana, Dawn, Gywn, Emma, Ghania, Dody, Tetyana, Hafiza, Jennifer, Ellen, Margaret and Paul.

I am also grateful for the help of my other supervisory committee members – Kirsten Skov, Martin Trotter and Colin Fyfe. Their interest and assistance in this project has been most appreciated.

In addition, my family and friends have been wonderful during this time, whether it was in the form of hilarious emails, Nicholson feasting free-for-alls, hikes to the peaks or parties in the city. Mom and Dad, Weasel, David, G and G, Western Nicholsons, mid-Western Nicholsons, Shena, Vanessa, Sarah, Kelly, Grace, Rich, Trev, Sean and Karen, Jen, Shawn, Gord, Tom, Terry, Emma and Clare – THANK YOU!!!!

And to the person whose heart appears on every one of these sheets of paper – this is for you Malte.

Dedication

This thesis is dedicated to: Connie Brown

A voyageur in the world of science all her life.

B.A. (1926), University of Western Ontario

M.A. (1928), University of Western Ontario

Chapter 1

Introduction

1.1 Combination gene therapy: past predictions/future prospects

Within the last decade, dramatic developments in genomic research have emphasized the potential for gene therapy to provide the next generation of novel therapeutics. Diverse strategies using gene therapy for *in vivo* human applications have been proposed each with its own advantages and disadvantages (1-4). Great expectations were placed on gene therapy to offer improved therapeutic products that retained the advantages of conventional therapies. It was predicted that gene therapy could offer a safe, cost-effective and versatile method for correcting the genetic defects responsible for many diseases, including cancer. Although significant advances have been made in the development of viable gene therapy strategies for the treatment of cancer, this technology has yet to realize these expectations for replacing conventional therapies such as chemotherapy, the use of radiation or surgery. Future research studies in the field of gene therapy may not focus on the development of novel methodology *per se*, but on its use in combination with other modalities to achieve improved patient response.

1.2 Hypothesis Statement

Working in synergy to benefit cancer treatment, gene therapy will be utilized in combination with previously identified treatment modalities, such as

chemotherapy, radiation and surgery. It is proposed that the advantages of gene therapy can be exploited and its effects enhanced with the use of selected chemotherapeutic drugs as a combined therapeutic strategy. The drugs may enhance transgene expression by inducing DNA damage and its associated repair or by synchronizing cells. Chemotherapeutic agents may also alter cellular processing of gene transfer components, perhaps by limiting access to the degradative lysosomal pathway. This restriction would thereby increase the potential for endosomal escape required by lipid-based plasmid delivery systems. **It is reasonable to hypothesize that enhanced transgene expression of a reporter gene can be achieved if lipoplex-mediated gene transfer is used in combination with chemotherapeutic agents.**

To examine the hypothesis that selected drugs can enhance lipoplex-mediated gene transfer, experimental models have been established for the transfer of a marker gene via a delivery vehicle capable of transfecting cells *in vitro* and *in vivo*. Lipoplexes, cationic liposomes complexed with plasmid DNA containing a suitable reporter gene, will mediate this gene transfer. Lipoplex-mediated gene transfer will be preceded by exposure to a variety of chemotherapeutic agents that have potential to augment transfection.

1.3 Advances in gene therapy

The effective management and treatment of inherited or acquired disease using genetic material requires detailed knowledge of genetic and cellular processes, in addition to the pathology of the disease. Genetic intervention involves alterations of either somatic or germline target cells with the introduction of genetic material capable of repairing a

defective gene or regulating production of a specific protein. Clinical advances in this field include: transferring normal transmembrane conductance regulator cellular DNA (cDNA) to the airway epithelium of cystic fibrosis patients (5), introducing antisense oligonucleotide sequences to inhibit translation of human immunodeficiency virus messenger RNA (6) and transferring the herpes simplex virus thymidine kinase suicide gene into cells, thereby rendering them susceptible to ganciclovir (7). These are only a few selected examples of the vast numbers of strategies that exist to treat various genetic defects that can be linked to acquired and inherited disease.

Gene therapy strategies rely upon techniques that permit a cDNA sequence linked to an appropriate promoter, to be introduced into a cell via a gene transfer agent (8). A fundamental requirement of successful gene therapy is efficient delivery of genes to target cells. This can be achieved by a variety of viral and non-viral techniques.

1.4 Methods of gene transfer

The introduction and expression of a foreign gene in a host cell can be achieved using viral (9-11) or non-viral (plasmid) (12-14) vectors. When assessing the merits of each type of vector, several parameters should be considered. They include: transfection efficiency, stability of gene product and longevity of its expression, ability to target specific cells/organs for delivery, capacity to generate cell-specific gene expression, toxicity, host immune response, ease of preparation/application and cost.

1.4.1 Viral vectors

DNA and RNA viruses have the ability to cause a transformation within a host cell via transfer of their genetic material (15). It was speculated that this inherent ability could be exploited for the purpose of transferring genes. There have been an extensive number of viruses studied for gene transfer potential, including retroviruses (16, 17), adenoviruses (18, 19) and herpesviruses (20, 21).

1.4.1.1 Retroviruses

By integrating genetic information directly into the genome of the host cell, retroviruses confer an altered genotype that is maintained within target cell progeny (22, 23). The retroviral genome contains the gene of interest and sequences that permit reverse transcription of RNA within the host cell and integration of the DNA into the host genome. This infectious vector is replicative-deficient due to the engineered absence of coding sequences required for viral genomic RNA production and packaging of the RNA into a virion. Introduction of this retroviral vector to a complementary cell line that is capable of replication and packaging but infectious-deficient, results in the assembly of a virus particle. Within the cytoplasm, viral RNA is converted using reverse transcriptase into viral DNA, which randomly integrates into the host cell genome. The altered gene is transcribed into mRNA and translated to yield the protein of interest. Some modifications may have to be performed during these processes to achieve a functional protein product.

These vectors are advantageous due to the large range of host cells that they may target and the efficiency and stability associated with this transfection. However, problems with retroviruses arise due to insertion gene size limitations and their capacity to transfect only dividing cells. As with all viral-based therapies, retroviral use is limited because of residual viral elements within the vector that may induce an immunogenic response within the host (24). Additional problems exist by virtue of their random integration within the host genome. This increases the potential for genetic rearrangement, germline infection and recombination events that could convert a replicative-defective vector to an infectious agent.

1.4.1.2 Adenoviruses

Adenoviral vectors are capable of transferring genetic information into the nucleus of the host cell but do not permit direct integration into the genome. As with retroviruses, this is achieved using a complementary packaging cell line (18). The adenoviral genome contains the gene of interest and structural sequences for assembling the genome into a virion, but lacks the early (E1) gene sequences necessary for replication. Introducing the adenoviral genome into a packaging cell line containing these early sequences results in assembly of an adenovirus vector. Entry of this large DNA vector via receptor-mediated endocytosis positions the vector within a cytoplasmic endosome. Disruption of the endosome allows adenoviral migration into the nucleus where the epichromosomal adenovirus genome directs production of the protein of interest (25).

This technique infects post-mitotic cells, thus allowing gene transfer to occur in a large number of replicative and non-replicative cells. Due to its epichromosomal nature,

the risk of altering host cell genotype or creating unwanted changes due to insertional mutagenesis is avoided. However, there remains a risk of recombination with wild type viruses, which could restore replicative and infectious abilities to the adenoviral genome. Due to the non-integrating, non-replicating nature of the adenoviruses, transgene expression is limited in duration and may require subsequent administration of the virus for sustained expression. This increases the potential for immunogenic viral coat proteins to trigger non-specific inflammation and T lymphocyte-mediated cellular immunity (26).

1.4.1.3 Herpesviruses

This family includes several classes of viruses, of which only the herpes simplex virus (HSV) has been utilized successfully as a gene therapy agent. Upon infection of post-mitotic cells, HSV establishes latent infections, resulting in permanent residence of the viral genome in host cells. HSV has a predisposition towards infecting neural cells in a manner that does not interfere with the host cell genome or its metabolism. HSV vectors can accept large gene inserts and can carry a unique promoter that enables gene expression during the HSV latency period of its life cycle. Problems using this type of vector for gene therapy occur as a result of the uncontrollable nature of this latent life cycle, in addition to HSV eliciting an immune response (27, 28).

1.4.1.4 Other viral systems

Adeno-associated (29), vaccinia (30), polio (31) and RNA viruses (32) are among the many other families of viruses that have been assessed for potential to transfer genes

in an efficient and reliable manner. Each was found to have its own advantages and disadvantages when employed as gene transfer agents. For example, adeno-associated viruses accept only small gene inserts and require adenoviruses for propagation. However, they can integrate into specific genomic regions of non-mitotic cells (18).

1.4.2 Plasmid expression vectors

Non-viral delivery systems attempt to incorporate many biological aspects of viral systems, but are fundamentally different in their gene delivery and expression processes (12-14). Plasmids are self-replicating molecules of DNA found in a variety of bacterial, fungal, plant and other species. Free plasmids exist in linear, relaxed and supercoiled configurations. Since rate of plasmid migration depends on degree of coiling, the supercoiled (migrates the furthest), relaxed (migrates the least) and linear (migrates in between) configurations can be resolved using gel electrophoresis (Figure 1). It is generally thought that optimal transfection is attained using supercoiled plasmid DNA (33). This is most likely due to relaxed and linearized plasmid DNA being more accessible to endonuclease digestion.

Prokaryotic plasmid DNA, containing the origin of replication and any other sequences necessary for transfecting eukaryotic host cells is isolated and purified by ion-exchange chromatography or density gradient ultracentrifugation. In addition to any promoter/enhancer sequences, a cDNA sequence vector that codes for production of the protein of interest is inserted into the plasmid DNA construct. Due to the DNA phosphate backbone, the resulting plasmid expression vector has a negative charge.

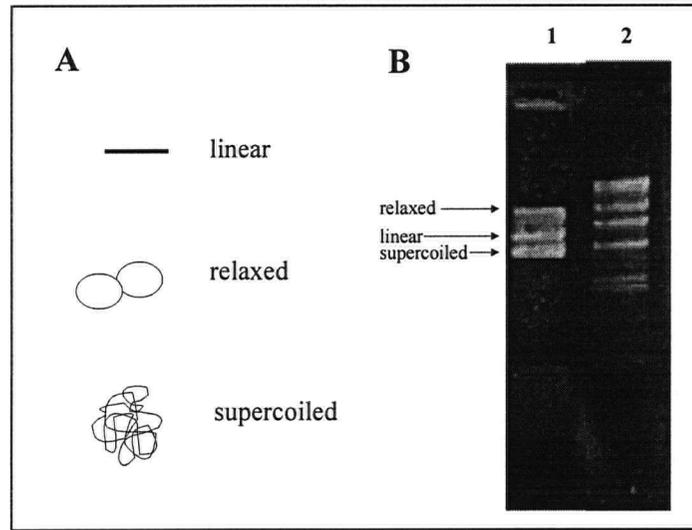


Figure 1. (A) Schematic representation of linear, relaxed and supercoiled plasmid DNA. (B) Agarose gel electrophoresis for separation of plasmid DNA. Lane 1, plasmid DNA in relaxed, linear and supercoiled configurations; lane 2, molecular weight marker (λ phage DNA digested with *Hind*III) (Figure derived from Harvie *et al.* (34)).

Upon addition of cationic peptides or other polyvalent cations, DNA is thought to condense into small toroid or rod-like structures (35). It has been suggested that plasmid DNA condensation occurs when 90% of the negative DNA phosphate charge is neutralized by counterions. Large aggregates have also been reported as a result of condensation reactions. At this time, it is not yet clear which condensed DNA structure is important for transfection. In fact, it has not yet been determined if DNA condensation is always necessary for transfection using non-viral gene transfer systems. Novel hydrophobic lipid-DNA complexes that transfect cells reasonably well have been recently formed with DNA in an uncondensed state (36).

Plasmid expression vectors require a suitable delivery system, capable of introducing the vector into a host cell where it is processed to yield the protein of interest. Since they lack the infectious ability of viruses, plasmid vectors can be introduced into

host cells by mechanical, chemical and carrier-based techniques such as electroporation, microinjection, calcium phosphate precipitation, in addition to polymer-, lipid- and composite vector-based delivery systems.

1.4.2.1 Polymer-based delivery systems

There has been a considerable amount of interest in the use of synthetic DNA delivery systems, originating with studies of polylysine-based vectors (37). Their numerous cytotoxic disadvantages drew attention to a class of non-linear vectors known as dendrimers (38). These highly branched compounds possess a number of surface amine groups that enable electrostatic charge interactions with plasmid vectors and cell surface molecules. These interactions facilitate uptake of the dendrimer complex into target cells. This method of gene delivery can be augmented by the addition of peptides into the dendrimer to enhance endosomal release of the DNA into the cytoplasm (39). It is thought that dendrimers transfect cells in a manner similar to polylysine vectors but have improved efficiency due to their greater solubility and more defined architecture.

1.4.2.2 Lipid-based delivery systems

Upon hydration, lipids spontaneously form spherical structures that possess an ideal surface to which DNA may bind. Liposomes that have been prepared from lipids containing cationic headgroups are capable of binding plasmid DNA, forming a self-assembling "lipoplex" complex. Prepared easily as liposome-DNA aggregates (40) or lipid-DNA particles (41), lipoplexes provide a safe, economical and simple vehicle for

gene delivery. Lipid-based delivery systems are widely used for DNA transfer as they offer low toxicity, the ability to transfer large gene sequences and a potential for cell-type specific targeting. They also have no risk for self-replication and are typically non-immunogenic due to the absence of immunogenic proteins (42).

For the studies outlined in this thesis, a lipid-based delivery system was used to mediate delivery of plasmid expression vectors containing a reporter gene. Specifically, the lipoplexes generated for use in this study incorporate liposomes and DNA in aggregate form. Details of this delivery system will be discussed in greater depth in section 1.5.

1.4.2.3 Composite “artificial” viral vectors

Genetically altering viral vectors to reduce some of their negative characteristics has led to the synthesis of a new class of vectors. There have been a variety of composite or “artificial” viral vectors produced, which combine viral vectors with other compounds (43, 44). For example, one delivery system incorporates a targeted molecular conjugate coupled to an adenovirus (45). Plasmid DNA can be linked to a polylysine-conjugated antibody and this complex can be bound to an adenovirus vector. This method incorporates the advantages of efficient adenoviral gene transfer with specific cell/organ-type targeting, as mediated by the antibody conjugate.

1.5 Lipid-based delivery systems

Many of the viral and non-viral delivery systems described in section 1.4 are utilized in gene transfer studies and may benefit from the synergistic effects of combination gene therapies. However, the work presented in this thesis focuses on the characteristics of lipid-based delivery and in particular, liposomes prepared with cationic and neutral lipids that are complexed with plasmid DNA expression vectors.

1.5.1 Lipids

Lipids are an essential component of cellular and intracellular membranes, critical for normal cell function. Composed primarily of phospholipids, cholesterol and small quantities of glycolipids, lipids are essential for membrane formation, active transport and some enzymatic activity (46). Most abundant are the polar phospholipids, which are composed of a phosphate headgroup and fatty acids esterified to a glycerol backbone. Cholesterol is the principal sterol of eukaryotic membranes. This neutral lipid is comprised of a rigid sterol ring and polar 3- β -hydroxyl group.

Most membrane lipids typically arrange themselves as a bilayer, forming a natural permeability barrier (47). This configuration can be attributed to the amphipathic characteristics of the lipids. The hydrophilic headgroup and hydrophobic fatty acyl chains serve to orient the lipid molecules, such that the headgroup interacts with the aqueous environment and non-polar acyl chains face one another (Figure 2A).

A

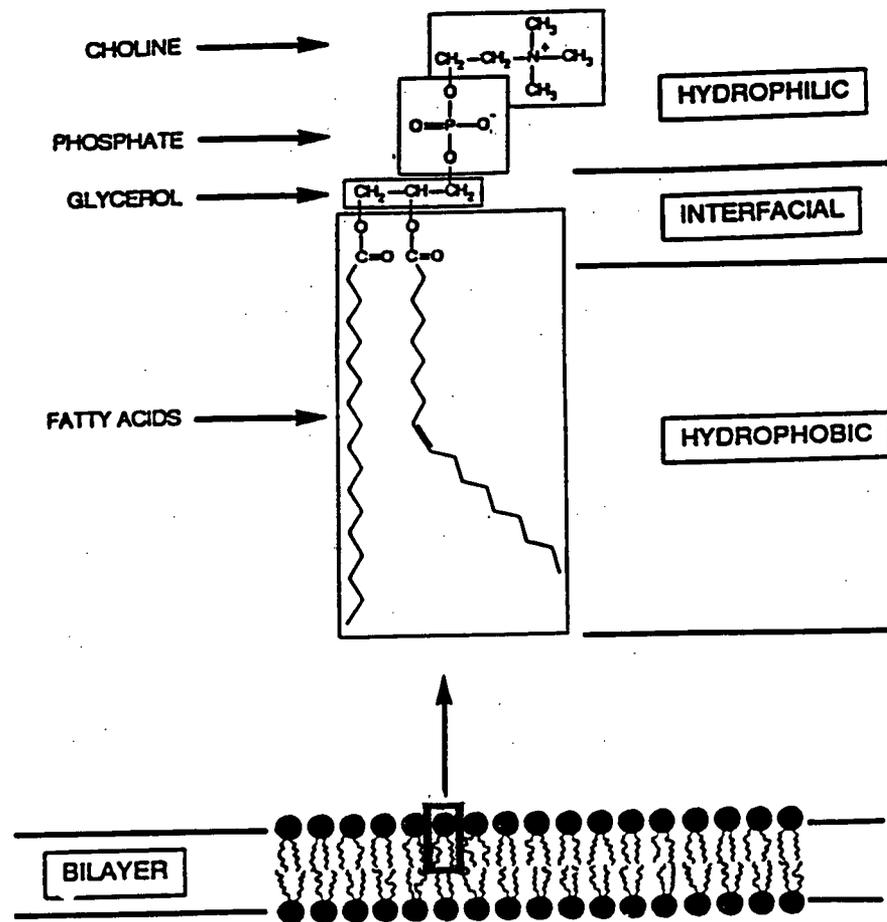
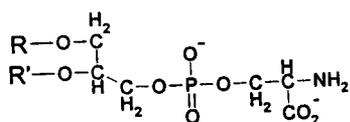


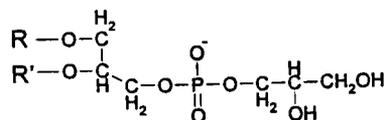
Figure 2A. Structure of phosphatidylcholine, a common bilayer-forming phospholipid.

The charge properties of the lipid headgroup greatly influence lipid behaviour and are critical in mediating interactions between the lipid and other charged compounds.

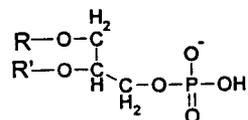
Membrane lipids are categorized on the basis of these charge properties. Some of the more common phospholipids are those comprised of anionic (*e.g.* phosphatidylserine, phosphatidylglycerol, phosphatidic acid, phosphatidylinositol) and neutral (*e.g.* phosphatidylethanolamine, phosphatidylcholine (Figure 2A)) headgroups (Figure 2B).

B

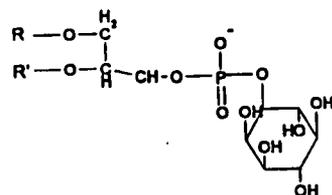
Phosphatidylserine



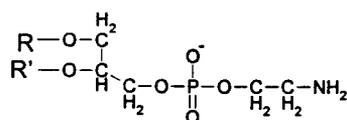
Phosphatidylglycerol



Phosphatidic acid



Phosphatidylinositol



Phosphatidylethanolamine

Figure 2B. Common phospholipid headgroup components.

In addition to the characteristics imparted by the lipid headgroup, the composition of acyl chains also plays an important role in lipid behaviour. Fatty acids are carboxylic acids with long-chain hydrocarbon side groups that exist in saturated or unsaturated form. The length and degree of saturation of hydrocarbon chains determines their efficiency in packing to fill space.

As previously mentioned, most lipids typically adopt a bilayer structure. This behaviour can be related to the generalized shape concept of lipids. Lipid shape depends on area calculated at the hydrophobic and hydrophilic interfaces, volume of the lipid molecule and its length (48). A cylindrical shape describes lipids whose hydrated

headgroups are approximately the same width as their tails. The steric requirements of packing cylindrical-shaped lipids together promote bilayer structures. However, this phase behaviour may deviate in response to variations in lipid headgroup and acyl chains, temperature or other extrinsic factors (48). Under specific conditions, some lipids prefer non-bilayer structures and will adopt micellar or hexagonal (H_{II}) morphology (Figure 3).

Lipid	Phase	Molecular Shape
phosphatidylcholine * phosphatidic acid phosphatidylserine phosphatidylglycerol phosphatidylinositol	bilayer	cylindrical
phosphatidylethanolamine * phosphatidic acid (in the presence of divalent cations, low pH) phosphatidylserine (in the presence of divalent cations, low pH)	hexagonal	cone

Figure 3. Polymorphic phases and corresponding molecular shape of various lipids.

* commonly used in lipid-based gene transfer delivery systems.

(Figure derived from Cullis *et al.* (1986) (48)).

H_{II} structures arise due to a net cone shape in which the tail of the lipid is wider than the hydrated headgroup. Headgroups are oriented inwards, encircling an aqueous core. The acyl chains exclude themselves from the aqueous environment by interacting with the acyl chains of neighbouring lipids.

The ability of lipids to adopt non-bilayer morphology suggests that these structures play a functional role in membrane-mediated events such as membrane fusion (40). This interbilayer event, in which two lipid bilayer membranes are fused together to form a single lipid bilayer via an intermediary structure, is of particular interest to lipid-based delivery systems that may require lipid membrane fusion. Aggregation and subsequent lipid mixing of membranes during the transfection process may lead to phase separation of lipid components. Production of a non-bilayer intermediate as a result of this separation is most likely precursory for membrane fusion.

1.5.2 Liposomes

Liposomes are spherical vesicles comprised of a bilayer lipid membrane surrounding an aqueous region. Upon hydration of lipids, large (>1000nm) multilamellar vesicles (MLVs) composed of numerous bilayers separated by aqueous channels will spontaneously form. Small (SUVs, <50nm) or large (LUVs, 50-200nm) unilamellar vesicles may be derived from MLVs, yielding single bilayer liposomes (Figure 4).

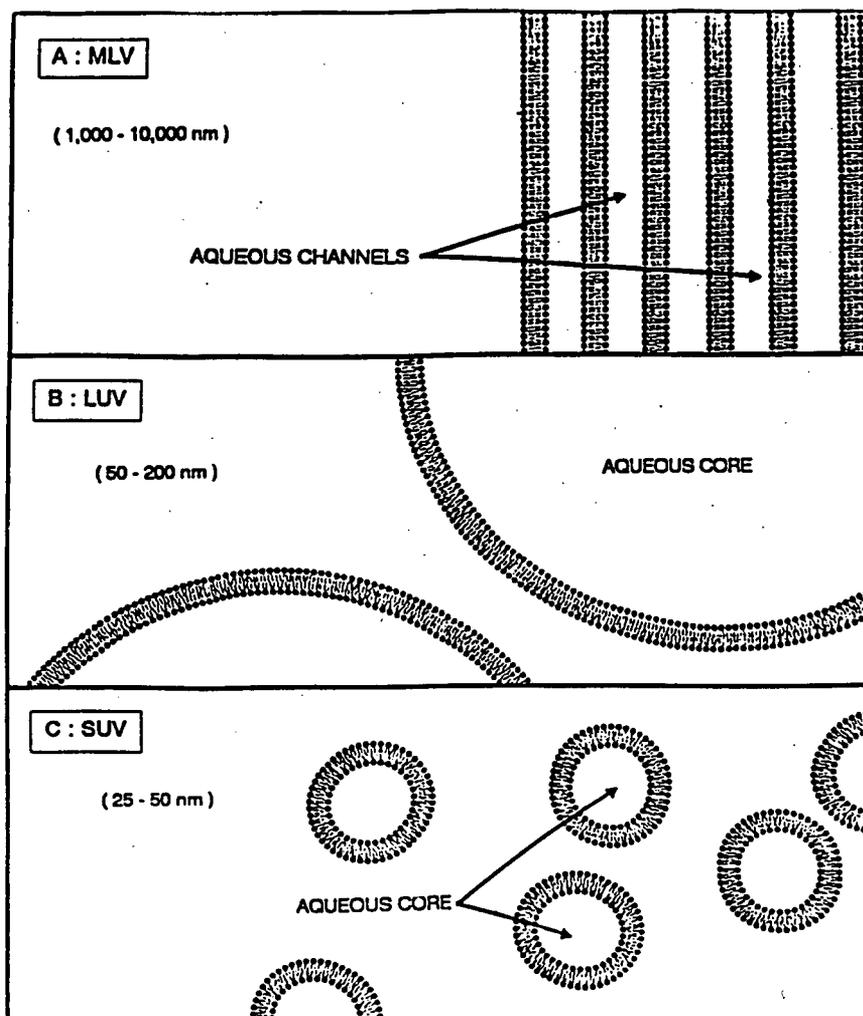


Figure 4. Schematic representation of (A) multilamellar, (B) large unilamellar and (C) small unilamellar vesicles. (49)

Initially, liposomes were used as models of biological membranes. This enabled the study of lipid structure and function during such events as fusion (50), multidrug resistance (51) and protein interaction (52). Liposomes have also been employed for the purpose of delivering a variety of effectors such as chemotherapeutic (53) or radioactive (54) agents, viruses (55), and oligonucleotide sequences (56). Another area of early liposomal research focused upon lipid-based DNA delivery systems and their capacity for gene transfer (57, 58). Investigators explored the use of liposomes as carriers of DNA into cells and found that DNA encapsulated into liposomes remained intact and resistant

to deoxyribonuclease digestion. They also determined that negatively charged liposomes, as compared to neutral liposomes, were more efficient as DNA transfer agents (59). The synthesis of cationic liposomes in 1987 by Felgner *et al.* (60) greatly aided gene therapy research by providing a DNA delivery system that was versatile, simple to use and maintained adequate transfection capabilities.

1.5.3 Cationic liposomes

Cationic liposomes are usually formulated as SUVs or LUVs and most often contain a combination of cationic (Figure 5) and neutral lipids. Manipulation of basic headgroups and fatty acyl chains resulted in the commercial synthesis of a vast selection of cationic lipids, ideal for lipid-based gene transfer. Cationic lipids currently used in gene transfer include: dioleoyldimethylammoniumchloride (DODAC), dioleoyltrimethylammonium propane (DOTAP), dimethyldioctadecylammonium bromide (DDAB), N[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl-ammonium chloride (DOTMA), 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl-ammonium bromide (DMRIE), dioctadecylamidoglycylspermidine (DOGS) and 3 β -[N-(dimethylaminoethane)-carbonyl]-cholesterol (DC-chol). The protonated amine group(s) of the cationic lipid attract negatively charged plasmid expression vectors via ionic interaction (61). Membrane fusion or lipid mixing between liposomes occurs upon DNA binding, resulting in reorganization of the lipid structure. During this process, DNA also undergoes structural changes and may collapse into a condensed structure as described in section 1.4.2.

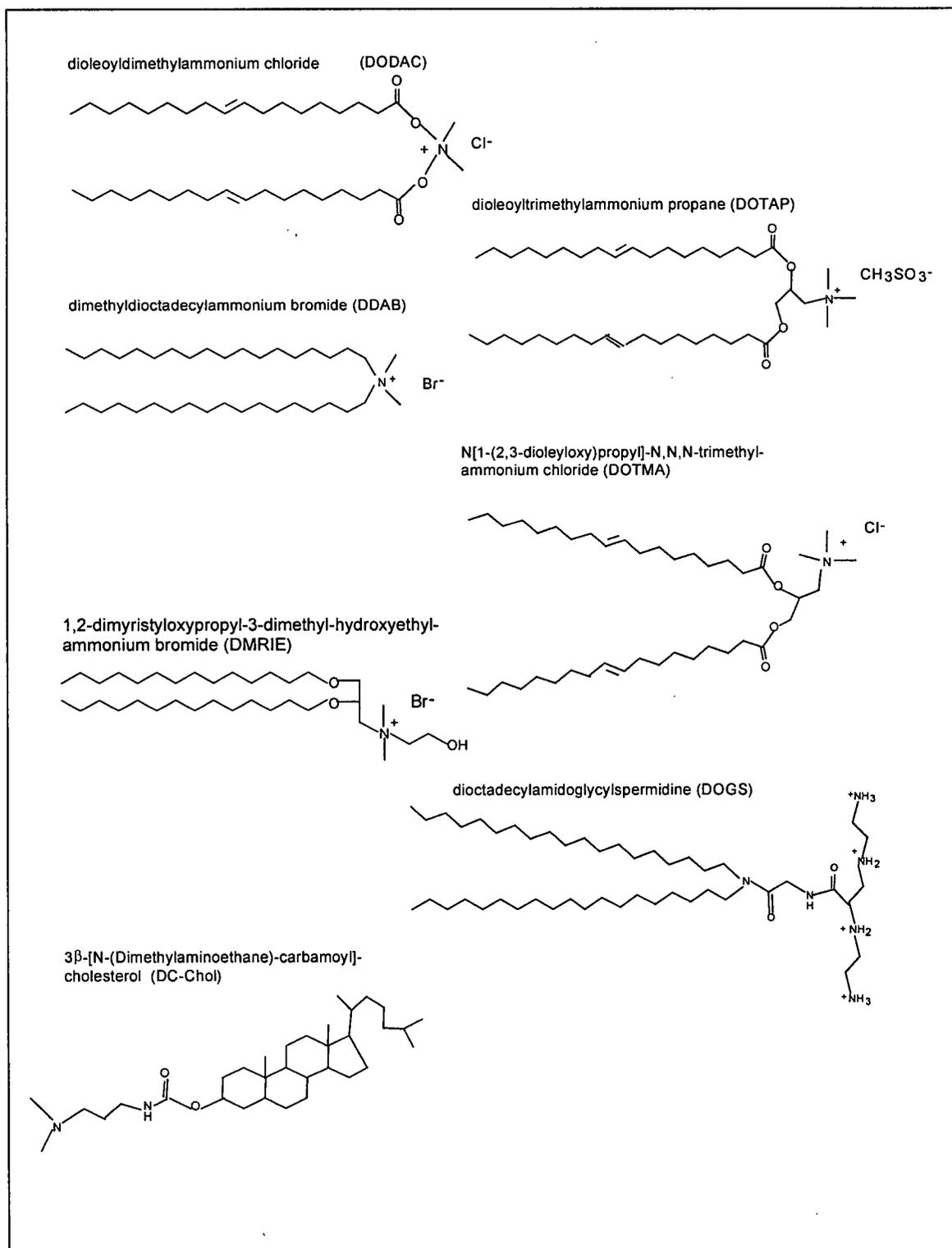


Figure 5. Chemical structures of cationic lipids used in lipid-based gene transfer delivery systems.

Neutral lipids, such as dioleoylphosphatidylethanolamine (DOPE) and cholesterol are often incorporated into the liposome formulation to serve as helper lipids. Some helper lipids, in the absence of bilayer-forming lipid, will adopt an H_{II} configuration as previously described in section 1.5.1. However, biological membranes composed of a mixture of bilayer and non-bilayer lipids are usually constrained within bilayer morphology. The non-bilayer lipids can be stabilized and an overall bilayer formation can be maintained with the presence of 20-50 mol% bilayer lipids (48). Inclusion of a helper lipid is thought to play a role in enhancing gene transfer, assisting during several stages of gene delivery such as lipoplex destabilization and endosomal release of DNA (62). Further consideration of this unique lipid group and in particular, the influence DOPE imparts upon gene transfer is given in section 1.5.4.2.

Pre-formed cationic liposomes are capable of complexing with plasmids via ionic interactions. Through simple mixing, positively charged lipids and negatively charged DNA attract one another, triggering the lipoplex self-assembly process. The resulting aggregated complex is competent at delivering plasmid DNA, facilitating transfection in a variety of cellular systems.

1.5.3.1 Lipoplexes

It has been shown that efficiency of gene delivery is influenced in part by the composition of the lipoplex (63). Specifically, the cationic group to anionic phosphate group charge ratio of the lipoplex, the relative amount of lipids present in the liposome and the absolute concentration of each component in the lipoplex are important factors for optimal lipoplex-mediated transfection efficiency (40). An excess of positive

liposomal charge insures complete binding of DNA, while retaining a residual charge to facilitate lipoplex binding to typically anionic cell plasma membranes. Assessment of different lipoplex formulations with respect to their transfection ability is hampered by a host of factors, including quantitative variations between cell types and the absence of standardized measurement protocols.

For the purpose of this thesis, cationic liposomes were prepared using DODAC and DOPE in a 1:1 mol ratio as described in section 2.2.3. They were then complexed with plasmid DNA in a 10:1 (nmol of lipid/ μ g of DNA) ratio (section 2.2.4). This specific lipoplex formulation was optimized for maximum transfection efficiency within this laboratory and is known to correspond with a 1.62:1.00 (positive cationic lipid to negative DNA phosphate group) charge ratio (64).

1.5.3.2 Lipoplex structures

Lipoplexes have been observed using a variety of visualization techniques (*e.g.* freeze-fracture electronmicroscopy, X-ray diffraction, optical microscopy) and are typically described as a diverse mixture of heterogenous structures. Lipoplex morphology has been reported as: lipid globules comprised of an alternating lipid bilayer/intercalated DNA monolayer lattice array (65); DNA strands coated by either a lipid bilayer (spaghetti-like structure) (66) or a lipid monolayer which complexes with many other monolayer-coated DNA structures in an inverted hexagonal array (honeycomb structure) (67) and other structures not yet understood (Figure 6). It has not been determined which lipoplex structure is the “active” component that contributes to lipoplex uptake and intracellular delivery of DNA to the nucleus.

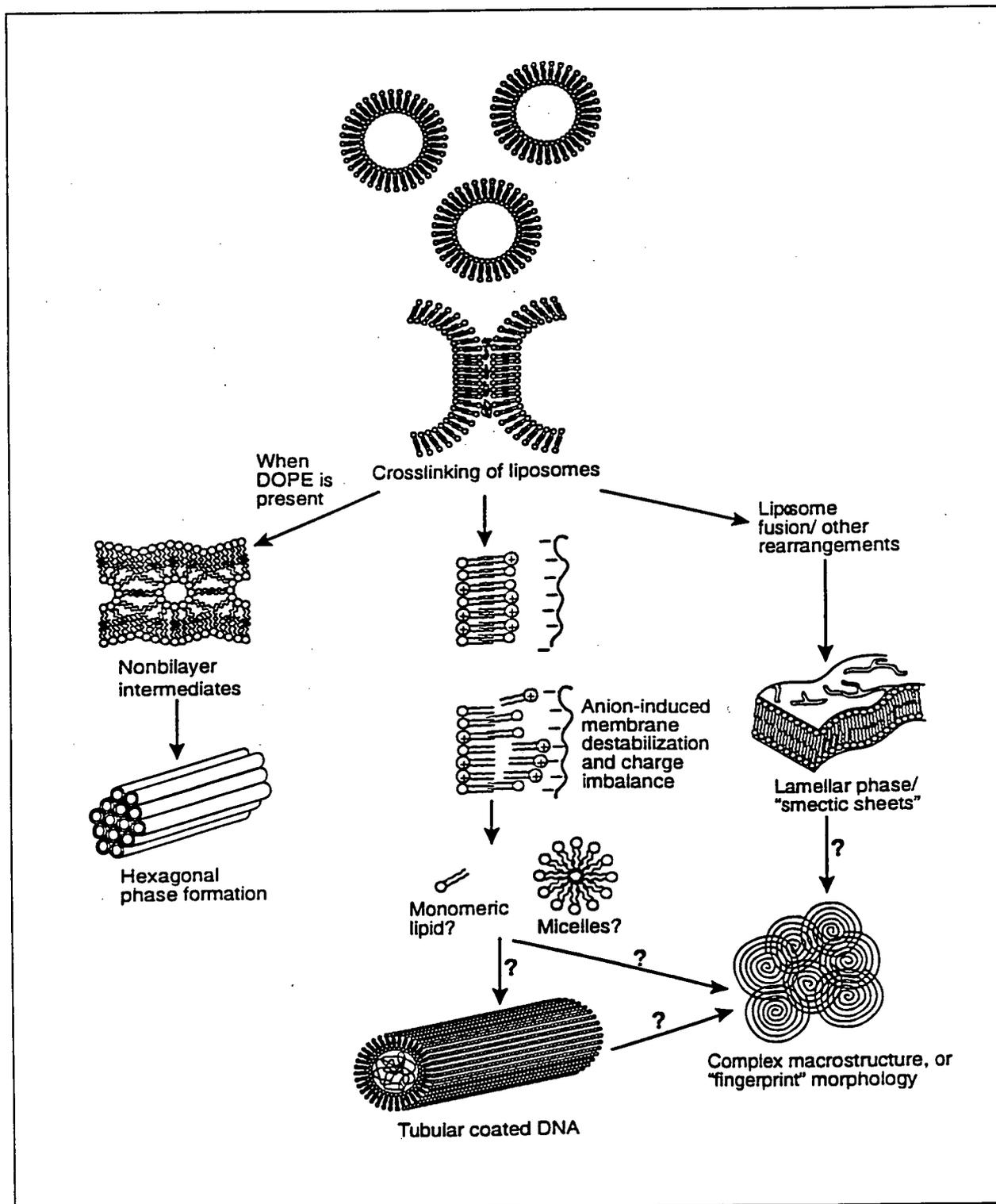


Figure 6. Models proposed for the structures of lipoplexes formed following addition of DNA to cationic lipids. Liposome-DNA complexes are thought to resemble honeycomb structures, rods of DNA wrapped in lipid, or lamellar sheets (68).

1.5.4 Mechanism of gene transfer

The ultimate goal of gene therapy is the expression of a transferred gene, resulting in the production of a protein designed to elicit a therapeutic response. Utilizing lipoplex-mediated gene transfer, these results can only be achieved upon successful completion of the following events. Cationic lipids and plasmid DNA are complexed together to form lipoplexes which are administered to cells and taken up by the endocytic pathway. DNA released from the endosome is transported into the nucleus. Transcription and translation of the foreign gene are necessary for production of a functional protein (Figure 7).

1.5.4.1 Uptake of lipoplexes

Upon addition to either *in vitro* cultured cells or *in vivo* systems, it is thought that lipoplexes gain access inside the host cell primarily by endocytosis (69) (Figure 7). The excess of positive charge on the lipoplex surface facilitates adhesion, via ionic interactions, between the lipoplex and negatively charged molecules within cell membranes (41). The accumulation of lipoplexes on the cell surface triggers the endocytic pathway, at which time, portions of the plasma membrane bud inwards, thus engulfing lipoplexes within endosomal vesicles (70, 71).

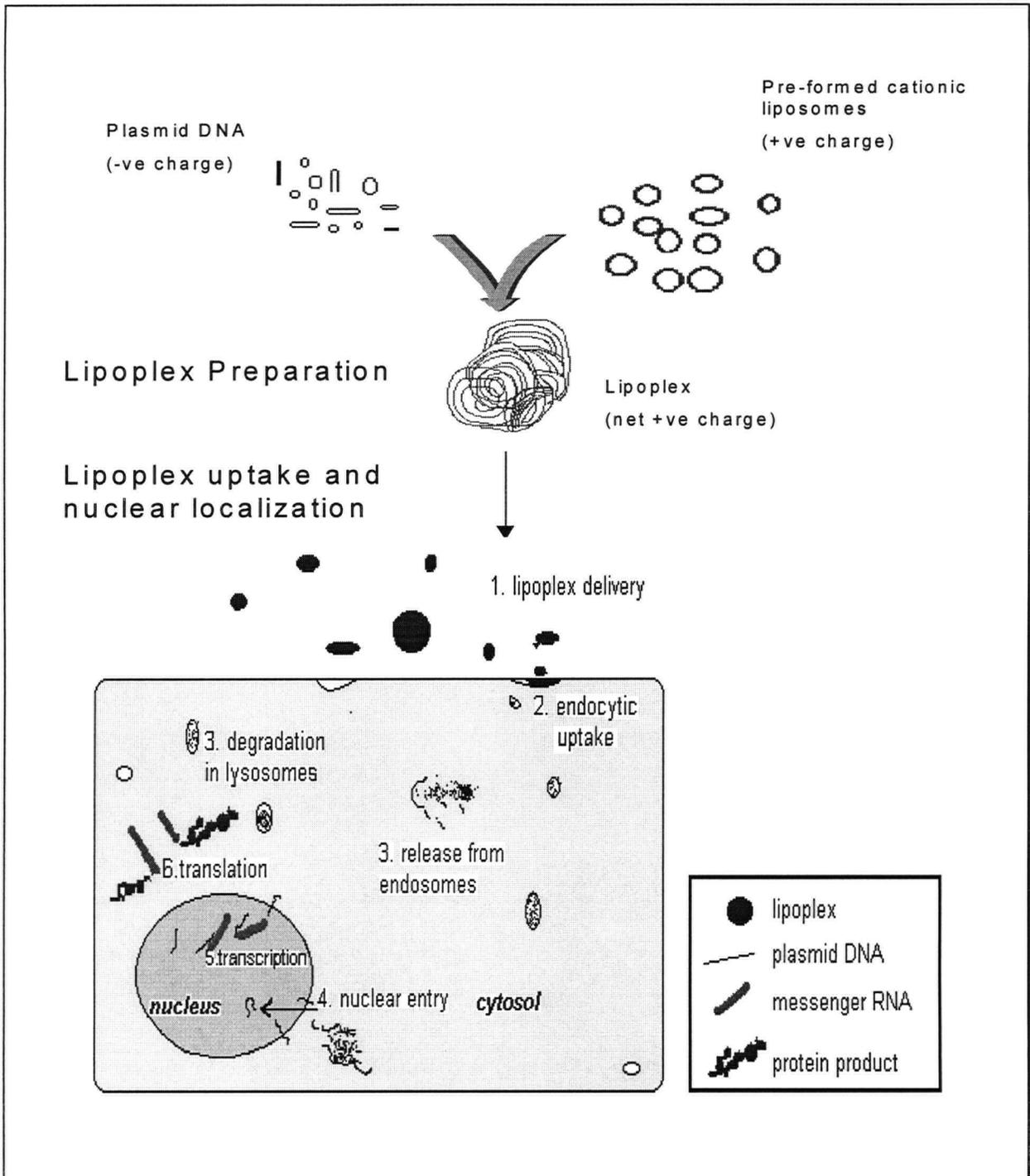


Figure 7. Lipoplex-mediated gene transfer. This process is initiated with the administration and uptake of lipoplexes into host cells. Nuclear localization is followed by transcription of plasmid DNA. Translation of the resulting messenger RNA completes production of the protein of interest (Figure provided by Ellen Wasan).

1.5.4.2 Nuclear Localization

It is known that plasmid DNA must dissociate from cationic liposomes (69) and be transported into the nucleus for expression (Figure 7). However, the mechanism of dissociation is not fully understood. One theory postulates that the internalized complex initiates endosomal membrane destabilization (72). This disruption causes the cytoplasmic-facing monolayer of the endosome to undergo specific structural changes. The anionic phospholipids predominantly located within this monolayer “flip-flop” and diffuse into the lipoplex. Forming charge-neutralized ion pairs with the cationic lipids, the DNA dissociates from the complex and is released into the cytoplasm.

A second theory suggests that the presence of helper lipids in the cationic liposome may also induce fusion of the lipoplex with endosomal membranes, thereby releasing DNA into the cytoplasm (73). For example, DOPE could act as a fusogen, promoting fusion between lipoplexes and internal membranes of endosome. Upon internalization, interactions between lipoplexes and endosome membranes induce cationic lipid phase separation. This generates DOPE-enriched regions within the lipoplexes, prompting formation of a liposome membrane H_{II} configuration. This triggers membrane disruption or fusion between the liposome and endosome, thereby releasing DNA from the lipoplex and into the cytoplasm.

Recent reports have further implicated DOPE as playing a role in facilitating DNA release from cationic lipid complex (34). Just as the amine groups of phosphatidylethanolamine may interact with non-esterified oxygen of lipid phosphate groups to induce polymorphic phase transition, a similar event may occur with DNA phosphate groups. These interactions are thought to weaken the binding interactions

between cationic lipids and DNA, thereby making them more susceptible to dissociation. Perhaps the presence of DOPE in lipoplex formulations enhances DNA release into the cytoplasm by influencing the destabilization process in this manner.

Much of the original DNA taken up by the host cell is degraded within lysosomes, however a small portion may dissociate and escape into the cytoplasm. Only a fraction of the DNA will avoid enzymatic digestion within the cytoplasm and gain entry to the nucleus via highly regulated localization signals. Upon entry into the host cell nucleus, the structural genes on the foreign DNA are transcribed onto complementary strands of messenger RNA. The resulting cytosolic messenger RNAs transiently associate with ribosomes, which initiate the translation process. Ribosomes catalyze the formation of peptide bonds between the amino acids specified by the messenger RNA sequence. In order to generate a functional protein, some polypeptides may require post-transcriptional modifications such as capping, polyadenylation or exon splicing. Expression of a functional protein completes the gene transfer process of exogenous DNA.

1.6 Evaluating gene transfer efficiency

Gene therapy involves the transfer of either marker or therapeutic genes for the treatment of disease. Assessing efficiency of this transfer has traditionally relied upon the empirical measurement of a transgene product (74, 75). Clinical trials monitoring therapeutic genes include: Epstein-Barr virus-mediated delivery of therapeutic genes to human B-lymphoblastoid cells (76); using the multidrug resistance-1 gene promoter for chemotherapy-inducible expression of therapeutic genes in cancer gene therapy (77); induction of apoptosis upon transgene expression of p53 (78) and increased cytotoxic

activity of cytokine-induced killer cells transfected with the interleukin-7 gene (79). Evaluating quantitative changes in transgene product for basis research can be simplified with the use of a marker gene such as chloramphenicol acetyltransferase (CAT) (80), luciferase (81), β -galactosidase (82), green fluorescent protein (83) or CD44 (84). Upon transfection into host cells, a reporter gene will ideally produce a sufficiently stable product that can be detected by means of a sensitive, inexpensive and rapid assay.

For the purpose of evaluating lipoplex-mediated gene transfer in this study, two separate reporter genes were used. Transgene expression of CAT and luciferase genes yielded products that were monitored using quantitative assays.

1.6.1 Chloramphenicol acetyltransferase

CAT is an enzyme typically found in *Streptomyces* and *Corynebacterium*, which catalyzes the acetylation of chloramphenicol to yield chloramphenicol 3-acetate. A two-phase extraction assay for CAT activity has been developed based on the enzymatic acetylation of radiolabeled [^{14}C]-chloramphenicol (^{14}Cm) (80). In the presence of n-butyryl Coenzyme A and ^{14}Cm , transfected CAT enzyme cleaves the n-butyryl Coenzyme A and transfers a butyryl group to the ^{14}Cm . The greater hydrophobicity of butyrylated ^{14}Cm allows for discrimination between the free and butyrylated ^{14}Cm . A simple phase extraction of the upper organic layer enables butyrylated ^{14}Cm to be quantified in a scintillation counter.

1.6.2 Luciferase

A luciferase plasmid DNA can be constructed to contain the gene that encodes for firefly luciferase. This is a monomeric protein containing reactive sulfhydryl groups that are responsible for catalytic activity (81). Upon translation, luciferase catalyzes the oxidation of beetle luciferin to produce a photon. Under experimental conditions, oxidation occurs via a luciferyl-AMP enzyme intermediate. The light intensity of this photon emission is a measure of luciferase catalysis rate and is stable for several minutes. Under ideal conditions, this assay may achieve 100-fold greater sensitivity than quantification using the CAT assay.

1.7 Problems with lipoplex-mediated gene therapy

In order for lipoplex-mediated gene therapy to become a viable alternative to conventional therapies, several problems need to be addressed. Efficient delivery plays a crucial role in successful transfection, therefore it is important to establish the rate-determining step and mechanistic details of this complicated process. These unknowns have hampered the process of optimizing conditions for successful gene transfer systems *in vitro* and *in vivo* (85). Another challenge impeding lipoplex-mediated gene therapy is the lack of target specificity. This problem is currently being addressed by modifications to the lipoplex, such as the addition of a targeting ligand (86).

The transient expression of most genes delivered by lipoplexes is of great concern, as is the relatively low level of transgene expression. Although sufficient transgene expression can be achieved using lipoplex-mediated gene transfer, levels are

still greatly surpassed by viral vectors. All of these problems represent important aspects of lipoplex-mediated gene transfer that must be resolved.

Although there are numerous hurdles to overcome, this technology is advancing in the treatment of cancer (87, 88). For example, Phase I and Phase II studies have been initiated for the treatment of metastatic tumours using an HLA-B7/beta2-microglobulin gene (89) and major histocompatibility complex genes (90). Due to the limitations of this technology, treatment strategies are currently focused upon regional administration of lipoplexes. It has been postulated that pre-exposure of tumour cells *in vitro* to chemotherapeutic agents may sensitize them to lipoplex-mediated gene transfer. This approach would address one of the concerns of current lipoplex protocols, by potentially enhancing transgene expression.

1.8 Chemotherapeutic Agents

There are several major classes of drugs, including the alkylating agents (91) (*e.g.* cyclophosphamide, nitrogen mustard), antimetabolites (92) (*e.g.* methotrexate, 5-fluorouracil) and natural products (93-95) (*e.g.* anthracyclines, vinca alkaloids, taxol). In addition to these major classes, there are some drugs such as platinum-based agents (96) that cannot be classified within any of these groups. Each class of drugs act by specific mechanisms of action such as attaching alkyl groups to DNA bases (alkylators), inhibiting production of DNA or RNA synthesis precursors (antimetabolites), damaging or preventing DNA synthesis (doxorubicin), preventing microtubule formation and subsequent cell division (vincristine), creating crosslinks between DNA strands

(cisplatin) or generating free radicals capable of inducing DNA strand breaks (bleomycin).

For the purpose of this study, chemotherapeutic drugs from several of these groups were chosen on the basis of these varying cytotoxic mechanisms, in addition to their potential to augment gene transfer through mechanisms briefly mentioned in section 1.2. The chemotherapeutic agents employed in this study include cisplatin, doxorubicin, vincristine and bleomycin.

1.8.1 Combination therapy strategies

A clinical approach that combines several modalities of therapy is often the manner by which human disease is managed. Cancer is one disease that demands combination treatments to ensure maximal therapeutic effect (97, 98). Cancer patients routinely undergo surgical treatment for localized tumour excision, followed by adjuvant radiation and/or chemotherapy to combat metastases or residual disease at the site of surgical resection. Early reports by Goldie and Coldman (1984) (99) suggested that spontaneous genetic mutations were involved in tumour resistance to chemotherapeutic cure and more effective treatment would be achieved with combinations of non-cross resistant agents. This strategy of combining several chemotherapeutic agents that utilize different cell kill mechanisms in order to avoid tumour resistance is routinely practiced in cancer treatment (100, 101). As chemotherapy is capable of sensitizing a variety of cells, this well-documented anticancer strategy is an ideal choice for augmenting gene transfer.

Preliminary reports support the notion that cytotoxic drugs could enhance transgene expression. As an example, research performed by Son and Huang (102)

revealed enhanced CAT reporter transgene expression when cisplatin exposure preceded the liposome-mediated gene transfer of human ovarian carcinoma cells. Numerous studies utilizing the techniques of gene therapy in combination with selected drugs or radiation to enhance therapeutic response have been conducted. Strategies exemplifying this diversified research include: osteocalcin promoter-based suicide gene therapy in combination with methotrexate to treat an osteosarcoma model (103); wild-type p53 and radiotherapy used to restore G1 checkpoint and apoptotic pathway in squamous cell carcinoma (104) and enhancement of DNA-mediated gene transfer efficiency in mutated CHO cells by radiation (105).

1.8.2 Cisplatin

cis-Diamminedichloroplatinum (II) (cisplatin) is a commonly used chemotherapeutic agent for cancer treatment (106, 107). It is a divalent inorganic platinum compound (Figure 8A) that targets DNA, primarily forming 1,2-intrastrand crosslinks that covalently link the platinum to the N⁷ position of adjacent purine bases (Figure 8B). To a lesser extent, cisplatin may also induce 1,3- or longer intrastrand crosslinks, in addition to interstrand or protein crosslinks (Figure 8C, D). These adducts are known to interfere with normal replication and transcription (108). Consequently, cisplatin-damaged cells have been shown to evoke the following reactions; cell cycle arrest and induction of DNA repair and/or apoptosis (109), changes in intracellular glutathione and metallothionein levels and fluctuations in other efflux pump mechanisms (110, 111) and alterations to protein kinase and other enzyme levels (112).

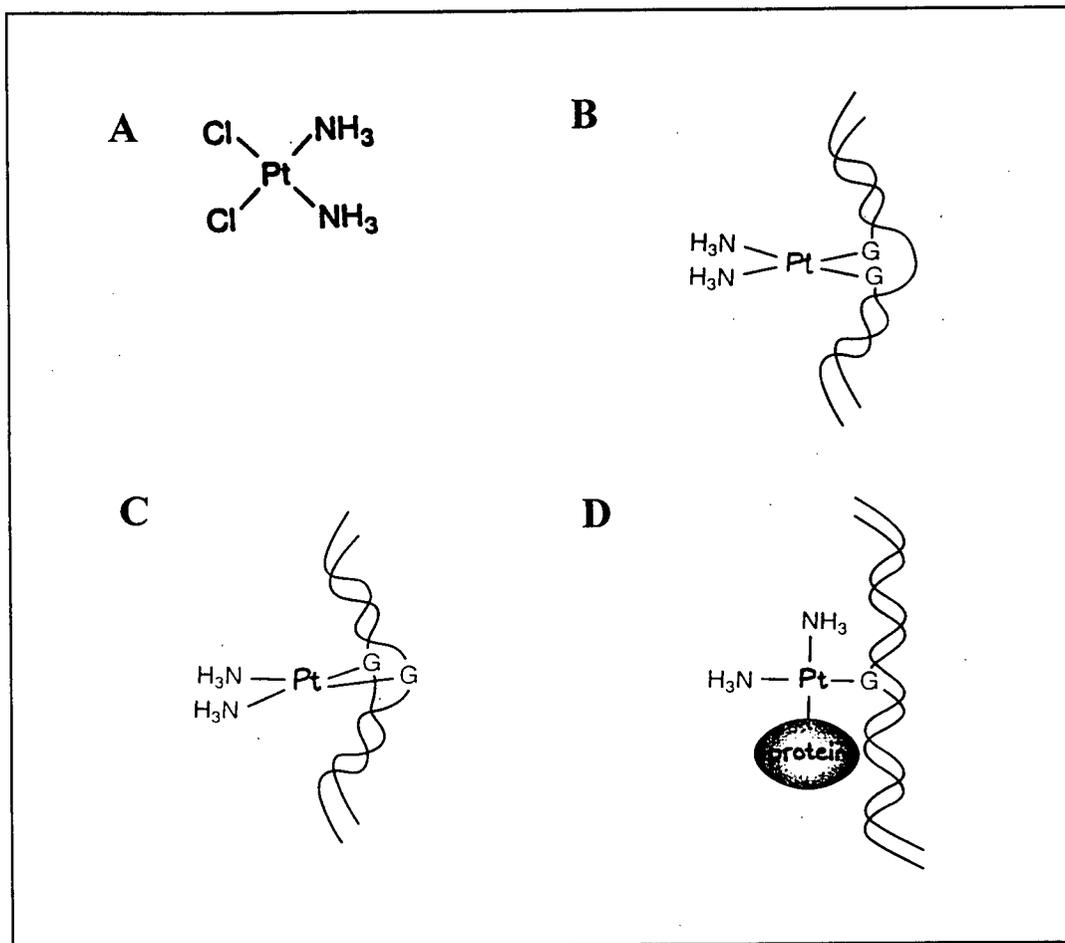


Figure 8. Structure of (A) cisplatin and (B-D) cisplatin-DNA adducts.

Cisplatin may form (B) intrastrand, (C) interstrand or (D) protein crosslinks with purine bases of DNA (107).

1.8.3 Doxorubicin

Another important group of antineoplastic agents is the anthracycline antibiotics that include doxorubicin. This hydrochloric salt of a glycoside antibiotic produced by *Streptomyces peucetius* (var. *caesius*) is an amphiphilic molecule comprised of tetracycline ring structures attached to an amino sugar moiety, via a glycosidic linkage (113) (Figure 9).

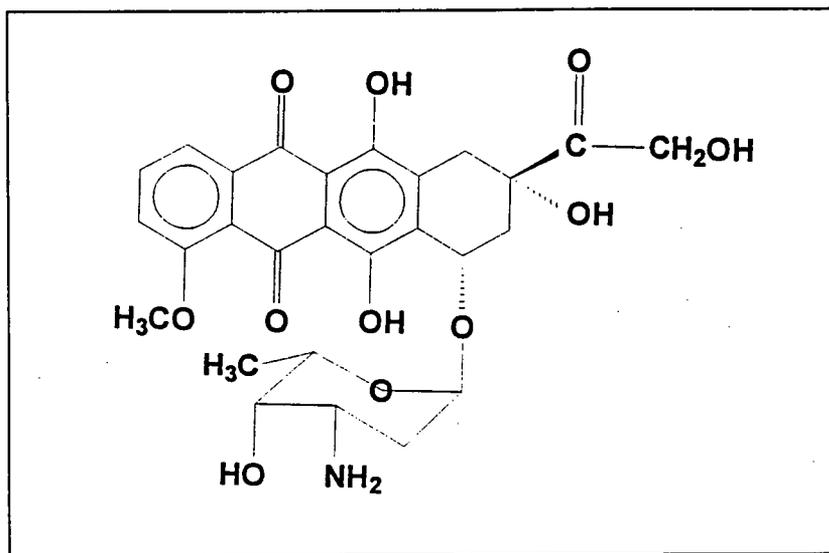
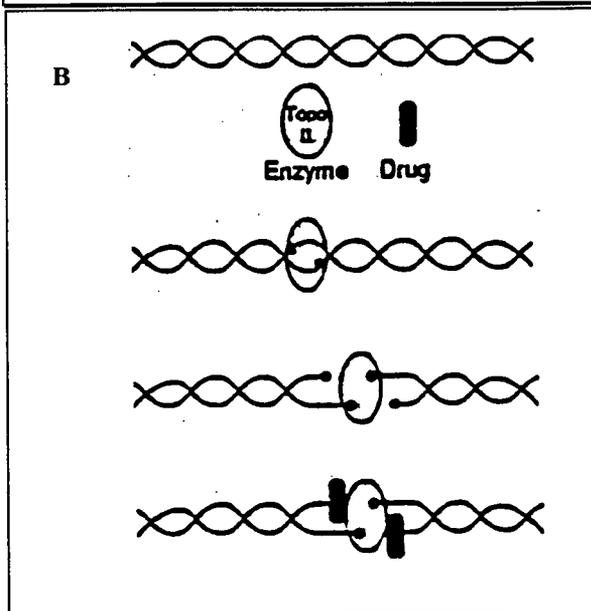
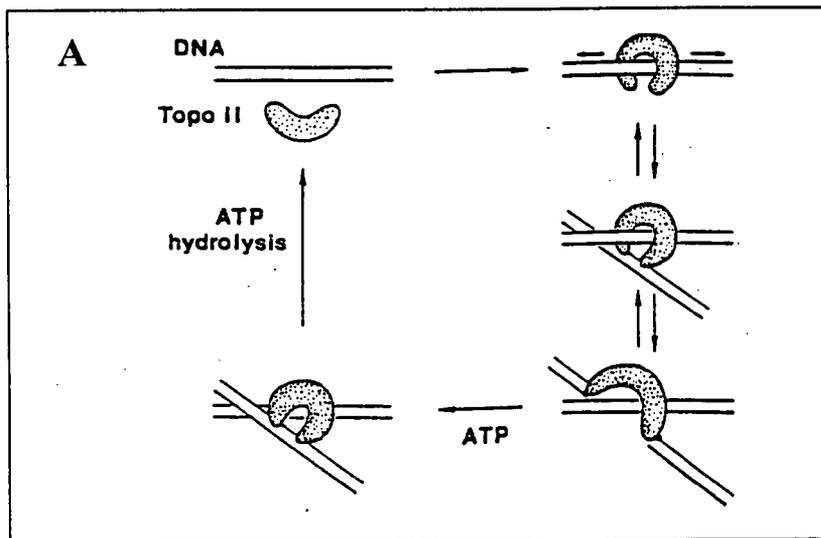


Figure 9. Structure of doxorubicin.

Its mechanism of cytotoxicity has been linked, in part to the DNA strand breakage and resealing actions of topoisomerase II (Topo II) (114) (Figure 10A). Covalently binding to duplex DNA, Topo II migrates along one of the strands until encountering a region at which the two helices meet. Remaining associated with the first strand, Topo II binds to and cleaves the second strand, thus producing a transient cleaved complex. This allows the two strands to pass by one another, thereby unwinding the helix, as required during DNA synthesis. In the presence of ATP, re-ligation of the DNA is completed, allowing Topo II to dissociate from the helix. Protein-associated DNA breaks are caused by doxorubicin effects upon Topo II. Doxorubicin is thought to intercalate into the DNA double helix, thereby altering DNA conformation. Topo II activity is arrested at the point of DNA cleavage, thus preventing the re-ligation of the cleaved DNA (114) (Figure 10B). DNA strand breaks caused by the interaction of doxorubicin with the Topo II-DNA



Binding of Topo II to DNA allows reversible double strand breakage.

DNA strand passage and unwinding.

Binding of doxorubicin to Topo-DNA complex prevents re-ligation of DNA.

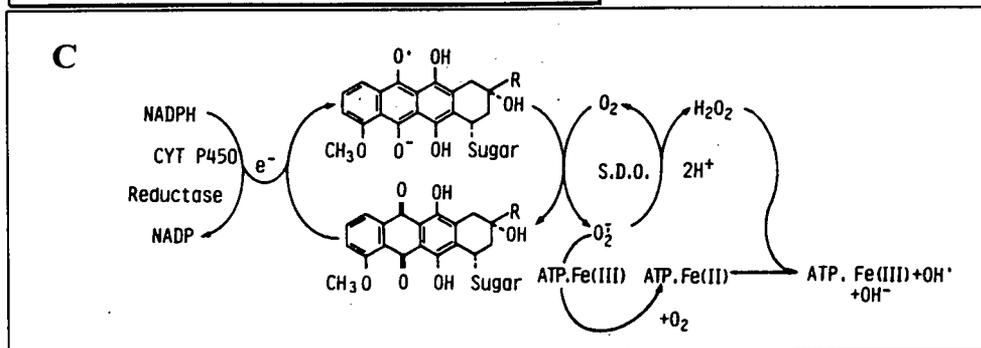


Figure 10. Schematic illustration of (A) Topo (II) involvement in DNA strand breakage and resealing; (B) doxorubicin interference with Topo II-DNA interactions; (C) enzymatic activation of doxorubicin and subsequent activation of superoxide anion, hydrogen peroxide and hydroxyl radicals. (Figure derived from Piccinini *et al.* (115)).

complex result in inhibition of nucleic acid synthesis and may lead to induction of the apoptotic pathway.

Alternatively, doxorubicin may be converted to a free radical intermediate and this leads to oxygen radical-mediated DNA damage (116) (Figure 10C). Reduction of doxorubicin to a semiquinone free radical is mediated by oxidoreductases (*e.g.* cytochrome P450). In the presence of free oxygen, this radical donates its electron, thereby generating a superoxide anion ($O_2^{\cdot-}$). While not toxic itself, superoxide can undergo reactions leading to the generation of hydrogen peroxide. Hydroxyl radical formation occurs by hydrogen peroxide reacting with ferrous iron or the semiquinone radical. These radical oxygen species (hydrogen peroxide and hydroxyl radical) are reactive and destructive, capable of DNA cleavage and peroxidation of membrane lipids.

1.8.4 Vincristine

The antineoplastic agent, vincristine is the sulfate salt of an alkaloid isolated from *Vinca rosea* (periwinkle). As with all of the vinca alkaloids, vincristine is composed of a dihydroindole nucleus (vindoline) that is linked to an indole nucleus (catharanthine) (94) (Figure 11).

The cytotoxicity of this cell cycle-specific drug has been attributed to its high binding affinity to microtubule proteins, thus inhibiting tubulin polymerization and preventing formation of the spindle apparatus in mitosis. This induces an arrest of cells at the G2/M phase, potentially leading to additional signal transduction phosphorylation events capable of initiating apoptosis (117).

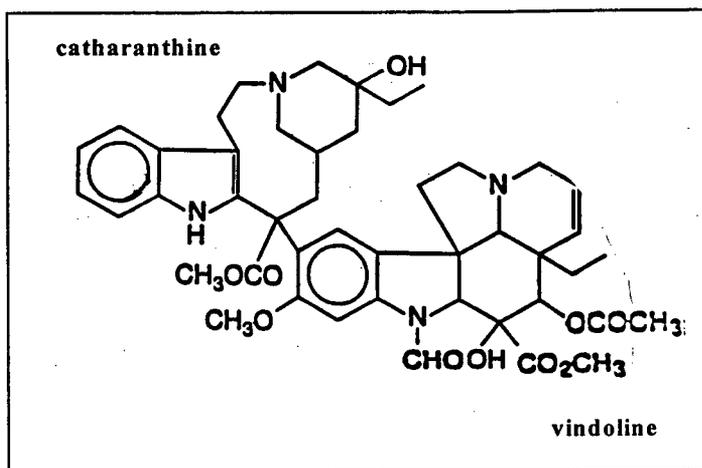


Figure 11. Structure of vincristine.

It is also important to note that this microtubule inhibitor has the potential to interfere with endosomal transport within the cytoplasm of affected cells. It has been postulated that the breakdown of the nuclear membrane facilitates DNA delivery to the nucleus (118). As this is a normal mitotic event, cell cycle arrest in the mitotic phase may therefore alter transgene delivery.

1.8.5 Bleomycin

Consisting of a mixture of peptides isolated as fermented products of *Streptomyces verticillus*, bleomycin is clinically administered as a mixture of the A₂ and B₂ compounds, which differ only in their terminal amine (119) (Figure 12). The structure of these peptides consist of a DNA binding fragment (boxed region) linked to an iron-binding structure.

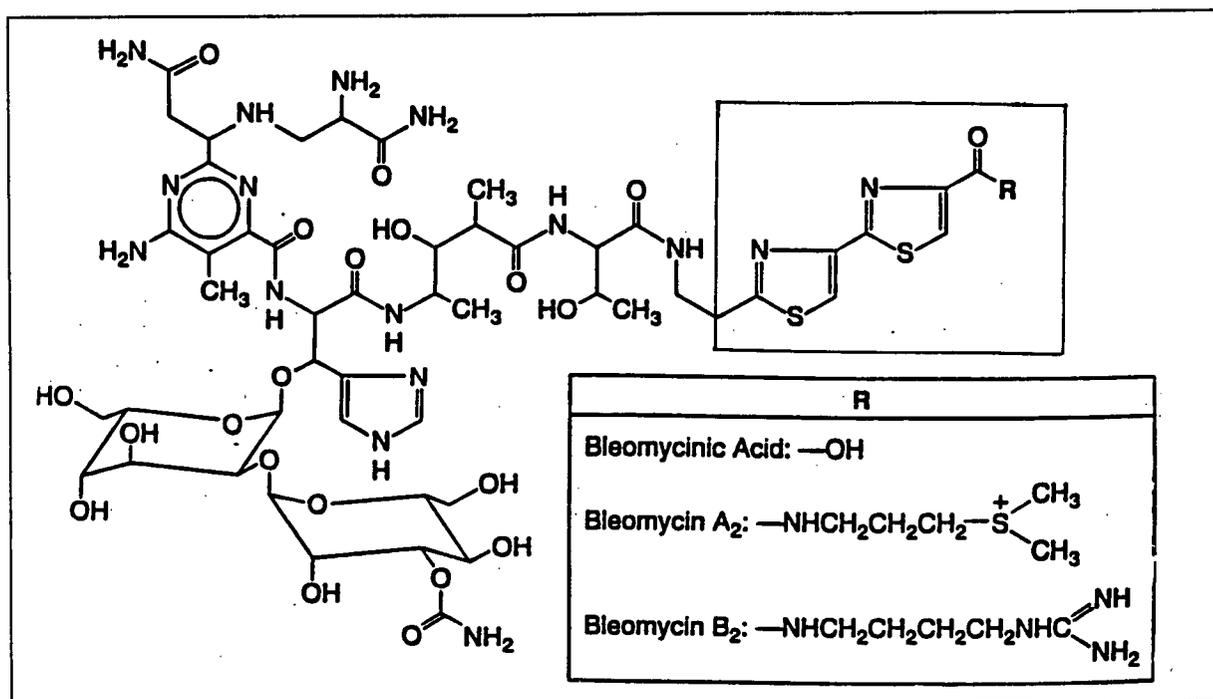


Figure 12. Structure of bleomycin.

Bleomycin toxicity is attributed to production of single- and double-strand breaks in DNA, leading to an arrest primarily in the G₂/M phase (120, 121). Ferrous iron binds to nitrogen-containing components of bleomycin and undergoes spontaneous reduction to yield an electron. Similar to the doxorubicin radical reaction described in section 1.8.3, the liberated electron is accepted by free oxygen to generate a superoxide anion (O₂^{·-}). The superoxide can undergo reactions leading to the generation of hydrogen peroxide or hydroxyl radicals. These radicals damage bleomycin-bound regions of DNA by attacking the phosphodiester bonds between guanine and other bases. Oxidation of nucleic acid moieties leads to their release from DNA, therefore causing strand breaks.

1.9 Thesis Objectives

Since gene therapy will become more prevalent in patient care, it is an utmost priority to ensure that combination strategies deliver beneficial results. The experiments outlined in this thesis evaluate lipoplex-mediated gene transfer in combination with four clinically relevant chemotherapeutic agents (cisplatin, doxorubicin, vincristine and bleomycin). These chemotherapeutic agents were selected due to their fundamentally different mechanisms of action. Three specific experimental objectives were defined:

- (i) to demonstrate that *in vitro* addition of lipoplexes did not alter the cytotoxic activity of the selected drugs
- (ii) to determine *in vitro* whether exposure to these chemotherapeutic agents enhanced/inhibited/did not affect transgene expression of a reporter gene
- (iii) to determine *in vivo* whether these agents enhanced/inhibited/did not affect transgene expression of a reporter gene.

Chapter 2

Materials and Methods

2.1 Materials

Dioleoyldimethylammoniumchloride (DODAC) was synthesized and supplied by Dr. Steven Ansell of Inex Pharmaceuticals Corp. (Vancouver, BC, CAN).

Dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Radiolabeled [³H]-cholesteryl hexadecyl ether (³H-CHDE) was obtained from Dupont NEN. Extrusion filters were purchased from Poretics Products Corp. (Mississauga, ON, CAN) and Spectral/Por dialysis membrane tubing was from Spectrum Medical Industries Inc. (Houston, TX, USA). Cisplatin was kindly donated by Dr. Kirsten Skov (BC Cancer Research Centre, Vancouver, BC, CAN). Doxorubicin, vincristine and bleomycin were purchased from the BC Cancer Agency (Vancouver, BC, CAN).

The 4.4 kilobase plasmid pINEXCatv2.0 contained the *Escherichia coli* chloramphenicol acetyltransferase (CAT) cDNA under the control of the cytomegalovirus promoter and alfalfa mosaic virus enhancer. The plasmid DNA was purified using a Qiagen Plasmid Purification Kit (Qiagen, Chatsworth, CA, USA). The gene expression plasmid vector, pINEX L018, contained the full coding region of the firefly *P. Pyralis* luciferase cDNA driven by the human cytomegalovirus promoter. This 5.7 kilobase plasmid also encoded a polyadenylation signal and enhancer, both which were simian virus 40 related. It was purified by cesium chloride density gradient ultracentrifugation.

Both of these plasmids were constructed and provided by Inex Pharmaceuticals Corp. (Vancouver, BC, CAN).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bicinchonic acid solution (BCA), copper (II) sulfate and bovine serum albumin (BSA) protein standard were all obtained from Sigma (St. Louis, MO, USA). All luciferase assay components were supplied in a kit from Promega (Madison, WI, USA) except for purified luciferase, which was purchased separately from Boehringer Mannheim Canada (Laval, PQ, CAN). All other chemicals were at least reagent grade and all solvents were high performance liquid chromatography grade.

2.2 Methods

2.2.1 Cell culture

The murine B16/BL6 melanoma cell line, obtained from NCI Tumour Repository (Bethesda, MD, USA) was cultured in RPMI 1640 media (Stemcell Technology, Vancouver, BC, CAN) and supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). SKOV-3 cells (human ovarian tumour cells, ATCC #HTB-77) were maintained in 10% FBS McCoy's media (Stemcell Technology, Vancouver, BC, CAN). Both cell lines were maintained as monolayers in 75cm² tissue culture flasks at 37°C (5% CO₂ atmosphere) with no antibiotics. Cells were washed twice with pre-warmed Hank's Balanced Salt Solution (HBSS) (Stemcell Technology, Vancouver, BC, CAN) and trypsinized with 0.25% trypsin-EDTA (Gibco/BRL, Grand Island, NY, USA). They were seeded at approximately 6x10³ (B16/BL6) and 1x10⁴ (SKOV-3) cells per well in 96-well

flat bottom microtiter plates (Costar, Cambridge, MA, USA) and incubated for 24h.

Cells were supplemented with 5% FBS media for the duration of the experimental period.

2.2.2 Chemotherapeutic agents

Stock solutions of doxorubicin, vincristine and bleomycin were prepared in 5% FBS media at room temperature (RT), in the dark. Cisplatin powder was dissolved in 150mM NaCl and rotated in a 37°C environment for 30 minutes (min) before being filtered using sterile 0.2µm Millex-pf filters. Serial dilutions for all of the drugs were made at RT using 5% FBS media to provide an appropriate dose range (cisplatin: 0.1-10µM, doxorubicin: 0.1-10µM, vincristine: 5×10^{-2} -10nM and bleomycin: 5×10^1 - 1×10^5 ng/ml).

Medium was aspirated from each of the wells containing plated cells, prior to addition of a 100µl aliquot of drug solution. Control cells were treated with 100µl 5% FBS media. Plates were then returned to the incubator for 24h.

2.2.3 Liposome preparations

DODAC/DOPE (1:1 mol ratio) liposomes were prepared according to the extrusion method of Hope *et. al* (122). Briefly, lipids were dissolved in chloroform (20mg/ml) and radiolabeled with a non-exchangeable liposomal lipid marker, ³H-CHDE (1-2 µCurie/20mg specific activity). The lipid solution volume was reduced under a stream of nitrogen gas and evenly dried as a thin layer film under vacuum at <76cm Hg for a minimum of 4h. Multilamellar vesicles (MLVs) were formed by hydrating films at

RT with 300mM lactose (0.22 μ M filtered). The MLVs were then vortexed and cooled until the solution became translucent. The hydrated lipid solution was passed ten times through an extruder (Lipex Biomembranes, Vancouver, BC, CAN) that had been fitted with three stacked (0.08 μ m pore size) polycarbonate membranes. The resulting large unilamellar vesicles (LUVs) were evaluated for liposomal specific activity as determined by ^3H -CHDE levels measured with a Canberra Packard 1900 TR Liquid Scintillation Counter (Packard Instrument Company, Downes Grove, IL, USA). The [^3H] radioactivity, expressed as disintegrations per minute (dpm), of a known lipid amount was used to calculate specific activity (dpm/nmol). This value was then used to calculate lipid concentration of the liposomes. Liposome size (100-120nm) was measured by quasi-electric light scattering using a Model 270 Nicomp Submission Particle Sizer (Pacific Scientific, Santa Barbara, CA, USA) operating at 632.8nm. All liposomes were dialyzed against distilled, demineralized water (ddH₂O) overnight and diluted to 40mM stock solutions with 300mM lactose.

2.2.4 Lipoplex preparations

All reagents and supplies were cooled to 4°C prior to preparation of liposome-DNA aggregates (lipoplexes). Liposomes and plasmid DNA (either pINEXCatv2.0 CAT plasmid or pINEX L018 luciferase plasmid) were diluted in ddH₂O to a concentration of 200 μ M and 20 μ g/ml, respectively. In a sterile glass tube, 500 μ l of diluted liposomes were mixed, dropwise, with 500 μ l of diluted plasmid DNA and incubated at 4°C for 30min. Free DNA was prepared in ddH₂O at a concentration of 10 μ g/ml plasmid DNA,

corresponding with the amount of plasmid DNA present in the lipoplexes. Lipoplexes were sized to ensure a homogeneous preparation with a mean diameter of approximately 200-400nm. The optimal ratio of DODAC/DOPE liposomes to plasmid DNA for the selected cell types had been determined in this laboratory (64) as 100nmol/ml lipid:10µg/ml DNA, which corresponds to a 1.62:1.00 (positive cationic lipid to negative DNA phosphate group) charge ratio.

2.2.5 *In vitro* transfection analysis

Lipoplexes were added to each of the experimental sample wells and one group of the control wells in 20µl aliquots using a glass SMI Micro/Pettors Model C capillary pipettor (Dade International Inc., Miami, FL, USA). Free DNA or 5% FBS media was added as a 20µl aliquot to the remaining sets of control wells. Cells were incubated for 4h, prior to addition of 80µl 5% FBS media to all wells. Cells were incubated for 48h, following which, they were subjected to cell viability assessment, transfection analysis and protein quantification.

2.2.5.1 MTT cytotoxicity analysis

The number of viable cells was determined by the MTT assay (123). MTT working solution was prepared by diluting the MTT stock solution (5mg MTT/ml phosphate buffered saline (pH 7.2) 1:5 (vol ratio) with pre-warmed 10% FBS media. Working solution (50µl) was added to each of the wells and incubated for 4h. All but ~15µl supernatant was aspirated before 150µl dimethyl sulfoxide was added to each well

to solubilize the blue MTT-formazan crystals. The solutions were re-suspended to ensure uniform colour and 100µl from each well was transferred to a new 96-well flat-bottomed microtiter plate. Optical density values were determined using a MRX Microplate Reader (Dynex Technologies Inc., Chantilly, VA, USA) at 570nm. Cell viability, recorded as percent of drug-free control sample, was determined in triplicate at each drug concentration.

2.2.5.2 *In vitro* luciferase expression analysis

Assays were performed using the Luciferase Assay System Kit in accordance with the manufacturer's protocol. Culture medium was aspirated and cells were washed twice with pre-warmed HBSS. Cells were lysed by incubation with 50µl 1X cell culture lysis reagent (125mM Tris (pH 7.8 with H₃PO₄), 10mM CDTA, 10mM DTT, 50% glycerol and 5% Triton X-100 diluted 1:4 (vol ratio) with ddH₂O from 5X stock solution) for 15min. The cell lysates from each well were transferred to microcentrifuge tubes and spun briefly in an IEC Micromax Microcentrifuge (Fischer Scientific Ltd., Nepean, ON, CAN) at a relative centrifugal force of 6000 x g to pellet large debris. A portion of cell lysate (20µl) was added to 100µl Luciferase Assay Reagent (lyophilized Luciferase Assay Substrate was reconstituted with 10ml/vial Luciferase Assay Buffer). Luciferase activity was detected using a Tropic Luminometer (Bio/Can Scientific, Mississauga, ON, CAN) with a 30 second measuring time. Background values obtained from non-transfected control cells were ~90 relative light units (RLU) and subtracted from all experimental values. Purified luciferase was serially diluted with Luciferase Lysis Buffer (1mg purified

luciferase $\sim 10^7$ arbitrary luciferase units) and used to establish a standard curve of RLU produced from a known amount of luciferase. Luciferase expression for control and experimental samples was determined using the slope from the linear portion of this standard curve. These values were all normalized for cellular protein amount as described next, and expressed as pg luciferase per mg of protein.

The cell lysates were assayed for protein content using the BCA colourimetric method in accordance to the manufacturer's protocol (124). Stock BCA solution (200 μ l) (Cu²⁺ sulfate diluted 1:50 (vol ratio) with BCA) was added to each cell lysate sample (cell lysate diluted 1:60 (vol ratio) with ddH₂O) and protein standard samples. Protein standard samples were prepared from a 1mg/ml BSA stock and diluted with ddH₂O to an appropriate protein concentration range (0-18 μ g protein/well). After sufficient incubation with the BCA solution, optical density values were read at 570nm. Cell lysate protein concentration values were calculated using the slope of the protein standard curve.

2.2.5.3 *In vitro* CAT expression analysis

Upon completion of the 48h transfection period, medium was aspirated from each well and cells were rinsed twice with HBSS. Lysis buffer was prepared by the addition of 20mM n-octyl beta-D-glucopyranoside to complete homogenization buffer [incomplete homogenization buffer (15mM Tris-HCl (pH 8.0), 60mM KCl, 15mM NaCl, 5mM EDTA (pH 8.0)) is combined with 0.15mM spermine, 1.0mM DTT, 35 μ g/ml PMSF, 0.5 μ g/ml leupeptin, 0.5 μ g/ml aprotinin and 5 μ M paraoxon]. Lysis buffer (400 μ l) was added to each well, which were scraped before transferring the cell lysates into

microcentrifuge tubes. Samples were centrifuged at 6000 x g for 10min at RT. Control sample supernatants were pooled, diluted 1:50 (vol ratio) in BSA buffer (2mg/ml BSA added to complete homogenization buffer) and used to establish a standard curve. Stock CAT (1×10^4 mU/ μ l) was serially diluted using BSA buffer to establish an appropriate concentration range (0-0.8mU/ μ l). A portion (10 μ l) from each diluted CAT solution was added to a 100 μ l aliquot of diluted control sample. Experimental samples were also diluted 1:50 (vol ratio) in BSA before 110 μ l was removed for further analysis.

Standard curve controls and experimental samples were heated at 65°C for 15min to inactivate deacetylase activity and then centrifuged at 6000 x g for 10min at RT. A portion of the supernatant (55 μ l) was removed and combined with 50 μ l radiolabeled [14 C]-chloramphenicol (14 Cm) working solution (D-threo-[dichloroacetyl-1,2- 14 Cm] diluted 1:10 (vol ratio) in BSA buffer) and 25 μ l n-butyryl Coenzyme A (diluted to 5mg/ml in ddH₂O) before being incubated for 2h in a 37°C water bath.

At RT, 300 μ l of mixed xylenes were added and each sample was vortexed for 30s and centrifuged for 3min at 6000 x g. The upper phase was transferred to a new tube containing 750 μ l incomplete homogenization buffer. Samples were vortexed and centrifuged for 3min at 6000 x g before 100 μ l of the upper phase was removed and added to a scintillation counter vial containing 5ml Picofluor scintillant (Packard Instrument Co., Meridian, CT, USA). The [14 C] radioactivity (expressed as dpm) was measured using the Canberra Packard 1900 TR Liquid Scintillation Counter (Packard Instrument Company, Downes Grove, IL, USA). Using the values obtained for the standard curve controls, the amount of CAT activity was calculated for each of the experimental

samples. One unit of enzyme activity can be defined as the amount of enzyme that converts 1nmole of substrate to product in 1min at 37°C. Using the BCA method for determining protein content (described in section 2.2.5.2), experimental values were converted and expressed as mU CAT activity per mg of protein.

2.2.6 *In vivo* analysis

2.2.6.1 Intraperitoneal model

C57BL/6J female mice were purchased at 7 weeks from Jackson Laboratories (Bar Harbor, MA, USA). They were injected intraperitoneally (i.p.) as groups of four with 1×10^5 B16/BL6 cells. Seven days after tumour cell inoculation, all experimental groups received a single intravenous (i.v.) injection of the selected cytotoxic agent. This was administered at a maximum therapeutic dose designed to provide the greatest therapeutic activity (cisplatin: 10mg/kg, doxorubicin: 20mg/kg, vincristine: 3mg/kg and bleomycin: 0.4U/kg). Three days later, all experimental groups were i.p. injected with plasmid as free DNA or formulated with DODAC/DOPE liposomes. Lipoplexes were prepared at 4°C by mixing pINEXCatv2.0 and DODAC/DOPE liposomes (1:1 mol ratio) in 300mM lactose at 1:10 (vol ratio), such that 35µg plasmid DNA was injected in a 500µl lactose volume. The lipoplexes were kept on ice for 30min and warmed to RT before i.p. administration. Free DNA was also prepared such that 35µg plasmid DNA was injected in a 500µl lactose volume. B16/BL6 tumours in the peritoneal cavity were harvested 24h after plasmid injection and CAT activity was measured. All of the mice appeared to be healthy for the duration of experimental period in each *in vivo* study.

2.2.6.2 Intravenous model

CD1 female mice were purchased at 5 weeks from Charles River Laboratory (St. Contant, PQ, CAN) and served as control mice for an i.v. model. Lipoplexes were prepared as described in section 2.2.6.1, except that 25µg plasmid DNA was injected in a 200µl lactose volume. Free DNA was also prepared such that 25µg plasmid DNA was injected in a 200µl lactose volume. Without any pre-exposure to chemotherapeutic agents, mice were injected via the lateral tail vein. As groups of four, mice were injected with free DNA or lipoplexes, or left untreated. Twenty-four hours post-transfection, various tissues (spleen, heart, kidney, liver and bone marrow) were harvested.

CD1 mice in the experimental groups of the i.v. model were injected with a single dose of chemotherapeutic agent at the maximum therapeutic dose (cisplatin: 10mg/kg, doxorubicin: 20mg/kg, vincristine: 3mg/kg and bleomycin: 0.4U/kg) on days 0, 7, 14 or 18, to correspond with 21, 14, 7 or 3 days drug exposure. On day 22, mice were injected via the lateral tail vein with 25µg plasmid as free DNA or formulated with DODAC/DOPE liposomes in a 200µl lactose volume. Only bone marrow and spleen were harvested 24h later.

2.2.6.3 *In vivo* CAT expression analysis

Fresh tissues were weighed and placed on ice until homogenization in an appropriate amount of complete homogenization buffer (20% homogenate for liver and 10% homogenate for spleen, kidney, heart and tumour). Bone marrow samples were flushed with 6ml PBS, centrifuged at 4°C for 10min at 1000 x g using a Centronics S-

103NAR Centrifuge (NCS Medical Inc., Delta, BC, CAN) and re-suspended in 500µl complete homogenization buffer. Cells were lysed by three freeze/thaw cycles (frozen in liquid nitrogen and thawed in a 37°C water bath) and cell lysates were centrifuged at RT for 10min at 6000 x g. A portion (110µl) of each experimental sample supernatant and 100µl aliquots of the pooled control supernatant were transferred to new tubes. Diluted CAT stock was made up in BSA buffer to an appropriate concentration range (0-0.8mU stock CAT/µl). A portion (10µl) of the diluted CAT was added to each of the pooled control supernatants. All samples were heat-inactivated and assayed for CAT activity according to the protocol described in section 2.2.5.3.

2.2.7 Statistical analysis

In vitro assays were performed in triplicate and each experiment was repeated independently at least three times. *In vivo* data were generated from four mice per experimental group, with selected experiments repeated at least two times. All *in vitro* and *in vivo* data shown are the mean ± standard error of the mean (SEM) for all experiments. Quantitative data generated for cell viability, CAT and luciferase expression and protein amount were statistically assessed using the Statistica computer program (Statistica Software Inc., Tulsa, OK, USA). Significance, as determined by a probability of less than 0.05 ($p < 0.05$) was tested using post-hoc comparison of means (Scheffe's test).

Chapter 3

Results

3.1 Examination of cytotoxicity

In order to determine the effects of combination therapy on transgene expression, it was important to establish initially whether addition of lipoplexes changed the toxicity of the chemotherapeutic agents selected. This was assessed using *in vitro* cytotoxicity assays, where cell viability was determined by the MTT assay. The results of this assessment, shown in Figure 13, clearly indicate for SKOV-3 cells that the concentration of drug that caused toxicity or inhibition of cell proliferation was not affected by the presence of lipoplexes. Cell viability for each drug dose was determined as a percentage of control sample not exposed to chemotherapeutic agent. Treatment with increasing concentrations of cisplatin (Figure 13A), for example, indicated that the IC_{50} (concentration of drug causing 50% cell death or 50% inhibition of cell proliferation) for cisplatin was approximately $8.0\mu\text{M}$ in the presence of lipoplexes and approximately $9.5\mu\text{M}$ in the absence of the transfecting agent. A similar dose-dependent decrease in cell viability was observed for all selected drugs, and no significant differences were noted between lipoplex-transfected and non-transfected cells. This experiment was repeated using B16/BL6 cells in the presence of cisplatin and similar results were achieved (Table 1).

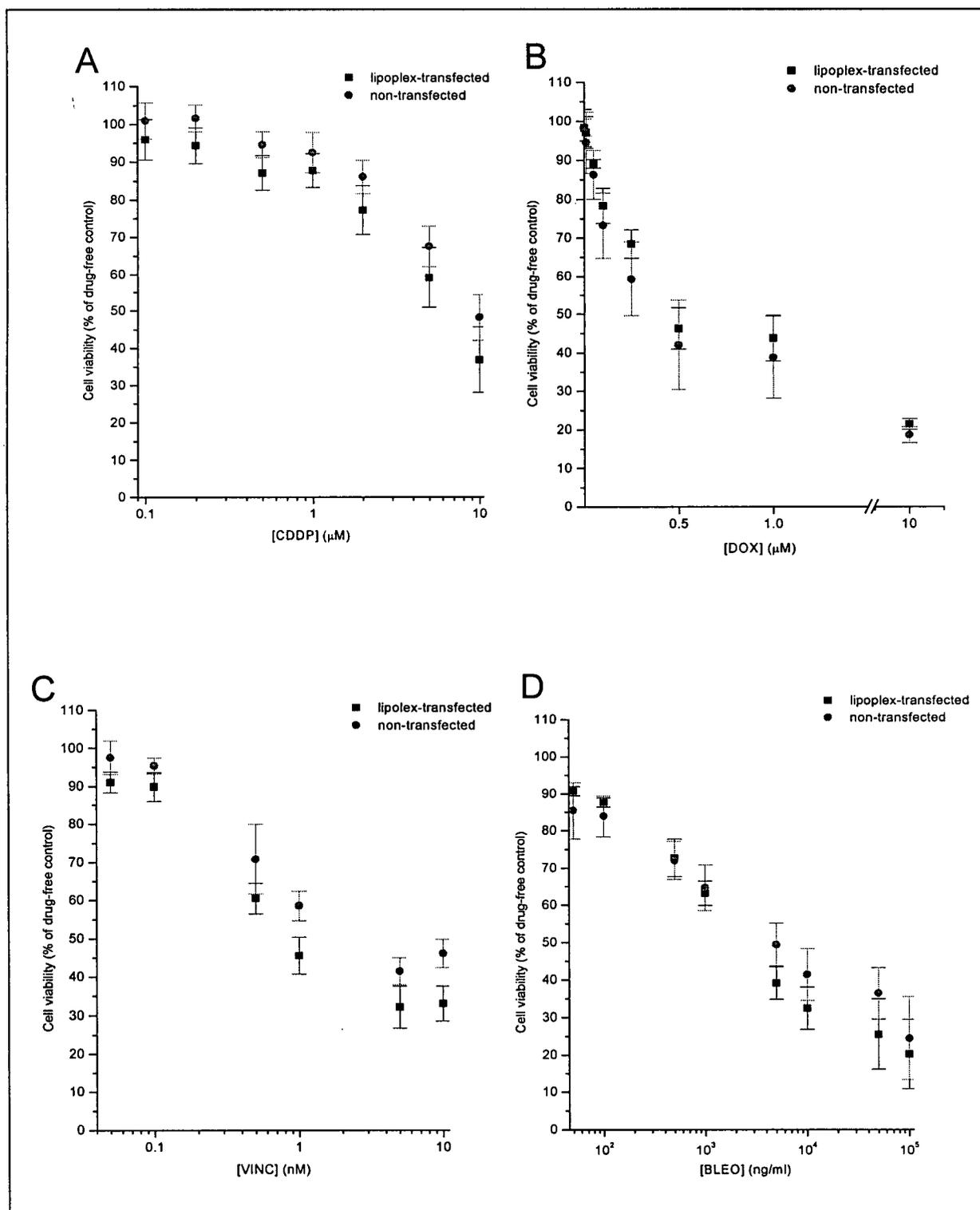


Figure 13. SKOV-3 cytotoxicity of selected anticancer agents in the presence and absence of added lipoplexes. SKOV-3 cells were plated (1×10^4 cells/well) and treated with varying concentrations of (A) cisplatin, (B) doxorubicin, (C) vincristine or (D) bleomycin in the presence (■) or absence (●) of lipoplexes. Cell viability was determined as described in section 2.2.5.1. The data points were determined from the mean \pm SEM of triplicate values obtained from at least 3 separate experiments.

Table 1. IC₅₀ for selected drugs and cell lines in the presence and absence of lipoplexes.^a

Cell Line	Cytotoxic Drug	IC ₅₀ (μM) ^b	
		- Lipoplexes	+ Lipoplexes
SKOV-3	Cisplatin	9.5	8.0
	Doxorubicin	0.4	0.5
	Vincristine	3.0 ^c	1.0 ^c
	Bleomycin	2.5x10 ³ ^d	2x10 ³ ^d
B16/BL6	Cisplatin	10.0	10.0

^a Lipoplexes were prepared as described in section 2.2.4 using DODAC/DOPE liposomes and pINEXL018 at a ratio of 10nmole lipid/1μg DNA.

^b IC₅₀ is estimated as the concentration of drug causing 50% cell death or 50% inhibition of cell proliferation.

^c units for vincristine are given as nM.

^d units for bleomycin are given as ng/ml.

3.2 *In vitro* lipoplex-mediated transfection in the presence of selected chemotherapeutic agents

A variety of parameters were considered when evaluating any influence that chemotherapeutic agents may have on the ability of host cells to express exogenous DNA. Transgene expression of two separate reporter genes under the control of the same eukaryotic promoter was assessed in both human SKOV-3 ovarian carcinoma and murine B16/BL6 melanoma cells.

3.2.1 Luciferase transgene expression in SKOV-3 cells

In order to test the effects of chemotherapeutic agent on lipoplex-mediated transfection, luciferase transgene expression was evaluated in SKOV-3 cells following a 24h exposure to different concentrations of each selected chemotherapeutic agent (as described in the Methods). Emphasis was placed upon use of drug concentrations that

were not cytotoxic (*i.e.* less than the IC₅₀ or concentrations at which less than 50% cell toxicity or inhibition of cell proliferation was observed). However, at least one drug concentration equivalent to a value that would result in significant cell toxicity (>50%) was also evaluated. Results shown in Figure 14 indicate that transgene expression could increase, decrease or remain the same relative to lipoplex only controls, depending upon the drug and its concentration. Transgene expression, normalized to cellular protein levels, was established following the application of free DNA, lipoplexes or a combination of chemotherapeutic agent and lipoplexes. Free DNA (dashed line) and lipoplex only (solid line) controls were averaged for all experiments and expressed as a constant mean value \pm SEM on each panel. There was a significant increase in luciferase transgene expression for lipoplex only transfected cells (67 ± 8 pg luciferase/mg protein), as compared to cells transfected with free DNA (0.04 ± 0.04 pg luciferase/mg protein).

Figure 14A shows that pre-exposure to cisplatin at all drug concentrations evaluated, whether toxic ($>8.0\mu\text{M}$) or non-toxic ($<8.0\mu\text{M}$), resulted in a minimum 8.5-fold increase in transgene expression, as compared to lipoplex only controls. These differences were significant ($p<0.05$) at all concentrations except $10\mu\text{M}$. Pre-treatment with cytotoxic doses of doxorubicin (Figure 14B) caused a dose-dependent decrease in transgene expression values. When SKOV-3 cells were exposed to doxorubicin concentrations below the IC₅₀, there was an apparent 2-3-fold increase in transgene expression measured, but these increases were not significantly different than lipoplex only controls. In comparison, when cells were pre-exposed to vincristine there appeared to be a dose-dependent increase in transgene expression (Figure 14C). At vincristine

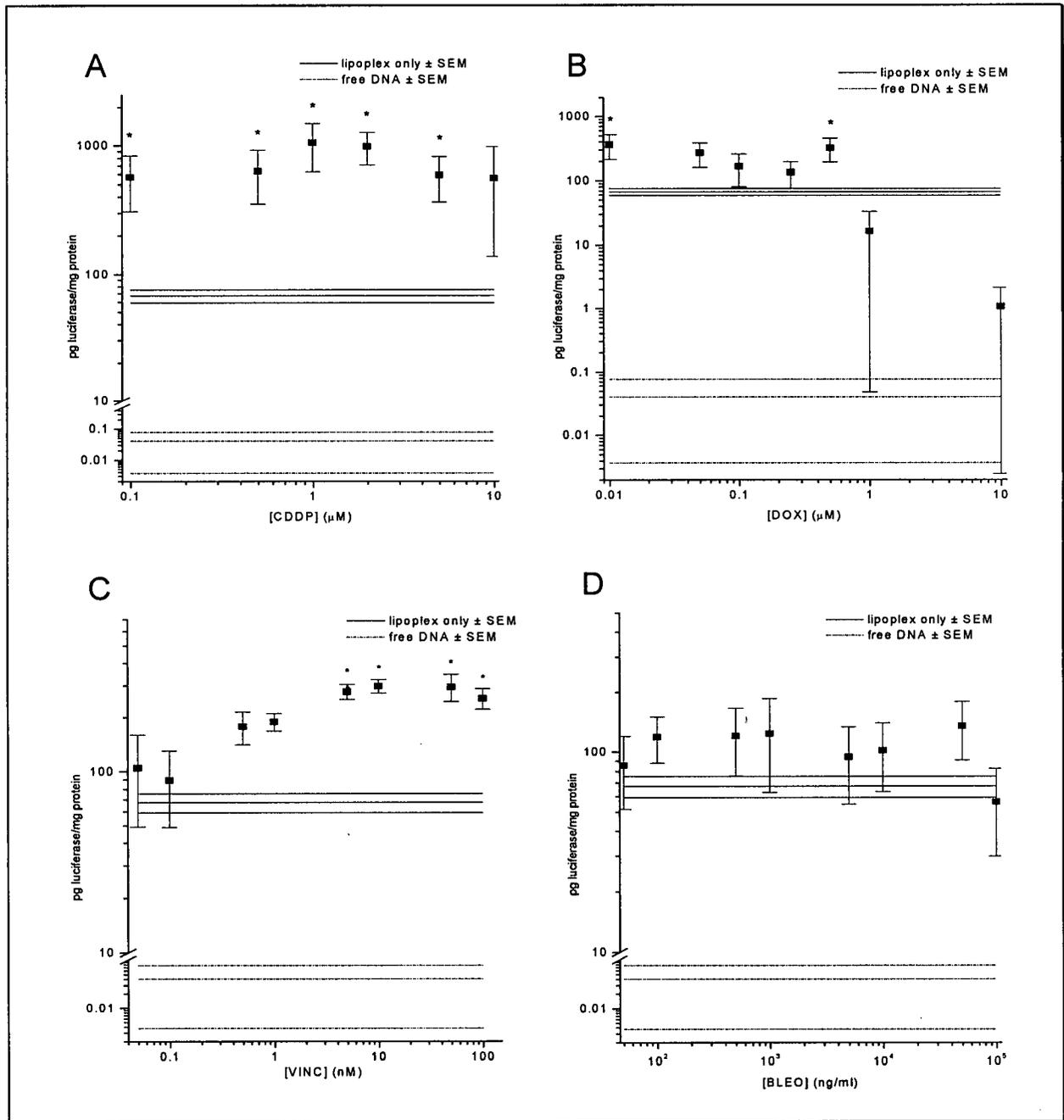


Figure 14. Luciferase transgene expression in SKOV-3 cells following lipoplex-mediated gene transfer. SKOV-3 cells were plated (1×10^4 cells/well), treated with varying concentrations of (A) cisplatin, (B) doxorubicin, (C) vincristine or (D) bleomycin and transfected with luciferase plasmid as free DNA or formulated with DODAC/DOPE liposomes. Free DNA (dashed line) and lipoplex only (solid line) controls were averaged and are depicted as a constant mean value \pm SEM. Data obtained in the presence of various drug concentrations were averaged from triplicate samples performed at least 3 times and are also expressed as mean value \pm SEM. Statistical analysis of reporter gene activity was evaluated for all *in vitro* experiments using post-hoc comparison of means (Scheffe's test) to show statistical difference.

* indicates significant ($p < 0.05$) compared to lipoplex only transfected controls.

concentrations greater than the IC_{50} there were significant 4-fold increases in luciferase expression over lipoplex only transfected values. Exposure of SKOV-3 cells to varying concentrations of bleomycin (Figure 14D) had no effect on transgene expression as compared to controls.

3.2.2 Luciferase transgene expression in B16/BL6 cells

B16/BL6 cells were also evaluated following transfection with lipoplexes in the presence of three different concentrations of each of the selected cytotoxic agents. This study was conducted to assess whether transfection is dependent upon cell type. The data from this study have been summarized in Figure 15. Similar to results obtained with the SKOV-3 cells, the level of transgene expression increased significantly when the luciferase expression vector was added as a DODAC/DOPE liposome formulation (solid line), compared to free DNA (dashed line). The transfection results obtained with B16/BL6 cells exposed to the various drugs were not comparable to those obtained with the SKOV-3 cells, suggesting that drug-induced effects on transgene expression may be cell type-specific.

In the presence of cisplatin at non-toxic ($<IC_{50}$) concentrations of 0.1 and 1.0 μM , there was no significant change in luciferase expression. However, a significant 14-fold suppression in luciferase expression was noted at the cytotoxic dose of 10 μM cisplatin. When B16/BL6 cells were pre-exposed to doxorubicin, there was a significant 135-fold decrease in luciferase transgene expression at all drug concentrations tested. Comparable to data obtained with SKOV-3 cells, a trend towards enhanced transgene expression was observed when B16/BL6 cells were pre-treated with a range of vincristine concentrations.

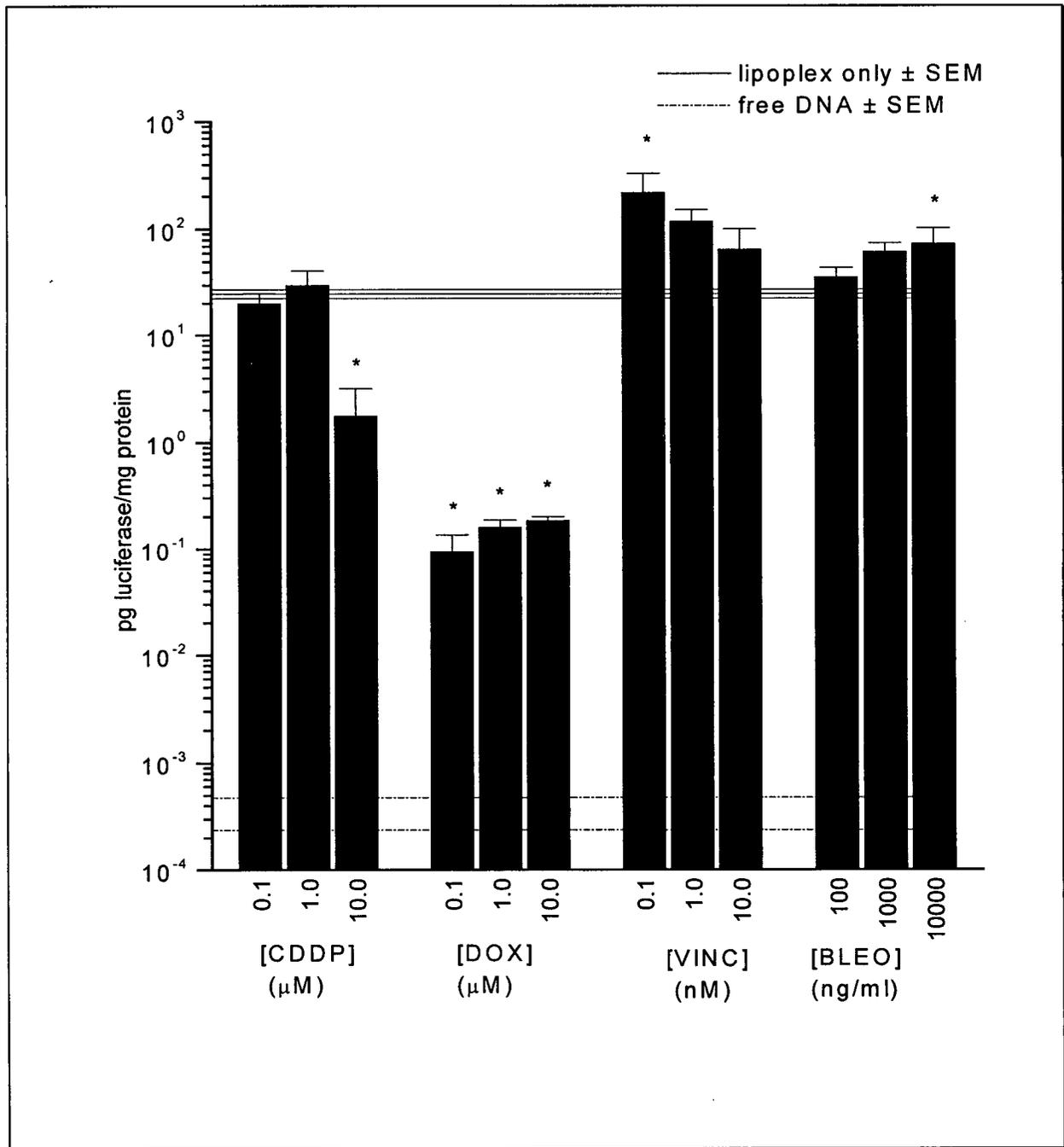


Figure 15. Luciferase transgene expression in B16/BL6 cells following lipoplex-mediated gene transfer. B16/BL6 cells were plated (6×10^3 cells/well), treated with the indicated concentrations of cisplatin, doxorubicin, vincristine or bleomycin and transfected with luciferase plasmid as free DNA or formulated with DODAC/DOPE liposomes. Free DNA (dashed line) and lipoplex only (solid line) controls were averaged and are depicted as a constant mean value \pm SEM. Data obtained in the presence of various drug concentrations were averaged from triplicate samples performed at least 3 times and are also expressed as mean value \pm SEM. Statistical analysis of reporter gene activity was evaluated for all *in vitro* experiments using post-hoc comparison of means (Scheffe's test) to show statistical difference.

* indicates significant ($p < 0.05$) compared to lipoplex only transfected controls.

Also comparable to SKOV-3 cells, pre-treatment of B16/BL6 cells with bleomycin caused no change or only slight increases in luciferase expression as compared to lipoplex only controls.

3.2.3 CAT transgene expression in B16/BL6 cells

In order to establish whether the impact of selected chemotherapeutic agents was transgene-specific, a limited study was conducted using B16/BL6 cells. They were pre-exposed to cisplatin and transfected using lipoplexes that had been formulated with a CAT gene plasmid. There were three specific reasons for conducting this particular study. Firstly, *in vivo* studies summarized in the following sections were restricted to evaluations of CAT gene plasmid because *in vivo* measurements of luciferase proved to be difficult and not very reproducible when compared to measurements of CAT. Secondly, *in vivo* studies summarized in the following sections evaluated transfection of B16/BL6 tumours following i.p. administration of lipoplexes. Transfection of B16/BL6 tumours *in vivo* has been well characterized in this laboratory (64). It has been determined that this model is more convenient than one based upon *in vivo* transfection of SKOV-3 tumours (60-80 days is required to establish SKOV-3 tumours in comparison to 7 days for the B16/BL6 tumours). Thirdly, a report published by Son and Huang (1994) (102) suggested that pre-exposure to cisplatin enhanced CAT transgene expression following lipoplex-mediated transfection.

Summarized in Table 2, transgene expression values were quantified by assessing CAT activity that had been normalized for cellular protein. As expected, the levels of CAT expression were significantly greater when cells were transfected with lipoplexes, in

comparison to free DNA. Incubation of B16/BL6 cells with concentrations of cisplatin below the IC₅₀ (<10µM) caused no significant change in transgene expression levels. When the cisplatin concentration was equivalent to the IC₅₀, there was a significant 24-fold reduction in expression. These results are quite comparable to those obtained when B16/BL6 cells were transfected with the luciferase plasmid (Figure 15).

Table 2. CAT transgene expression in B16/BL6 cells following transfection with DODAC/DOPE lipoplexes prepared using pINEXCatv2.0, in the presence and absence of cisplatin^a.

Cisplatin Concentration (µM)	Plasmid Formulation	Transgene Expression ± SEM (mU CAT activity/mg cell protein) ^b
0	Free DNA	0.40 ± 0.4
0	Lipoplex	880 ± 50
0.2	“	780 ± 90
2.0	“	1540 ± 260
10.0	“	37 ± 10 ^c

^a Lipoplexes were prepared as described in section 2.2.4 using DODAC/DOPE liposomes and pINEXCatv2.0 at a ratio of 10nmole lipid/1µg DNA.

^b CAT expression was measured as described in section 2.2.5.3.

^c Significantly different (p<0.05) from lipoplex only control.

3.3 *In vivo* lipoplex-mediated transfection in the presence of selected chemotherapeutic agents

To evaluate the influence of pre-treatment with chemotherapeutic agents on *in vivo* transfection activity, two models were developed. The first model evaluated transgene expression of CAT reporter gene in mice bearing B16/BL6 tumours, where drug treatment was given i.v., prior to i.p. administration of free plasmid or plasmid formulated with DODAC/DOPE lipoplexes. The characteristics of this i.p. tumour model and factors that influence lipoplex transfection have been evaluated previously in this laboratory (125). The second model evaluated transgene expression of CAT reporter gene in selected tissues isolated from mice administered lipoplexes via the lateral tail

vein. It is known that following i.v. administration, lipoplexes are rapidly eliminated from the circulation with a concomitant delivery to tissues such as the spleen, liver and lung (63). As shown in section 3.3.2, transfection is most consistently observed in the spleen and the bone marrow following i.v. administration of lipoplexes.

3.3.1 Transfection of B16/BL6 tumours following intraperitoneal administration of lipoplexes

Previous research in this laboratory has demonstrated that B16/BL6 tumours can be reproducibly transfected when lipoplexes (35µg plasmid DNA in 500µl lactose volume) are administered early in the development of the tumour (tumour weight between 50 and 200mg) (125). A similar model was used in this study to assess how chemotherapeutic drugs affected transfection following i.p. administration of DODAC/DOPE lipoplexes formulated with the pINEXCatv2.0 plasmid. Seven days after tumour cell inoculation in C57BL/J6 mice, a single i.v. injection of the selected chemotherapeutic agent was administered at a dose that should have provided maximum therapeutic activity (maximum therapeutic dose). Three days later, the mice received a single i.p. injection of the plasmid as free DNA or formulated with DODAC/DOPE liposomes. B16/BL6 tumours in the peritoneal cavity were harvested 24h after plasmid injection and the level of CAT expression was measured. Figure 16 emphasizes two important results from this study. Firstly, transgene expression levels in the isolated B16/BL6 tumours showed a significant 48-fold elevation when mice were injected with the lipoplex-formulated plasmid, in comparison to free plasmid. Secondly, pre-treatment with the selected chemotherapeutic agents had no significant effect on the level of CAT expression, achieved following lipoplex-mediated transfection. A 2-fold reduction in

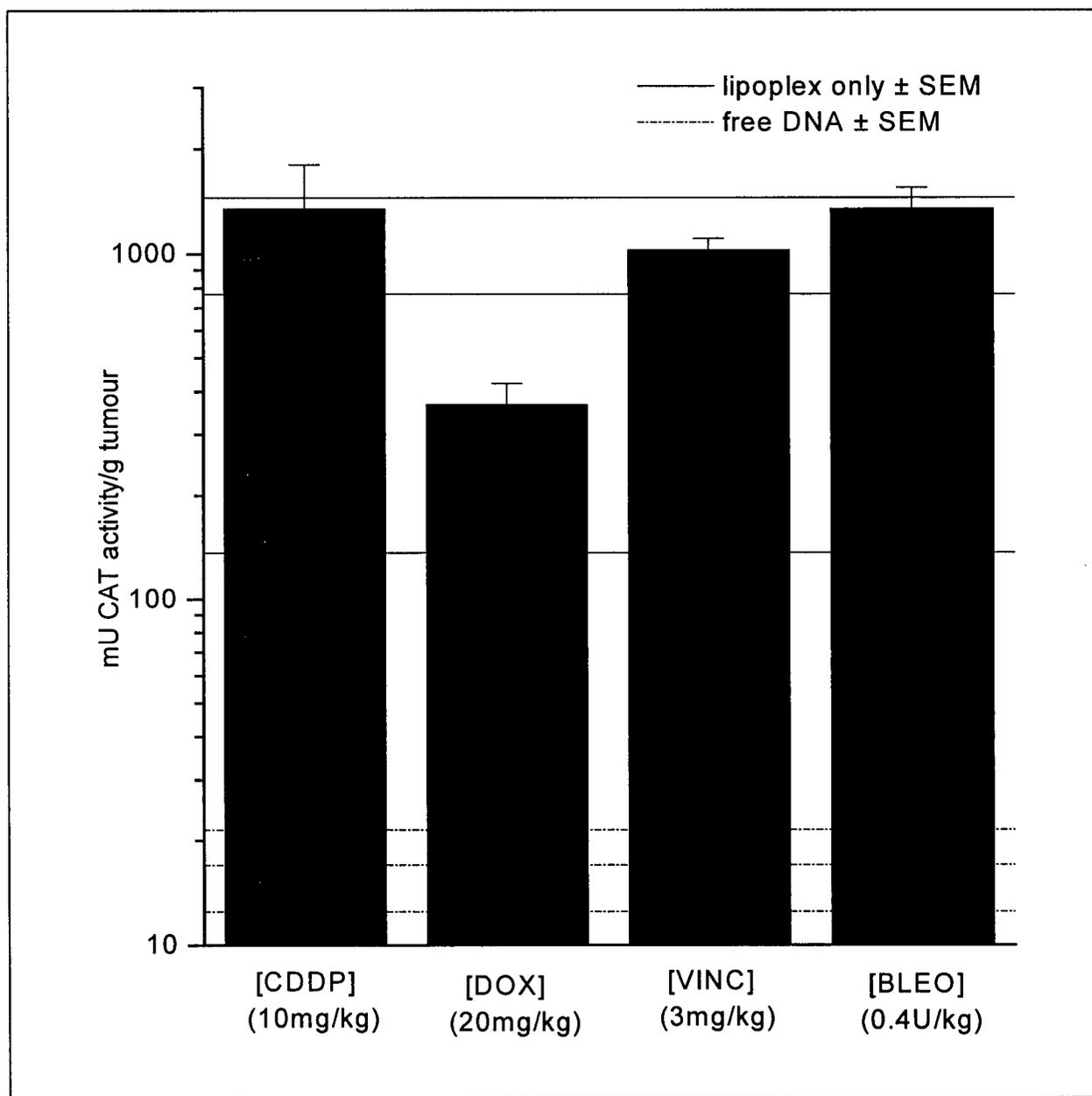


Figure 16. CAT transgene expression in intraperitoneal B16/BL6 tumours following intraperitoneal administration of lipoplexes. C57BL/6J mice were injected i.p. with 1×10^5 B16/BL6 cells seven days prior to treatment with a single i.v. dose of cisplatin (10mg/kg), doxorubicin (20mg/kg), vincristine (3mg/kg) or bleomycin (0.4U/kg). Three days after drug administration, CAT plasmid, as free DNA or formulated with DODAC/DOPE liposomes was injected i.p. Mice were killed 24h post-transfection and tumours were harvested. Cellular extracts were assayed for CAT activity and normalized by tumour weight as described in section 2.2.6.3. Free DNA (dashed line) and lipoplex only (solid line) controls were averaged and are depicted as a constant mean value \pm SEM. Other data points are averaged from four mice and expressed as mean value \pm SEM. Statistical analysis of reporter gene activity was evaluated for all *in vivo* experiments using post-hoc comparison of means (Scheffe's test) to show statistical difference.

expression was observed in tumours isolated from doxorubicin-treated animals, but this difference was not significant.

3.3.2 Transfection following intravenous administration of DODAC/DOPE lipoplexes

Following i.v. administration, lipoplexes are eliminated rapidly from the plasma compartment and distributed to a number of organs. In this study, in order to assess which organs expressed the transgene, CD1 mice were given a single i.v. injection of CAT plasmid as free DNA or formulated with DODAC/DOPE liposomes, via the lateral tail vein. Various tissues were harvested 24h after administration and CAT activity was measured. The results, shown in Figure 17, indicate that spleen, bone marrow, heart and kidney from mice injected i.v. with lipoplexes all expressed CAT transgene at levels significantly above that which was observed following injection of free DNA. The level of CAT activity was highest in the spleen and bone marrow, where expression levels relative to tissues isolated from free DNA-injected animals were 360-fold and 80-fold greater, respectively. For this reason, studies assessing the influence of chemotherapy on transgene expression following i.v. administration of lipoplexes focused upon these two tissues.

The studies that assessed the influence of chemotherapy pre-treatment on transgene expression in spleen and bone marrow following i.v. administration of lipoplexes have been summarized in Figure 18. These experiments evaluated transgene expression following drug exposure and plasmid injection of a 21 day time course. This extensive time course was completed because it was known that significant suppression of spleen and bone marrow cellularity would be observed at the selected drug doses.

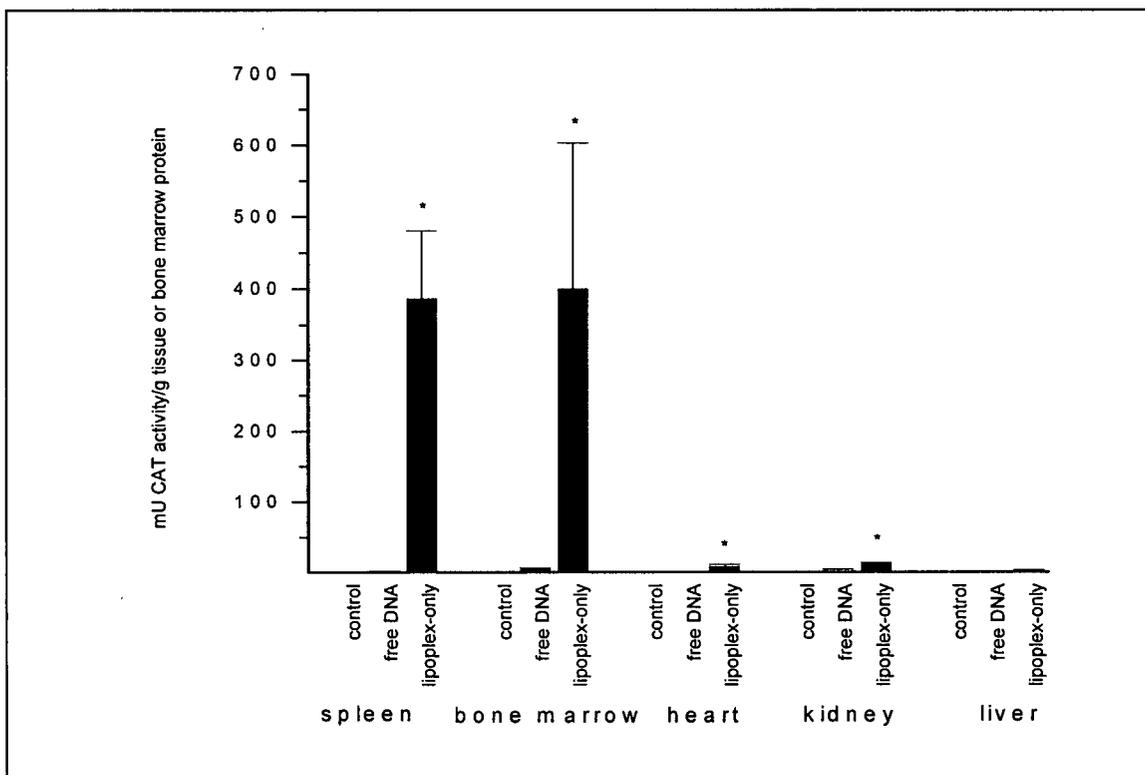


Figure 17. CAT transgene expression in various tissues following intravenous administration of lipoplexes. CD1 mice were administered CAT plasmid as free DNA or formulated with DODAC/DOPE liposomes, via the lateral tail vein. After 24h, the spleen, bone marrow, heart, kidney, lung and liver were harvested. Tissues were assayed for CAT activity and normalized for tissue weight or cellular protein (bone marrow) as described in section 2.2.6.3. Data points were averaged from four mice and shown as mean value \pm SEM. Statistical analysis of reporter gene activity was evaluated for all *in vivo* experiments using post-hoc comparison of means (Scheffe's test) to show statistical difference.

* indicates significant ($p < 0.05$) compared to lipoplex only transfected animals.

Previous experience with doxorubicin, for example, has demonstrated significant suppression, with a nadir in cell number occurring between 1 and 3 days after drug administration (126). Recovery of the spleen weight and bone marrow cellularity to control levels occurred over a 21 day time period. For this reason it was important to evaluate expression levels at time points where marrow suppression was greatest (3 days) and at time points during the recovery phase (7, 14 and 21 days). Mice were given a

single i.v. injection of the indicated chemotherapeutic agent on either day 0, 7, 14 or 18, corresponding to 21, 14, 7 or 3 day drug exposure. On day 22, free DNA or lipoplexes containing the CAT gene were administered by i.v. injection. Twenty-four hours later, spleen and bone marrow samples were harvested.

For simplicity the results for each drug will be described separately. In addition, it is important to emphasize that expression levels were always determined 24h after lipoplex administration (*i.e.* the time course is not measuring stability of expression following plasmid injection). Expression levels in the spleen (Figure 18A) and bone marrow (Figure 18B) were not significantly affected by pre-treatment with cisplatin, regardless of the time-point after drug administration. Similar results were obtained when the mice were pre-treated with bleomycin, where there was a 2-5-fold reduction in spleen transfection and a less than 2-fold reduction in bone marrow transfection at time points 3, 7, and 14 days after drug administration. These differences were not statistically significant. A similar tendency towards decreased transgene expression levels was also observed following administration of vincristine. For example, 3 days after drug administration there was a 31-fold decrease in transgene expression in the bone marrow, however this was not statistically significant due to the small experimental sample number. Typically, a 2-5-fold decrease in expression level was observed in spleen and bone marrow recovered from animals treated with vincristine, 7 and 14 days before lipoplex injection. When animals were pre-treated with doxorubicin there was a significant decrease in expression level observed in spleen tissue and a tendency to reduced transfection in the bone marrow. This observation was greatest at days 3 and 7 after drug administration, after which, the suppression tended to diminish by days 7 and

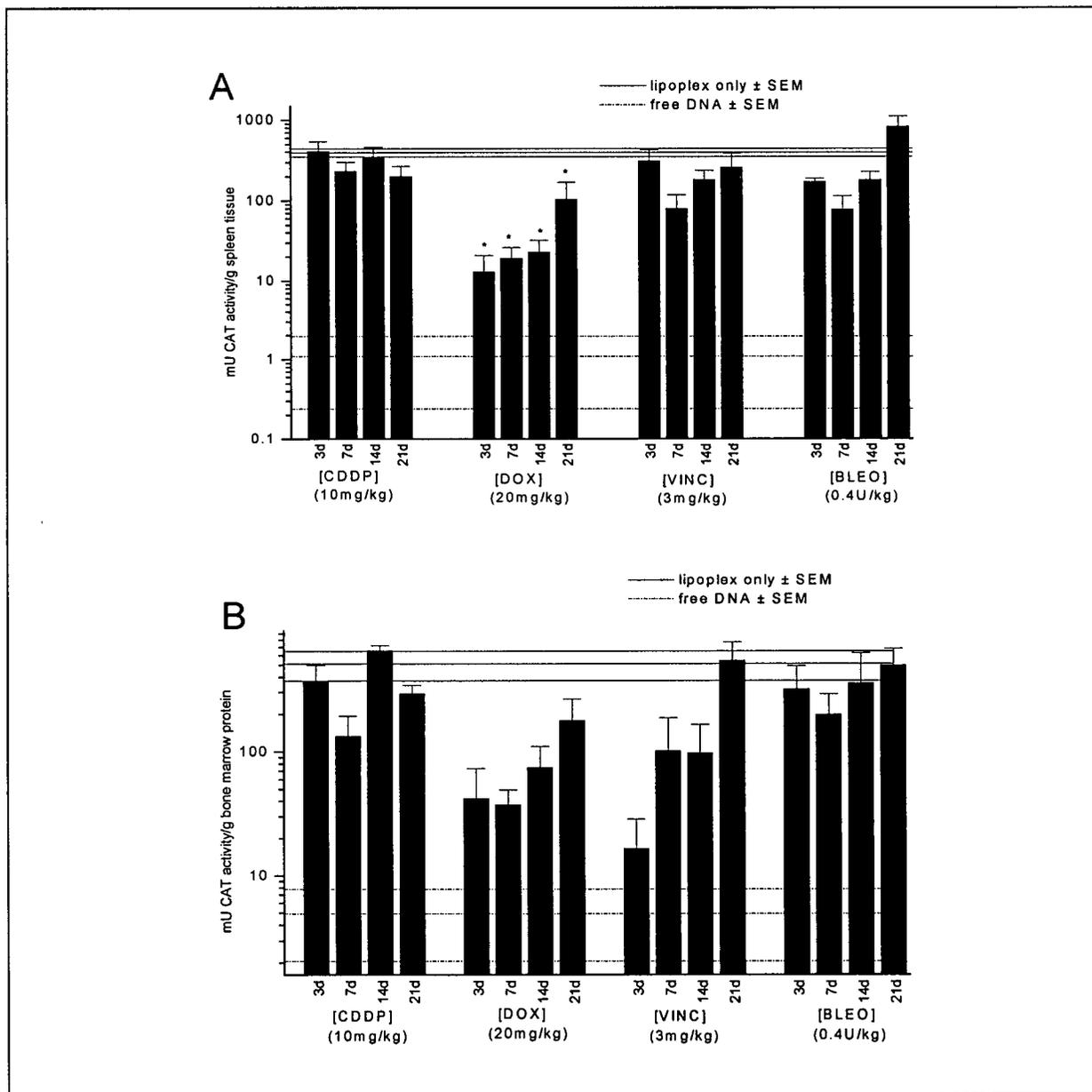


Figure 18. CAT transgene expression in spleen (A) and bone marrow (B) following intravenous administration of lipoplexes. CD1 mice were injected i.v. with a single dose of cisplatin (10mg/kg), doxorubicin (20mg/kg), vincristine (3mg/kg) or bleomycin (0.4U/kg). At 3, 7, 14 or 21 days post-drug injection, mice were administered CAT plasmid as free DNA or formulated with DODAC/DOPE liposomes, via the lateral tail vein. After 24h, the spleen and bone marrow were harvested. Tissues were assayed for CAT activity and normalized for tissue weight or cellular protein (bone marrow) as described in section 2.2.6.3. Free DNA (dashed line) and lipoplex only (solid line) controls were averaged and are depicted as a constant mean value \pm SEM. Other data points were averaged from four mice and shown as mean value \pm SEM. Statistical analysis of CAT activity was evaluated using post-hoc comparison of means (Scheffe's test) to show statistical difference.

* indicates significant ($p < 0.05$) compared to lipoplex only transfected animals.

21. However, even 21 days after doxorubicin administration there was a significant 4-fold reduction in transgene expression observed in spleen tissue and a 3-fold (but not statistically significant) reduction in expression levels in bone marrow.

Chapter 4

Discussion

The studies described in this thesis concerning the influence of chemotherapy on lipoplex-mediated gene transfer were conducted for two specific reasons. Firstly, previous studies have suggested that transgene expression may be enhanced when viral or non-viral transfecting agents are used in combination with cytotoxic drugs (102, 127) or radiation (105, 128). Secondly, it is strongly believed that in the future, gene therapy protocols for cancer treatment will be used in combination with other modalities. Due to its complicated nature, cancer is currently treated by elaborate therapeutic strategies involving a combination of surgery, radiation and chemotherapeutic agents. Typically, the use of chemotherapeutic agents involves administration of a variety of drugs that act via unique mechanisms to ensure maximal tumour cell kill while reducing the potential for development of resistance mechanisms. It is anticipated that the “drugs” that arise through the development of selected gene therapy protocols will add to the existing armament of drugs already in use to treat cancer. The results summarized in this thesis suggest that under specific conditions, pre-treatment of cells with a chemotherapeutic agent can result in significant increases in transfection when using a lipid-based formulation for the delivery of a plasmid expression vector. However, under other defined conditions, significant suppression of transgene expression may occur. In this discussion, common cellular mechanisms by which chemotherapeutic agents may enhance or suppress transgene expression will be addressed.

Transgene expression may be influenced by one or a combination of the following:

- (i) DNA damage and associated repair
- (ii) cell cycle effects
- (iii) altered intracellular processing

While each of these will be discussed in turn, evidence herein support the concept that drug-induced changes in intracellular processing of lipoplexes are the most likely means by which transgene expression can be altered.

Postulated as a mechanism capable of influencing transgene expression, damage to the host cell DNA and its associated repair processes may be induced by a variety of techniques, including some chemotherapeutic agents (129, 130) and radiation (131). Damage to the DNA may present itself in the form of single- and double-strand breaks and/or crosslinks between the strands. Lesions that are not lethal to the cell induce DNA repair processes. Numerous complementary signaling pathways serve to prompt cell cycle arrest and activation of DNA repair events. Previous studies reporting enhanced transgene expression following lipoplex-mediated transfection, implicated DNA damage and repair events as likely effectors of this change (102). It was stated that exposure of cells to cisplatin prior to gene transfer increases their transfection ability and that cisplatin-induced DNA damage may be responsible for this result. Similarly, Stevens *et al.* (1996) (132) stated that DNA-damaging agents such as ionizing radiation enhance the transfection efficiency of a transgene integrating into the host genome. The anticipated repair processes that accompany DNA damage were thought to affect recombination events, thereby improving integration of the transgene. However, this theory has recently been disputed by Jain and Gewirtz (1999) (128). Their work irradiating cultured human

breast tumour cells 24h before transfection with a liposomal complex containing the luciferase gene, essentially ruled out direct effects of DNA damage as the contributing factors to enhancement of transgene expression.

Three of the selected chemotherapeutic agents in this study, cisplatin, doxorubicin and bleomycin are known DNA-damaging agents whose mechanisms of action are quite different. Cisplatin forms platinum-DNA crosslinked adducts. Doxorubicin interacts with Topo II-DNA complexes, preventing re-ligation of DNA and therefore, causing strand breaks. Doxorubicin may also damage DNA via formation of reactive oxygen species such as hydrogen peroxide and hydroxyl radical. Bleomycin is activated through interactions with various metals to produce reactive oxygen species that are capable of fragmenting DNA via double strand breaks. The most compelling evidence against DNA damage and repair events contributing to alterations in transgene expression centered on the use of bleomycin in combination with lipoplex-mediated gene transfer. Results from *in vitro* and *in vivo* studies consistently reported no significant changes in transgene expression levels when pre-treatment of bleomycin preceded gene transfer. Although quantitative measurements of DNA damage were not performed during these experiments, it can be reasonably stated that this cellular mechanism does not result in conditions that favour transgene expression enhancement or suppression.

Another theory to account for changes in lipoplex-mediated transgene expression is drug-induced cell cycle alterations. The sequence of events that occurs during the lifetime of eukaryotic cells can be generally divided into four stages. Excluding the variable quiescent stage, cells cycle through a lengthy G1 (gap) phase into an S (synthesis) phase, during which they undergo DNA replication. The G2 phase is

followed by a brief M (mitosis) phase, which is responsible for mitotic activity and cell division. It is during the latter stages of this M phase that the nuclear membrane is disrupted, allowing for chromosome segregation into two daughter cells. A new cycle commences with the transition from the M phase to the G1 phase of the next cycle.

Doxorubicin is thought to inhibit normal cell cycling by interfering with events associated with DNA synthesis and arresting cells during the S phase. In comparison, vincristine exerts its influence during mitotic events and arrests cells in the M phase. By temporarily interrupting normal cell cycling, these drugs may act to synchronize cells. If intracellular events are cell cycle-specific, synchronization may alter transgene expression levels. It has been observed that some cells undergoing mitosis experience greater expression of lipoplex-mediated transgene than cells in any other stage (133). These studies also showed that when lipoplex-treated cells arrested in G1 were permitted to resume cycling, transgene expression correlated to the transition period between G2/M and the G1 phase of the next cycle. The highest level of transgene expression was noted when cells were exposed to lipoplexes during mitosis or just prior to this stage. These results hinted at possible cell cycle-dependent mechanisms that might regulate lipoplex delivery. Therefore, exposure to drugs such as doxorubicin or vincristine may serve to synchronize cells, thus regulating the number of cells available for lipoplex processing. In the case of vincristine, cell cycle arrest during M phase and the ensuing synchronization upon resumption of normal cycling, may increase the number of cells experiencing nuclear membrane disruption.

The observed enhancement of transgene expression following vincristine pre-treatment *in vitro* may be a consequence of cell cycle synchronization and in particular, a

greater amount of DNA evading the nuclear envelope barrier. The results observed *in vitro* with doxorubicin pre-treatment suggested that arresting cells in S phase could prevent a large percentage of the plasmid from accessing the nucleus, due to an intact membrane barrier. The conclusion of this study is that *in vitro* cell cycle synchronization is possibly involved in altering transgene expression. However, this is not a likely mechanism by which transgene expression of cells *in vivo* are influenced by chemotherapeutic agents, as cell synchronization is extremely difficult to maintain beyond a single cycle (134).

Alternatively, the effects of chemotherapy on transgene expression may be a consequence of changes to the intracellular processing of lipoplexes. Lipoplexes taken up by host cells must escape from endosomal vesicles if they are to escape degradation via the lysosomal pathway. Plasmid DNA dissociates from liposomes and is released from the endosome. It must then be transported from the cytosol into the nucleus and processed to yield the protein of interest. Chemotherapeutic agents causing changes to transgene expression may target any of these intracellular processing events. For example, cisplatin-induced DNA damage activates repair events that are linked to signaling pathways such as the protein kinase C cascade (135). Activation of this enzyme in the presence of physiological concentrations of calcium and membrane phospholipids causes cellular protein phosphorylation and feedback inhibition of some growth factor receptors. This inhibition may culminate in the internalization of these receptors via the endocytic pathway (136). As such, this could increase the number of lipoplexes taken up into the cell. Therefore, cisplatin potentially affects transgene expression via indirect activation of the endocytic process. Repair processes induced by

cisplatin damage are also involved in other signaling pathways, whose downstream effectors are coupled with transcription factors. For example, the mitogen-activated protein kinase (112) and jun kinase/stress-activated protein kinase (137) pathways activate transcription factors, c-jun and c-fos. Perhaps cisplatin affects transgene expression by altering transcription or influencing the stability of plasmid message.

Vincristine is not categorized as a DNA-damaging agent as its mechanism of action targets tubulin, thus blocking polymerization of tubulin into microtubules and arresting cell division in metaphase. In the absence of an intact mitotic spindle, chromosomes are not properly segregated to opposing poles and will abnormally clump or remain dispersed in the nucleus. It has been postulated that the breakdown of the nuclear envelope accompanying G2/M transition could facilitate DNA transport into the nucleus (58). Therefore, a microtubule inhibitor such as vincristine could directly affect transgene expression by arresting cells in a state of nuclear membrane disruption, thereby facilitating access of the transgene to the nucleus.

The results generated with the use of vincristine in this study provide compelling evidence that intracellular processing is a likely candidate for the mechanistic basis behind altered transgene expression. The mitotic arrest induced by vincristine resulted in elevated *in vitro* expression levels, most likely due to improved DNA transport into the nucleus. However, cisplatin results also *in vitro* are more ambiguous as they display changes in transgene expression levels that appear to be specific to cell type. These results serve to highlight the complexities associated with drug-induced interference of intracellular processing. Chemotherapeutic agents may induce a multitude of events related to intracellular events, each influencing transgene expression in a different

manner. For example, drugs that have the ability to interfere with endosomal processing or block lysosomal degradation would assist endosomal escape, potentially elevating transgene expression through greater delivery of plasmid DNA to the cytoplasm. Alternatively, these drugs could also inhibit intracellular processing by blocking internalization of lipoplexes via the endocytic pathway, culminating in decreased transgene expression levels.

The use of chemotherapeutic agents in combination with lipoplex-mediated gene transfer had a varied response *in vitro* depending on the selected drug, its concentration and the cell type. However, it was observed *in vivo* that pre-treatment with the selected drug of B16/BL6 tumours or i.v. transfection of spleen and bone marrow cells, caused a tendency towards suppression of transgene expression. Unlike the uniform population *in vitro*, the population of cells transfected *in vivo* is not known. Long term suppression of transgene expression in spleen and bone marrow following doxorubicin and vincristine exposure suggests drug-induced cell death of a cell population capable of being transfected.

This study has focused upon the effects of chemotherapy in combination with plasmid-based expression vectors and the possible role of drug-induced cellular processing events. Chemotherapeutic effects upon internalization and processing events may differ when employing viral-based vectors. Drug-induced enhancement of a dividing cell population would benefit gene transfer from viral-based vectors such as retroviruses. Studies conducted by Szilvassy *et al.* (1989) (138) used 5-fluorouracil to isolate a dividing progenitor marrow population from murine hematopoietic cells and reported increases in retrovirus-mediated gene transfer.

It can be concluded from this study that some chemotherapeutic agents are capable of influencing the transgene expression achieved following lipoplex-mediated gene transfer and that the presence of lipoplexes does not alter the toxicity of these drugs. Several explanations as to how selected drugs enhance or suppress transgene expression have been postulated in this thesis. The data generated in this study support the notion that alterations to transgene expression are most likely due to changes in cellular processing events, as a result of drug treatment.

Further research to strengthen this theory might include quantitative assays to determine the actual concentration of drug taken up into the cell. In the case of DNA-damaging agents, it would be beneficial to quantify the amount of damage the host cell incurs. Additional research covering a broader range of chemotherapeutic agents might also provide supportive evidence for this theory. Alternatively, a wider selection of drugs may uncover additional mechanisms of drug action that impose some influence on gene transfer. Focusing upon cellular processing events, it would be of interest to unravel the finer mechanistic details of doxorubicin and vincristine and their effects upon lipoplex-mediated gene transfer. In particular, if the suppression achieved *in vivo* was due to elimination of a population of cells capable of transfection, what were those cells and how can they be protected and induced to proliferate?

Combination therapy continues to advance in clinical use and so it is imperative that basic research continues to seek combination therapies that will translate into clinically therapeutic strategies. It is important to stress that suppression of transgene expression was noted at cytotoxic doses of some drugs, which is the dosage at which clinical treatments are conducted. As such, the results obtained in this study should serve

as a critical reminder for the potentially hazardous effects of lipoplex-mediated gene therapy in combination with chemotherapy. Cautionary mandates should be placed when applying this combination strategy to clinical situations. Future efforts in basic research should acknowledge the inhibitory potentials realized in this study and use this information to develop strategies that enable drug-induced enhancement of gene transfer.

Chapter 5

References

1. Crystal RG. (1995) Transfer of genes to humans: early lessons and obstacles to success. [Review] *Science*. 270:404-10.
2. Yanez RJ and Porter AC. (1998) Therapeutic gene targeting. [Review] *Gene Therapy*. 5:149-59.
3. Nabel EG, Simari R, Yang Z, San H and Nabel GJ. (1997) *In vivo* gene transfer: a biological tool. [Review] *Annals of the New York Academy of Sciences*. 811:289-92.
4. McCabe ER. (1993) Clinical application of gene therapy: emerging opportunities and current limitations. [Review] *Biochemical Medicine & Metabolic Biology*. 50:241-53.
5. Rosenfeld MA, Yoshimura K, Trapnell BC, Yoneyama K, Rosenthal ER, Dalemans W, Fukayama M, Bargon J, Stier LE and Stratford-Perricaudet L. (1992) *In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell*. 68:143-55.
6. Veal GJ, Agrawal S and Byrn RA. (1998) Synergistic inhibition of HIV-1 by an antisense oligonucleotide and nucleoside analog reverse transcriptase inhibitors. *Antiviral Research*. 38:63-73.
7. Smythe WR, Hwang HC, Amin KM, Eck SL, Davidson BL, Wilson JM, Kaiser LR and Albelda SM. (1994) Use of recombinant adenovirus to transfer the herpes simplex virus thymidine kinase (HSVtk) gene to thoracic neoplasms: an effective *in vitro* drug sensitization system. *Cancer Research*. 54:2055-9.
8. Mulligan RC. (1993) The basic science of gene therapy [Review] *Science*. 260:926-32.
9. Cristiano RJ, Xu B, Nguyen D, Schumacher G, Kataoka M, Spitz FR and Roth JA. (1998) Viral and nonviral gene delivery vectors for cancer gene therapy. *Cancer Detection & Prevention*. 22:445-54.
10. Jolly D. (1994) Viral vector systems for gene therapy [Review] *Cancer Gene Therapy*. 1:51-64.
11. Robbins PD, Tahara H and Ghivizzani SC. (1998) Viral vectors for gene therapy. [Review] *Trends In Biotechnology*. 16:35-40.
12. Felgner PL. (1997) Nonviral strategies for gene therapy. *Scientific American*. 276:102-6.

13. Cotton M and Wagner E. (1993) Non-viral approaches to gene therapy [Review] *Current Opinion in Biotechnology*. 4:705-10.
14. Ledley FD. (1995) Nonviral gene therapy: the promise of genes as pharmaceutical products. [Review] *Human Gene Therapy*. 6:1129-44.
15. Pastan I. (1979) Cell transformation. *Methods in Enzymology*. 58:368-70.
16. Gordon EM and Anderson WF. (1994) Gene therapy using retroviral vectors. [Review] *Current Opinion in Biotechnology*. 5:611-6.
17. Uckert W and Walther W. (1994) Retrovirus-mediated gene transfer in cancer therapy. [Review] *Pharmacology & Therapeutics*. 63:323-47.
18. Berns KI and Giraud C. (1995) Adenovirus and adeno-associated virus as vectors for gene therapy. [Review] *Annals of the New York Academy of Sciences*. 772:95-104.
19. Tong X, Engehausen DG, Freund CT, Agoulnik I, Guo Z, Oehler MK, Kim TE, Hasenburg A, Contant CF, Woo SL and Kieback DG. (1998) The efficacy of adenovirus-mediated gene therapy of ovarian cancer is enhanced by using the cytomegalovirus promoter. *Anticancer Research*. 18:719-25.
20. Latchman DS. (1994) Herpes simplex virus vectors for gene therapy. [Review] *Molecular Biotechnology*. 2:179-95.
21. Oldfield EH, Ram Z, Culver KW, Blaese RM, DeVroom HL and Anderson WF. (1993) Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir. *Human Gene Therapy*. 4:39-69.
22. Vile RG, Tuszynski A and Castleden S. (1996) Retroviral vectors. From laboratory tools to molecular medicine. [Review] *Molecular Biotechnology*. 5:139-58.
23. Osborne WR. (1991) Retrovirus-mediated gene expression in mammalian cells. [Review] *Current Opinion in Biotechnology*. 2:708-12.
24. Boris-Lawrie K and Temin HM. (1994) The retroviral vector. Replication cycle and safety considerations for retrovirus-mediated gene therapy. [Review] *Annals of the New York Academy of Sciences*. 716:59-70.
25. Brody SL and Crystal RG. (1994) Adenovirus-mediated *in vivo* gene transfer. [Review] *Annals of the New York Academy of Sciences*. 716:90-101.

26. Han JS, Qian D, Wicha MS and Clarke MF. (1998) A method of limited replication for the efficient *in vivo* delivery of adenovirus to cancer cells. *Human Gene Therapy*. 9:1209-16.
27. Glorioso JC, DeLuca NA and Fink DJ. (1995) Development and application of herpes simplex virus vectors for human gene therapy. [Review] *Annual Review of Microbiology*. 49:675-710.
28. Glorioso JC, Goins WF, Schmidt MC, Oligino T, Krisky DM, Marconi PC, Cavalcoli JD, Ramakrishnan R, Poliani PL and Fink DJ. (1997) Engineering herpes simplex virus vectors for human gene therapy. [Review] *Advances in Pharmacology (New York)*. 40:103-36.
29. Berns KI and Giraud C. (1996) Biology of adeno-associated virus. [Review] *Current Topics in Microbiology & Immunology*. 218:1-23.
30. Peplinski GR, Tsung K and Norton JA. (1998) Vaccinia virus for human gene therapy. [Review] *Surgical Oncology Clinics of North America*. 7:575-88.
31. Alexander L, Lu HH, Gromeier M and Wimmer E. (1994) Dicistronic polioviruses as expression vectors for foreign genes. *AIDS Research & Human Retroviruses*. 10:S57-60.
32. Palese P. (1998) RNA virus vectors: where are we and where do we need to go? [Review] *Proceedings of the National Academy of Sciences of the United States of America*. 95:12750-2.
33. Hirose S and Suzuki Y. (1988) *In vitro* transcription of eukaryotic genes is affected differently by the degree of DNA supercoiling. *Proceedings of the National Academy of Sciences of the United States of America*. 85:718-22.
34. Harvie P, Wong FMP and Bally MB. (1998) Characterization of lipid DNA interactions. I. Destabilization of bound lipids and DNA dissociation. *Biophysical Journal*. 75:1040-51.
35. Bloomfield VA. (1996) DNA condensation. [Review] *Current Opinion in Structural Biology*. 6:334-41.
36. Reimer DL, Zhang Y, Kong S, Wheeler JJ, Graham RW and Bally MB. (1995) Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA. *Biochemistry*. 34:12877-83.
37. Kollen WJ, Midoux P, Erbacher P, Yip A, Roche AC, Monsigny M, Glick MC and Scanlin TF. (1996) Gluconoylated and glycosylated polylysines as vectors for gene transfer into cystic fibrosis airway epithelial cells. *Human Gene Therapy*. 7:1577-86.

38. Haensler J and Szoka FC Jr. (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chemistry*. 4:372-9.
39. Kukowska-Latallo JF, Bielinska AU, Johnson J, Spindler R, Tomalia DA and Baker JR Jr. (1996) Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proceedings of the National Academy of Sciences of the United States of America*. 93:4897-902.
40. Gao X and Huang L. (1995) Cationic liposome-mediated gene transfer. [Review] *Gene Therapy*. 2:710-22.
41. Wong FMP, Reimer DL and Bally MB. (1996) Cationic lipid binding to DNA: characterization of complex formation. *Biochemistry*. 35:5756-63.
42. Scherman D, Bessodes M, Cameron B, Herscovici J, Hofland H, Pitard B, Soubrier F, Wils P and Crouzet J. (1998) Application of lipids and plasmid design for gene delivery to mammalian cells. [Review] *Current Opinion in Biotechnology*. 9:480-5.
43. Jacoby DR, Fraefel C and Breakefield XO. (1997) Hybrid vectors: a new generation of virus-based vectors designed to control the cellular fate of delivered genes [editorial]. *Gene Therapy*. 4:1281-3.
44. Schwarzenberger P, Hunt JD, Robert E, Theodossiou C and Kolls JK. (1997) Receptor-targeted recombinant adenovirus conglomerates: a novel molecular conjugate vector with improved expression characteristics. *Journal of Virology*. 71:8563-71.
45. Trubetskoy VS, Torchilin VP, Kennel S and Huang L. (1992) Cationic liposomes enhance targeted delivery and expression of exogenous DNA mediated by N-terminal modified poly(L-lysine)-antibody conjugate in mouse lung endothelial cells. *Biochimica et Biophysica Acta*. 1131:311-3.
46. Dowhan W. (1997) Molecular basis for membrane phospholipid diversity: why are there so many lipids?. [Review] *Annual Review of Biochemistry*. 66:199-232.
47. Blaurock AE. (1982) Evidence of bilayer structure and of membrane interactions from X-ray diffraction analysis. [Review] *Biochimica et Biophysica Acta*. 650:167-207.
48. Cullis PR, Hope MJ and Tilcock R (1986) Lipid polymorphism and the role of lipids in membranes. *Chemistry and Physics of Lipids*. 40:127-44.
49. Hope MJ and Kitson CN. (1993) Liposomes. A perspective for dermatologists. [Review] *Dermatologic Clinics*. 11:143-154.

50. Duzgunes N, Wilschut J, Fraley R and Papahadjopoulos D. (1981) Studies on the mechanism of membrane fusion. Role of head-group composition in calcium- and magnesium-induced fusion of mixed phospholipid vesicles. *Biochimica et Biophysica Acta*. 642:182-95.
51. Romsicki Y and Sharom FJ. (1997) Interaction of P-glycoprotein with defined phospholipid bilayers: a differential scanning calorimetric study. *Biochemistry*. 36:9807-15.
52. Kimelberg HK. (1976) Protein-liposome interactions and their relevance to the structure and function of cell membranes. [Review] *Molecular & Cellular Biochemistry*. 10:171-90.
53. Mayer LD. (1998) Future developments in the selectivity of anticancer agents: drug delivery and molecular target strategies. [Review] *Cancer & Metastasis Reviews*. 17:211-8.
54. Suntres ZE and Shek PN. (1996) The pulmonary uptake of intravenously administered liposomal alpha-tocopherol is augmented in acute lung injury. *Journal of Drug Targeting*. 4:151-9.
55. Bernardes C, Valdeira ML, Ramalho-Santos J and de Lima MC. (1995) Fusion activity of African swine fever virus towards target membranes: lipid dependence and effect of dehydrating agents. *Biochemistry & Molecular Biology International*. 37:481-8.
56. Soni PN, Brown D, Saffie R, Savage K, Moore D, Gregoriadis G and Dusheiko GM. (1998) Biodistribution, stability, and antiviral efficacy of liposome-entrapped phosphorothioate antisense oligodeoxynucleotides in ducks for the treatment of chronic duck hepatitis B virus infection. *Hepatology*. 28:1402-10.
57. Fraley RT, Fornari CS and Kaplan S. (1979) Entrapment of a bacterial plasmid in phospholipid vesicles: potential for gene transfer. *Proceedings of the National Academy of Sciences of the United States of America*. 76:3348-52.
58. Nicolau C and Sene C. (1982) Liposome-mediated DNA transfer in eukaryotic cells. Dependence of the transfer efficiency upon the type of liposomes used and the host cell cycle stage. *Biochimica et Biophysica Acta*. 721:185-90.
59. Rizzo WB, Schulman JD and Mukherjee AB. (1983) Liposome-mediated transfer of simian virus 40 DNA and minichromosome into mammalian cells. *Journal of General Virology*. 64:911-9.

60. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM and Danielsen M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proceedings of the National Academy of Sciences of the United States of America*. 84:7413-7.
61. Gershon H, Ghirlando R, Guttman SB and Minsky A. (1993) Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. *Biochemistry*. 32:7143-51.
62. 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. *Biophysical Journal*. 71:590-9.
63. Song YK, Liu F, Chu S and Liu D. (1997) Characterization of cationic liposome-mediated gene transfer *in vivo* by intravenous administration. *Human Gene Therapy*. 8:1585-94.
64. Reimer DL, Kong S and Bally MB. (1997) Analysis of cationic liposome-mediated interactions of plasmid DNA with murine and human melanoma cells *in vitro*. *Journal of Biological Chemistry*. 272:19480-7.
65. 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. *Science*. 275:810-4.
66. 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 Letters*. 356:361-6.
67. May S and Ben-Shaul A. (1997) DNA-lipid complexes: stability of honeycomb-like and spaghetti-like structures. *Biophysical Journal*. 73:2427-40.
68. Wasan EK, Harvie P, Edwards K, Karlsson G and Bally MB. (1998) A multi-step lipid mixing assay to model structural changes in cationic lipoplexes used for *in vitro* transfection. [Submitted: *Biochimica et Biophysica Acta*.]
69. Zabner J, Fasbender AJ, Moniger T, Poellinger KA and Welsh MJ. (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. *Journal of Biological Chemistry*. 270:18997-19007.
70. Friend DS, Papahadjopoulos D and Debs RJ. (1996) Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. *Biochimica et Biophysica Acta*. 1278:41-50.

71. Miller CR, Bondurant B, McLean SD, McGovern KA and O'Brien DF. (1998) Liposome-cell interactions *in vitro*: effect of liposome surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes. *Biochemistry*. 37:12875-83.
72. Xu Y and Szoka FC Jr. (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry*. 35:5617-23.
73. Farhood H, Serbina N and Huang L. (1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochimica et Biophysica Acta*. 1235:289-95.
74. Tseng WC, Haselton FR and Giorgio TD. (1997) Transfection by cationic liposomes using simultaneous single cell measurements of plasmid delivery and transgene expression. *Journal of Biological Chemistry*. 272:25641-7.
75. 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 Therapy*. 4:226-37.
76. Banerjee S, Livanos E and Vos JM. (1995) Therapeutic gene delivery in human B-lymphoblastoid cells by engineered non-transforming infectious Epstein-Barr virus. *Nature Medicine*. 1:1303-8.
77. Walther W, Wendt J and Stein U. (1997) Employment of the *mdr1* promoter for the chemotherapy-inducible expression of therapeutic genes in cancer gene therapy. *Gene Therapy*. 4:544-52.
78. Nguyen DM, Wiehle SA, Koch PE, Branch C, Yen N, Roth JA and Cristiano RJ. (1997) Delivery of the p53 tumor suppressor gene into lung cancer cells by an adenovirus/DNA complex. *Cancer Gene Therapy*. 4:191-8.
79. Finke S, Trojaneck B, Lefterova P, Csipai M, Wagner E, Kircheis R, Neubauer A, Huhn D, Wittig B and Schmidt-Wolf IG. (1998) Increase of proliferation rate and enhancement of antitumor cytotoxicity of expanded human CD3+ CD56+ immunologic effector cells by receptor-mediated transfection with the interleukin-7 gene. *Gene Therapy*. 5:31-9.
80. Shaw WV. (1967) The enzymatic acetylation of chloramphenicol by extracts of R factor-resistant *Escherichia coli*. *Journal of Biological Chemistry*. 242:687-93.
81. de Wet JR, Wood KV, DeLuca M, Helinski DR and Subramani S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Molecular & Cellular Biology*. 7:725-37.

82. Pardy K. (1994) Reporter enzymes for the study of promoter activity. *Molecular Biotechnology*. 2:23-7.
83. Misteli T and Spector DL. (1997) Applications of the green fluorescent protein in cell biology and biotechnology. [Review] *Nature Biotechnology*. 15:961-4.
84. Foster LC, Arkonac BM, Sibinga NE, Shi C, and Perrella MA and Haber E. (1998) Regulation of CD44 gene expression by the proinflammatory cytokine interleukin-1beta in vascular smooth muscle cells. *Journal of Biological Chemistry*. 273:20341-6.
85. Ledley TS and Ledley FD. (1994) Multicompartment, numerical model of cellular events in the pharmacokinetics of gene therapies. *Human Gene Therapy*. 5:679-91.
86. Kao GY, Change LJ and Allen TM. (1996) Use of targeted cationic liposomes in enhanced DNA delivery to cancer cells. *Cancer Gene Therapy*. 3:250-6.
87. Dachs GU, Dougherty GJ, Stratford IJ and Chaplin DJ. (1997) Targeting gene therapy to cancer: a review. [Review] *Oncology Research*. 9:313-25.
88. Son K and Huang L. (1996) Factors influencing the drug sensitization of human tumor cells for *in situ* lipofection. *Gene Therapy*. 3:630-4.
89. Heo DS, Yoon SJ, Kim WS, Lee KH, Seol JG, Lee SG, Jung CW, Cho EK, Kim CW, Park MH, Sung MW, Kim KH, Bang YJ and Kim NK. (1998) Locoregional response and increased natural killer activity after intratumoral injection of HLA-B7/beta2-microglobulin gene in patients with cancer. *Human Gene Therapy*. 9:2031-8.
90. Hui KM, Ang PT, Huang L and Tay SK. (1997) Phase I study of immunotherapy of cutaneous metastases of human carcinoma using allogeneic and xenogeneic MHC DNA-liposome complexes. *Gene Therapy*. 4:783-90.
91. Berger NA. (1993) Alkylating agents. [Review] *Cancer Chemotherapy & Biological Response Modifiers*. 14:26-36.
92. Kaye SB. (1998) New antimetabolites in cancer chemotherapy and their clinical impact. [Review] *British Journal of Cancer*. 78:1-7.
93. Namer M. (1993) Anthracyclines in the adjuvant treatment of breast cancer. [Review] *Drugs*. 45:4-9.
94. Rahmani R and Zhou XJ. (1993) Pharmacokinetics and metabolism of vinca alkaloids. [Review] *Cancer Surveys*. 17:269-81.

95. Aisner J and Cortes-Funes H. (1997) Paclitaxel in head and neck and other cancers: future prospects. [Review] *Seminars in Oncology*. 24:S2-113-S2-115.
96. Gordon M and Hollander S. (1993) Review of platinum anticancer compounds. [Review] *Journal of Medicine*. 24:209-65.
97. Prosnitz LR, Brizel DM and Light KL. (1997) Radiation techniques for the treatment of Hodgkin's disease with combined modality therapy or radiation alone. [Review] *International Journal of Radiation Oncology, Biology, Physics*. 39:885-95.
98. Stephens FO. (1990) Developments in surgical oncology: induction (neoadjuvant) chemotherapy - the state of the art. [Review] *Clinical Oncology (Royal College of Radiologists)*. 2:168-72.
99. Goldie JH and Coldman AJ. (1984) The genetic origin of drug resistance in neoplasms: implications for systemic therapy. *Cancer Research*. 44:3643-53.
100. Hait WN, Lazo JS, Chen DL, Gallichio VS and Filderman AE. (1988) Antitumor and toxic effects of combination chemotherapy with bleomycin and a phenothiazine anticalmodulin agent. *Journal of the National Cancer Institute*. 80:246-50.
101. Pereira FL, Pedreira CE and de Sousa JB. (1995) A new optimization based approach to experimental combination chemotherapy. *Frontiers of Medical & Biological Engineering*. 6:257-68.
102. Son K and Huang L. (1994) Exposure of human ovarian carcinoma to cisplatin transiently sensitizes the tumor cells for liposome-mediated gene transfer. *Proceedings of the National Academy of Sciences of the United States of America*. 91:12669-72.
103. Cheon J, Ko SC, Gardner TA, Shirakawa T, Gotoh A, Kao C and Chung LW. (1997) Chemogene therapy: osteocalcin promoter-based suicide gene therapy in combination with methotrexate in a murine osteosarcoma model. *Cancer Gene Therapy*. 4:359-65.
104. Chang EH, Jang YJ, Hao Z, Murphy G, Rait A, Fee WE Jr, Sussman HH, Ryan P, Chiang Y and Pirollo KF. (1997) Restoration of the G1 checkpoint and the apoptotic pathway mediated by wild-type p53 sensitizes squamous cell carcinoma of the head and neck to radiotherapy. *Archives of Otolaryngology - Head & Neck Surgery*. 123:507-12.
105. Perez CF and Skarsgard LD. (1986) Radiation enhancement of the efficiency of DNA-mediated gene transfer in CHO UV-sensitive mutants. *Radiation Research*. 106:401-7.

106. Rosenberg B. (1985) Fundamental studies with cisplatin [Review] *Cancer*. 55:2303-16.
107. Zamble DB and Lippard SJ. (1995) Cisplatin and DNA repair in cancer chemotherapy. [Review] *Trends in Biochemical Sciences*. 20:435-9.
108. Pillaire MJ, Hoffmann JS, Defais M and Villani G. (1995) Replication of DNA containing cisplatin lesions and its mutagenic consequences. [Review] *Biochimie*. 77:803-7.
109. Ormerod MG, O'Neill C, Robertson D, Kelland LR and Harrap KR. (1996) cis-Diamminedichloroplatinum(II)-induced cell death through apoptosis in sensitive and resistant human ovarian carcinoma cell lines. *Cancer Chemotherapy & Pharmacology*. 37:463-71.
110. Kasahara K, Fujiwara Y, Nishio K, Ohmori T, Sugimoto Y, Komiya K, Matsuda T and Saijo N. (1991) Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. *Cancer Research*. 51:3237-42.
111. Mutoh M, Sumizawa T, Furukawa T, Haraguchi M, Tani A, Saijo N, Kondo T and Akiyama S. (1998) An active efflux system for heavy metals in cisplatin-resistant human KB carcinoma cells. *Experimental Cell Research*. 240:312-20.
112. Shishodia S, Sodhi A and Shrivastava A. (1998) Involvement of Ras and MAP kinase (ERK-1) in cisplatin-induced activation of murine bone marrow-derived macrophages. *Biochemistry & Molecular Biology International*. 45:527-34.
113. Speth PA, van Hoesel QG and Haanen C. (1988) Clinical pharmacokinetics of doxorubicin. [Review] *Clinical Pharmacokinetics*. 15(1):15-31.
114. Osheroff N, Corbett AH and Robinson MJ. (1994) Mechanism of action of topoisomerase II-targeted antineoplastic drugs. [Review] *Advances in Pharmacology (New York)*. 29:105-26.
115. Piccinini F, Monti E, Paracchini L and Perletti G. (1990) Are oxygen radicals responsible for the acute cardiotoxicity of doxorubicin? *Advances in Experimental Medicine & Biology*. 264:349-52.
116. Rowley DA and Halliwell B. (1983) DNA damage by superoxide-generating systems in relation to the mechanism of action of the anti-tumour antibiotic adriamycin. *Biochimica et Biophysica Acta*. 761:86-93.
117. Owellen RJ, Hartke CA, Dickerson RM and Hains FO. (1976) Inhibition of tubulin-microtubule polymerization by drugs of the Vinca alkaloid class. *Cancer Research*. 36:1499-502.

118. Wilke M, Fortunati E, van den Broek M, Hoogeveen AT and Scholte BJ. (1996) Efficacy of a peptide-based gene delivery system depends on mitotic activity. *Gene Therapy*. 3:1133-42.
119. Vloon WJ, Kruk C, Pandit UK, Hofs HP and McVie JG. (1987) Synthesis and biological properties of side-chain-modified bleomycins. *Journal of Medicinal Chemistry*. 30:20-4.
120. Twentyman PR. (1983) Bleomycin - mode of action with particular reference to the cell cycle. [Review] *Pharmacology & Therapeutics*. 23:417-41.
121. Nakayama C, Adachi N and Koyama H. (1998) Bleomycin enhances random integration of transfected DNA into a human genome. *Mutation Research*. 409:1-10.
122. Hope MJ, Bally MB, Webb G and Cullis PR. (1985) Production of large unilamellar vesicles by rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochimica et Biophysica Acta*. 812:55-65.
123. Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 65:55-63.
124. Lane RD, Federman D, Flora JL and Beck BL. (1986) Computer-assisted determination of protein concentrations from dye-binding and bicinchoninic acid protein assays performed in microtiter plates. *Journal of Immunological Methods*. 92:261-70.
125. Reimer DL, Kong S, Monck M, Wyles J, Tam P, Wasan EK and Bally MB. (1998) Liposomal lipid and plasmid DNA delivery to B16/BL6 tumors following intraperitoneal administration of cationic liposome DNA aggregates. [In Press: *Journal of Pharmacology and Experimental Therapeutics*].
126. Bally MB, Nayar R, Masin D, Cullis PR and Mayer LD. (1990) Studies on the myelosuppressive activity of doxorubicin entrapped in liposomes. *Cancer Chemotherapy & Pharmacology*. 27:13-9.
127. McPherson JP, Deffie AM, Jones NR, Brown GA, Deuchars KL and Goldenberg GJ. (1997) Selective sensitization of adriamycin-resistant P388 murine leukemia cells to antineoplastic agents following transfection with human DNA topoisomerase II alpha. *Anticancer Research*. 17:4243-52.
128. Jain PT and Gewirtz DA. (1999) Sustained enhancement of liposome-mediated gene delivery and gene expression in human breast tumour cells. *International Journal of Radiation Biology*. 75:217-23.

129. Whitaker SJ. (1992) DNA damage by drugs and radiation: what is important and how is it measured? [Review] *European Journal of Cancer*. 28:273-6.
130. Kubota M. (1991) Generation of DNA damage by anti-neoplastic agents. [Review] *Anti-Cancer Drugs*. 2:531-41.
131. Link CJ Jr, Burt RK and Bohr VA. (1991) Gene-specific repair of DNA damage induced by UV irradiation and cancer chemotherapeutics. [Review] *Cancer Cells*. 3:427-36.
132. Stevens CW, Zeng M and Cerniglia GJ. (1996) Ionizing radiation greatly improves gene transfer efficiency in mammalian cells. *Human Gene Therapy*. 7:1727-34.
133. Mortimer I, Tam P, MacLachlan I, Graham RW, Saravolac EG and Joshi PB. (1999) Cationic lipid mediated transfection of cells in culture requires mitotic activity. [In Press, *Gene Therapy*].
134. Klein HO, Toermer HJ, Christian E, Coerper C, Lennartz KJ and Akokan G. (1980) Experimental investigations on a sequential combination chemotherapy protocol. *Journal of Cancer Research & Clinical Oncology*. 96:65-78.
135. Ohmori T and Arteaga CL. (1998) Protein kinase C epsilon translocation and phosphorylation by cis-diamminedichloroplatinum(II) (CDDP): potential role in CDDP-mediated cytotoxicity. *Cell Growth & Differentiation*. 9:345-53.
136. Iacopetta B, Carpentier JL, Pozzan T, Lew DP, Gorden P and Orci L. (1986) Role of intracellular calcium and protein kinase C in the endocytosis of transferrin and insulin by HL60 cells. *Journal of Cell Biology*. 103:851-6.
137. Potapova O, Haghghi A, Bost F, Liu C, Birrer MJ, Gjerset R and Mercola D. (1997) The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. *Journal of Biological Chemistry*. 272:14041-4.
138. Szilvassy SJ, Fraser CC, Eaves CJ, Lansdorp PM, Eaves AC and Humphries RK. (1989) Retrovirus-mediated gene transfer to purified hemopoietic stem cells with long-term lympho-myelopoietic repopulating ability. *Proceedings of the National Academy of Sciences of the United States of America*. 86:8798-802.