6

"Diffusible-PEG-Lipid Stabilized Plasmid Lipid Particles"

Ian MacLachlan* **and Pieter Cullis*,†**

*Protiva Biotherapeutics Incorporated, Burnaby, BC, Canada V5G 4Y1
†Inex Pharmaceuticals Inc., Burnaby, BC, Canada V5J 5J8

ABSTRACT

Many viral and non-viral gene transfer systems suffer from common pharmacological issues that limit their utility in a systemic context. By application of the liposomal drug delivery paradigm, many of the limitations of the first generation non-viral delivery systems can be overcome. Encapsulation in small, long-circulating particles called stabilized plasmid lipid particles (SPLP) results in enhanced accumulation at disease sites and selective protein expression. This work compares the detergent dialysis method of SPLP manufacture with an alternative method, spontaneous vesicle formation by ethanol dilution. The

Advances in Genetics, Vol. 53
0065-2660/05 $35.00
DOI: 10.1016/S0065-2660(05)53006-2

pharmacology of SPLP, as determined by monitoring lipid label and quantitative real time PCR, is also presented.

I. INTRODUCTION

Current efforts in gene transfer research focus on the development of genetic drugs capable of treating acquired diseases such as cancer, inflammation, viral infection or cardiovascular disease. The disseminated nature of these diseases requires the development of vector systems capable of accessing distal sites following systemic or intravenous administration. Unfortunately, most vectors have limited utility for systemic applications. Viral vectors, for example, are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver and spleen. In addition, many viruses induce immune responses that compromise potency upon subsequent administration. In the case of most non-viral vectors such as plasmid DNA-cationic lipid complexes (lipoplexes), the large size and positively charged nature of these systems also results in rapid clearance upon systemic administration with the highest expression levels observed in first-pass organs, particularly the lungs (Huang and Li, 1997; Hofland *et al.*, 1997; Templeton *et al.*, 1997; Thierry *et al.*, 1995). In addition, lipoplexes often give rise to significant toxicities both *in vitro* and *in vivo* (Harrison *et al.*, 1995; Li and Huang, 1997; Tousignant *et al.*, 2000, 2003). In spite of these limitations, non-viral gene transfer systems offer specific clinical and commercial advantages as therapeutics. Because non-viral systems use synthetic or highly purified components, they are chemically defined and free of adventitious agents. Non-viral systems can be manufactured under controlled conditions, relatively unconstrained by the biological considerations that define the scale-up of viral production in mammalian cell culture. These advantages have encouraged a number of investigators to focus on the development of non-viral gene transfer systems that have utility in a systemic context (Dzau *et al.*, 1996; Li and Huang, 1997; Templeton *et al.*, 1997; Wheeler *et al.*, 1999; Zhu *et al.*, 1993). Here we will describe one system that specifically attempts to address the inability of current vector systems to overcome the first barrier to systemic gene delivery, delivery to the disease site and the target cell.

II. PROPERTIES OF A PLASMID DELIVERY SYSTEM FOR THE TREATMENT OF SYSTEMIC DISEASE

A. Definition of an appropriate vector

We propose the following definition of an ideal carrier for systemic gene transfer: The ideal vector will (i) be safe and well tolerated upon systemic administration;

(ii) have the appropriate pharmacokinetic attributes to ensure delivery to disseminated disease sites; (iii) deliver intact DNA to target tissue and mediate transfection of that tissue; (iv) be non-immunogenic; and (v) be stable upon manufacture to facilitate production at commercial scale with uniform, reproducible performance specifications.

Gene-based drugs must maximize the benefit to patient health while minimizing the risks associated with treatment. Accordingly, gene transfer systems must be safe and well tolerated. Attempts to bypass the inherent pharmacology of a given vector by invoking elaborate or invasive treatment methodologies are likely to result in an increased, potentially unacceptable, risk to the patient. Methods such as 'hydrodynamic injection' or direct portal vein infusion may continue to generate exciting preclinical results, but translation of these methods to a clinical setting will be limited. Gene-based drugs will be adopted more readily if they can be delivered in a manner analogous to conventional medicines, for example by intravenous injection or in oral form.

The toxicity associated with systemic administration of poorly tolerated compounds is exacerbated by accumulation in non-target tissue and can be reduced by optimizing delivery to the target site. In the case of gene-based drugs, 'delivery' is determined by physical and biochemical properties including stability, size, charge, hydrophobicity, interaction with serum proteins and non-target cell surfaces, as well as the mechanism of action of the nucleic acid payload. In the context of a disease site, effective delivery requires that a vector overcome obstacles associated with heterogeneous cell populations that are often proliferating rapidly, at different stages of the cell cycle and not conforming to the patterns of organization established during the development of normal tissue. As demonstrated in this work, these challenges, and other potential barriers to transfection, can represent opportunities for conferring a degree of selectivity greater than that associated with the use of conventional therapeutics.

B. Overcoming the barriers to transfection

The barriers to transfection include the pharmacological barriers inhibiting delivery to the target cell, and the intracellular barriers that inhibit nuclear delivery and expression of the plasmid DNA construct. An effective delivery system must be able to confer stability to the nucleic acid payload in the blood despite the presence of serum nucleases and membrane lipases. Systemic delivery requires the use of a 'stealthy' delivery system, since indiscriminate interaction with blood components, lipoproteins or serum opsonins, can cause aggregation before the carrier reaches the disease site. This is especially important in the case of systemic delivery systems containing large polyanionic molecules such as plasmid DNA, which have a greater potential for inducing toxicity through interaction with complement and coagulation pathways (Chonn *et al.*, 1991).

Other barriers to gene delivery may include the microcapillary beds of the "first pass" organs, the lung and the liver, and the phagocytic cells of the reticuloendothelial system. Accessing target cell populations requires extravasation from the blood compartment to the disease site. Carriers of appropriate size can pass through the fenestrated epithelium of tumor neovasculature and accumulate at the tumor site via the "enhanced permeation and retention" (EPR) effect (Mayer *et al.*, 1990), also referred to as "passive" targeting or "disease site" targeting. In order to take advantage of the EPR effect, which can result in accumulation of up to 10% of the injected dose per gram of tumor tissue, the gene carriers must be small (diameter on the order of 100 nm) and long-circulating (circulation lifetimes of 5 h or more following intravenous injection in mice). Clearly, nucleic acids require pharmaceutical enablement in the form of appropriate carriers that confer: protection from degradation, an extended circulation lifetime, appropriate biodistribution and delivery facilitation of the nucleic acid payload to the disease site.

While delivery of intact plasmid DNA to a target cell is a *prerequisite*, it in no way *guarantees* transfection. Once at the cell surface, vectors are confronted with a number of physical and biochemical barriers, each of which must be overcome in order to effect transfection and transgene expression. The first physical barrier to transfection is the plasma membrane, protected by the carbohydrate coating, or glycocalyx, formed by the post-translational glycosylation of transmembrane proteins. Although early models of lipid-mediated transfection invoked a putative fusion event between the plasma membrane and the membrane of the lipid vesicle, it is now generally agreed that the majority of intracellular delivery occurs through endocytosis.

Endocytosis is a complex process by which cells take up extracellular material. This occurs through a number of discrete pathways, reviewed elsewhere in this volume. While there is some evidence that non-viral vectors may be taken up by caveolae, syndecan-mediated endocytosis or other clathrin-independent pathways, the classical endocytic pathway involves the activity of cell surface clathrin-coated pits, invaginations in the plasma membrane that are subsequently pinched off into the cytoplasm (Goldstein *et al.*, 1985). When this occurs, internalized material remains trapped on the exoplasmic side of the internalized vesicle, without direct access to the cytoplasm or the nucleus. Endocytic vesicles undergo a series of biochemical changes that represent escape opportunities for a non-viral vector. The first such change occurs within 5 min of uptake as internalized vesicles form the early endosome containing the "Compartment of Uncoupling of Receptor and Ligand" (CURL) (Geuze *et al.*, 1983). Early endosomes are transiently fusogenic (Dunn and Maxfield, 1992) with a pH close to that of the exoplasm, while late endosomes have a significantly lower luminal pH (Murphy *et al.*, 1993). As endosomes mature to form lysosomes they experience a further decrease in internal pH and an increase in fusogenicity.

Although the process of clatharin-dependent endocytosis has been well characterized, the processing and release of internalized non-viral vectors or their DNA payload is not well understood. Even less clear is the relative import of clathrin-independent uptake through mechanisms that share some, but not all of the features of the classical pathway. Improvements in our understanding of these alternative pathways, and their role in non-viral gene transfer, will be important for the rational design of more effective intracellular delivery strategies for non-viral vectors.

Following uptake, plasmid DNA spends some indeterminate residency time in the cytoplasm prior to gaining entry to the nucleus. Unlike viral systems that have evolved specific mechanisms to traverse this barrier, untargeted non-viral vectors rely on diffusion to facilitate interaction with the nuclear envelope (Kopatz *et al.*, 2004). However the cytoplasm, rather than an empty space, is a highly organized compartment containing networks of cytoskeletal elements and membrane-bound organelles that have the potential to interact with and accumulate vector systems that arrive at the cytosol intact. When plasmid DNA is delivered by direct microinjection into the cytosol of mammalian cells it is rapidly degraded by divalent-cation-dependent cytosolic nucleases (Howell *et al.*, 2003; Lechardeur *et al.*, 1999). This has implications for vector design. Vector systems that either protect the DNA payload from degradation following endosome release or effectively minimize the cytoplasmic residency time are to be expected to yield improved transfection efficiencies.

The final physical barrier to transfection is delivery to the nucleus. The nucleus has evolved as a means of organizing, isolating and protecting the genome of eukaryotic cells from adventitious agents such as viruses or transposons. The nuclear uptake of DNA is limited by the presence of an intact nuclear envelope and as such non-viral transfection is considerably more efficient in highly mitotic cells (Mortimer *et al.*, 1999; Wilke *et al.*, 1996). Strategies to overcome this barrier to transfection take one of two forms: either targeting transfection reagents to cell populations with a high degree of mitotic activity, such as tumor tissue; or enhancing the low level of transfection that occurs in quiescent cells by using either nuclear targeting technologies or condensing agents that compact plasmid DNA to a size more amenable to uptake through the nucleopore complex (Blessing *et al.*, 1998; Sebestyen *et al.*, 1998).

C. Proposed mechanism of stabilized plasmid lipid particle mediated transfection

1. Delivery to the target cell

The demands imposed upon vectors used for systemic applications are conflicting. First, the carrier must be stable and long-circulating, circulating long enough to facilitate accumulation at disease sites via the EPR effect.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.