

# The challenge of liposomes in gene therapy

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## Summary

Recently, liposomes have gained a special interest as gene delivery systems: over 30 human clinical trials for gene delivery using cationic liposomes have been approved; all these delivery methods use intratumoral, subcutaneous and other local delivery but not systemic delivery due to the toxicity of cationic lipids. Stealth liposomes (coated with polyethyleneglycol to camouflage the liposome and evade detection by the immune system) have a remarkable longevity in body fluids, have negligible toxicity with respect to their lipid components, reduce the toxicity of the encapsulated drug, and can deliver efficiently their doxorubicin payload (DOXIL) or cis-platin to tumor lesions. The mechanism of stealth liposome accumulation in tumors involves their extravasation through gaps in the endothelium of tumor vessels. DOXIL can sustain a much higher concentration of Doxorubicin in tumor tissue compared to free drug administration at comparable doses. Liposomes tagged with folate-PEG or with antibodies can target specific tissues. We propose that "stealth" liposomes, could find future applications to systemically deliver plasmid DNA with therapeutic genes (*p53*, *HSV-tk*, *angiostatin*) to primary tumors and their metastases leading to complete cancer eradication.

### Abbreviations:

AUC, area-under-the-plasma concentration vs time curve

CHOL, cholesterol

CL, cardiolipin

DDAB, dimethyldioctadecylammonium bromide

DOGS, dioctadecylamidoglycylspermine

DOPE, dioleoyl phosphatidylethanolamine

DOSPA, (2,3-dioleoyloxy-N-[20(2,5-bis(3-aminopropyl)amino)-1-oxypentyl]amino)ethyl]-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-1-propanaminium trifluoroacetate

DOTMA or lipofectin, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride

DOX, doxorubicin

DOXIL, "stealth" liposomes loaded with doxorubicin

DSPC, distearoyl phosphatidyl choline

DXR, doxorubicin

EPC, egg phosphatidyl choline

EPG, egg phosphatidyl glycerol

HSPE, hydrogenated soy phosphatidyl ethanolamine

MPS, mononuclear phagocyte system,

PC, phosphatidyl choline

PEG, polyethyleneglycol

SM, sphingomyelin

## I. Introduction

Liposome-mediated drug delivery has clear advantages compared to administration of free drugs: (i) Because of the slow releasing of drugs, encapsulated into the lumen of their lipid bilayer, into the blood stream of animals including humans. Soon after Alec Bangham and his colleagues first described liposomes in the mid 1960s as closed vesicular structures able to envelop water soluble molecules, pharmacologists recognized their potential value in drug delivery (Bangham and Horne, 1964); the rationale was simple: use liposomes as a safe vehicle for delivering drugs more specifically to sites of disease while limiting exposure of normal tissues. (ii) Because of the minimization of allergic and other untoward reactions caused by drugs and proteins after their encapsulation in

liposomes (Gregoriadis and Neerunjun, 1975). Cytotoxic anti-tumor agents were of particular interest as these in general have a narrow therapeutic window, i.e., dose-limiting side effects limit their therapeutic utility. (iii) Because the liposome uptake by cells warrants entrance of chemicals and other molecules into otherwise inaccessible cells (Segal et al, 1974). (iv) Because of the tendency of "stealth" liposomes to preferentially accumulate in tumor tissues (Gabizon and Papahadjopoulos, 1988; Gabizon et al, 1994); during tumor growth and vascularization of the cell mass blood vessels are formed from epithelial cells which protrude inside the tumor cell mass at sites digested with collagenases; the new blood vessels need a maturation time to attain the vein/artery-type of wall; during this time the vessel wall can be penetrated by liposomes. (v) Because of the possibility of adding various

substances on their surface to target particular cell types. Liposomes tagged with folate-PEG (Lee and Low, 1995) or with antibodies (Straubinger et al, 1988; Ahmad et al, 1992, 1993) are promising vehicles for drug and gene delivery.

Liposomes can be prepared by various methods including (i) reverse phase evaporation, (ii) dehydration-rehydration, (iii) detergent dialysis, and (iv) thin film hydration followed by sonication or repeated extrusions through membranes of 400 down to 50-nm diameter pores.

Liposomes have found wide applications. As an exotic example, liposomes are also being used for the delivery of water-soluble antibiotics and of the chemotherapeutics trimethoprim and sulfamethoxazole to the larvae of aquatic animals, such as to nauplii of *Artemia franciscana*, which are uptaken and concentrated into larvae tissues; these larvae are the main food source of marine fish larvae delivering the drugs to treat infectious diseases in fish cultures (Touraki et al, 1995, 1996).

Liposomes can be divided into two major classes: nearly neutral or "true" liposomes and cationic liposomes or complexes of cationic lipids with plasmid DNA. Cationic liposomes, because of their instant interaction with the strongly anionic DNA, have been used widely in gene delivery. The field of drug and gene delivery using liposomes has been reviewed by Lasic and Martin, 1995; Lasic and Papahadjopoulos, 1995; Ledley, 1995; Boulikas, 1996a, 1998a; Martin, 1997.

There is no limit on the size of DNA to be delivered to cells with liposomes compared with the upper limit of 7.5 kb that can be accommodated into viral/retroviral vectors because of packaging limitations.

Several key steps can be conceptualized for effective gene transfer to somatic cells using liposomes: (i) choice of type of lipid, size of liposome, and type of complex with plasmid DNA which will determine the time for its clearance from body fluids, biodistribution in tissues, and efficacy of delivery; (ii) interaction of the gene-lipid complex with components in the serum or body fluids (plasma proteins, macrophages, immune response cells); (iii) targeting to the cell type, organ, tumor, and binding to the cell surface; (iv) mode of entrance to the cell (poration through the cell membrane, receptor-mediated endocytosis); (v) release from cytoplasmic compartments (endosomes, lysosomes) and release of the plasmid DNA from its lipid complex. The remaining of the steps (nuclear import, maintenance of the plasmid as an extrachromosomal element or integration into the chromosomes of the cell, transcription, splicing and processing of the transcript to mature mRNA, export to the cytoplasm, translation onto polyribosomes into protein, posttranslational modification of the protein, and in some cases, addition of a signal peptide for export of the protein outside of the cell) would depend on the type of DNA

control elements added to the therapeutic gene and not on the liposome. All steps can be experimentally manipulated and improvements in each one of them can enormously enhance the level of expression and therapeutic index of a gene therapy approach.

In this article we shall elaborate on the use of stealth liposomes for the delivery of the antineoplastic drug Adriamycin (also called Doxorubicin) to tumors. We will then review the use of cationic liposomes in gene delivery, the ongoing Clinical trials using cationic lipids, and speculate on possible future applications of stealth liposome technology on systemic delivery of plasmid DNA with therapeutic genes for the treatment of primary tumors and their metastases.

## II. Drug delivery with conventional liposomes

### A. Doxorubicin as an antineoplastic drug

Doxorubicin (DOX, DXR) is one of the most widely used anticancer drugs with the broadest spectrum of antitumor effects. For example, DOX combined with 5-fluorouracil has been used for the treatment of D3 stage of prostate cancer causing 50% reduction in PSA in 11 out of 18 patients. However, this treatment, like other cytotoxic chemotherapies (e.g. cyclophosphamide plus granulocyte-macrophage colony stimulating factor supplementation), either alone or in combination with endocrine therapy, have shown only marginal survival benefits; hormone refractory prostate cancer is resistant to cytotoxic agents likely via a mechanism involving overexpression of the *MDR1* gene by prostate cancer cells in the advanced stage of the disease (reviewed by Hsieh and Simons, 1993).

DOX has also been linked to monoclonal antibodies or proteins in order to reduce its toxicity. The chemistry includes ester bond formation and C-N linkages between 14-bromodaunorubicin and proteins or poly-L-amino acids, and the use of enzyme-sensitive or acid sensitive spacer arms (for references see Nagy et al, 1996).

For drug targeting to specific cell types, the 2-pyrrolino-doxorubicin, a derivative 500-1000 times more potent than doxorubicin, was coupled to agonistic and antagonistic analogs of luteinizing hormone-releasing hormone (LH-RH); this coupling preserved the binding capacity for rat pituitary LH-RH receptors; the highly cytotoxic 2-pyrrolino-DOX/LH-RH analogs could constitute anticancer drugs for various tumors expressing LH-RH receptors (Nagy et al, 1996).

However the clinical applications of DOX are limited because of its gastrointestinal and cardiac toxicity, suppression to bone marrow cells, and other side effects.

## **B. Encapsulation of antineoplastic drugs into liposomes reduces toxicity**

Among the dozens of liposome-encapsulated anti-tumor agents studied in animal models, the anthracycline antibiotics, in particular doxorubicin and daunorubicin, emerged as benefiting substantially from liposome encapsulation (Gabizon et al., 1990). Animals were able to tolerate greater doses of a variety of formulations of liposome-encapsulated doxorubicin compared with the free drug and antitumor activity was, in general, maintained.

Clinical trials and animal studies, or studies with cells in culture using liposomes as carriers of DOX show a reduction of complications and side effects, enhanced antitumor activity, and improved therapeutic index (Mayer et al, 1989). These advantages are thought to arise from a sustained release of the liposomal drug into the blood stream (Bally et al, 1990).

## **C. Interaction of liposomes with the mononuclear phagocyte system (MPS)**

Liposomes are rapidly removed from blood by elements of the MPS, fixed macrophages residing in liver, spleen, lung and bone marrow. It is believed that binding of plasma proteins (lipoproteins, immunoglobulins, complement) to the liposome surface triggers such rapid macrophage uptake (Lasic et al., 1991).

Despite the lack of true targeting, internalization of liposome-encapsulated anthracyclines by MPS cells was found to diminish exposure of certain tissues to the toxic effects of such drugs. For example, doxorubicin-related nausea/vomiting and cardiomyopathy are believed to be related to the drug's peak levels in plasma. By using liposome encapsulation to sequester the majority of an injected dose in the MPS, in theory, initial plasma levels of free drug are attenuated and safety improved. The drug is eventually released from MPS organs and distributes to peripheral tissues in free (i.e., unencapsulated) form. In this case, the pharmacokinetic pattern would be intended to mimic that of doxorubicin administered as a prolonged infusion, a regimen known to reduce drug-related side effects (Bielack et al., 1989).

Indeed, it has been shown that administration of liposome-encapsulated doxorubicin reduces the drug's acute and chronic toxicities in preclinical animal models. Moreover, results from animal models indicate that

doxorubicin delivered in this fashion retains its activity against systemic tumors (Olsen et al., 1982). The pharmacokinetics and safety of various clinical formulations of liposomal doxorubicin have been reported in the scientific literature (Kumai et al., 1985; Sells, et al., 1987; Delgado et al., 1989; Cowens et al., 1989, 1990, 1993; Rahman et al., 1990; Treat et al., 1990; Creaven et al., 1990; Gabizon et al., 1990, 1991, 1992; Akamo et al., 1991; Owen et al., 1992; Batist et al., 1992; Mazanet et al., 1993; Conley et al., 1993; Embree et al., 1993). Clinical pharmacokinetic measurements confirm that conventional liposome formulations are cleared rapidly from plasma. These data also suggest that a considerable amount of encapsulated doxorubicin is released into plasma prior to MPS uptake (Gabizon et al., 1991; Conley et al., 1993).

## **D. The liposome-anthracycline family tree**

Armed with the knowledge that MPS uptake can provide favorable safety advantages for encapsulated doxorubicin, formulation scientists began to optimize liposome carriers for this purpose. As shown in **Figure 1**, the first major branch of the liposome anthracycline family tree was represented by these "MPS Targeted" formulations. Two alternative formulation approaches (sub-branches) soon emerged. The first, relied upon acidic lipids incorporated into the liposome bilayer (such as cardiolipin (CL) and egg phosphatidyl glycerol (EPG) to bind doxorubicin (which is positively charged at physiological pH) to the membrane itself (Gabizon et al., 1992; Rahman et al., 1990). Formation of such "ion-pairs" between the drug and an acidic membrane component provided strong association and robust formulations that were stable in vitro and that could be freeze dried for long-term storage.

The second approach, represented by TLC D-99, used true encapsulation of doxorubicin into the aqueous compartment of the liposome and employed a clever technique to circumvent the problem of leakage (Cowens et al., 1993). In this case doxorubicin is loaded into the liposomes immediately prior to administration (in a hospital pharmacy) by adding an aqueous solution of doxorubicin (at neutral pH 7.0) to liposomes containing a low pH internal buffer (pH 4.0). The pH gradient thus formed across the liposome membrane leads to mobilization of doxorubicin to the liposomes. Once inside, the low pH environment traps the drug preventing it from leaking out (as long as the pH gradient is maintained).

**Figure 1:** Family Tree illustrating the relationship between formulation strategy and the development of liposomal anthracycline products.

The ion pair formulations have been tested clinically but have not progressed beyond phase 1-2 studies. TLC-D99 is in advanced phase 3 trials in metastatic breast cancer.

Recognizing that MPS uptake represented the main obstacle to targeting, another branch of liposomes developed were liposomes that resist binding/interaction with plasma proteins (opsonization) with a view toward prolonging liposome blood residence times and targeting potential. Early work suggested that a modest degree of “MPS avoiding” activity could be obtained by formulations composed of high phase transition lipids and cholesterol. Size was also a critical parameter, the smaller the liposome the longer it circulated: 300-nm in diameter liposomes are cleared from the blood approximately three times faster than small 100-nm liposomes (Huang et al, 1992).

This “pure lipid” subbranch arrived at two formulations of small diameter (~50nm), one composed of DSPC/cholesterol (Presant et al, 1990) and the other of sphingomyelin/cholesterol (Webb et al, 1995) both of which showed relatively slow MPS clearance. DaunoXome, a DSPC/cholesterol formulation of daunorubicin, is the only product to emerge from this pure lipid approach. DaunoXome is approved in the US and Europe for the treatment of AIDS-related Kaposi’s sarcoma (see below).

### **III. “Stealth” liposomes**

#### **A. Polyethylene glycol (PEG)-coated liposomes circulate for long periods in body fluids**

Coating the surface of liposomes with inert materials designed to camouflage the liposome from the body’s host defense systems was shown to increase remarkably the plasma longevity of liposomes. The biological paradigm for this “surface modified” subbranch was the erythrocyte, a cell which is coated with a dense layer of carbohydrate groups, and which manages to evade immune system detection and to circulate for several months (before being removed by the same type of cell responsible for removing liposomes).

The first breakthrough came in 1987 when a glycolipid (the brain tissue-derived ganglioside GM<sub>1</sub>) was identified which, when incorporated within the lipid matrix, allowed liposomes to circulate for many hours in the blood stream (Allen and Chonn, 1987). A second glycolipid, phosphatidylinositol, was also found to impart long plasma residence times to liposomes and, since it was extracted from soy beans, not brain tissue, was believed to be a more pharmaceutically acceptable excipient (Gabizon et al, 1989).

A major advance in the surface-modified subbranch was the development of polymer-coated liposomes (Allen et al, 1991). Polyethylene glycol (PEG) modification had been used for many years to prolong the half-lives of biological proteins (such as enzymes and growth factors) and to reduce their immunogenicity (e.g. Beauchamp et al, 1983). It was reported in the early 1990s that PEG-coated liposomes circulated for remarkably long times after intravenous administration. Half-lives in the order of 24 h were seen in mice and rats and over 30 hours in dogs. The term “stealth” was applied to these liposomes because of their ability of evade interception by the immune system (in much the same way as the stealth bomber was able to evade radar) (Gabizon and Papahadjopoulos, 1988;

Klibanov et al, 1990; Papahadjopoulos et al, 1991; Senior et al, 1991; Huang et al, 1994). The increased hydrophilicity of the liposomes after their coating with the amphipathic PEG5000 leads to a reduction in nonspecific uptake by the reticuloendothelial system.

Whereas the half-life of antimyosin immunoliposomes was 40 min, their coating with PEG increased their half-life to 1000 min after intravenous injection to rabbits (Torchilin et al, 1992).

### **B. Mechanism of loading of doxorubicin into “stealth” liposomes**

DOXIL, the PEG-coated liposomes packed with doxorubicin, (also called CAELYX in Europe) is the first product to emerge from the surface-modified liposome subbranch. It, too, is approved in the US and Europe for treatment of Kaposi's sarcoma.

The mechanism of doxorubicin loading into liposomes is explained in **Figure 2**. Liposomes composed of HSPC:CHOL (1:1) and 5% PEG-DSPE of a diameter of 85 nm are prepared in high ammonium sulfate; these are then brought into a solution of high concentration of Doxorubicin in ammonium chloride at a higher pH.

Loading is mediated via exchange of ammonia molecules with uncharged doxorubicin; ammonia molecules pass from the inside of the liposomes to the outside, whereas doxorubicin enters liposomes. Loading is driven by the gradient of a chemical potential across the membrane of the liposome, the efflux of ammonia to a larger external volume, the precipitation of doxorubicin inside the liposome, and the pH gradient.

The reactions in the outside volume between liposomes involve removal of a proton from the doxorubicin-ammonium chloride complex and its binding to ammonia converting it into ammonium ions; the neutral doxorubicin-ammonia complex crosses the liposome bilayer. The reactions inside the liposome involve protonation of the doxorubicin-ammonia complex from a hydrogen ion removed from the ammonium ion and its precipitation as doxorubicin sulfate (Lasic, 1995).

High resolution cryo-electron microscopy has shown a precipitate of Doxorubicin molecules with sulfate ions inside liposomes into fibrillar colloidal complexes which align into bundles (**Figure 3**). The structure has been confirmed by small angle X-ray scattering where the periodicity of 2.7 nm observed was thought to represent the thickness of the gel fibers (Lasic et al, 1992).

**Figure 2.** The mechanism of loading of DOX into liposomes.

**Figure 3.** The coffee bean appearance of the precipitated DOX sulfate within liposomes. Courtesy of Dan Lasic.

**Figure 4.** Cut-away view of a DOXIL particle.

**Figure 4** shows schematically a cut-away view of a DOXIL particle.

DOX encapsulated into PEG-coated liposomes is cleared 450 times slower than free DOX (Gabizon et al, 1994). For example, uptake of DOX encapsulated into liposomes coated with PEG-folate was found to be uptaken by KB cells in culture, which express high levels of the folate receptor, at a rate 45-fold higher than liposomal DOX although at a rate only 1.6 times higher than that of free DOX (Lee and Low, 1995). The enhanced release of DOX from folate-PEG-liposomes internalized into the acidic endosomal organelles known as caveolae seemed to be responsible for the increased cytotoxicity of DOX to tumor cells (Lee and Low, 1995).

### **C. Preclinical antitumor activity of DOXIL**

The efficacy of DOXIL has been evaluated in a variety of different tumor models, including several human xenograft models (Papahadjopoulos et al, 1991; Huang et al, 1992; Vaage et al, 1992, 1993, 1994; Williams et al, 1993; Siegal et al, 1995; Amantea et al, 1997). In every model examined DOXIL was more effective than the same doses of doxorubicin **(i)** at inhibiting or halting tumor growth, **(ii)** at effecting cures and/or **(iii)** at prolonging survival times of tumor-bearing animals. Most often, all three endpoints were improved by DOXIL, and in no case was DOXIL less effective than doxorubicin. DOXIL was more active in both solid and dispersed tumors, and was more effective than doxorubicin in preventing spontaneous metastases from intramammary implants of two different mammary tumors in mice. These findings are also supported by studies done with DOXIL in several murine tumor models and human xenograft models (**Figure 5**).

**Figure 5:** Growth kinetics of human lung cancer xenografts (**A**) and human prostate cancer (PC-1) xenografts (**B**) implanted in SCID mice. Groups of 10 animals were engrafted in the flank with  $2 \times 10^6$  TL-1 cells at day 0 and treated weekly (via tail vein injection) for 10 weeks starting one week post engraftment with saline (circles), doxorubicin (also called Adriamycin) (3 mg/kg, diamonds) or DOXIL (3 mg/kg, squares). Adapted from Siegal et al, 1995.

**Figure 6.** Electron micrographs showing colloidal gold (arrows) in the intracellular vesicles of a typical mononuclear phagocyte. The particles are often seen within the endosomes (lower insert)

and secondary within lysosomes (upper insert) of macrophages at the border of the liver-implanted tumor.

In general, the efficacy of doxorubicin in these models was limited by its toxicity at high doses. Typically, DOXIL could be used at a higher dose, offering an increased therapeutic advantage. Pharmacokinetic and tissue distribution studies suggest that the greater persistence, particularly in tumor tissue, achieved with DOXIL compared to conventional doxorubicin also contributes a therapeutic advantage. The efficacy of DOXIL compared to that of conventional liposomal (non-Stealth) doxorubicin indicated that DOXIL was significantly more effective than conventional liposomal doxorubicin, demonstrating the impact of the long-circulating Stealth liposome. Based on the results of these nonclinical studies, DOXIL appears to be an effective agent for the treatment of both solid and dispersed tumors.

Tissue distribution of sterically-stabilized liposomes was studied by Huang et al, (1992). Following tail vein injection the microscopic localization of liposomes labeled with encapsulated colloidal gold was found predominantly in Kupffer cells (macrophages) of the liver (not in hepatocytes) (**Figure 6**) and within macrophages of the bone marrow. Electron microscopy showed the presence of gold in endosomes and lysosomes of fixed sinusoidal lining macrophages in the liver.

#### **D. Combinations of DOXIL with other anticancer drugs are effective anticancer regimens in preclinical studies**

Humanized monoclonal antibodies directed against receptors overexpressed on malignant cells such as HER2 or EGFR have been used in the treatment of malignancies but are not active on their own (Chrysogelos et al, 1994; Baselga et al, 1996). **Figure 7** shows that the combination of such anticancer regimens with DOXIL results in a synergistic antitumor activity: a combination of the DOXIL with the C225 EGFR antibody had a spectacular

effect in inhibiting growth of human breast cancer cells in nude mice.

## **IV. Clinical trials using DOXIL**

### **A. Pharmacokinetics of DOXIL in human patients**

Population pharmacokinetic analysis has been conducted on a group of 83 patients receiving DOXIL at doses ranging from 10 to 60 mg/m<sup>2</sup> (17 females, 66 males) (Gabizon et al, 1994; Northfelt et al, 1996). At doses ranging from 10 to 40 mg/m<sup>2</sup>, DOXIL pharmacokinetics were linear. At dosages above 40 mg/m<sup>2</sup>, DOXIL displayed nonlinear pharmacokinetics as evidenced by a disproportionate increase in the area-under-the-plasma concentration versus time curve (AUC) with increasing dose amounts. In general, drugs that display nonlinear pharmacokinetics have a potential to accumulate to toxic levels in the plasma if not monitored regularly (e.g., phenytoin). In the case of DOXIL, this is not a concern since the drug is administered a minimum of every three weeks, after which time no drug is detectable in the plasma of patients. **Table 1** lists the statistics of selected pharmacokinetic parameters for all 83 patients. There was no evidence of accumulation at dose intervals of  $\geq 3$  weeks.

Utilizing the fitted pharmacokinetic parameter results from this analysis, simulated plasma concentration versus time profiles of DOXIL were generated at doses of 10 – 60 mg/m<sup>2</sup> (**Figure 8**). The nonlinearity of DOXIL pharmacokinetics at higher doses is most evident at doses greater than 40 mg/m<sup>2</sup>.

No correlations were observed between pharmacokinetic parameters and age, weight, body surface area, tumor type, sex, and renal (as determined by serum

creatinine) and hepatic function (as determined by total bilirubin levels).

DOXIL has also been used as single-agent therapy for advanced breast cancer among elderly patients in a phase clinical trial (Ranson et al, 1997) and as primary therapy for refractory ovarian cancer also in a phase II study

**Figure 7.** Growth kinetics of human xenograft of A431 breast tumor implanted subcutaneously in nude mice. Groups of animals were treated via tail vein injection as indicated in the figure. C225 is a monoclonal antibody against epidermal growth factor receptor EGFR)

**Table 1: DOXIL pharmacokinetic parameter estimates n = 83**

Statistic	V <sub>ss</sub> (L/m <sup>2</sup> )	CL <sub>i</sub> (L/h/m <sup>2</sup> )	K <sub>m</sub> (mg/L)	AUC <sub>50</sub> (mg/L · h)
Mean	3.40	0.108	2.01	3260
CV%	18.2	54.2	66.3	54.8
Median	3.42	0.0950	1.85	3018
Minimum	2.20	0.0269	0.428	535
Maximum	5.67	0.393	8.84	9520

V<sub>ss</sub> – volume of distribution at steady-state, CL<sub>i</sub> – intrinsic clearance, K<sub>m</sub> – Michaelis Menton Constant, AUC<sub>50</sub> – area-under-the-curve normalized for a 50 mg/m<sup>2</sup> dose of DOXIL

(Muggia et al, 1997). While generally manageable in both type of trials, epithelial cell toxicity manifesting itself as palmar-plantar erythrodysesthesia (PPE, hand-foot syndrome) may limit the amount of DOXIL patients are able to tolerate.

### **B. Amount of non-liposomal Doxorubicin in plasma**

Several lines of evidence support the conclusion that the majority of the doxorubicin (between 93% and 99%) in plasma is encapsulated within the liposome after i.v. administration of DOXIL. The most convincing come

from work by Gabizon et al who conducted a pilot pharmacokinetic of DOXIL (Druckmann et al, 1989). In this study the fraction of the liposome-encapsulated and free, non-liposomal drug in circulation after DOXIL administration was quantitated directly using a Dowex column separation method that is able to accurately and reproducibly quantitate ≥ 7% free drug in the plasma (Speth et al, 1988). Using this method, essentially all the doxorubicin measured in plasma was liposome-associated (**Figure 9**). These findings suggest that at least 90 to 95% of the doxorubicin measured in plasma, and possibly more, is liposome-encapsulated.

**Figure 8:** Simulated plasma clearance kinetics of doxorubicin after a single 30 minute infusion of DOXIL at doses ranging from 10 to 60 mg/m<sup>2</sup>.

**Figure 9:** Clearance over a one week period of total vs. encapsulated doxorubicin after a single 50 mg/m<sup>2</sup> dose of DOXIL in cancer patients. Data points represent mean values  $\pm$  standard deviation for 14 patients in the DOXIL group and 4 patients in the doxorubicin group (adapted from Druckmann et al, 1989). The method described in Speth et al, 1988 was used to separate the encapsulated from released drug fractions.

The amount of doxorubicin that remains liposome-associated while circulating in plasma is an important point that deserves further emphasis from a safety perspective. Acute adverse reactions associated with doxorubicin administration including nausea and vomiting and chronic cardiotoxicity are believed to be directly related to peak concentrations of the drug in plasma. As pointed out above, while in the circulation, DOXIL liposomes remain intact, retaining virtually all of the doxorubicin in encapsulated form (Speth et al, 1987a). Although total plasma levels of doxorubicin may be relatively high for several days after DOXIL administration, the majority of the dose is sequestered within the liposome during this period and thus is not bioavailable to distribute (as free drug molecules) to

tissues, including the GI tract and myocardium. With respect to level of available drug in plasma, DOXIL resembles more that of a 96 hour continuous infusion of doxorubicin than the usual 30 minute infusion. Prolonged infusion of doxorubicin is known to reduce cardiotoxicity and GI irritation.

### **C. Comparison of pharmacokinetic parameters: DOXIL vs. Doxorubicin**

According to literature reports, an i.v. bolus injection of doxorubicin in humans produces high plasma concentrations of doxorubicin that decline quickly due to rapid and extensive distribution into tissues (Greene et al, 1982). Apparent volumes of distribution range from 1400

to 3000 L, reflective of the drug's extensive tissue distribution. The doxorubicin plasma concentration-time curve in humans is biphasic, with a distribution half-life of 5 to 10 minutes and terminal phase elimination half-life of 30 hours (Benjamin et al, 1984; Speth et al, 1987a, b). A triphasic curve has also been described with a terminal plasma half-life of approximately 30 hours (Benjamin et al, 1977). Clearance of doxorubicin after doxorubicin administration ranges from 24 to 73 L/hour (Greene et al, 1982). No accumulation in plasma occurs after repeated injections (Benjamin et al, 1984; Speth et al, 1987a, b).

The pharmacokinetics of DOXIL are significantly different from those reported for doxorubicin. Administration of DOXIL results in a significantly higher doxorubicin area-under-the-plasma concentration vs time curve (AUC), lower rate of clearance (approximately 0.1 L/hour) and smaller volume of distribution (5 to 7 L) relative to administration of doxorubicin (**Figure 10**). The first phase of the biexponential plasma concentration-time curve after DOXIL administration is relatively short (approximately 5 hours), and the second phase, which represents the majority of the AUC, is prolonged (half-life 50 to 55 hours).

**Figure 10.** DOXIL can generate over 100 times higher concentration of drug in plasma than Adriamycin after single doses of 50 mg/m<sup>2</sup> (body surface area) to patients.

Doxorubicin C<sub>max</sub> after DOXIL administration is 15- to 40-fold higher than after the same dose of doxorubicin, and the ratio quickly increases as doxorubicin is rapidly cleared from circulation. Importantly, the vast majority of the total plasma doxorubicin remains liposome-encapsulated after DOXIL treatment. Because of the high percentage of liposome encapsulation in DOXIL, the amount of free (i.e., "bioavailable") drug in the plasma appears to be significantly lower than that measured after administration of an equal dose of doxorubicin.

This conclusion is supported by the same type of calculations presented above, which derive the apparent concentration of free doxorubicin based on the reported relationship between doxorubicinol and doxorubicin concentrations in plasma. For example, five minutes after

the end of the infusion, the mean doxorubicinol level following a 20 mg/m<sup>2</sup> dose of DOXIL was approximately 22 ng/mL. Using the doxorubicinol:doxorubicin concentration ratio reported in the literature, as described above, predicted free doxorubicin concentration at this time point would be 54 ng/mL in DOXIL-treated patients (the total plasma concentration measured at this time point was 8863 ng/mL). Comparatively, patients in the Northfelt et al study (1996) who received a dose of doxorubicin 20 mg/m<sup>2</sup>, had initial plasma concentrations of doxorubicin of approximately 500 ng/mL.

Studies on human cancer xenografts in nude mice demonstrate an increased accumulation of Doxorubicin at the lesion after DOXIL treatment compared to administration of free Adriamycin (**Figures 11, 12**).

**Figure 11.** Increased accumulation of Doxorubicin in prostate cancer xenografts (**A**) and pancreatic carcinoma xenografts (**B**) after DOXIL treatment compared to administration of free Adriamycin.

**Figure 12.** Increased accumulation of Doxorubicin in C26 tumors in mice after DOXIL treatment compared to administration of free Adriamycin at different time intervals.

### D. Doxorubicin levels in KS lesions

Biopsies of KS lesion tissue and adjacent normal skin were obtained in 22 patients (**Table 2**) (Amantea et al, 1997). Doxorubicin levels in KS lesions were higher than the levels in normal skin in 20 of the 22 patients; in 14 patients normal skin levels were below the lower limit of quantitation (0.4  $\mu\text{g/g}$  tissue), whereas all KS lesion levels were quantifiable.

Forty-eight hours after DOXIL administration, median doxorubicin levels in biopsies of KS lesions ranged from 3-fold to 16-fold higher than in normal skin from the same patients. The median doxorubicin concentration in KS lesions was 1.3  $\mu\text{g/g}$  tissue in 7 patients receiving 10  $\text{mg/m}^2$  DOXIL and 15.2  $\mu\text{g/g}$  tissue in 7 patients receiving 20  $\text{mg/m}^2$  DOXIL; normal skin concentrations were 0.4 and 0.9  $\mu\text{g/g}$  tissue in the 10 and 20  $\text{mg/m}^2$  dose groups, respectively.

Biopsy data 48 hours after DOXIL injection in the 7 patients receiving 20  $\text{mg/m}^2$  are shown in **Figure 13**. Ninety-six hours after drug treatment, KS lesion doxorubicin levels were 3-fold and 5-fold greater than in

normal skin from the same patients in the 10 and 20  $\text{mg/m}^2$  groups, respectively. Median doxorubicin concentration in KS lesions was 4.3 and 3.3  $\mu\text{g/g}$  tissue in 4 patients receiving 10  $\text{mg/m}^2$  and 4 patients receiving 20  $\text{mg/m}^2$  dose, respectively; median concentration in the normal skin was 1.4  $\mu\text{g/g}$  tissue for the 10  $\text{mg/m}^2$  dose group, and 0.7  $\mu\text{g/g}$  tissue in the 20  $\text{mg/m}^2$  group.

Although too few time points were studied to allow determination of an AUC for doxorubicin in KS lesions or skin, these data suggest that doxorubicin accumulates in KS lesions after DOXIL treatment.

The levels of Doxorubicin were substantially higher in KS lesions after DOXIL compared to free Adriamycin administration (**Figure 14**).

**Table 2:** Concentration of doxorubicin in KS lesions and normal skin after DOXIL administration

Time after Infusion	No. of Patients	Dose ( $\text{mg/m}^2$ )	Doxorubicin Concentration ( $\mu\text{g/g}$ tissue)		KS/Normal Skin
			Median (range)		
			KS Lesion	Normal Skin	
48 hr	7	10	1.32 (0.17-22.43)	0.40 (0.26-1.55)	2.43
	7	20	15.21 (2.98-25.56)	0.92 (0.38-1.74)	20.89
96 hr	4	10	4.26 (1.91-36.44)	1.42 (0.70-2.78)	3.20
	4	20	3.28 (1.03-4.17)	0.73 (0.55-1.14)	4.94

**Figure 13:** Doxorubicin concentration in KS lesion tissue and adjacent normal skin tissue. Seven KS patients were given a 20 mg/m<sup>2</sup> dose of DOXIL and, 96 hours later, biopsies were taken of a representative cutaneous KS lesion and normal skin near the lesion. The tissue was homogenized, extracted with solvents and total doxorubicin measured by HPLC.

**Figure 14.** DOXIL compared to Adriamycin sustains a higher concentration of Doxorubicin in Kaposi's sarcoma lesions in human patients. From Northfelt et al (1996) *J Clin Pharmacol* 36, 55-63.

### **E. Combinations of DOXIL with other anticancer drugs in clinical trials**

The dose-limiting toxicity of Navelbine (vinorelbine tartrate) is granulocytopenia. In combination with doxorubicin, Navelbine produced a 57% overall objective response rate as first-line therapy of advanced breast cancer, however, the incidence of grade 4 granulocytopenia was 83%, with 8% requiring hospitalization due to febrile neutropenia and one septic death (Hochster, 1995). Substitution of doxorubicin with DOXIL in this combination is being explored as a means of maintaining the favorable tumor response of the combination while reducing the incidence of hematological toxicity. Navelbine would not be expected to contribute to the skin toxicity seen with DOXIL.

The excitement generated by Gianni et al (1995) who reported a greater than 90% objective response rate in metastatic breast for a taxol/doxorubicin combination is tempered by the rather unfavorable side effects profile of this regimen. Severe, febrile neutropenia was common and peripheral neuropathy occurred in one third of the patients. Perhaps more troubling was the development of reversible congestive heart failure (CHF) in 18% of

women after a median of 480 mg/m<sup>2</sup> doxorubicin, results which raise the specter of taxol-related enhancement of doxorubicin cardiotoxicity.

These encouraging preclinical findings are supported by results of a pilot clinical study done by Berry et al (1996). These authors have reported the results of endomyocardial biopsies performed on a series of AIDS-KS patients who received cumulative doses of DOXIL ranging from 469 to 860 mg/m<sup>2</sup>. These findings support the rationale for combining taxol (paclitaxel) with DOXIL. Both drugs have demonstrated activity in breast and ovarian cancer. With respect to toxicities, the incidence of severe neutropenia and peripheral neuropathy are lower for DOXIL than taxol. Preclinical and early clinical biopsy results strongly suggest that DOXIL produces less damage to the myocardium relative to comparable cumulative doses of doxorubicin. Thus the cardio-protective effect of DOXIL may translate into a reduced risk of cardiotoxicity relative to the highly active taxol-doxorubicin combination. Moreover, taxol causes relatively little skin toxicity. Based on these considerations, several phase I dose-finding trials of DOXIL and taxol have been launched.

## **V. Extravasation of “stealth” liposomes into tumors**

### **A. Mechanism of enhanced DOXIL accumulation in tumors**

An understanding of the mechanisms by which liposome-encapsulated doxorubicin accumulates within solid tumors after DOXIL administration, and how this deposition pattern and subsequent slow release of drug improve the antitumor activity of DOXIL relative to treatment with the free drug, is now emerging (refer to **Figure 15**).

### **B. Plasma stability and long plasma residence times are critical requirements**

DOXIL liposomes are intended to carry their payload of doxorubicin directly to tumors. So, any premature release of the drug, while the liposomes are still in route (i.e., in the circulation), would detract from the total amount of encapsulated doxorubicin able to reach the desired target. This requirement highlights the importance of engineering plasma stability into DOXIL liposomes. As mentioned earlier, conventional liposome formulations of doxorubicin have been shown to release a significant proportion of their payload into the bloodstream soon after injection (Gabizon et al, 1991; Conley et al, 1993). Drug release appears to follow protein adsorption/intercalation into the liposome which disrupts the barrier properties of the membrane. Moreover, the liposomes, together with any remaining drug, are removed by cells of the MPS

within several minutes to a few hours after injection. As a consequence of this rapid clearance, doxorubicin delivered in conventional liposomes has little opportunity to reach tumors in encapsulated form.

By virtue of the PEG groups grafted to their surface, DOXIL liposomes are stable in plasma and release very little drug while in the circulation (see discussion above). Moreover, the PEG coating provides slow clearance; after a single injection, DOXIL can be detected in the circulation for 2-3 weeks. Slow clearance kinetics provide an opportunity for these liposomes to reach sites of disease such as tumors. Measurements made in tumor-bearing animals and cancer patients indicate that uptake of pegylated liposomes by tumors is also a slow process. In preclinical tumor models, the peak uptake of DOXIL is reached 24-48 hours after injection (Vaage et al, 1993; Working et al, 1994).

In cancer patients given <sup>111</sup>Indium encapsulated in pegylated liposomes of the same composition and size as DOXIL, peak uptake in tumors is seen 48-72 hours after injection (**Figure 16**, Stewart Simon, personal communication, May 20, 1997). Slow uptake in tumors highlights the importance of long circulation times; if liposomes are to have an opportunity to reach and enter tumors in significant numbers, they must circulate for periods of days after injection.

**Figures 17 and 18** also show localization of <sup>111</sup>In-labeled Stealth liposomes into a T4 squamous cell carcinoma of the tongue and a squamous cell carcinoma of the lung, respectively.

**Figure 19** shows complete eradication of a KS lesion after six cycles of treatment with DOXIL.

**Figure 15:** Proposed mechanism for DOXIL accumulation in tumors. ① Liposomes containing doxorubicin circulate for 2-3 weeks after injection. During this period virtually all of the drug remains encapsulated. The liposomes pass many times through the blood vessels feeding growing tumors. ② Intact liposomes extravasate through defects/gaps present in newly sprouting

vessels and enter the tissue compartment; lodging in the tumor interstitium near the vessel. ③ Drug molecules are released from the extravasated liposomes. Liposome leakage is believed to be the consequence of conditions present in the interstitial fluid surrounding tumors which lead to physical/chemical breakdown of the liposome membrane (low pH, oxidizing agents, enzymes, uptake by macrophages). ④ Free drug

molecules penetrate deeply into the tumor and enter tumor cells. ⑤ Doxorubicin molecules bind to nucleic acids and kill tumor cells. Note that such a mechanism does not require a close physical encounter between a liposome and target cell, since free drug molecules are able to diffuse through barriers that may intercept liposomes.

**Figure 16:** Gamma scintigraphic image of a lung cancer patient 48 and 96 hours after administration of DOXIL liposomes containing  $^{111}\text{In}$ . Note that both images are posterior views. Uptake of the radioactive liposomes is seen in certain normal tissues including spleen, liver, bone marrow. The activity visible in the central chest (substernal) and upper abdomen represent liposomes that are still circulating in the heart and major vessels at these time points. The liposomes are taken up by a large tumor in the left upper lung. The density of radioactivity is as high or higher in the tumor than in any normal organ.

**Figure 17.** Plain anterior view scintigrams of a patient with T4 squamous cell carcinoma of the tongue injected with  $^{111}\text{In}$ -labeled “stealth” liposomes. The image at 4 h postinjection shows the blood pool, early uptake by the liver reticuloendothelial system, and EDTA-chelated  $^{111}\text{In}$  in the bladder. The tumor is seen clearly (white arrow) 72 hours after injection and is still visible at 10 days. From Stewart and Harrington, 1997 with their kind permission and the permission of Oncology.

**Figure 18.** Anterior scintigram of a patient with squamous cell carcinoma of the lung together with SPECT images taken at 72 hours injected with  $^{111}\text{In}$ -labeled “stealth” liposomes. The tumor is seen clearly (arrow) in all images. Prominent  $^{111}\text{In}$  activity can also be seen in the liver, spleen, and bone marrow. From Stewart and Harrington, 1997 with their kind permission and the permission of Oncology.

**Figure 19.** Therapy of a KS lesion with DOXIL.

### **C. Liposomes extravasate through gaps in the endothelium of tumor vessels**

Stealth liposomes of the same size and lipid composition as DOXIL, but containing entrapped colloidal gold designed to serve as a marker to follow liposome distribution by microscopic techniques, have been shown to enter solid colon tumors implanted in mice (Huang et al, 1992) and KS-like lesions in HIV-transgenic mice (Huang et al, 1993) (**Figure 20**). In these mouse models, movement of liposomes from the vascular lumen into the tumor interstitium was visualized by light and electron microscopy. Transcytosis of liposomes from the lumen of blood vessels, through endothelial cells, and into the extravascular compartment of KS lesions was seen, as was intracellular uptake of liposomes by some spindle cells within lesions. However, these processes appear to be restricted to a minority of the particles entering the tumor (Huang et al, 1993). The vast majority of the liposomes were seen to enter through gaps in the endothelial cell wall.

This finding is consistent with results reported by Yuan, et al who used pegylated liposomes ranging in size from 100-600 nm to probe the cut off size of the gaps present in a human adenocarcinoma xenograft implanted in nude mice (Yuan et al, 1995). This tumor was permeable to liposomes up to 400 nm in diameter,

**Figure 20.** Histological preparation of KS-like lesion nodule. Early lesion and adjacent normal skin in transgenic mice by liposome-encapsulated colloidal gold. A-C: Sections of KS-like lesion nodule were from a 16-month old F2 mouse that had a localized 5-mm spherical erythematous lesion on its back. The sections reveal that the gold particles are localized predominantly in the lesion region. Arrows in C show labeling of spindle cells. D: Section of an early lesion invisible to the naked eye in a 8-month-old C4 mouse showing that the gold marker is scattered extravasated erythrocytes in the collagenous dermis. E: Normal skin adjacent to the tumor shown in A. From Huang et al, 1993.

suggesting the cut off size in this tumor is between 400-600 nm. Given their small size (85 nm) and long circulation times, DOXIL liposomes would be expected to extravasate in tumors that exhibit gaps of such dimensions. Gaps/defects are known to be present in solid tumors (Seymour, 1992; Jain, 1989) and KS lesions (Francis et al, 1986; Vogel et al, 1988). Indeed, fluorescent pegylated liposomes of <100 nm in diameter have been visualized by video microscopy extravasating in real time into the interstitium of implanted tumors using window chamber models (Yuan et al, 1994; Huang et al, 1995; Dewhirst and Needam, 1995).

### **D. Release of drug following extravasation**

Encapsulated doxorubicin is released from the DOXIL liposomes after extravasation in tumors (Dewhirst and Needam, 1995). Several possible factors may contribute to liposome breakdown and drug release in tumors: (i) conditions present in the interstitial fluid surrounding tumors may cause breakdown of the liposomes, such as low pH, (Stubbs et al, 1992) and lipases released from dead or dying tumor cells (Sakayama et al, 1994); (ii) inflammatory cells (which are often found in tumors (Dvorak et al, 1981) may release factors that lead to liposome destabilization such as enzymes or superoxide and other oxidizing agents (Cobbs et al, 1995); or (iii)

phagocytic cells residing in tumors (Pupa et al, 1996) which are known to engulf liposomes (Huang et al, 1995), may digest the lipid matrix intracellularly and release doxorubicin (or its active metabolites) back into the interstitial fluid (Gabizon et al, 1991). A combination of these possibilities may well be responsible for the observed release of doxorubicin after extravasation of DOXIL liposomes in tumors (Gabizon et al, 1995).

The rate of release of doxorubicin within a tumor has yet to be measured directly. In order to do so, it would be necessary to separate encapsulated drug (i.e., drug molecules that have not been released from intact liposomes) from free drug in a solid tissue. Although such a separation is possible in biological fluids (such as plasma; Druckmann et al, 1989) it is technically difficult to conduct in solid tissues such as tumors; the conditions needed for quantitative extraction of doxorubicin lead to liposome disruption. Despite the difficulty of directly measuring release kinetics, indirect methods suggest that the release of doxorubicin from DOXIL liposomes occurs over a period of days to perhaps weeks following administration. In a recent study using a human pancreatic xenograft model in nude mice, Vaage et al showed that tumor levels of doxorubicin peak at 24-48 hours after DOXIL, and fall slowly over a period of a week (Vaage et al, 1997). These results suggest that the liposomes entering the tumor release their drug locally at quite a slow rate.

The improved antitumor activity of DOXIL relative to a comparable dose of free doxorubicin can be partially attributed to these slow in situ release kinetics. Consider the distribution kinetics after a dose of free doxorubicin. Drug molecules enter the tumor (and other tissues) quickly, reaching maximal exposure (i.e., peak concentrations) within minutes (Working et al, 1994). During the subsequent 24 hours, tumor doxorubicin concentration drops precipitously to undetectable levels. During this brief "pulse" of doxorubicin, those cells not exposed to a cytotoxic concentration for a sufficient amount of time, or which are not at a sensitive point in the cell cycle, can escape therapy and continue to proliferate. A typical course of doxorubicin is given on a three week cycle. This length of time between injections is needed to allow for recovery from the hematologic toxicity associated with doxorubicin therapy. Following such a schedule, it is quite likely that tumor cells are exposed to cytotoxic levels of drug for only a few hours during the 3 week interval between injections. In the case of DOXIL which is also given in a 2-4 week cycle, not only does more drug reach the tumor, but, by virtue of the slow in situ release kinetics provided by the liposomes, tumor cells are exposed to drug over a period of several days to perhaps a week or more after a single dose. Such a release pattern may contribute to DOXIL's antitumor response.

## **E. Tumor cell penetration and cytotoxicity**

Given its amphipathic nature, a doxorubicin molecule that is released from a liposome can quickly diffuse through surrounding fluids and connective tissue, enter tumor cells, bind to nucleic acids and inhibit DNA synthesis. Indeed, it is quite likely that drug molecules released from DOXIL can penetrate many cell layers into the tumor, well beyond the point that the liposome itself has reached. Early findings suggest that penetration of "free" drug in this fashion may be essential for DOXIL's antitumor activity.

As mentioned above, microscopic observations indicate that liposomes extravasate in tumors at particular sites; primarily through vessels forming at the advancing edge of angiogenesis (Yuan et al, 1994). The deposition of extravasated liposomes in these areas is perivascular and focal, occurring primarily at the roots of capillary sprouts where weak spots (possibly defects or gaps) in the endothelium are believed to occur. Given the geometry of the system, liposomes that enter through such gaps may not be able to penetrate deeply into the tumor interstitium. Liposome penetration may be limited by a range of physical obstacles including tight cell-cell junctions (often found in highly differentiated epithelial cell tumors), dense connective tissue stroma, small extracellular volume and high interstitial fluid viscosity (that may be caused by fibrin cross-linking) (Nagy et al, 1995). Ideally all tumor cells, regardless of their proximity to blood vessels or the liposome depots that may form near them, would be exposed to a cytotoxic dose of drug. So, the observation that drug molecules released from focal, perivascular deposits of liposomes are able to penetrate deeply into the tumor mass may be a critical requirement for expression of DOXIL's antitumor activity.

## **VI. Encapsulation of other drugs into stealth liposomes**

### **A. cis-Platinum (SPI-77)**

Cisplatin (Platinol) is active alone and or combination chemotherapy regimens against a wide range of epithelial malignancies including testicular, ovarian, head and neck, lung, bladder, and cervical cancers (Loehler and Einhorn, 1984). Cisplatin chemotherapy is often limited by side effects that prohibit continued treatment. In addition, some tumors are initially resistant or acquire cisplatin resistance with continued exposure. The major dose-limiting cisplatin-induced toxicity in humans is renal toxicity, although significant nausea and vomiting, ototoxicity, peripheral neuropathy and myelotoxicity are also induced by cisplatin administration. Attempts to ameliorate cisplatin-induced toxicity and/or resistance have focused on the development of platinum derivatives that are less toxic and/or more active than the parent

compound (Schilder et al, 1994; Kelland and McKeage, 1994). Alternative approaches include altering the pharmacology of the drug by altering the treatment schedule, hydrating patients prior to and during therapy, or administering renal protectant therapy. Encapsulating the drug within liposomes has shown improved therapeutic capacity (Steerenberg et al, 1987; Potkul et al, 1991).

SPI-77 is a formulation of cisplatin encapsulated in virtually the same type of liposome as DOXIL. SPI-77 exhibits plasma pharmacokinetics characteristic of sterically stabilized (Stealth) liposomes, with long circulation, high  $C_{max}$  and area-under-the-plasma concentration vs time curve (AUC), and low clearance and volume of distribution compared to non-liposomal cisplatin (**Figure 21**). In vitro leakage studies suggest that plasma levels of platinum primarily or solely represent liposomal cisplatin, i.e., drug that is in liposomes and free or bound to proteins.

The therapeutic activity of SPI-77 has been evaluated and compared to non-liposomal cisplatin in various tumor models, including the C26 colon carcinoma in Balb/c mice and a xenograft of the NCI-H82 small cell lung tumor in athymic mice (**Figure 22**). SPI-77 showed meaningful anti-tumor activity in these tumor models. Cisplatin was only effective in the NCI-H82 xenograft model; carboplatin (Paraplatin) was ineffective in both. SPI-77 only occasionally produced complete tumor responses, but

did cause a persistent inhibition of tumor growth during and after treatment. In many animals, tumors grew slowly to intermediate size and then were apparently arrested, with little additional growth evident. Although cisplatin treatment resulted in better inhibition of tumor growth in both trials in the NCI-H82 xenograft models, SPI-77 was more effective in producing a prolonged response to treatment, with persistent inhibition of tumor growth.

## **B. Stealth Vincristine**

Vincristine is used clinically both as a single agent and in combination regimens, for the treatment of hematological malignancies, head and neck cancer, Kaposi's sarcoma and lung cancer. Early work with conventional liposomal Vincristine showed no improvement in safety or therapeutic activity relative to the free drug (Layton and Trouet, 1980).

Stealth liposome-encapsulated Vincristine (S-Vinc) prolonged the drug's distribution phase plasma half-life in rats from 0.22 to 10.5 hours. While there was no significant difference in  $LD_{50}$  between encapsulated and free drug (at doses of  $\approx 2.5$  mg/kg, given by i.v. injection), mice given sublethal doses of S-Vinc experienced significantly less weight loss compared to animals receiving the same dose of Vincristine. Compared to free

**Figure 21:** Plasma clearance of cisplatin (circles) or SPI-77 (squares) after single intravenous injection in rabbits.

**Figure 22.** Tumor growth kinetics of a human small cell lung cancer xenograft (NCI-H82) implanted subcutaneously in athymic mice. Groups of tumor-bearing animals were treated via tail vein injection with 100 mg/kg carboplatin (circles), saline (inverted triangles), 6 mg/kg cisplatin (squares) or 6 mg/kg SPI-77 (triangles).

**Figure 23.** Tumor volume in BLAB/c mice given multiple tail vein injections of saline, 1.3 mg/kg Vincristine (Oncovin) or 1.3 mg/kg Stealth liposomal Vincristine (S-VINC). Treatment was given on days 10,17 and 24 after implantation of the murine C26 colon carcinoma. (NMT = no measurable tumor)

drug, S-Vinc was more active against intraperitoneally and subcutaneously implanted tumors. In a subcutaneously-implanted murine colon tumor model, multiple doses of free drug did little to retard tumor growth, but S-Vinc slowed tumor growth and improved long-term survival in several dosing regimens (**Figure 23**; Allen et al, 1995). Stealth liposomes extravasate preferentially to tumors by leaking through new vessels during the process of angiogenesis of the tumor (Papahadjopoulos et al, 1991; Huang et al, 1992, 1993; Gabizon et al, 1994).

Steric hindrance by coating the liposome surface with PEG can inhibit recognition of targeting ligands, such as antibodies, by cell membrane proteins on the targeted cell (Mori et al, 1991; Torchilin et al, 1992). This obstacle can be in part overcome by conjugating a water-soluble drug at the end of the PEG polymer. For example, 66-nm in diameter liposomes can be efficiently targeted to tumor cells that express folate receptors (KB cells) via conjugation of the folate to a PEG spacer of 25 nm in length; shorter PEG spacers were not efficient in

mediating binding of the liposomes to KB cells (Lee and Low, 1995). Antibodies attached to long PEG spacers can give stealth liposomes that are effective in target binding and exhibit prolonged circulation times (Papahadjopoulos et al, 1991; Blume et al, 1993).

## VII. Cell targeting with liposomes

### A. IgG-coated liposomes can target specific cell types

Injected liposomes are localized mainly in the fixed macrophages of the liver and spleen tissue; indeed the reticuloendothelial system of the body rapidly removes liposomes from the blood. Gangliosides and sphingomyelin, when included into the lipids of the liposome, act synergistically to diminish the rate of uptake of liposomes by macrophages of the host defense system; this results in extended circulation times of these large unilamellar liposomes (Allen and Chonn, 1987).

Attempts to generate cell-targeting have focused primarily on the addition of monoclonal antibodies to the surface of the liposome. Liposomes tagged on their surface with IgG immunoglobulins directed against a variety of cell membrane proteins and desialylated fetuin which binds to the parenchymal cells of the liver can deliver bleomycin and mediate selective cellular uptake of the entrapped drug (Gregoriadis and Neerunjun, 1975). Apparently the hydrophobic IgG regions penetrate the lipid bilayers whereas the immunologically active portions are facing the exterior of the liposomes and are available for interaction with cells.

Immunoliposomes tagged with monoclonal antibodies against c-ErbB2 (other names Neu or HER2), product of the protooncogene *c-erbB2*, a growth factor receptor-tyrosine kinase, were bound preferentially to breast cancer cells in culture which overexpress this receptor; loading these immunoliposomes with doxorubicin made them more toxic to cell lines overexpressing the *c-erbB2* oncogene; furthermore, when this immunoliposome bullet was injected into SCID mice bearing human breast tumor xenografts it was able to deliver the cytotoxic doxorubicin to the tumor cells (Park et al, 1995).

More recently, production of cell-targeting ligands has been achieved by cell-binding peptides specific for different cell types in culture; these peptides are selected through several rounds of binding to a particular cell type from random peptide-presenting phage libraries (Devlin et al, 1990; Cwirla et al, 1990; Barry et al, 1996).

Antibodies have been attached to neutral liposomes (Straubinger et al, 1988; Ahmad et al, 1992, 1993). A disadvantage of using antibodies that are bulky in liposome formulations is the increase in the volume of the liposome: small liposomes extravasate at the site of a tumor more readily than large liposomes and larger

liposomes are captured more frequently by macrophages in animal and human studies; thus, keeping the size of the liposome small offers a clear advantage for its use as a delivery system.

Often antibodies are loaded to preassembled liposomes in order to avoid exposure of the antibody to organic solvents; in other cases antibodies are reacted with preassembled liposomes containing lipids with activated head groups (Heath et al, 1983; Matthay et al, 1989). Antibodies have also been conjugated to N-glutaryl-phosphatidylethanolamine in aqueous dispersions and have been reassembled with the drug bullet and bilayer lipids by detergent dialysis into targeting liposomes (Maruyama et al, 1990; Lundberg et al, 1993); however, the encapsulation efficiency with hydrophilic drugs is very low.

### B. Liposomes tagged with folate receptor and the caveolae vesicle

#### 1. Caveolae

The purpose of this approach is to bypass the lysosomal compartment that could modify and degrade foreign DNA during DNA delivery.

Caveolae, also known as plasmalemmal vesicles, are cell membrane organelles appearing under transmission electron microscopy as 50-100 nm invaginations of the plasma membrane (Bundgaard et al, 1979; Montesano et al, 1982). Caveolae are abundant in endothelial cells and are rich in glycosyl-phosphatidylinositol (GPI); caveolae concentrate specific proteins that bind to GPI lipids and mediate a unique transcytosis or potocytosis mechanism where the engulfed material is not presented to lysosomes but to Golgi or is emptied to the cytoplasm. Proteins interacting with the GPI lipid components include SRC tyrosine kinases, an anchorage mediated by their palmitoylation (Robbins et al, 1995), the folate receptors  $\alpha$ ,  $\beta$ , and  $\gamma$ , and G protein-coupled receptors, and may thus constitute integral components of the signal transduction from the cell exterior to cytoplasm and the nucleus across the cell membrane (reviewed by Anderson, 1993a,b; Lisanti et al, 1994).

Cholera toxin trafficking, observed by fluorescence confocal microscopy, might occur via caveolae directed to the Golgi compartment (Bastiaens et al, 1996); cationic amphiphilic drugs (also cationic liposomes?) inhibit the internalization of cholera toxin to the Golgi (Sofer and Futerman, 1995). These studies support a model for internalization of cationic or amphiphilic liposomes via caveolae.

#### 2. The GPI anchor

The mechanism of GPI anchoring involves covalent attachment of the glycosyl-phosphatidylinositol moiety to

the C-terminus of the protein through an ethanolamine linkage. The GPI anchor precursor is synthesized in the endoplasmic reticulum and linked to protein post-translationally. This occurs soon after the protein synthesis; the GPI anchor is added in the lumen of the endoplasmic reticulum (Takahashi et al, 1996) and might involve either a protease linked to a transferase or a single transpeptidase which breaks a peptide bond at the C-terminus and forms the amide bond to the ethanolamine (Ferguson and Williams, 1988). Synthesis of the GPI anchor involves several steps; the first reaction is transfer of the N-acetyl-glucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI); deacetylation of this molecule is then followed by the sequential addition of three mannosyl residues (Man); the last step involves transfer EtN-P to the third mannose from phosphatidylethanolamine. Most of the genes involved in GPI synthesis have been cloned (see Takahashi et al, 1996). The core backbone of the GPI anchor is conserved from yeast to mammals and has the structure: ethanolamine-P-6Man<sub>1</sub>2,2Man<sub>1</sub>6,6Man<sub>1</sub>4GlcNAc<sub>1</sub>6myoinositol1-P-lipid (Ferguson and Williams, 1988; Takahashi et al, 1996).

The phosphatidylinositol glycan of complementation class B (PIG-B) is a ER transmembrane protein involved in transferring the third mannose; about 60 aa are to the cytoplasmic site and the large C-terminal portion of 470 aa, that contains the active site, lies within the lumen of the ER (Takahashi et al, 1996). A somatic mutation in the X-linked *PIG-A* gene involved in the first step in GPI synthesis results in defective GPI anchor and is a somatically acquired genetic disease known as paroxysmal nocturnal hemoglobinuria; the defect arises from afflicted clonal hematopoietic cells (Takeda et al, 1993).

The nascent proteins that are to be GPI anchored have a signal peptide sequence at their C-terminus; this C-terminal peptide is cleaved and the new C-terminus is linked to the ethanolamine of the GPI anchor (see Takahashi et al, 1996 for more references). Once attached to the GPI anchor, proteins are transported to the plasma membrane by vesicular transport via the Golgi apparatus; protein molecules with GPI anchors are more mobile in the lipid bilayer than proteins with a transmembrane domain and such proteins are thought to be localized at specialized regions of the plasma membrane. The importance of the GPI anchor is obvious in the case of the neural acetylcholinesterase, also attached to the membrane via a GPI anchor: rapid destruction of acetylcholine in the region of the synapse triggers neurotransmission. Other protein molecules attached to membranes via a GPI anchor include alkaline phosphatase, 5' nucleotidase, alkaline phosphodiesterase, the lymphoid antigens Thy-1 and RT-6 and others (reviewed by Ferguson and Williams, 1988).

The GPI anchor can be broken by proteases as in the case of folate receptor (Lacey et al, 1989) or by activation of phospholipase C (PLC) in response to triggering at the

cell surface; one of the cleavage products of phosphoinositides by PLC is diacylglycerol which stimulates protein kinase C and another cleavage product is inositol phosphate that triggers the release of Ca<sup>++</sup> from intracellular stores. This has led to the idea that breakdown of GPI anchors might be a component of receptor mediated triggering pathway.

A number of membrane proteins are known to be associated with caveolae. The protein-tyrosine kinase p59<sup>hck</sup> is first myristoylated and then palmitoylated at another site, cysteine-3; palmitoylation targets p59<sup>hck</sup> to caveolae vesicles (Robbins et al, 1995). The FYN tyrosine kinase is also anchored to caveolae membrane via its palmitoylation at cysteine-3 (Shenoy-Scaria et al, 1994). Among the SRC family of tyrosine kinases, myristoylation is a prerequisite for their anchorage to the cell membrane and mutations at the N-terminal glycine where myristoylation takes place results in the exclusive retain of the kinase in the cytoplasm (Resh, 1994). Different types of interactions have been evoked to explain anchorage of the SRC family of tyrosine kinases to the cell membrane including insertion of the myristate moiety into the lipid bilayer, electrostatic protein-lipid interactions, and interactions between the anchor part of the SRC proteins with protein domains already embedded in the cell membrane (Resh and Ling, 1990; Sigal et al, 1994). This suggests that caveolae participate in the transduction of signals across the plasma membrane (Anderson, 1993a,b; Lisanti et al, 1994).

### 3. Folate as an essential cofactor in purine/pyrimidine biosynthesis

Folic acid, broadly distributed in plant leaves, is essential for mammals (vitamin) supporting cell growth; its reduced form, tetrahydrofolate, serves as an intermediate carrier of hydroxymethyl (-CH<sub>2</sub>OH), formyl (-CHO), or methyl (-CH<sub>3</sub>) groups in a large number of enzymatic reactions in particular those involved in the intermediary metabolism of purines, pyrimidines, and amino acids; tetrahydrofolate (THF) is composed of the two condensed ring compound 2-amino-4-hydroxy-6-methyltetrahydro pteridine linked to p-aminobenzoic acid which is esterified with the amino group of glutamic acid. N<sup>5</sup>-methyl-THF is formed by removal of the -CH<sub>2</sub>OH group from serine and its reduction to -CH<sub>3</sub>; the methyl group is then donated to homocysteine to form methionine. N<sup>10</sup>-formyl-THF is a cofactor of the enzyme phosphoribosylaminoimidazole-carboxamide formyltransferase; N<sup>5</sup>, N<sup>10</sup>-methylene-THF is a cofactor of the enzyme phosphoribosylglycinamide formyltransferase; both derivatives of THF donate a formyl group to two different intermediates during biosynthesis of inosinic acid, the precursor of adenylic and

guanylic acids (AMP and GMP) during the building of the purine ring on D-ribose-5-phosphate. N<sup>5</sup>, N<sup>10</sup>-methylene-THF is also a cofactor of the enzyme thymidylate synthetase which catalyzes methylation of the 5 position of deoxyuridylic acid (dUMP) to deoxythymidylic acid (dTMP); the antifolate drugs aminopterin and amethopterin, used as antineoplastic drugs, are competitive inhibitors of dihydrofolate reductase (DHFR) that converts DHF into THF (Lehninger, 1975).

#### **4. Folate receptor (FR) is overexpressed in tumor cells**

The FR molecule is maximally expressed on the surface of cells cultured in low folate medium and mediates the high affinity accumulation of 5-methyltetrahydrofolate in the cytoplasm of these cells. Because of their increased metabolic rates tumor cells have increased needs for folate and overexpress folate receptor (Matsue et al, 1992; Weitman et al, 1992; Mayor et al, 1994). A special interest for the FR emerged from the finding that its density on the cell membrane is considerably (more than 20-fold) higher in tumor than in normal cells especially ovarian adenocarcinoma and cervical carcinoma cell lines; FR expression, albeit at lower levels, was detected in normal bone marrow, spleen, thymus and ovarian and uterine carcinoma tissue explants (Weitman et al, 1992). FR is also expressed in subsets of breast, lung, and colon cancer, in neuroendocrine carcinomas and rare gliomas (Garin-Chesa et al, 1993). Folate receptor (also called folate-binding protein) was identified by cDNA cloning as the ovarian cancer-associated antigen recognized by the monoclonal antibody MOv18; this monoclonal antibody was used for immunodiagnosis of ovarian cancers. FR was not amplified in 16 out of 16 carcinoma cell lines examined and thus the overexpression of this gene in ovarian cancer involves other mechanisms (Campbell et al, 1991). The overexpression of FR in cancer cells has raised the possibility of targeting tumor cells with folate attached to different ligands such as PEG-liposome-encapsulated doxorubicin (Lee and Low, 1995, see below).

The folate receptor (FR) is a membrane protein linked to glycosyl-phosphatidylinositol. The anchor to GPI of the protein molecule is a C-terminal 19 aa residue segment: WAAWPFLSLALMLLWLLS (Lacey et al, 1989; Coney et al, 1991). Thus FR is not a transmembrane protein since it lacks a cytoplasmic tail. The protein is released from the membrane by cleavage of its anchor with phosphatidylinositol phospholipase C, apparently enriched in plasma and responsible for a soluble form of the FR in plasma as well as milk. The cDNA cloning also revealed a signal peptide at the N-terminus responsible for targeting to the lumen of the endoplasmic reticulum: MAQRMTTQLLLLLVWVAVVGEAQT, with the hydrophobic core of the sequence underlined (Lacey et al,

1989). It is noted here that the FR possesses a putative weak nuclear localization signal AKHHKEKPGPEDK; thus, a possible cleavage of the membrane anchor of the molecule inside the cytoplasm, if occurring at all under physiological or pathological conditions, could give a soluble form of the folate receptor similar to that found in human and cow milk, able to enter the nucleus (Boulikas, 1996b).

The part of the cell membrane with a high density of RFs (clusters of about 750 protein molecules; Rothberg et al, 1990) is potocytosed forming a special type of vesicle known as caveolae; according to a model proposed by Rothberg and coworkers (1990) caveolae, enclosing the RF with the folate bound to it, remain attached to the membrane at the cytoplasmic side of the cell; the pH inside the caveolae vesicle drops by one unit as a result of a proton pump on the vesicle increasing the concentration of H<sup>+</sup> inside the vesicle and causing the dissociation of the folate from its receptor; folate then moves across the caveolae membrane via the transporter using the energy generated by the H<sup>+</sup> gradient; finally, folate is modified by a chain of glutamic acid residues, a modification entrapping it into the cytoplasm, and the caveolae unseals and presents the receptor to the exterior of the cell for another cycle.

About 600,000 RF molecules per cell have been estimated for MA104 monkey kidney epithelial cells in culture; these are grouped into about 800 clusters per cell each containing 750 RF molecules (Rothberg et al, 1990).

#### **5. Folate-PEG-liposomes in tumor therapy**

The folate receptor has been predicted from cDNA molecular cloning and sequencing to be anchored in the membrane via a glycosyl-phosphatidylinositol (GPI) linkage (Lacey et al, 1989). GPI in membranes has a special function (Low and Saltiel, 1988) and molecules internalized into cells via GPI-enriched caveolae do not pass to the lysosomal compartment as do clathrin-coated pits (Rothberg et al, 1990).

One additional advantage of the folate-PEG-liposome is that the conjugation of folate-PEG-distearoylphosphatidyl ethanolamine (DSPE) is performed prior to liposome assembly and is thus compatible with the different methods of liposome preparation (Lee and Low, 1995).

### **VIII. Cationic liposomes in gene delivery**

#### **A. Principle of cationic liposome-mediated gene transfer**

Cationic liposomes have gained wide recognition as delivery vehicles for plasmid DNA in somatic cell gene

transfer often circumventing the shortcomings of the viral and retroviral systems (Lasic and Papahadjopoulos, 1995; Ledley, 1995; Aliño et al, 1996; Cao et al, 1995); one advantage using cationic liposomes is that there is no limit on the size of DNA to be delivered to cells compared with the upper limit of 7.5 kb that can be accommodated into viral/retroviral vectors. The elimination of therapeutically important cells from the body by the immune system due to expression of viral proteins after *ex vivo* delivery of genes with recombinant adenovirus seems to be an additional drawback of viral methods (Dai et al, 1995).

Two approaches have been used for the liposomal delivery of genes: (i) encapsulation of plasmids (Kaneda et al, 1989) and oligonucleotides (Thierry and Dritschilo, 1992) into true liposomes and (ii) formation of a complex between liposomes composed of cationic lipids and plasmid DNA (Aliño et al, 1996; Cao et al, 1995). Use of pH-sensitive liposomes (Wang and Huang, 1987) or liposomes with folate ligands exposed on their surface (Lee and Low, 1995) have been used to circumvent the cumbersome uptake of such complexes into endosomes (lysosomes) by phagocytosis resulting in DNA degradation.

Important parameters affecting cationic liposome-mediated transfection efficiency are (i) the type of lipid, (ii) the ratio of lipid to DNA, (iii) the presence of DNA condensing agents such as spermine, polylysine, histones, (iv) whether cells in culture or somatic cells in animals *in vivo* are being targeted, (v) presence of fusogenic peptides in the complex (Wagner et al, 1992), and (vi) the type of control elements that drive the reporter or therapeutically important gene. The physicochemical properties of such complexes and their interaction with the cell surface are not well understood (Lasic and Papahadjopoulos, 1995).

The calcium phosphate coprecipitation method and high molecular weight polycations (dextran) are still extensively used for the introduction of plasmid DNA into

cells; however, these methods display a high variability in transfection, are toxic to cells, and result in the introduction of many copies of DNA into a single cell whereas the majority of cells may not be transfected at all. Furthermore, multiple copies of foreign DNA may become integrated into the host's genome; the mechanisms involved have not been fully elucidated.

Compared to viral vectors, liposomes are safer to prepare, their toxicity can be monitored, and the risk of pathogenic and immunological complications is diminished; a great variety of cationic lipids is available for transfection studies (**Table 3**); liposome compositions can be matched with the appropriate liposome mean diameter which is controlled by ultrasonication or extrusion through membranes of various pore sizes.

*In vivo* studies have shown that cationic liposome-plasmid complexes are cleared rapidly from the blood stream of animals and do not circulate beyond one pulse of the heart when injected into the tail vein in mice (Huang, SK, SEQUUS, personal communication). Cationic liposome-plasmid complexes are rapidly taken up by endothelial cells; this explains why the primary tissue target is lung, which has by far the largest surface area of vascular endothelium, followed by liver and heart (Huang, SK and Danilo Lasic, personal communications).

The mechanism of internalization of cationic-liposome-plasmid complexes by cells *in vivo* is not thoroughly understood; cationic liposomes could electrostatically bind to the slightly negatively-charged surface of the cells followed by endocytosis leading to the enclosure of the liposome-plasmid complex into endosomes and lysosomes. According to a different model (Danilo Lasic, personal communication) cationic liposome-plasmid complexes enter rapidly the cell like "bullets". It is believed that only a tiny fraction of the plasmid reaches

**Table 3.** Cationic and neutral lipids used in liposome formulations and other polymers for gene transfer

Abbreviated name	Full name	Reference
DC-CHOL	3β [N-(N',N'-dimethylaminoethane)carbonyl]cholesterol	Gao and Huang, 1991; Litzinger et al, 1996; Zuidam and Barenholz, 1997
DDAB	dimethyldioctadecyl ammonium bromide	e.g. Lappalainen et al, 1997
DMRIE	N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide	Felgner et al, 1994
DMTAP	1,2-dimyristoyl-3-trimethylammonium propane	Song et al, 1997; Filion and Phillips, 1997
DODAC	Dioctadecyldimethylammonium chloride	Behr et al, 1989
DOGS	Dioctadecylamidoglycylspermine (Transfectam, Promega)	Behr et al, 1989
DOPC	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine	Zuidam and Barenholz, 1997
DOPE	dioleoyl phosphatidylethanolamine (neutral fusogenic lipid)	
DOSPA	2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-	Lappalainen et al, 1997

	dimethyl -1-propanaminium trifluoroacetate	
DOTAP	1,2-dioleoyloxypropyl-3-(trimethylammonium)propane N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride	
DOTMA	N-[1-(2,3-dioleoyloxy) propyl]-n,n,n-trimethylammonium chloride	Felgner et al, 1987
DOTMA:DOPE 1:1	Lipofectin (GIBCO BRL)	Yoshimura et al, 1992; Zhu et al, 1993; Hyde et al, 1993
DPPES	Dipalmitoyl phosphatidylethanolamidosperrmine	Behr et al, 1989
DPTAP	1,2- dipalmitoyl-3-trimethylammonium propane	Song et al, 1997; Filion and Phillips, 1997
DSPE	distearoyl phosphatidylethanolamine (neutral lipid)	Felgner et al, 1987
DSTAP	1,2-disteroyl-3-trimethylammonium propane	Song et al, 1997; Filion and Phillips, 1997
ExGen (PEI)	Polyethylenimine	Ferrari et al, 1997

intact the cytoplasmic compartment and even a smaller fraction is imported into nuclei (Boulikas, 1998b).

There is no upper limit in plasmid DNA size to be complexed with cationic liposomes as opposed to adenoviruses, AAV, and retroviruses that can accommodate a maximum of 7.5 kb of foreign DNA because of packaging limitations. This factor is of utmost importance when large genomic regions need to be transferred in order to obtain correct developmental expression after transduction of fetuses or newborn animals.

Positively-charged liposomes containing distearoyl phosphatidylethanolamine, a lipid which promotes fusion of liposomes with membranes, has been used for the transfer of plasmid DNA into cells (Felgner et al, 1987; Felgner and Ringold, 1989). Liposomes containing head groups able to form discrete complexes with DNA induce wrapping of DNA around unilamellar 80- to 100-nm in diameter vesicles in a way reminiscent of the wrapping of 167 bp of DNA around the 55-nm core histone octamer forming nucleosomes (see Behr et al, 1989).

The positively-charged groups of the lipids interact with DNA causing both a condensation of the plasmid by diminishing the negative electrostatic repulsions on DNA as well as electrostatic binding of DNA. The liposome-plasmid DNA complex is then presented to cells in culture or is injected into animals intravenously, intraperitoneally, subcutaneously, intratracheally, or via other routes.

## B. Studies on cationic liposome-mediated gene transfer

A number of studies in gene therapy have used cationic liposomes as means of delivering DNA. Examples include transfer of prostaglandin G/H synthase to protect lungs in rabbits against endotoxin-induced inflammation and pulmonary hypertension (Conary et al 1994); of  $\alpha_1$ -antitrypsin cDNA to protect lungs (Canonica et al, 1994)

or  $\alpha_1$ -antitrypsin cDNA encapsulated into negatively-charged liposomes to protect connective tissue from the lytic action of the leukocyte neutrophil elastase (Aliño et al, 1996); of the human *CFTR* (cystic fibrosis transmembrane conductance regulator) gene in lungs in *CFTR*-deficient transgenic mice (Hyde et al, 1993; Alton et al, 1993) or in normal mice (Yoshimura et al, 1992) by tracheal instillation for the transduction of airway epithelial cells for cystic fibrosis; of the MHC class I HLA-B7 heavy chain gene for the treatment of cancer (Lew et al, 1995); of tyrosine hydroxylase (*TH*) gene in order to alleviate degeneration of dopaminergic nigrostriatal neurons in rat models of Parkinson's disease (Jiao et al, 1993; Cao et al, 1995); of the wild-type *p53* gene to treat nude mice inoculated with breast carcinoma cells (Lesoon-Wood et al, 1995); and of the *IL-2* gene for prostate cancer therapy (Vieweg et al, 1995).

Cationic liposomes have also been used for arterial gene transfer (Nabel et al, 1990, 1993, Takeshita et al, 1994).

Stable cationic lipid/DNA complexes were formed by solubilizing DOSPA:DOPE in 1% octylglucoside, 10 mM Tris pH 7.4 followed by the addition of DNA and exhaustive dialysis of the complex against 10 mM Tris, pH 7.4, 5% dextrose; the lipid-DNA complex had a storage life of up to 3 months after formation with respect to its ability to transfect tissue culture cells and was competent of transfecting cells in the presence of 15% fetal bovine serum (FBS) whereas convenient cationic liposome-DNA complexes are unable to transfect cells in the presence of FBS; a precipitate of the stable cationic lipid-DNA complex formed after 14 days on shelf storage at 4°C could be pelleted retaining all the transfection efficiency and displaying lower toxicity because of the removal of the free uncomplexed lipid in the supernatant (Hofland et al, 1996).

## DOTAP

DOTAP, a monocationic lipid, has been used to transfer efficiently the lacZ reporter gene and CFTR cDNA into mice without any inflammatory response (McLachlan et al, 1995).

### Lipofectin

Lipofectin (a 1:1 mixture of the cationic DOTMA with the neutral DOPE) has been shown to deliver reporter genes to the rodent airways after direct intratracheal injection (Yoshimura et al, 1992) or after intravenous administration (Zhu et al, 1993). Lipofectin has been used to alleviate the symptoms of cystic fibrosis in transgenic CF mice after transfer of the *CFTR* gene (Hyde et al, 1993). Lipofectin has been used for the transfer of the *CCK* gene to suppress audiogenic epileptic seizures, as well as for the transfer of reporter genes directly injected into mouse brain (Ono et al, 1990; Roessler and Davidson, 1994).

### DOGS (Transfectam)

DOGS appears to be more efficient than Lipofectin and DOTAP for the transfer of the luciferase gene to polyp and tracheal lung epithelial cells in culture; this may be due to the presence of a secondary amine with a  $pK_a=5.4$  in the DOGS molecule which might be able to buffer the acidic endosomes and protect the plasmid DNA from degradation; DOGS, however, was inefficient for gene transfer to submucosal gland cells which are active in producing sticky mucus in CF patients and should be the target cells to be corrected by CFTR gene transfer (Ferrari et al, 1997).

Compaction of plasmid DNA with dipalmitoyl phosphatidylethanolamidosperrmine (DPPES) and dioctadecylamidoglycylspermine (DOGS), collectively known as lipospermines, gave lipid-coated plasmid DNA rather than liposome-plasmid complexes; lipospermines interact strongly with DNA eventually promoting coating of supercoiled DNA plasmids and promote binding of the complex to the cell membrane (Behr et al, 1989). When dispersed in water (either by sonication or by dilution of an ethanol solution) they form unilamellar vesicles of 80-100 nm, unstable in ionic media, and interacting cooperatively with plasmids because of the strong affinity of the spermine group for DNA ( $10^5-10^7 M^{-1}$ ). Small unilamellar vesicles formed between DOGS and egg yolk lecithin were unable to mediate transfection and thus DOGS-mediated transfection is not a liposome-mediated process but a process mediated by cationic lipid-coated plasmid (Behr et al, 1989).

Although the *in vitro* conditions for transfection seem to favor a ratio of positive charges of lipids to negative phosphate charges on DNA of about 1:8, the *in vivo* optimal conditions are 1 lipid molecule/30 phosphates

using DDAB:DOPE and DDAD:Chol (Boulikas et al, in preparation). Previous studies by Behr and coworkers (1989; reviewed by Behr, 1994) and Schwartz et al (1995) using DOGS have also shown that the *in vivo* optimal conditions require a lower lipid:DNA charge ratio; the explanation might be that anionic proteins in the blood serum or in the extracellular matrix could interact with cationic lipid particles inhibiting their uptake by the cells in tissues.

The *in vivo* efficacy for transferring episomally replicating plasmids containing the human papovavirus BKV origin of replication/early regulatory region and the large T antigen gene, as well as the luciferase reporter gene under control of RSV promoter were investigated by Thierry and coworkers (1995); dioctadecylamidoglycylspermidine:DOPE liposomes hydrated in the presence of plasmid DNA, injected into the vein of mice, sustained expression in the liver, lung, spleen, heart, and other tissues for up to three months postinjection. These vectors were found to replicate extrachromosomally in the lung tissue; use of nonepisomal vectors under the same conditions gave only transient expression of the luciferase gene which disappeared after about 4-5 days.

Cationic liposomes have been used for targeting brain cells after direct intracranial injection of the plasmid-liposome complex. DOGS:DOPE liposomes have successfully transferred the luciferase reporter gene under control of CMV promoter to striatal parenchyma and paraventricular brain cells in neonatal mice; however, the expression of the transgene, although significant at early times postinjection diminished over time (Schwartz et al, 1995).

## C. Advantages and drawbacks using cationic lipids for gene delivery

Cationic lipids may show low efficiency of transfection despite the relatively large amounts of DNA used.

Cationic lipid-DNA complexes with a net positive charge may interact with circulating serum proteins or anionic components of the extracellular matrix in the various tissues; this interaction reduces their bio-availability (Schwartz et al, 1995). In addition positively-charged complexes activate complement and complement-dependent phagocytosis by macrophages in the reticuloendothelial system and are, thus, cleared rapidly from body fluids (Plank et al, 1996). Thus, although the optimal lipid-DNA ratio may be to an excess of positive charges for the transfection of cells in culture (mediated by the ability of cationic lipids to interact with the relatively negatively-charged external surface of the cell membrane and higher poration through the membrane) the optimal ratio *in vivo* is nearly that which gives a neutral

complex (Boulikas et al, in preparation, see Boulikas 1998a).

#### D. Entry of liposome-DNA into cells

Neutral liposomes are internalized via the endocytic uptake mechanism. However, cationic liposome-plasmid complexes (Capaccioli et al, 1993; Boutorine and Kostina, 1993) as well as Sendai virus-derived liposomes (Compagnon et al, 1992; Morishita et al, 1993) seem to permit a direct passage of the oligonucleotide load through the cell membrane (see also Bongartz et al, 1994).

#### E. Enhancement of cationic liposome delivery

A number of methods have been invented to augment the efficiency of internalization or release from endosomes of DNA-liposome complexes, or to enhance nuclear import of the plasmid after its release in the cytoplasm. The external cell surface has a net negative charge and thus liposome-DNA particles with a net positive charge can be electrostatically anchored to the external cell membrane.

Liposomes are internalized into endosomes which degrade the plasmid; use of folate attached to ligands on the surface of the liposome (Gottschalk et al, 1993) changes the route of internalization and the particles are taken up by the caveolae vesicles rather than endosomes which lack nucleases and release more readily their content to the interior of the cell compared with endosomes.

Use of pH-sensitive liposomes, such as DOPE: cholesterol: oleic acid liposomes (Wang and Huang, 1987; Huang et al, 1987) seem to induce their break-down at the lower pH of the endosomes releasing the DOPE to the endosome; DOPE then induces endosome membrane breakdown and release of its content to the cytoplasm.

Use of nuclear proteins in complex with plasmid DNA encapsulated into true (as opposed to cationic) liposomes has been found to increase transfection efficiency; DNA was rapidly transported into the nuclei and its expression reached a maximum within 6-8h after transfection (Kaneda et al, 1989; Kato et al, 1991). According to this procedure Sendai virus was used to fuse DNA-loaded ganglioside liposomes with protein-containing membrane vesicles purified from red blood cells; cointroduction of HMG-1 protein showed rapid uptake of plasmids by nuclei whereas with BSA in the place of HMG the grains of the in situ hybridization were located in the cytoplasm after 6 h reaching the nucleus only after about 24h (Kaneda et al, 1989).

#### F. Problems and advantages for liposomal delivery of genes

During *in vivo* delivery foreign DNA can be attacked by macrophages, lymphocytes, or other components of the immune system and the vast majority is cleared from blood, intracellular, or other body fluids before it is given the chance to reach the membrane of the cell target; the half-life of naked plasmids injected intravenously into animals is about 5 min (reviewed by Boulikas, 1998a). On the other hand "stealth" liposomes persist in the body fluids for days as seen in the whole body scintigraphs of empty radioactively-labeled "stealth" liposomes (Figures 17-19); the encapsulated plasmid DNA would also circulate for long times. However, tumor cells, where the "stealth" liposomes are localized are very often of epithelial origin (brain, colon, breast, head & neck, prostate tumors) and not engaged in the uptake of liposomes or other colloidal particles. Thus, stealth liposomes remain in the extracellular space and slowly releasing their material after lysis over days. One strategy to circumvent this bottleneck would be to include cationic lipids in the lipid bilayer and devise methods for PEG coating to fall off; the cationic lipids are then expected to mediate rapid poration through the cell membrane or uptake, as cationic lipids are known to penetrate rapidly most types of cells.

The elimination of therapeutically important cells from the body by the immune system due to expression of viral proteins after *ex vivo* delivery of genes with recombinant adenovirus (Dai et al, 1995) would not apply to liposomal delivery of genes.

#### IX. Clinical trials using liposome-mediated gene transfer

All RAC-approved protocols for gene transfer to human patients with liposomes use cationic lipids (Table 4). Because of the toxicity of cationic lipids none of these protocols employs systemic intravenous injection of the cationic liposome-plasmid complex; instead the type of administration involves (i) direct intratumoral injection for immunotherapy of melanoma, lymphoma, renal carcinoma and a great variety of metastatic malignancies; (ii) subcutaneous injection for glioblastoma antisense-IGF therapy, or for immunotherapy of melanoma, colon with hepatic metastases, renal, breast, and small cell lung cancer; (iii) intranasal delivery for CFTR and alpha-1-antitrypsin deficiency; (iv) intradermal injection for the immunotherapy of ovarian cancer and advanced or metastatic prostate cancer using *IL-2* cDNA; and (v) intraperitoneal and intrapleural delivery of the adenoviral *E1A* gene to control the regulation of the *HER-2/neu* oncogene in ovarian and breast cancer (Table 4).

## X. Prospects

To-date, only very few conventional (radiation/chemotherapy) regimens on cancer patients lead to tumor eradication. Most treatments, after cancer is detected at an advanced stage, prolong the life of the patient by few months but reduce its quality because of the adverse effects of antineoplastic treatments. Gene therapy offers strong hopes to millions of desperate people, both patients and relatives.

Although "stealth" liposomes extravasate preferentially in solid tumors, they remain in the extracellular space and are not readily internalized by tumor cells; liposomes loaded with doxorubicin concentrate the drug in the solid

tumor and release their content over a period of several days. Use of substance P, a peptide known to increase vascular permeability, increased liposome extravasation in tissues that possess receptors for substance P (trachea, esophagus, and urinary bladder); extravasated liposomes remained in the extracellular space beyond the postcapillary venular endothelium at early times. The colloidal gold particles encapsulated into liposomes were localized intracellularly in both endothelial cells and macrophages at 24 h postinjection (Rosenecker et al, 1996).

**Table 4. RAC-approved human gene therapy protocols using liposomes**

(For clinical trials using viruses see **Appendix 1** in Boulikas, 1998, pages 159-172 of this volume).

	<b>Protocol number &amp; human disease</b>	<b>Gene and route of administration</b>	<b>Investigators/ Affiliation</b>	<b>Title of protocol &amp; date of RAC/NIH approval</b>
	9202-013 (Closed) Gene Therapy /Phase I /Cancer /Melanoma /Adenocarcinoma / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DC-Chol /HLA-B7 /Beta-2 Microglobulin cDNA /Intratumoral / <b>Direct Injection</b> /Catheter Delivery to Pulmonary Nodules	Nabel, Gary J.; University of Michigan, Ann Arbor, Michigan	Immunotherapy of Malignancy by In Vivo Gene Transfer into Tumors. RAC Approval: 2-10-92 /NIH Approval: 4-17-92 Closed: 11-19-92 (Replaced by Protocol #9306-045)
	9306-045 (Open) Gene Therapy /Phase I /Cancer / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /HLA-B7 /Beta-2 Microglobulin cDNA / Intratumoral / <b>Direct Injection</b> /Catheter Delivery to Pulmonary Nodules	Nabel, Gary J.; University of Michigan Medical Center, Ann Arbor, Michigan	Immunotherapy for Cancer by Direct Gene Transfer into Tumors. RAC Approval: 6-7-93 /NIH Approval: 9-3-93
1.	9306-052 (Open) Gene Therapy /Phase I /Cancer / Glioblastoma /Antisense	<b>In Vitro</b> /Autologous Tumor Cells /Lethally Irradiated / Cationic Liposome Complex /Lipofectin (Gibco BRL) /Insulin-like Growth Factor Antisense / <b>Subcutaneous Injection</b>	Ilan, Joseph; Case Western Reserve University School of Medicine and University Hospitals of Cleveland, Cleveland, Ohio	Gene Therapy for Human Brain Tumors Using Episome-Based Antisense cDNA Transcription of <b>Insulin-Like Growth Factor I</b> . RAC Approval: 6-8-93 /NIH Approval: 12-2-93
2.	9309-053 (Open) Gene Therapy /Phase I /Cancer / Small Cell Lung Cancer / <b>Immunotherapy</b>	<b>In Vitro</b> /Autologous Tumor Cells /Lethally Irradiated / Cationic Liposome Complex /Lipofectin (Gibco BRL) /Cytokine / <b>Interleukin-2 cDNA</b> /Neomycin Phosphotransferase cDNA / <b>Subcutaneous Injection</b>	Cassileth, Peter; Podack, Eckhard R.; Sridhar, Kasi; University of Miami; and Savaraj, Niramol; Miami Veterans Administration Hospital, Miami, Florida	Phase I Study of Transfected Cancer Cells Expressing the Interleukin-2 Gene Product in Limited Stage Small Cell Lung Cancer. RAC Approval: 9-9-93 /NIH Approval: 12-2-93
3.	9312-063 (Open) Gene Therapy /Phase I /Cancer /Melanoma / <b>Immunotherapy</b>	<b>In Vitro</b> /Allogeneic Tumor Cells /Lethally Irradiated /Cationic Liposome Complex /Lipofectin (Gibco BRL) /B7 (CD80) cDNA /Neomycin Phosphotransferase cDNA / <b>Subcutaneous Injection</b>	Sznol, Mario; National Institutes of Health, Frederick, Maryland	A Phase I Trial of B7-Transfected Lethally Irradiated Allogeneic Melanoma Cell Lines to Induce Cell Mediated Immunity Against Tumor-Associated Antigens Presented by HLA-A2 or HLA-A1 in Patients with Stage IV Melanoma. RAC Approval: 12-3-93 /NIH Approval: 4-19-94
4.	9312-064 (Closed) Gene Therapy /Phase I /Cancer /Colon /Hepatic Metastases / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL-1005 /HLA-B7 /Beta-2 Microglobulin cDNA /Intratumoral /Hepatic Injection	Rubin, Joseph; Mayo Clinic, Rochester, Minnesota	Phase I Study of Immunotherapy of Advanced Colorectal Carcinoma by Direct Gene Transfer into Hepatic Metastases. <b>Sponsor:</b> Vical, Incorporated RAC Approval: 12-3-93 /NIH Approval: 4-19-94 Closed: 3-16-95
5.	9312-066 (Open) Gene Therapy /Phase I /Monogenic Disease	In Vivo /Nasal Epithelial Cells /Cationic Liposome Complex /DMRIE-DOPE /Cystic Fibrosis	Sorscher, Eric J. and Logan, James L.; University of Alabama, Birmingham,	Gene Therapy for Cystic Fibrosis Using Cationic Liposome Mediated Gene Transfer: A Phase I Trial of Safety and

	<b>/Cystic Fibrosis</b>	Transmembrane Conductance Regulator cDNA /Intranasal	Alabama	Efficacy in the Nasal Airway. RAC Approval: 12-3-93 /NIH Approval: 1-4-95
6.	9403-070 (Open) Gene Therapy /Phase I /Monogenic Disease / <b>Alpha-1-Antitrypsin Deficiency</b>	In Vivo /Nasal Epithelial Cells /Respiratory Epithelial Cells /Cationic Liposome Complex /DC-Chol-DOPE /Alpha-1 Antitrypsin cDNA /Intranasal /Respiratory Tract Administration (Bronchoscope)	Brigham, Kenneth; Clinical Research Center at Vanderbilt University Medical Center, Nashville, Tennessee	Expression of an Exogenously Administered Human Alpha-1-Antitrypsin Gene in the Respiratory Tract of Humans. <b>Sponsor:</b> Gene Medicine, Inc. RAC Approval: 3-3-94 /NIH Approval: 10-25-94
7.	9403-071 (Closed) Gene Therapy /Phase I /Cancer /Renal Cell / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL-1005 /HLA-B7 /Beta-2 Microglobulin cDNA /Intratumoral / <b>Direct Injection</b>	Vogelzang, Nicholas; the University of Chicago, Chicago, Illinois	Phase I Study of Immunotherapy for Metastatic Renal Cell Carcinoma by Direct Gene Transfer into Metastatic Lesions. <b>Sponsor:</b> Vical, Incorporated RAC Approval: 3-4-94 /NIH Approval: 4-19-94 Closed: 4-5-95
8.	9403-072 (Closed) Gene Therapy /Phase I /Cancer /Melanoma / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL-1005 /HLA-B7 /Beta-2 Microglobulin cDNA /Intratumoral / <b>Direct Injection</b>	Hersh, Evan; Arizona Cancer Center, Tucson, Arizona; and Akporiaye; Harris; Stopeck; Unger; and Warneke; University of Arizona, Tucson, Arizona	Phase I Study of Immunotherapy of Malignant Melanoma by Direct Gene Transfer. <b>Sponsor:</b> Vical, Incorporated RAC Approval: 3-4-94 /NIH Approval: 4-19-94
9.	9409-086 (Open) Gene Therapy /Phase I /Cancer /Breast / <b>Immunotherapy</b>	<u>In Vitro</u> /Autologous Tumor Cells /Lethally Irradiated /Cationic Liposome Complex /Avectin™ /Cytokine / <b>Interleukin-2 cDNA</b> / <b>Subcutaneous Injection</b>	Lyerly, H. Kim; Duke University Medical Center, Durham, North Carolina	A Pilot Study of Autologous Human Interleukin-2 Gene Modified Tumor Cells in Patients with Refractory or Recurrent Metastatic Breast Cancer. RAC Approval: 9-12-94 /NIH Approval: 10-25-94
10.	9412-095 (Open) Gene Therapy /Phase I /Solid Tumors /Lymphoma / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL-1102 /Cytokine / <b>Interleukin-2 cDNA</b> /Intratumoral / <b>Direct Injection</b>	Hersh, Evan; Arizona Cancer Center, Tucson, Arizona; and Rinehart, John; Scott and White Clinic; Temple Texas.	Phase I Trial of Interleukin-2 Plasmid DNA /DMRIE /DOPE Lipid Complex as an Immunotherapeutic Agent in Solid Malignant Tumors or Lymphomas by Direct Gene Transfer. <b>Sponsor:</b> Vical, Incorporated RAC Approval: 12-1-94 /NIH Approval: 3-2-95
11.	9506-108 (Open) Gene Therapy /Phase I /Cancer /Renal Cell /Melanoma / <b>Immunotherapy</b>	<u>In Vitro</u> /Autologous Tumor Cells /Lethally Irradiated /Cationic Liposome Complex /DMRIE-DOPE Vical VCL-1005 /HLA-B7 /Beta-2 Microglobulin cDNA / <b>Subcutaneous Injection</b>	Fox, Bernard A. and Urba, Walter J.; Earle A. Childs Research Institute, Providence Medical Center, Portland, Oregon	Adoptive Cellular Therapy of Cancer Combining Direct HA-B7 /β-2 Microglobulin Gene Transfer with Autologous Tumor Vaccination for the Generation of Vaccine-Primed Anti-CD3 Activated Lymphocytes. RAC Approval: 6-9-95 /NIH Approval: 9-30-95
12.	9506-110 (Open) Gene Therapy /Phase I /Cancer /Ovarian / <b>Immunotherapy</b>	<u>In Vitro</u> /Autologous Tumor Cells /Lethally Irradiated /Cationic Liposome Complex /DDAB-DOPE /Cytokine / <b>Interleukin-2 cDNA</b> / <b>Intradermal Injection</b>	Berchuck, Andres and Lyerly, H. Kim; Duke University Medical Center, Durham, North Carolina	A Phase I Study of Autologous Human Interleukin-2 (IL-2) Gene Modified Tumor Cells in Patients with Refractory Metastatic Ovarian Cancer. RAC Approval: 6-10-95 /NIH Approval: 9-30-95
13.	9508-115 (Open) Gene Therapy /Phase II /Cancer /Metastatic Malignancies (Breast Adenocarcinoma, Renal Cell Carcinoma, Melanoma, Colorectal Adenocarcinoma, non-Hodgkin's Lymphoma) / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL 1005 /HLA-B7 /Beta-2 Microglobulin cDNA /Direct <b>Intratumoral Injection</b>	Chang, Alfred E.; Univ of Michigan; Hersh, Evan; Arizona Cancer Center; Vogelzang, Nicholas; University of Chicago; Levy, Ronald; Stanford University; Redman, Bruce; Wayne State University; Figlin, Robert; UCLA; Rubin, Joseph; Mayo Foundation; Rinehart, John J.; Scott and White Hospital, Texas A & M University; Doroshow, James H.; City of Hope; Klasa, Richard; British Columbia Cancer Agency; Sobol, Robert; Sidney Kimmel Cancer Center	Phase II Study of Immunotherapy of Metastatic Cancer by Direct Gene Transfer. <b>Sponsor:</b> Vical, Incorporated Sole FDA Review Recommended by NIH /ORDA: 8-2-95
14.	9508-121 (Open) Gene Therapy /Phase	In Vivo /Autologous Tumor Cells /HLA B7	Figlin, Robert A.; University of California Los Angeles	Phase I Study of HLA-B7 Plasmid DNA /DMRIE /DOPE Lipid Complex as an

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I/Cancer/Renal Cell/ <b>Immunotherapy</b>	cDNA/ <b>Intratumoral</b> /Concurrent Interleukin-2 Therapy	Medical Center, Los Angeles, California	Immunotherapeutic Agent in Renal Cell Carcinoma by Direct Gene Transfer with Concurrent Low Dose Bolus IL-2 Protein Therapy. <b>Sponsor:</b> Vical, Incorporated Sole FDA Review Recommended by NIH/ORDA: 8-14-95
15. 9509-127 (Open) Gene Therapy /Phase I /Monogenic Disease / <b>Cystic Fibrosis</b>	In Vivo /Nasal Epithelial Cells /Cationic Liposome Complex /DOPE /Cystic Fibrosis Transmembrane Conductance Regulator cDNA; <b>Intranasal Administration</b>	Welsh, Michael J. and Zabner, Joseph; Howard Hughes Medical Institute, University of Iowa College of Medicine, Iowa City, Iowa	Cationic Lipid Mediated Gene Transfer of CFTR: Safety of a Single Administration to the Nasal Epithelia. <b>Sponsor:</b> Genzyme Corporation Sole FDA Review Recommended by NIH /ORDA: 9-26-95
16. 9510-132 (Open) Gene Therapy /Phase I /Cancer /Locally Advanced or Metastatic Prostate / <b>Immunotherapy</b>	<u>In Vitro</u> /Autologous Tumor Cells /Lethally Irradiated /Cationic Liposome Complex /Cytokine / <b>Interleukin-2 cDNA /Intradermal Injection</b>	Paulson, David; and Lyerly, H. Kim; Duke University Medical Center, Durham, North Carolina	A Phase I Study of Autologous Human Interleukin-2 (IL-2) Gene Modified Tumor Cells in Patients with locally Advanced or Metastatic Prostate Cancer. Sole FDA Review Recommended by NIH /ORDA: 10-19-95
17. 9512-137 (Open) Gene Therapy /Phase I /Cancer /Ovarian, Breast /Oncogene Regulation /HER-2 /neu	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DC-Chol-DOPE /E1A /Intraperitoneal, <b>Intrapleural Administration</b>	Hortobagyi, Gabriel N.; Lopez-Berstein, Gabriel; and Hung, Mien-Chien; MD Anderson Cancer Center, Houston, Texas; Kilbourn, Robert, Rush-Presbyterian /St. Luke's Medical Center, Chicago, Illinois; Weiden, Paul, Virginia Mason Medical Center, Seattle, Washington	Phase I Study of E1A Gene Therapy for Patients with Metastatic Breast or Ovarian Cancer that Overexpresses Her-2 /neu. <b>Sponsor:</b> Targeted Genetics Corporation RAC Approval: 12-4-95 /NIH Approval: 2-2-96
18. 9512-142 (Open) Gene Therapy /Phase I /Gene Therapy /Cancer /Head and Neck Squamous Cell Carcinoma / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL 1005 /HLA-B7 /Beta-2 Microglobulin cDNA /Direct <b>Intratumoral Injection</b>	Gluckman, Jack L.; University of Cincinnati Medical Center, Cincinnati, Ohio	Allovectin-7 in the Treatment of Squamous Cell Carcinoma of the Head and Neck. Sole FDA Review Recommended by NIH /ORDA: 12-15-95
19. 9608-156 (Open) Gene Therapy /Phase I /Cancer /Breast / <b>Immunotherapy</b>	In Vitro /Allogeneic Tumor Cells /Lethally Irradiated /Cationic Liposome Complex /B7(CD80) cDNA / <b>Subcutaneous Injection</b>	Urba, Walter J., Providence Portland Medical Center, Portland, Oregon	Phase I Trial Using a CD80-Modified Allogeneic Breast Cancer Line to Vaccinate HLA-A2-Positive Women with Breast Cancer. Sole FDA Review Recommended by NIH /ORDA: 8-6-96
20. 9609-161 (Open) Gene Therapy /Phase I /Cancer /Small Cell Lung Cancer / <b>Immunotherapy</b>	<u>In Vitro</u> /Autologous Tumor Cells /Lethally Irradiated /Cationic Liposome Complex /Lipofectin(GibcoBRL) /B7-1(CD80) cDNA / <b>Subcutaneous Injection</b>	Antonia, Scott J., H. Lee Moffitt Cancer Center, Tampa, Florida	Treatment of Small Cell Lung Cancer Patients In Partial Remission Or At Relapse With B7-1 Gene-Modified Autologous Tumor Cells As A Vaccine With Systemic Interferon Gamma. Sole FDA Review Recommended by NIH /ORDA: 10-10-96
21. 9610-162 (Open) Gene Therapy /Phase I /Cancer /Solid Tumors /Oncogene Regulation /HER-2 /neu	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DC-Chol-DOPE /E1A / <b>Intratumoral Injection</b>	LaFollette, Suzanne, Rush /Presbyterian /St. Luke's Medical Center, Chicago, Illinois; Murray, James L., M.D. Anderson Cancer Center, Houston, Texas; Yoo, George, Wayne State University, Detroit, Michigan	A Phase I Multicenter Study of Intratumoral E1A Gene Therapy for Patients with Unresectable or Metastatic Solid Tumors that Overexpress HER-2 /neu. <b>Sponsor:</b> Targeted Genetics Corporation Sole FDA Review Recommended by NIH /ORDA: 10-29-96
22. 9611-168 (Open) Gene Therapy /Phase II /Cancer /Melanoma / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL 1005 /HLA-B7 /Beta-2 Microglobulin cDNA /Direct <b>Intratumoral Injection</b>	Hersh, Evan M., Arizona Cancer Center, Tucson, Arizona; Klasa, Richard, British Columbia Cancer Agency, Vancouver, B.C., Canada; Gonzales, Rene, University of Colorado Cancer Center, Denver, Colorado; Silver, Gary, Northern California Melanoma Clinic, San Francisco, California; Thompson, John	Phase II Study of Immunotherapy of Metastatic Melanoma by Direct Gene Transfer. <b>Sponsor:</b> Vical, Incorporated NIH /ORDA Receipt Date: 11-26-96. Sole FDA Review Recommended by NIH /ORDA: 1-6-97

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| 23. 9611-169 (Open) Gene Therapy /Phase I /II /Cancer /Solid Tumors / <b>Immunotherapy</b>                             | In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL 1102 /Cytokine / <b>Interleukin-2 cDNA</b> /Direct <b>Intratumoral Injection</b> | A.,U. of Washington Medical Center, Seattle, Washington   | Phase I/II Trial of Interleukin-2 DNA /DMRIE /DOPE Lipid Complex as an Immunotherapeutic Agent in Cancer by Direct Gene Transfer.  |
|  |   | Hersh, Evan, M., Arizona Cancer Center, Tucson, Arizona; Rinehart, John, Scott and White Clinic, Temple, Texas; Rubin, Joseph, Mayo Clinic, Rochester, Minnesota; Sondak, Vernon K., University of Michigan Medical Center, Ann Arbor, Michigan; Gonzales, Rene, University of Colorado Cancer Center, Denver, Colorado; Sobol, Robert E., Sharp HealthCare, San Diego, California; and Forscher, Charles A., Cedars-Sinai Comprehensive Cancer Center, Los Angeles, California | <b>Sponsor:</b> Vical, Incorporated NIH /ORDA Receipt Date: 11-26-96. Sole FDA Review Recommended by NIH /ORDA: 1-17-97  |
| 24. 9612-170 (Open) Gene Therapy /Phase I /Monogenic Disease / <b>Cystic Fibrosis</b>                                  | In Vivo /Lung and Nasal Epithelial Cells /Cationic Liposome Complex /DOPE /CFTR cDNA / <b>Aerosol Administration</b>  | Sorscher, Eric, University of Alabama, Birmingham, Medical Center   | Safety and Efficiency of Gene Transfer of Aerosol Administration of a Single Dose of a Cationic Lipid /DNA Formulation for the Lungs and Nose of Patients with Cystic Fibrosis.  |
|  |   |   | <b>Sponsor:</b> Genzyme Corporation NIH /ORDA Receipt Date: 12-17-96. Sole FDA Review Recommended by NIH /ORDA: 1-6-97   |
| 25. 9703-184 (Open) Gene Therapy /Phase I /Cancer /Prostate Cancer / <b>Immunotherapy</b>                              | In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL-1102 /Cytokine / <b>Interleukin-2 cDNA</b> / <b>Intratumoral Injection</b>       | Belldegrun, Arie, University of California, Los Angeles, School of Medicine, Los Angeles, California  | A Phase I Study Evaluating the Safety and Efficacy of Interleukin-2 Gene Therapy Delivered by Lipid Mediated Gene Transfer (Leuvectin) in Prostate Cancer Patients.  |
|  |   |   | <b>Sponsor:</b> Vical, Inc. NIH /ORDA Receipt Date: 3-24-97. Sole FDA Review Recommended by NIH /ORDA: 5-21-97   |
| 26. 9704-186 (Open) Gene Therapy /Phase I /Monogenic Disease / <b>Cystic Fibrosis</b>                                  | In Vivo /Nasal Epithelial Cells /Cystic Fibrosis Transmembrane Conductance Regulator cDNA /Cationic Liposome Complex /EDMPC / <b>Intranasal Administration</b>    | Noone, Peadar G., Knowles, Michael R., University of North Carolina at Chapel Hill, North Carolina  | A Double-Blind, Placebo Controlled, Dose Ranging Study to Evaluate the Safety and Biological Efficacy of the Lipid-DNA Complex GR213487B in the Nasal Epithelium of Adult Patients with CysticFibrosis.  |
|  |   |   | <b>Sponsor:</b> Glaxo Wellcome Inc. NIH /ORDA Receipt Date: 4-23-97. Sole FDA Review Recommended by NIH /ORDA: 5-13-97   |
| 27. 9705-190 (Open) Gene Therapy /Phase I /Cancer /Squamous Cell Carcinoma of the Head and Neck / <b>Immunotherapy</b> | In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DOTMA-Cholesterol /Cytokine / <b>Interleukin-2 cDNA</b> / <b>Intratumoral Injection</b>               | O'Malley, Bert W., Johns Hopkins Medical Institutions, Baltimore, Maryland  | A Double-Blind, Placebo-Controlled, Single Rising-Dose Study of the Safety and Tolerability of Formulated hIL-2 Plasmid in Patients with Squamous Cell Carcinoma of the Head and Neck (SCCHN). Sponser: Gene Medicine, Inc. NIH /ORDA Receipt Date: 5-27-97. Sole FDA Review Recommended by NIH /ORDA: 6-16-97 |
|  |   |   | Phase II Study of Immunotherapy by Direct Gene Transfer with Allovectin-7 for the Treatment of Recurrent or Metastatic Squamous Cell Carcinoma of the Head and Neck  |
| 28. 9706-191 (Open) Gene Therapy /Phase II /Cancer /Head and Neck Squamous Cell Carcinoma / <b>Immunotherapy</b>       | In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE /Vical VCL-1005 /HLA-B7 /Beta-2 Microglobulin cDNA /Direct <b>Intratumoral Injection</b>   | Gluckman, Jack L., Gleich, Lyon L., University of Cincinnati Medical Center, Cincinnati, Ohio; Swinehart, James M., Colorado Medical Research Center, Denver, Colorado; Hanna, Ehab, University of Arkansas for Medical Sciences /Arkansas Cancer Research Center (UAMS), Little Rock,  | <b>Sponsor:</b> Vical, Inc. NIH /ORDA Receipt Date: 6-6-97. Sole FDA Review Recommended by NIH /ORDA: 7-7-97   |

		Arkansas; Castro, Dan J., University of California, Los Angeles, Los Angeles, California; Gapany, Markus, Veterans Affairs Medical Center, Minneapolis, Minnesota; Carroll, William, R., University of Alabama at Birmingham, Birmingham, Alabama; and Coltrera, Marc D., University of Washington Medical Center, Seattle, Washington	
29.	9709-210 (Open) Gene Therapy /Phase I-II /Cancer /Melanoma / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE /Vical VCL-1005 /HLA-B7 /b2-Macroglobulin cDNA /Direct <b>Intratumoral Injection</b>	Gonzales, Rene; University of Colorado Cancer Center, Denver, Colorado and Hersh, Evan; Arizona Cancer Center, Tucson, Arizona  Compassionate Use Protocol for Retreatment with Allovectin-7 Immunotherapy for Metastatic Cancer by Direct Gene Transfer  <b>Sponsor:</b> Vical, Inc. NIH /ORDA Receipt Date: 9-8-97. Sole FDA Review Recommended by NIH /ORDA: 9-26-97
30.	9708-211 (under review) Gene Therapy /Phase I /Monogenetic Disease / <b>Canavan Disease</b>	In Vivo /Autologous Brain Cells /Plasmid DNA /Adeno-associated Virus /Poly-L-Lysine /Cationic Liposome Complex /DC-Chol /DOPE /Aspartoacylase cDNA / <b>Intracranial</b> (Ommaya Reservoir) <b>Administration</b>	During, Matthew, J.; University of Auckland, New Zealand; Leone, Paola; and Seashore, Margretta, R.; Yale University, New Haven, Connecticut  Gene Therapy of Canavan Disease: Retreatment of Previously Treated Children NIH /ORDA Receipt Date: 8-28-97.
31.	9709-212 (Open) Gene Therapy /Phase I /Cancer /Melanoma / <b>Immunotherapy</b>	In Vivo /Autologous Tumor Cells /Cationic Liposome Complex /DMRIE-DOPE Vical VCL-1005 /HLA-B7 /Beta-2 Microglobulin cDNA /Vical-1102 / <b>Interleukin-2 cDNA /Intratumoral Injection</b>	Gonzales, Rene; University of Colorado Health Sciences Center, Denver, Colorado; and Hersh, Evan M.; Arizona Cancer Center, Tucson, Arizona  Phase I Study of Direct Gene Transfer of HLA-B7 Plasmid DNA /DMRIE /DOPE Lipid Complex (Allovectin-7) with IL-2 Plasmid DNA /DMRIE /DOPE Lipid Complex (Leuvectin) as an Immunotherapeutic Regimen in Patients with Metastatic Melanoma  <b>Sponsor:</b> Vical, Inc. NIH /ORDA Receipt Date: 9-18-97. Sole FDA Review Recommended by NIH /ORDA: 10-8-97
32.	9711-222 (under review) Gene Therapy /Phase I /Monogenetic Disease / <b>Canavan Disease</b>	In Vivo /Autologous Brain Cells /Plasmid DNA /Adeno-Associated Virus /Protamine /Cationic Liposome Complex /DC-Cholesterol-DOPE /Aspartoacylase cDNA / <b>Intracranial</b> (Ommaya Reservoir) <b>Administration</b>	Freese, Andrew; Thomas Jefferson University, Philadelphia, Pennsylvania  Gene Therapy of Canavan Disease NIH /ORDA Receipt Date: 11-12-97.

A breakthrough would be the encapsulation of plasmids with therapeutic genes (e.g. antitumor genes such as p53, HSV-tk, angiostatin) into Stealth liposomes with mechanisms inducing PEG fall off after their accumulation into tumors; when a certain amount of cationic liposomes is included into the lipid composition of the Stealth liposome, the PEG-free liposome is expected to be taken rapidly by tumor cells. Fusogenic peptides or other strategies could be combined to release the liposomes from endosomes. These regimens could be combined with nontoxic levels of antineoplastic drugs encapsulated into Stealth liposomes that might act synergistically with the tumor suppressor or tumor killer genes to eradicate cancer. The major advantage, no doubt, would be the systemic delivery of genes with this approach, able to hit, like no other gene therapy regimen currently available, the primary tumor and its metastases.

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