Cystic Fibrosis Gene Therapy: Methods for the optimisation of CFTR gene delivery.

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1 Introduction

1.1   Cystic Fibrosis: A Historical Perspective

Cystic fibrosis (CF) is one of the most common lethal autosomal recessive disorders, affecting approximately 1 in 2000 live births. CF is a disease affecting multiple organ systems including the lung and upper respiratory tract, gastrointestinal tract, pancreas, liver, sweat glands and the genitourinary tract [1].

One of the first references to CF is found in European folklore which says: "Woe to that child who when kissed in the forehead tastes salty. They are bewitched and soon will die" [2]. This observation refers to the salty sweat of CF patients, and its importance is still recognised today in the form of the gold-standard diagnostic test for CF, the sweat test.

The importance of CF as a genetic disease means that it has been intensively studied. Over the years there have been many breakthroughs in the understanding of CF, and it has also often provided a model for our understanding of inherited disorders in general.

Typical symptoms, now known to be related to CF, have been recorded since 1650. Meconium ileus relating to exocrine pancreatic failure was described by Landsteiner in 1905 [3]. In 1938 Dorothy Andersen reported the first systematic study of CF, describing the clinical, anatomical, pathological and epidemiological characteristics of the disease. The study, which involved 49 CF patients, showed neonatal deaths resulting from intestinal obstruction and childhood deaths as a result of respiratory failure [4]. In 1945 CF was described as mucoviscidosis, a term still used today, describing the disease generally as a 'state of thickened mucus' [5]. Since these early observations, intensive study has revealed many more details regarding all aspects of CF.

1.2   Molecular Genetics of CF

The gene involved in CF had been mapped to chromosome 7q31.2 by 1985 and was isolated by positional cloning in 1989 by Drs. Lap-Chee Tsui, John Riordan and Francis Collins, and was positively characterised by the identification of mutations in CF patients. The gene product was named the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR).

The CFTR gene is composed of at least 27 exons and covers approximately 250 kb of DNA with the gene being transcribed into an mRNA of 6.5 kb. The protein encoded by the mRNA contains exactly 1480 amino acids and has a predicted molecular mass of 168 kDa. Analysis suggests the presence of 12 transmembrane segments, clustered in two groups, and shows that CFTR belongs to the ATP binding cassette transporter superfamily of proteins [6] (See Figure 1-1).

1.3   CFTR

1.3.1   The gene

The expression of CFTR appears highly regulated with CFTR expression confined to certain tissues, where it displays cell type specificity-specifically, epithelial cells [7]. The full length CFTR mRNA transcript is derived from the splicing of 27 exons.

1.3.2   The protein

The product of the CFTR gene- the CFTR protein- belongs to a family of transmembrane proteins called adenosine triphosphate (ATP) binding cassette transporters, with the primary function of CFTR being as an apical Cl^- secretory channel. As its name suggests, CFTR also regulates other ion channels and is itself regulated by cAMP [8, 9].

NOTE:
This figure is included on page 4
of the print copy of the thesis held in
the University of Adelaide Library.

Figure 1-1: Structure of the CFTR protein

Model showing the proposed domain structure of CFTR including the 12 membrane spanning domains, two nucleotide-binding-domains and regulatory domains [10].

ABC transporters contain two nucleotide-binding domains and two membrane spanning domains. Nucleotide binding domains bind and hydrolyse ATP using the energy for active transmembrane substrate transport and/or to produce conformational changes in the membrane spanning domains (MDSs). The MSDs share little if any sequence homology between unrelated family members and confer specific transport functions on individual ABC transporters. The nucleotide binding domains can then be considered as conserved engines that power a wide variety of different machinery including pumps, regulators and in the case of CFTR- a channel, with ATP hydrolysis regulating channel gating. The regulatory domain of CFTR is unique among the ABC transporters and contains multiple phosphorylation sites and charged amino acids. Phosphorylation of the regulatory domain regulates channel activity.

CFTR gene expression levels can be modulated by cAMP, PKA, PKC and phorbol esters [11].

The regulation of the CFTR gene is not fully understood, and it appears to operate on many levels. It has been reported that phorbol 12-myristate 12-acetate (PMA), pharmacological agents that mobilise intracellular calcium and tumor necrosis factor-α (TNF- α) down regulate CFTR gene expression in epithelial cells.

TNF- α has been shown to down regulate CFTR gene expression post-transcriptionally by destabilising the transcripts, where as PMA - in addition to its transcriptional action- regulates the cellular CFTR content by altering the stability of the protein *via* activation of protein kinase C (PKC). PKC activation by PMA has been reported not only to modulate CFTR gene transcription but also to shorten the half life of the CFTR protein [12].

Interferon gamma (IFN-γ) has also been shown to down regulate CFTR. IFN- γ reduces the amount of CFTR protein and cAMP- dependant Cl- fluxes, suggesting that CFTR function is diminished. IFN- γ down

regulates CFTR gene expression at the post-transcriptional level since it does not affect the transcription rate. It also acts at least in part, by destabilising CFTR mRNA, reminiscent of the effect of TNF-α [12].

Up regulation of CFTR gene expression has been observed with estrogen and other pharmalogical agents that increase intracellular cAMP (eg. Forskolin) enhance CFTR gene expression in the female reproductive tract [12].

CFTR also acts as a regulator of other membrane proteins, with one such protein being the outwardly rectifying Cl^- channel (ORCC). The principal abnormality of the ORCC in CF cells is that cAMP agonists fail to activate ORCC channels. How CFTR regulates these channels is not known, however, studies suggest that NBD1 and the R domain are required for ORCC regulation. CFTR also regulates the release of cellular ATP with expression of CFTR in some way promoting ATP release from cells. As, in addition to CFTR, regulation of ORCC may require extracellular ATP, one hypothesis suggests that CFTR facilitated release of ATP, stimulates a purinergic receptor which acts *via* a second messenger pathway to regulate the ORCC.

CF airway epithelia show amiloride sensitive Na^+ absorption under voltage-clamped short-circuit conditions that is approximately two times that seen in non-CF airway epithelia. This increased activity of apical membrane amiloride sensitive Na^+ channels is most likely due to the epithelial sodium channel (ENaC). In non-CF airway epithelia, cAMP agonists are reported to reduce or not change amiloride short circuit current, whereas they are reported to increase this current in CF airways. These observations suggest an inhibitory or negative effect of CFTR on ENaC function. However, this relationship is different in different tissues. For example, the relationship is opposite in the sweat gland where activity of ENaC requires CFTR function [13].

CFTR also regulates K^+ channel function and the function of the Cl^-/HCO_3^- exchanger. Often CFTR channels function in parallel with a Cl^-/HCO_3^- exchanger to effect net HCO_3^- secretion and activation of CFTR by cAMP agonists stimulates Cl^-/HCO_3^- exchanger activity [14, 15]. The potential mechanisms by which CFTR may regulate other membrane channels and transporters is shown in Figure 1-2.

1.3.3   Mutations in the CFTR gene

Mutations in the CFTR gene are associated with the disease state of CF. There are more than 1500 reported mutations in the CFTR gene, and it is important to understand that the functional consequences of many of these mutations are poorly understood, and that a majority of these mutations are rare [16]. The different mutations can be divided into 5 classes. These are class I, which result in defective or reduced protein production; class II, which result in defective protein processing; classes III/IV, which result in defective regulation or conduction, and class V mutations that lead to low levels of mRNA, either by producing an unstable protein or due to abnormal splicing of pre-mRNA transcripts [17]. The majority of CFTR mutations associated with disease are those associated with mutations involving one to a few nucleotides. Amino acid substitutions (missense mutations) account for the majority (45 %) of CFTR mutations, frameshift mutations account for 22 %, splice site 16 %, nonsense 14 % and in-frame deletions (2%), of which the most common mutation ΔF508 (see below), is an example. One percent are genomic rearrangements such as deletions or insertions affecting hundreds of base pairs [1]. See Figure 1-3.
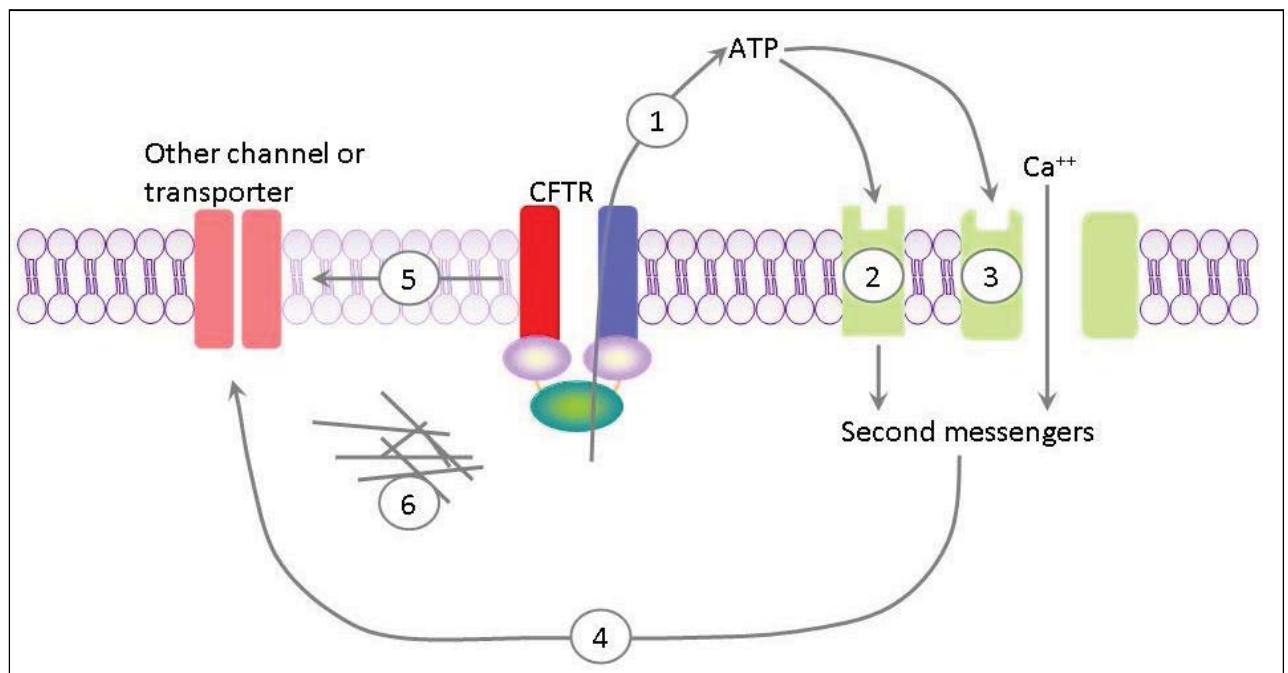
Figure 1-2: Potential mechanisms by which CFTR may regulate other membrane channels and transporters

(1) may influence the release of cellular ATP

(2) ATP can activate membrane receptors, such as P2Y receptors that increase cell Ca^{++} concentration

(3) ATP can activate P2X receptors that allow influx of extracellular Ca^{++} which serves as a 2nd messenger

(4) The effect of 2nd messengers may alter the function of membrane channels and transporters either directly or indirectly, for example *via* phosphorylation or influencing protein-protein interactions

(5) There may be direct associations between CFTR and other membrane channels and transporters that influences function

(6) Regulatory interactions maybe mediated by linker and adaptor proteins with connections to the cytoskeleton (eg. Proteins containing PDZ)

The most common mutation associated with CF is a three base pair deletion that produces an in-frame deletion of phenylalanine at position 508 (ΔF508). This mutation is associated with 70% of CF chromosomes [17].

The absence of phenylalanine at position 508 appears to be associated with the abnormal folding of the CFTR protein, which prevents it being transported to the cell surface [17].

The most common mutations after ΔF508 are the G551D missense mutation, in which residue 551 (glycine) is altered to asparagine, which accounts for 2.12% of CF mutations, the G542X nonsense mutation, which accounts for 2.43% of mutations, and the N1303K missense mutation (residue 1303 (aspartate) substituted by lysine), accounting for a further 1.25% of CF mutations [1].

1.3.4   Screening and diagnosis of CF

In the absence of an effective neonatal screening program, many individuals with CF are diagnosed after symptoms begin to appear; patients can present with respiratory symptoms, failure to thrive, steatorrhea and meconium ileus. Other symptoms include sinus disease, liver disease, obstructive azoospermia and electrolyte imbalance. These symptoms are frequently accompanied by conditions that may not be reversible, including impaired growth and chronic respiratory infection [1]. The median age for diagnosis based on when symptoms appear is greater than one year of age, with one quarter of patients identified after three years of age [1]. Current practice in Australia dictates that every new born child in every state undergoes screening for CF, whereas in countries such as the United States newborn screening is not carried out for every child at birth. This lack of screening at birth raises the median age at which patients are diagnosed and therefore the start of preventative treatment.
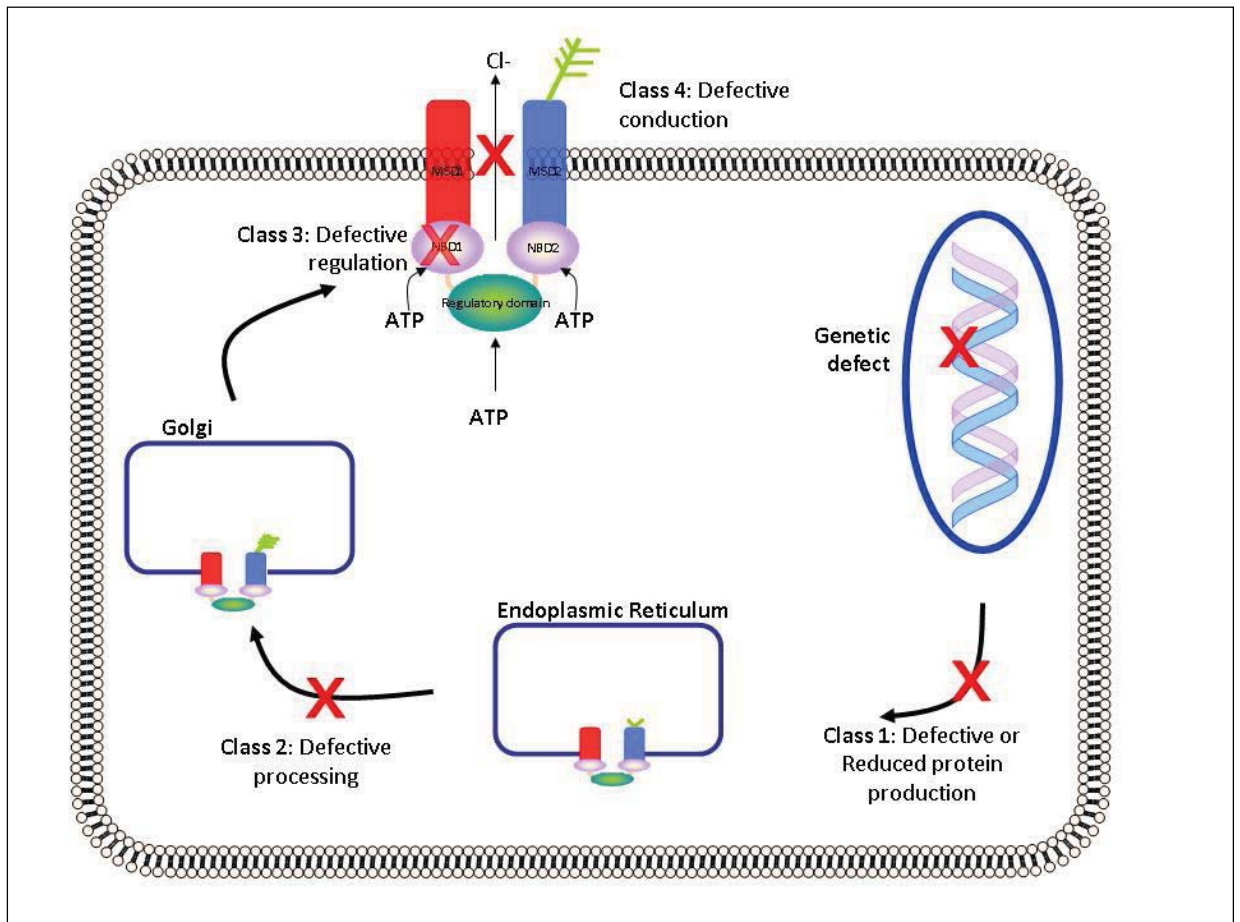
Figure 1-3: Mechanisms by which CF-associated mutations disrupt CFTR function

The sweat test remains the most widely used laboratory test, and the gold-standard, to confirm CF. In patients with CF, the absence of functioning CFTR restricts the reabsorption of chloride. As there is no other pathway for effective chloride reabsorption in the sweat duct, sodium is also poorly absorbed, and as a result the sweat of patients contains a high level of salt [18]. The sweat Cl⁻ concentration will accurately diagnose over 90 percent of cases [1] and a sweat level of greater than 60 mM on two separate occasions is considered diagnostic for CF. Levels higher than 160 mM are considered as erroneous, since this value is not physiologically possible.

Sweat is collected from patients using one of two methods; The Gibson Cooke method uses pilocarpine iontophoresis to stimulate sweat production with subsequent collection of sweat onto gauze or filter paper for analysis. More recently, many laboratories have adopted the Wescor apparatus, which also employs pilocarpine iontophoresis but with the sweat collected into microbore tubing.

Chloride concentration is determined by one of three methods, colormetric techniques (titration), ion selective electrodes and coulometric methods (which included the use of a chloridometer). Most laboratories reported the chloride concentration of a healthy population to be <40 mmol/L and the chloride reference for the CF population to be >60 mmol/L [19].

Genetic testing for CF also takes place, for one of two reasons: to assist a clinical evaluation or to evaluate an individual who may have an increased risk of carrying a CF allele. A wide range of mutation testing methods are currently used in diagnostic laboratories for CF genetic testing. The most frequently used mutation detection methods are heteroduplex analysis, restriction enzyme analysis, reverse dot-blot, the commercial kits INNO-LiPA CF2 (Innogenetics nv, Gent, Belgium), Elucigene CF4 and CF12 (AstraZeneca Diagnostics, Abingdon, Oxforshire, UK), and OLA Cystic Fibrosis Assay (PE Applied Biosystems, New Jersey, USA). The INNO-LiPA CF2 kit is based on reverse hybridisation. Amplified biotinylated DNA material is hybridised with oligonucleotide probes specifically designed to

correspond with the wild-type sequence and probes designed to correspond with the mutant sequence. These probes are immobilised as parallel lines on membrane based strips. After hybridisation, streptavidin labelled with alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. The test can be performed in 3.5 hours and eight mutations are screened [20].

The Elucigene CF4 detects mutations *via* a single tube assay optimised for use with DNA extracted from dried blood spots. The method uses fluorescent ARMS ™ allele specific amplification technology, which detects mutations or small deletions in DNA. The principle of ARMS ™ is that oligonucleotides with a 3' mismatched residue will not function as PCR primers under specified conditions. Selection of appropriate oligonucleotides allows specific mutant or normal DNA sequences to be amplified and detected [21].

The OLA (oligonucleotide ligation assay) detects 33 CF mutations using allele specific probes carrying inert mobility modifiers at the 5' end. A resultant mutant probe is 2 base equivalents longer than the normal probe. This assays detects the change in size between the mutants compared to normal, and can also detect normal genotype, heterozygous and homozygous genotypes [22].

Single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), two-dimensional DNA electrophoresis and sequencing are mostly used as CF mutation screening methods [23].

Since genetic analysis in CF is PCR-based, a minimal amount of DNA is required in most cases. A mouthwash sample or buccal scrape (in the case of babies) provides sufficient DNA. If this cannot be obtained a small blood sample of 2-5 mL will provide enough DNA for extensive analysis. Prenatal diagnosis of CF is usually carried out on a chorionic villus sample taken during the first trimester of

pregnancy. Amniocytes can also be used for molecular genetic analysis- either directly spun down from amniocentesis sample or after 10-14 days in culture. Direct analysis should be carried out with caution due to contamination from maternal cells [23].

Newborn screening for CF is now commonplace in first world countries, including all states within Australia. Newborn screening programs for CF are based on the determination of immunoreactive trypsinogen (IRT), a marker of pancreatic dysfunction, from a dried blood spot taken a few days following birth. Newborns with CF generally have an elevated IRT level as compared to normal individuals. However this test is not specific for CF as normal infants may have transient elevations in IRT levels. Therefore, diagnosis is confirmed by using mutation analysis and the sweat test [1]. The advantage of diagnosing CF early in life is that it allows early treatment, which is beneficial in terms of slowing the progression of the disease, with the intent of prolonging life.

1.4   CF: The multi-organ disease

1.4.1   Pancreas

Electrolyte transport by pancreatic ducts in CF patients is disrupted due to the loss of apical CFTR Cl^- channels and impaired Cl^-/HCO_3^- exchange [24]. The impaired ion exchange causes a decrease in the alkalinity of the secretion that normally hydrates, solubilises and inactivates the acinar secretions. The consequence of this is thickened secretions that obstruct the pancreatic ducts and leads to destruction of the organ [1].

Observations of altered and/or increased mucus production in the pancreas made it easy for investigators to conclude that the fundamental abnormality in CF is related to mucus thickening [25].

The progression of pancreatic disease in CF appears to have three levels of involvement. The first involves widening of the ducts, with intra- and interlobular fibrosis and microcysts appearing due to obstruction of the ducts. Acinar and ductal atrophy and disintegration accompanied by fatty infiltration follow, and finally, scar tissue forms surrounding isolated islets of Langerhans, with total loss of acinar and ductal structures.

Pancreatic disease can be managed with enzyme therapy and nutritional supplements as well as dietary counselling. The addition of 500 -2500 Units of Lipase/kg of food eaten leads to normal pancreatic functioning [1].

1.4.2   Gastrointestinal Tract

The biliary tree, the gallbladder and the intestine have all been reported as showing signs of increased mucus or thickened mucus production. Gastrointestinal problems related to CF begin from birth, with some newborns presenting with meconium ileus. In these infants, a thick, dehydrated mucoid plug blocks the small bowel. Microscopic analysis of intestinal epithelium shows dilated mucus glands with prominent mucus cells along the lumens that contain a 'stringy' secretion [26].

As patients with CF are prone to gastrointestinal problems throughout their lifetime, management of these problems increasingly emphasizes non-surgical approaches to relief of obstructions and prevention of obstruction by appropriate dietary and bowel regimens [1].

1.4.3   Reproductive Tract

Studies of the female reproductive tract have given further evidence of CF as a disease of altered mucus secretion. Mucus plugs have been found obstructing the cervical canal, rendering most women with CF infertile [27, 28].

In the male genital tract, thickened secretions plugging the small ducts cause sterility [1].

1.4.4   Complications of CF

A range of other complications are also associated with CF and are listed in Table 1-1.

Most complications associated with CF can now be managed through enzyme replacement therapies, prompt and accurate diagnosis with better pharmaceutical treatments and less invasive surgical measures. Lung complications however, remain the major challenge for better treatments and potential cures as the manifestations of the lung disease are now attributed with almost all the morbidity and mortality associated with CF.

1.5   CF: The Lung Disease

The most devastating effects of CF are found in the lung. The lung disease and the effects on airway epithelium are the most widely studied aspects of CF, due to the high morbidity and mortality associated with the lung aspects of the disease, and the successful treatment of the other aspects of the disease.

1.5.1   The physiology of the human airway

The lungs are comprised of the conducting airways, which include the trachea, bronchi, to the ends of the terminal bronchioles. The function of the conducting airways is to warm and humidify air as it is breathed in, and to distribute it to the areas where the exchange of gases occurs, that is the alveoli [29]. See Figure 1-4.

CF lung disease is associated with the conducting airways, and in particular the epithelial cells that line them. The conducting airways are lined with pseudostratified columnar epithelium consisting of non-ciliated columnar cells, ciliated epithelial cells, goblet and basal cells and submucosal glands.

The cilia present on the apical surface of the epithelial cells beats at ~12.5 – 15 beats/ second, which is effective in clearing foreign particles from the cell surface and keeping the epithelium as a sterile environment [30]. This surface is also covered by a layer of liquid known as the airway surface liquid (ASL). This consists of two parts, a periciliary liquid layer and a mucus layer. The periciliary layer has two important functions in mucus clearance: (i) it provides a low viscosity solution for ciliary beat and (ii) acts as a lubricant layer separating the secreted mucins in the mucus layer from the cell surface mucins that facilitate cough clearance [17].

1.5.2   Physiology of the CF lung

Although CF is a multi-organ disease, lung involvement is the major cause of morbidity and mortality-causing approximately 95% of CF related deaths [1].

Mucus is a significant problem in the CF lung, with hypersecretion associated with airflow obstruction. Mucus hypersecretion is also believed to cause prolonged colonization and infection of CF patients with bacterial pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [31]. Excess mucus in the airways appears to contribute to morbidity by increasing the frequency and severity of these pulmonary infections with bacterial pathogens [31].

Table 1-1: Outline of complications associated with cystic fibrosis

Pancreatic	pancreatic insufficiency (failure to produce enzyme for normal food digestion)
	chronic pancreatitis (inflammation of the pancreas, altering normal structure and function)
Hepatobiliary	focal biliary cirrhosis (progressive destruction of the small bile ducts)
	steatosis (abnormal retention of lipids within cells)
	cholelithiasis (gallstones)
Gastrointestinal	meconium ileus
	distal intestinal obstruction syndrome
	malabsorption (abnormal uptake of nutrients from digestive tract)
	gastrointestinal reflux
	rectal prolapsed
Renal	nephrolithiasis (kidney stones)
Endocrine	diabetes mellitus
	Osteoporosis
	delayed sexual development
	hypogonadism (lack of function of the ovaries/testes)
Genitourinary	obstructive azoospermia (males) (absence of live sperm in semen)
Respiratory	sinusitis (inflammation of the sinus lining)
	chronic pulmonary infection
	airway obstruction
	bronchiectasis (chronic dilation of the bronchi)
	hemoptysis (coughing blood)
	Pneumothorax
	respiratory failure
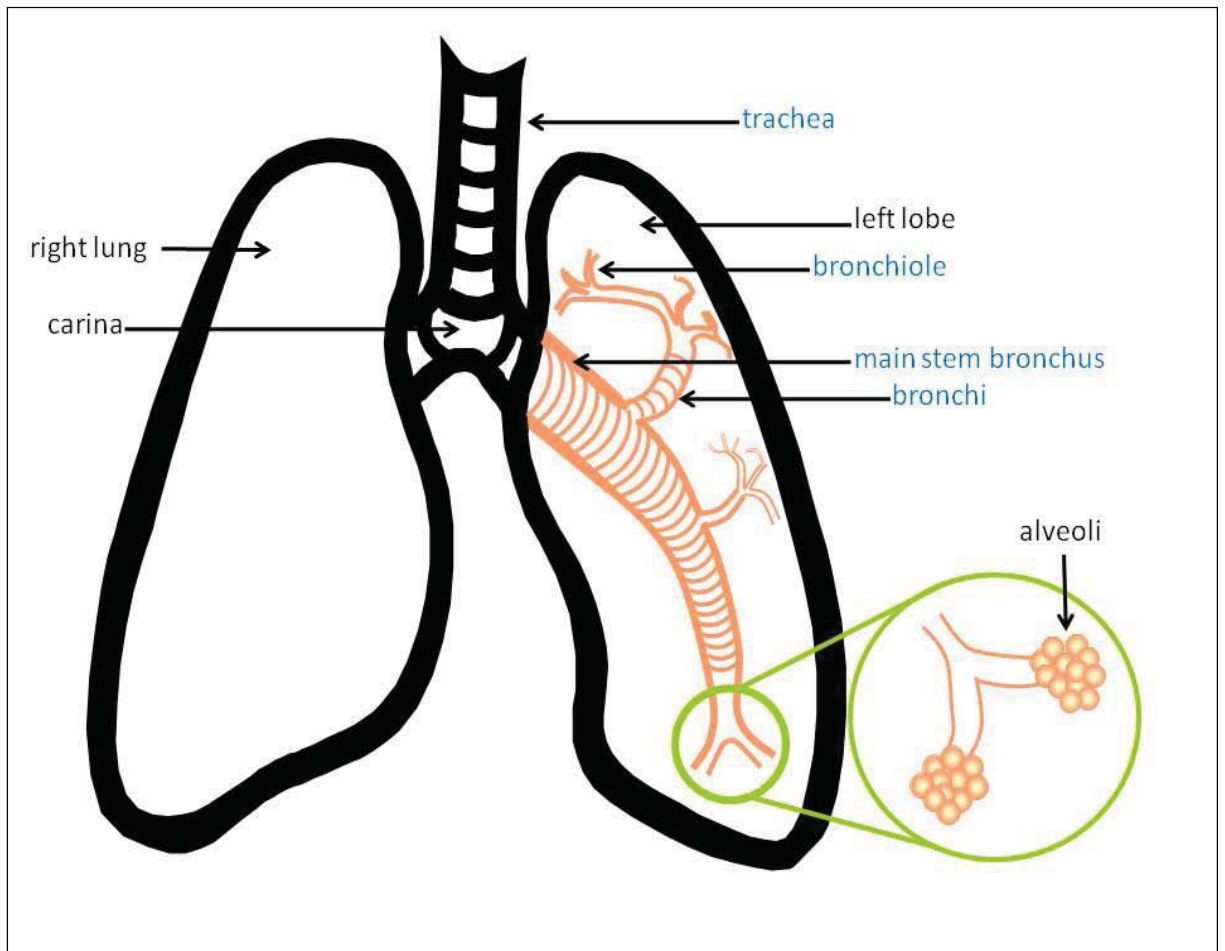
Figure 1-4: Structure of the human airways

Lungs are comprised of left and right sides. At the carina, the trachea bifurcates into the left and right main bronchus. The right and left main bronchi further branch into bronchi and divide again into segmental bronchi and finally to terminal bronchioles. The structures highlighted by blue text indicate the conducting airways. Terminal bronchioles divide into respiratory bronchioles which continue downstream as alveolar ducts completely lined with alveoli.

The impaired clearance of mucus causes worsening of lung disease by directly obstructing airway lumen, inducing an inflammatory state in the airways. In fatal cases of CF, total occlusion of the small airways with mucus plugging is evident [32]. CF can be classed as a genetic form of chronic bronchitis progressing to bronchiectasis and ultimately respiratory failure [17].

As lung disease progresses, there is increased injury to the structure of the airway. At the end stage of lung disease there is widespread destruction of the airway architecture, along with fibrosis of lung parenchyma surrounding the airway. Progression of the lung disease is caused by the inflammatory response to bacterial infection in CF individuals [1] with severe neutrophil-mediated inflammation a characteristic of CF. This inflammation occurs early in life and is exaggerated and prolonged compared to non-CF subjects. It is also somewhat ineffective and often fails to eradicate the microbes to which it is directed. Data suggests that the CF airways could be inherently pro-inflammatory; however, this idea is not entirely accepted within the CF community [33].

1.5.3   Ion transport in the normal lung

The ionic balance across the epithelium is important in maintaining a functional respiratory system, especially with regard to mucociliary clearance. The model currently used to describe normal ion transport by airway epithelia is as follows:

Sodium ions enter the cell across the apical membrane through amiloride sensitive ENaC. Sodium entry through ENaC is passive. Sodium is then pumped out across the basolateral membrane via the Na^+-K-ATPase. The Na^+-K-ATPase provides the energy for ion transport by maintaining low intracellular Na^+ levels. Potassium ions exit the cell passively through the basolateral membrane K^+ channel. The basolateral K^+ conductance and K^+ concentration gradient hyperpolarize the cell, providing the electrical driving force for Na^+ and Cl^- transport. There are likely to be two different basolateral K^+

channels, one type regulated by intracellular Ca^{2+} concentration and another stimulated when intracellular cAMP levels increase. Chloride ions enter the cell across the apical membrane through CFTR, which is localised to the apical membrane. The apical membrane also contains other Cl^- channels that appear to be regulated by Ca^{2+}. Chloride ions exit the cell through the Cl^- channels in the basolateral membrane. The basolateral membrane contains a neutral co-transporter that can couple the entry of 2 Cl^- to 1 Na^+ and 1 K^+. The Na^+ concentration gradient created by the basolateral Na^+-K-ATPase provides the driving force for the entry of Cl^- and K^+ [1]. See Figure 1-5.

1.5.4   CFTR dysfunction and ion transport in the CF lung

In airway epithelia, CFTR has been easiest to characterise as a Cl^- secretory channel [8, 9]. In this epithelia the major regulatory activity of CFTR involves tonic inhibition of ENaC [34]. The cell surface stability of ENaC is a major determinant of its regulation. ENaC, down regulated by CFTR is stabilised at the plasma membrane. In CF patients, there is a lack of this ENaC regulation and associated stabilisation [35].

Data obtained from *in vivo* basal transepithelial potential difference (TPD) measurements and *in vitro* studies of short circuit current and isotope flux [36], electrochemical driving forces and estimates of apical membrane Na^+ permeability [37, 38], have all provided evidence that a major functional defect in CF airway epithelia is excessive absorption of Na^+. The net effect of accelerated Na^+ transport is the excessive transport of NaCl and water from the CF airway surface [39-41]. The consequence of this is dehydration of the airway surface liquid, leading to thickened secretions on the airway. The ionic strength of the ASL (concentrations of Cl^- and Na^+) does not differ between CF and normal when tested in mice [42] and is generally thought not to differ significantly between normal individuals and CF patients [43] [44].
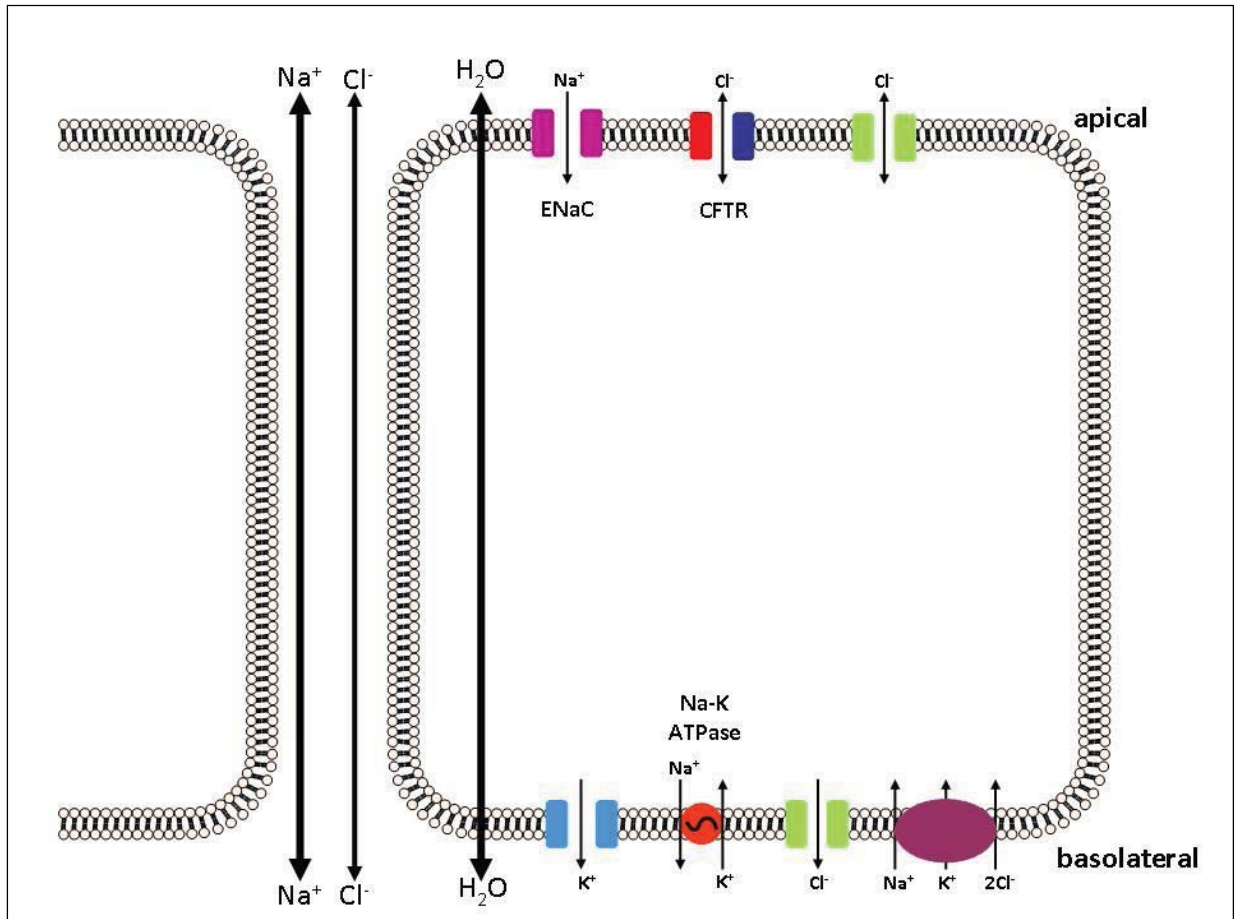
Figure 1-5: Model of ion transport in normal and CF airway epithelia

Apical CFTR Cl^- channels allow Cl^- ions to enter the cell, maintaining a normal ion balance within the cell. In the CF airway, the defective CFTR channel leads to increased Na^+ absorption, dehydrating the airway surface.

Airway surface liquid depletion is believed to cause ciliary collapse and loss of mucociliary clearance [40] [45]. Mucociliary transport is not completely absent in CF patients, as local mediators secreted onto the airway surface liquid play an important role in the dynamic regulation of the ASL volume. These can induce both CFTR-dependent and CFTR-independent chloride secretion [46]. For example, Respiratory Syncytial Virus (RSV) and possibly other viruses, increase ATPase activity in respiratory epithelium, which results in the breakdown of ATP and loss of the compensatory chloride secretion. This may explain in part the negative impact such viruses have on the airway clearance seen in CF patients [46].

1.5.5   Measurements of disease progression and severity

Measurements for the monitoring of the health of CF patients are critical, with a need for tests to be sensitive, and to allow detection of changes both long term and short term. Tests need to be repeatable and reproducible to small changes with confidence. For CF patients, testing needs to be minimally or non-invasive, as the lungs are already compromised and it is not beneficial to the patient to compromise them further. Testing also needs to be well tolerated and applicable to patients across all age groups and to patients of differing disease severity [33].

There are many types of sampling that can be used to characterize disease state or severity in CF patients. Outlined below are these samples and the characteristics of disease that can be measured from the samples.

I. **Sputum** in CF patients contains high levels of inflammatory cells, pro-inflammatory cytokines and proteolytic enzymes which can be easily measured, including: increased neutrophils, TNFα, IL-1β, IL-6, IL-8 and leukotrine B4 [47]. There is also a well described relative deficiency of anti-inflammatory cytokines such as interleukin 10 and anti-proteases that can be detected in CF patients [33].

II. **Bronchoalveolar lavage** (BAL) is often considered the accepted standard technique for airway sampling, and shows similar patterns of inflammation to that seen in sputum samples. It is however, invasive and not easily repeated, which renders it unfavourable for continued sampling in patients. A defining parameter of BAL measurements is characterized in healthy patients, who have BAL that is mostly macrophage dominated, whereas CF patients have a large excess of neutrophils [33].

III. **Exhaled breath** is easy and non invasive to obtain and can be used reproducibly even in young children. With this form of sampling, the level of exhaled nitric oxide (NO) is the principal focus and the levels of NO in CF patients are reduced from those found in normal subjects. A likely explanation for this is due to the known anti-inflammatory properties of NO, and the lack of anti-inflammatory activity in CF patients [33].

IV. **Exhaled breath condensate** is relatively easy to obtain, and each sample contains a small volume of airway lining fluid, the pH of which is abnormally low in CF patients. However this method does lack sensitivity [33].

V. **Blood and serum markers** including inflammatory cell counts and acute phase reactant such as C-reactive protein and immunoglobulin G are recommended as a test of efficacy and safety outcome measures in CF clinical trials. Testing blood markers is not a stand alone measure, and should be used as an adjunct to direct lung sampling techniques to get additional information about what is happening in the lung [33].

VI. **Urine** can be easily obtained, and gives an indication of increased tissue degradation products unique to CF patients. However, the levels in urine samples fluctuate rapidly and this limits the utility of the measures observed [33].

VII. **Imaging and radiology** have long been described to visualize the lungs.

o Plain chest radiography, although easily carried out, lacks sensitivity particularly in early stage disease.

     o   Computed tomography (CT) is more sensitive than chest radiography, however, the risk in younger patients from the increased radiation may outweigh the higher sensitivity available, particularly if CT assessment is proposed as a part of a regular monitoring process.

     o   Magnetic resonance imaging (MRI) lacks sufficient resolution in the lungs to detect obstruction and airway damage.

     o   Positron emission tomography (PET) is a relatively new technique in lung disease. Labeled glucose uptake indicates areas of inflammation, and in CF patients there is a greater uptake when compared to normal subjects due to the increased amount of inflammation in CF lungs [33].

VIII.   **Pulmonary Lung Function (PFT) testing** consistently provides evidence of an obstructive process leading to airflow limitation and elevation of lung volume. Although these tests are limited to patients 5 years and older, this is the most widely accepted approach to measuring the progress of lung function in CF patients. Forced expiratory volume in one second (FEV1) is the most useful measure as it is easily reproducible and relatively easily performed, and is the best predictor of survival. When FEV1 reaches 30% of normal, 2 year survival is less than 50% and listing for a lung transplant should take place [1].

1.6 Current Therapies

The treatment of CF is currently directed towards the downstream effects of the disease. As yet, there is no clinically available therapy designed to correct the underlying CFTR dysfunction.

The major non-pharmacologic approach is standard chest physiotherapy. Airway clearance techniques are used for the clearance of abnormally viscous airway secretions in the lungs and respiratory tract. This is based on the understanding that coughing clears mucus from large airways, while vibrations

move small airway secretions. Active techniques include the forced expiratory technique, autogenic drainage, positive expiratory pressure and airway oscillators. Passive techniques include high frequency chest compressions and an intrapulmonary percussive ventilator [1].

Bronchodilators such as beta-adrenergic agents and ipratropium bromide have been used in CF patients, with these patients showing improved pulmonary function over time [1]. Other treatments such as mucolytic agents, used to reduce the viscous mucus present in CF patients, antibiotic therapy for lung infections, anti-inflammatory treatments such as corticosteroids and nonsteroidal anti-inflammatory drugs, are all commonly used for treatment of the symptoms of CF and slow the progression of the disease.

An area of possible treatment for CF is the stimulation of alternative chloride channels. Chloride secretion in airway cells is not limited to CFTR. A calcium-dependent chloride channel also secretes chloride apically from epithelial cells. Increasing the activity of this channel may compensate for the lack of CFTR mediated chloride secretion. Two agents that stimulate the calcium dependent chloride channel either directly or through the purinergic P2Y receptor, denufosol and lancovutide, are currently in clinical trials [48].

An alternative approach to CFTR pharmacotherapy is to inhibit sodium absorption through the epithelial sodium channel. Interestingly, one such drug, Amiloride (an epithelial sodium channel blocker with a short half life) showed no clinical benefit and in fact showed a trend towards poorer lung function in patients [49]. The use of amiloride in a mouse model possessing signs of airway disease *via* βENaC overexpression, also showed a lack of efficacy whereas, if used before airway disease had developed, it appeared to prevent the development of lung disease [50].

Nebulised hypertonic saline has been shown to improve hydration of mucus in CF patients and improve mucus clearance from the airways [51]. Pulmozyme (dornase alfa) is a recombinant human DNase which hydrolyses the large amounts of DNA found in CF sputum and so reduces mucus viscosity [52]. Only a subset of patients responds to pulmozyme and there is no marker to predict who will or will not benefit from this treatment.

Mucoactive agents such as hyperosmolar agents look to be the most promising short term prospects, these are designed to encourage the flux of H_2O across the lung surface. Bronchitol (Phamaxis, Sydney Australia) is expected to have a significant effect if current phase III trials succeed. Bronchitol is an inhaled dry powder formulation of mannitol, comprised of hollow microspheres that penetrate lower airways more efficiently than nebulised products [53]. In phase II studies it delivered a 7.5% improvement in lung function over a three month dosing period, which is comparable to the efficacy of pulmozyme [54].

Several companies are looking at the problem of irregular salt transport by modulating other chloride or sodium channels in the lung. Phase II trials of denufosol tetrasodium inhalation solution are ongoing. Denufosol acts by stimulating the purinergic $P2Y_2$ receptor, which activates alternative chloride channels, theoretically compensating for the lack of CFTR activity in the airway. In trials, an improvement of 2.5% in lung function was seen over 24 weeks of treatment. Another pharmaceutical shown to produce compensatory chloride channel activation is the polycyclic peptide Moli1901 (Lancovutide). In phase II clinical trials 4 weeks of aerosolised Moli1901 lead to a 2% increase in lung function [55]. A randomised, double-blind, placebo-controlled, dose-finding study to evaluate the efficacy and safety of aerosolised Moli1901 in adolescents with CF has recently been completed, with results pending. Another compound, P-680 from Parion Sciences, blocks ENaC and was developed to retard sodium absorption and so restore hydration [56]. No data is currently available regarding this compound and its effectiveness in CF patients [54].

Since the mid 1980's a lung or heart/lung transplant has been the only therapeutic option available to patients with end-stage lung disease. For many sufferers of CF, a lung transplant is the only option for prolonging life. However, as with any type of organ transplant, the availability of organs remains an important limitation. There is also only a small time and health window within which a patient's health is poor enough to warrant a transplant, but good enough to survive and benefit form the substantial surgical procedures involved. The on-going side effects from organ transplantation also need to be considered, with rejection of the organ a major concern. Patients must be continuously treated with immunosuppressive drugs to minimise the risk of rejection, however, this leads to an increased risk of infection since the patients' immune system is suppressed globally.

While there is no denying that the ongoing development of new pharmaceuticals for CF have led to marked improvements in treatment, they do not represent a true cure for the disease. One long-term goal of the CF research community has been to effect a cure for the disease using gene therapy, by introducing a functional copy of the CFTR gene to the right cells. This should stop the disease progression where it is at, and if done early enough, effectively prevent the disease developing.

1.6.1 Gene Therapy

Gene therapy involves the introduction of a therapeutic nucleic acid sequence into a cell to modify the expression of a gene within that cell. Due to electrostatic repulsion within cells the entry of DNA, RNA and oligonucleotides is inhibited, therefore a vector is generally used to deliver the nucleic acid sequence. A vector can be a synthetic non-viral vector, with the nucleic acid sequences being associated with compounds such as cationic lipids or cationic polymers [57]. Viral gene therapy options include the use of vectors derived from adenovirus, adeno-associated virus, retroviruses and lentiviruses. Cystic Fibrosis has had among the highest number of gene therapy trials (for an inherited genetic disease), with many vector systems being trialled [58]. As with any gene therapy, it is critical

that a sufficient quantity of the gene therapy agent is delivered to affected cells and the expressed protein is functional for a duration that is therapeutically meaningful. For CF this goal has not yet been achieved.

One of the many challenges facing CF gene therapy is that it is not known how much CFTR is required to be delivered to cells to achieve a functional outcome. As CF is an autosomal recessive disease, only one functional copy of the CFTR gene is required for normal functioning. Based on this and data from mild CF patients it has been suggested that the amount of CFTR correction needed to improve clinical outcomes may be between 10-20% of normal [59]. However, in these examples this lower amount of CFTR is found in all cells. In addition, it has been reported that as little as 6% of airway epithelial cells dispersed within the epithelia is sufficient to correct chloride transport [60]. Whether higher levels of CFTR expression in fewer cells can compensate for low numbers of gene-corrected cells is unclear, especially as some *in vitro* evidence suggests that over-expression of CFTR can result in mislocalisation of CFTR to the basolateral membrane. This overexpression of CFTR showed some CFTR channels were present in the basolateral membrane, where they shunted Cl⁻ flow, reducing the net transepithelial Cl⁻ transport [59]. There have been other concerns that over-expression would "super-correct" Cl- transport and generate excessive fluid secretion. This concern appears unnecessary as shown by Zhang *et al* 2009 [61] that ion transport rates and ASL heights in CF human airway epithelia with CFTR over-expression did not exceed that measured in non-CF human airway epithelia [61]. These are important issues to consider in choosing the most appropriate vector to deliver the CFTR gene, as a strong promoter driving the CFTR gene may not give the best correction of Cl⁻ transport.

1.6.1.1 Non viral gene transfer

Non-viral gene transfer most commonly utilises synthetic polymers to deliver a therapeutic gene into a cell, with non-viral vectors including variants of liposomes. Plasmid DNA is too large to package into

regular liposomes, but larger particles can be made using positively charged lipids called lipoplexes which can interact with negative charges on cell membranes and on DNA. The use of lipoplexes improves delivery into the cell nucleus and this allows the possibility of incorporation into the chromosome. However, lipoplexes are less efficient than viral gene transfer agents. Attempts to improve their performance have included the incorporation of various viral signal proteins into the outer coat [62]. Non-viral delivery methods may appear to be advantageous as they may not induce, or at least have greatly reduced levels of the immune responses seen with some viral vectors [62], and as they are a synthetic compound their formulation can be strictly controlled. Non-viral approaches have already been used in therapy for CF, and the outcomes of some of the major trials testing the use of lipoplexes and cationic polymers to achieve gene transfer in CF are summarised in Table 1-2.

Table 1-2: Summary of clinical trials of non-viral gene therapy for cystic fibrosis: Adapted from [63]

Trial	Liposome	Number of Patients	Target	Route of administration	Efficacy	Side effects
Caplen, 1995	DC-Chol/DOPE	15	Nose	Aerosol	20% correction to normal	None
Noone, 2000	EDMPC-Chol	11	Nose	Aerosol	None	None
Alton, 1999	GL-67™/DOPE/DMPE-PEG$_{5000}$	16	Lungs	Nebulised	25% correction to normal	Mild, Flu like symptoms
Zabner, 1997	GL67™/DOPE	12	Nose	Direct instillation	Partial correction	None

Trials with lipoplexes (GL-67™/DOPE/DMPE-PEG$_{5000}$) showed partial correction of chloride transport, and also showed that transfected cells had significantly lower binding abilities to *Pseudomonas aeruginosa* measured in patients who received treatment *via* nasal aerosol or breathed into the lungs

[64]. This is a significant finding as chronic bacterial infection is involved in worsening CF pathology. However there is still much work needed before the use of lipoplexes for long-term, effective, gene treatment of CF becomes a reality due to their lack of persistent gene expression and their low efficiency of expression compared to viral vectors [65].

DNA conjugates are also a non viral form of gene therapy. DNA is conjugated to certain polycations (e.g. polylysine) in a complex with specific antibodies or proteins able to target the complex to a specific cell protein. This method allows DNA to be directed to a specific cell type *in vivo* [66]. Poly-L-lysine has been shown as an effective vector to transfer the CFTR gene into CF airway epithelial cells and correct the Cl- channel defect [67]. In another study, gluconoylated polylysine with β-D-GlcNAc residues showed gene transfer with CFTR similar to that achieved with polyethylenimine and 10-fold higher than that observed with lipofectin and lipofectamine [68].

Another method of gene transfer is *via* non-viral hybrids. This method allows for delivery of a plasmid with the appropriate replication signals (elucidated from HPV or Epstein-Barr virus sequences) and a therapeutic gene to allow extended periods of gene expression [66].

Despite initial speculation of non-viral gene therapy holding the answers to gene therapy for CF, there has been no clinical use due to the transient effects seen in patient trials. The short term results obtained from these trials do however give some support to the efficacy of gene therapy.

1.6.1.2   Viral gene therapy

Viral gene therapy involves the use of virus-mediated systems to introduce a functional copy of a defective gene into affected cells. The rationale for this approach is that evolution has produced organisms – viruses – capable of overcoming the various barriers that normally prevent exogenous

agents from reaching the interior of cells. Some of the most studied methods of gene therapy are based on virus-mediated systems such as adenovirus (AdV), adeno-associated virus (AAV) and retroviruses, including lentiviruses (LV).

1.6.1.2.1   *Adenovirus*

The first viral vectors tested in clinical trials of CF gene therapy were adenoviral vectors. Adenovirus is a relatively benign pathogen able to efficiently transduce non-dividing cells. The genome of AdV and its functions has been widely studied, it can be easily manipulated, and there are few limitations on the amount of vector that can be produced.

It has been found that the transduction efficiency of AdV is similar in cultures of epithelial cells from healthy and CF affected patients. Early studies by Drumm *et al* 1990, showed that an AdV vector containing the CFTR (AdV-CFTR) gene could produce a functional Cl⁻ channel *in vitro* [69]. Further research using AdV-CFTR was carried out *in vivo,* in particular in mouse models. AdV mediated gene transfer to the lower airways appears to be efficient in mice, however, when AdV is applied to an uninjured airway of rodents, primates and humans, gene transfer was found to be low [70].

As CF is a life-long disease, a requirement for gene therapy is for expression of the normal gene over a long period of time- a lifetime. The gene expression seen with adenovirus has been both inefficient and non-persistent [71] with gene expression unable to be detected 1 to 2 months following vector delivery. One reason for this is that AdV is a non-integrating virus and the genome is lost over time or is diluted as cells divide. Another factor which is thought to limit expression is the immune response to transduced cells. Further development of AdV vectors for CF has been slow due to the host immune response and inefficiency of gene transfer [72]. The host immune response is partly due to AdV being a common airway pathogen. Recipients of AdV-mediated gene therapy may already have circulating

antibodies to the virus making administration of these vectors difficult. Complexing AdV with polyethylene glycol (PEG) is able to mask the virus from the immune system, which can aid in gene transfer with AdV [73].

AdV vectors induce an immunologically-mediated inflammatory response as early as 6 hours post treatment, thus raising obvious concerns regarding their safety. This inflammatory response is due to the expression of late genes from the AdV vector as well as the immunogenic nature of the viral particles [71]. Due to the host immune response and safety issues regarding the use of AdV, further improvements to this technology are needed to make it a viable candidate for gene therapy for CF.

The safety of AdV vectors has been improved with the development of gutless AdV vectors. Gutless AdV are essentially AdV vectors devoid of all viral coding regions, and contain only the essential AdV replication elements (required for construction of the virus), packaging signal and the transgene expression cassette [74]. Theoretically, this design eliminates the immunological problems associated with the expression of Ad late genes in transduced cells. Gutless vectors are grown in the presence of a helper virus that supplies the structural proteins required for replication and packaging [75]. As a helper virus is used for the supply of structural proteins, there is always a risk of contamination with helper virus. In addition it is possible that recombination between the helper virus and the gutless vector within the packaging signal may be responsible for the generation of packaging competent helper virus. Studies by Sakhuja *et al* 2003, showed 3 types of helper virus contaminant in their gutless vector preparations [75]. These observations suggest that contamination may be a limiting factor in the use of gutless vectors for gene therapy.

Goldman *et al* 1995 showed gene transfer to the baboon lung using a gutless AdV. This second generation vector had an E1 deletion and contains a temperature sensitive mutation in the E2a gene

which encodes defective DNA-binding protein. Using this 2nd generation vector gene expression was prolonged and resulted in diminished levels of perivascular inflammation [76].

Although frequently used in early CF gene therapy clinical trials, in recent times the use of adenovirus vectors has decreased. This is in part attributed to the low transduction efficiency in human airway epithelial cells but is also due to the inability for effective re-administration [58].

1.6.1.2.2   Adeno-associated virus

Adeno-associated virus (AAV) is a virus capable of both stable integration and transduction of the airway epithelium [77]. AAV is a non-pathogenic parvovirus with a 4.7 kb genome and requires co-infection with a helper virus such as AdV or Herpesvirus for replication. Flotte *et al* 1992, showed that AAV vectors integrate efficiently in a CF bronchial epithelial cell culture and can be used to effectively deliver the CFTR gene [78]. Subsequently, vector genomes were found in the airway surface epithelia of treated rabbits from 3 days to 6 months. There were no pathological changes to suggest an inflammatory response had occurred, indicating that AAV-CFTR vectors may be non-toxic and safe for airway gene therapy [77]. A drop in the number of transduced cells was observed over time following transduction with AAV, suggesting that only the terminally-differentiated cells of the surface epithelium had been affected, and that gene transfer was being lost as these cells turned over. This finding suggests that AAV may not give the required persistence over time to correct the CFTR defect [77]. Despite this, there have been a number of clinical trials of AAV vectors. New serotypes of AAV and means of augmenting delivery may give some hope of producing clinically relevant levels of gene transfer [79].

1.6.1.2.3   Sendai virus

Recombinant Sendai virus (SeV) is a very efficient gene transfer agent for airway epithelial cells. Second generation transmission-incompetent SeV expressing CFTR has been shown to correct the chloride transport defect in CF knockout mice [80]. Expression mediated by SeV is however transient and requires repeat administration. Re-administration is inefficient and attempts at tolerization against SeV peptides was shown to be unsuccessful [81] [82]. Sendai virus may be useful for acute disease that only requires transient expression of a gene. Therefore, the use of SeV for CF lung disease is restricted in a similar fashion to that of AdV until the problem of repeat administration can be solved.

1.6.1.2.4   Lentivirus

The ability of lentiviruses to transduce non dividing cells renders them viable candidates for use in gene therapy [83]. The Lentivirus genus is a part of the Retroviridae family, and comprises of a number of viruses, of which Human Immunodeficiency Virus Type-1 (HIV-1) is the most well characterized [83]. As a HIV-1 based lentiviral vector was used in this study a more detailed description of HIV-1 is given.

The HIV-1 lentivirus genome has a number of open-reading frames which encode proteins, essential for replication. These include the *gag, pol* and *env* open reading frames, with *gag* directing synthesis of structural proteins, *pol* involved in the synthesis of viral cDNA and the integration of viral into cellular DNA, and the *env* open reading frame mediating attachment of the virus to the target cell [84]. *Gag, pol* and *env* open reading frames are a basic characteristic of all retroviral genomes, with a number of small open reading frames distinguishing lentivirus from other retroviruses, these include Tat and Rev. The Tat and Rev proteins regulate lentiviral gene expression at the transcriptional and post-transcriptional levels respectively [84]. The other minor proteins include Vpu, a late viral gene product that is an integral membrane phosphoprotein that associates mainly with the internal membranes of the cell. Nef is another minor protein encoded only by primate lentiviruses. Virion Infectivity factor

(Vif) acts during assembly to allow the formation of particles competent for the early steps in infection. The viral protein R (Vpr) is a short basic protein that associates with viral particles through an interaction with the C-terminal region of gag and accumulates in the nucleus of infected cells [85]. The involvement of these proteins in the HIV-1 lifecycle can be summarised as follows:

- Attachment and Entry

Interaction of the lentivirus with the target cell occurs *via* binding of the viral envelope protein to a specific receptor on the cell membrane, for HIV-1 this is CD4. Once bound to the cellular membrane, the viral membrane undergoes fusion with the cell membrane and delivers the viral core into the cytoplasm [86].

- Reverse transcription

Reverse transcription leads to the synthesis of double-stranded linear DNA from a single stranded RNA genome. The virally encoded reverse transcriptase catalyses this process [87].

- Integration

Following synthesis of viral DNA, viral integrase performs specific cleavages at the 5' and 3' termini and catalyses integration into the host genome. This step is essential for retroviral gene expression and allows the provirus to become a permanent genetic element in the host [87].

- Transcription and viral protein synthesis

Early transcription from the provirus results in the production of doubly spliced mRNAs encoding Rev, Tat and Nef. Translation of mRNA leads to accumulation of Tat and Rev. Tat and Rev then induce a shift to late transcription where unspliced and singly spliced RNA species are produced. These mRNA's encode the structural proteins necessary for the production of viral progeny [87].

- Virion assembly and release

The viral RNA and structural proteins are packaged into viral particles and released by budding at the plasma membrane. After the polyproteins *gag* and *gagpol* are cleaved, mature particles become infectious. In contrast to the wild-type lentivirus, in current lentivirus gene vector systems the proteins

required for the production of virus are provided in a number of packaging constructs and not the transfer construct. Therefore, once the transfer vector is integrated into the host cell, it no longer has the ability to direct production of viral proteins which ensures that no progeny virus can be produced i.e. the lentivirus vector is replication deficient [87].

The first real attempt to produce a viable HIV-1 derived gene transfer system was by Naldini *et al* in 1996 [88]. This vector was able to transduce growth arrested cells *in vitro* and *in vivo*. Development of lentiviral vectors progressed to include the deletion of minor proteins not required for production of a HIV-1 gene transfer vector- namely proteins Vif, Vpr, Vpu and Nef. The deletion of these proteins resulted in greatly decreased chances of pathogenic replication competent virus production as these proteins all play important roles in HIV-1 pathogenesis.

Third generation vectors are Tat independent [89]. Rev was also placed on a separate helper plasmid to increase the number of recombination events required to produce a replication competent virus.

Lentiviral vectors have been pseudotyped with varied envelope proteins and have shown efficient gene transfer in multiple scenarios. One such envelope is the Vesicular Stomatitis Virus G glycoprotein (VSV-G). VSV-G pseudotyped viruses have a broad tropism, are resistant to freeze/thaw cycles and can be easily concentrated by ultracentrifugation [90]. Other envelope proteins that have been used to pseudotype lentivirus vectors are the baculovirus GP64 glycoprotein, the Ebola Zaire glycoprotein, and those originating from murine leukemia virus, lymphocytic choriomeningitis virus, Ross River virus, gibbon ape leukemia virus and feline endogenous virus [91]. Pseudotyped lentiviral vectors have proven to be effective vehicles to enable efficient transduction of a variety of cell types including airway epithelia [92-96].

1.6.1.2.5   Gene therapy trials with viral vectors

There have now been more than 15 clinical trials involving viral mediated gene therapy in patients with CF. Most trials to date have utilized adenovirus as the gene delivery agent. In a trial conducted by Zabner *et al* 1993, 3 patients with CF were treated with an E1-deficient adenovirus encoding CFTR, with delivery to a defined area of the nasal epithelium. The treatment was seen to correct the Cl⁻ transport defect and achieve a normal response to cAMP agonists, which was detected for 3 weeks post treatment. This study did not assess long term gene transfer which is essential for gene therapy of an inherited disorder such as CF [97]. A further study by Crystal *et al* 1994, administered adenovirus containing human CFTR cDNA to the nasal and bronchial epithelium of 4 individuals with CF. Follow up testing at 6-12 months demonstrated no long term adverse effects due the virus or delivery methods and the authors suggest correction of the CF phenotype in the airway epithelium might have been achieved using this strategy [98]. A 1995 trial by Hay *et al* tested an adenovirus containing CFTR cDNA delivered to the nasal epithelium of 9 individuals with CF. Two weeks post administration, PD values decreased towards levels measured in normal subjects. This study was limited to the nasal epithelium and again shows it is possible to deliver CFTR cDNA to improve the abnormal CF bioelectric phenotype [99]. Knowles *et al* 1995 delivered an adenoviral vector containing CFTR cDNA in 4-logarithmically increasing doses. In this case adenovirus mediated transfer of the CFTR gene did not correct functional defects in nasal epithelium and local inflammatory responses limited the dose of adenovirus that could be administered. Long term sustained transduction was not surprisingly found to be a problem in this study [72].

In a 1996 study by Zabner *et al*, repeat dosing of an adenoviral vector was investigated in 6 CF patients with dosing to the nasal epithelia. There were no detectable adverse effects, with the vector partially correcting the defect in airway Cl⁻ transport in some subjects, with less correction noted with subsequent doses. No changes in PD were seen [100].

Trials with adenoviral vectors to deliver the CFTR gene to CF affected patients have so far been limited due to the lack of sustained gene transfer, this leads to the need for re-dosing, which is ineffective due to the immune response mounted by the patient, as often patients have had prior exposure to AdV and have developed antibodies that inhibit effective gene transfer. The use of other gene transfer agents- such as lentivirus, or determining methods to negate the immune response mounted against gene transfer agents is of priority for future clinical trials. Lentiviral gene delivery as a single-dose may reduce the need for incorporating other agents into the therapy to negate immune responses as maybe required for other forms of gene delivery.

There have been a number of clinical trials utilizing an AAV virus to deliver the CFTR gene. In one study, 10,000 replication units of virus or placebo was delivered into the maxillary sinus of 23 CF patients with a FEV_1 >40. During the trial period of 90 days, biopsy, blood cell counts TPD measurements, cultures of bodily secretions and visualization of the sinuses *via* a rhinolaryngofibroscope were carried out. There was no evidence of inflammation or host immune response to the vector treatment, however the study did not provide evidence for efficacy after gene transfer but did support the safety of AAVCFTR vector at the dosage levels given in the study [101].

Clinical trials of another tgAAV2-CFTR followed encouraging pre-clinical studies with the vector. Three phase I trials of single administration of the vector instilled into one lobe of the lung using a bronchoscope into the maxillary sinus or delivered to the whole lung *via* aerosol all showed that the vector was well tolerated. Vector DNA was detected up to 70 days after administration in the sinus or 90 days in the lung. Further clinical trials using AAV2-CFTR have taken place, with the safety profile of the virus giving encouraging results, however, no improvement was seen in lung function testing- a measure of gene transfer success. Secondary endpoints included IL-8 concentration in induced sputum and assessment by high-resolution chest CT. Decreases in IL-8 concentration were noted when compared to placebo groups, however, bronchial brushings in a number of patients did not reveal

mRNA expression and showed antibodies to the AAV2-CFTR (the AAV component). A decision was made to terminate clinical trials with AAV2-CFTR due to lack of sufficient evidence of efficacy [102].

To date there have not been any clinical trials for CF utilizing a lentiviral gene therapy system.

1.6.1.2.6   Lentiviral vector system used in this study

The lentiviral gene delivery system used in this study is a 5 plasmid system [103], consisting of the following constructs: pcDNA3Tat101ml, pHCMVwhvRevml, pHCMVwhvgagpolstml, phCMV-G and a vector plasmid containing the transgene. Several transgenes were used in these studies including LacZ, Luciferase and CFTR.

The marker gene used to visualise gene expression within cells, so as to enable identification of cell type, is a nuclear localised, codon-optimised version of the LacZ gene. This version of the LacZ gene was used because a major disadvantage of the cytoplasmic LacZ gene as a marker is its underestimation of gene transfer in some biological systems [104]. The codon-optimised version developed by Anson and Limberis 2004, and the further addition of a nuclear localisation signal to the vector used in this study, has been shown to give a 15-fold increase in the level of ß-gal expression, with staining present in the nucleus of transduced cells, making individually stained cells easier to differentiate from adjacent cells [105].

Another reporter gene which was used for some studies is a modified Firefly Luciferase gene (Promega), with the bioluminescence produced upon the addition of Luciferin used as a measure of gene transfer.

For studies using the therapeutic transgene CFTR, the human CFTR sequence was cloned into the transfer vector.

Production of lentivirus from the constructs described above, is achieved by transfection of 293T cells *via* calcium phosphate co-precipitation, with virus harvested 48 hours post transfection, and purified *via* ultrafitration and ultracentrifugation [103]. This method of virus production gives high titre, relatively pure virus, with minimal loss of virus particles through the purification process [103].

1.6.1.2.6.1 Viral titre, p24 and specific infectivity

Virus titre is generally determined as transducing units (TU) of virus per mL by marker gene expression of the vector, or *via* Real-time PCR, giving a vector copy number in transduced cells. Titre is cell dependent and therefore, needs to be carried out in a consistent manner. Studies have shown that titre as determined for the same virus, using different cell types, varied between the cell types used [106].

As described in 1.6.1.2.6, the lentivirus system used for this study is comprised of 5 helper plasmids, one of which contains the sequence encoding the HIV-1 *gag* protein. The *gag* protein is synthesised as a 55 kDa polyprotein, which is then cleaved into three proteins, of which p24 is one. This protein can be assayed using ELISA as a method for quantifying the amount of virus particles present. It should be noted that this simply gives a measure of the amount of particles, and not the number of infectious virus particles.

From the amount of virus particles (p24) and the number of transducing units (titre), the number of transducing units / ng p24, also known as the specific infectivity of the virus, can be determined. This is a measure of the relative number of infectious and non-infectious units of virus present. The specific

infectivity can be manipulated to an extent, by altering the amount of *gag* protein present during virus production [103].

1.6.1.2.6.2 Safety of lentiviral vectors

When using lentiviral vectors, safety is one of the most important aspects to consider. One major safety issue with the use of a lentivirus for gene therapy, particularly one derived from HIV-1, is the possibility of a replication-competent virus being inadvertently delivered or forming after delivery. The most likely route to the formation of replication-competent virus is the unintended recombination of the vector containing the transgene and the helper plasmids during virus production. The risk of this occurring is influenced by the number of helper plasmids and the amount of sequence overlap between the plasmids. The system used for this study utilised 5 plasmids with the only sequence overlap of note between the transfer vector and pHCMVwhvgagpolstml of 500 bp. This small overlap decreases the likelihood of replication competent virus forming compared to systems that have larger amounts of sequence overlap [107]. In addition, the homology is reduced by manipulation of the codon usage in the HCMVwhvgagpolstml coding sequence. Theoretically, replication competent virus could also arise *via* recombination with retroviral like sequences in the genome of the cells used for virus production or in the host cell.

In the first human gene therapy trials using a γ-retroviral vector was used to restore mutant genes in inherited immunodeficiencies [108] [109]. A retroviral vector introduces RNA and enzymes- namely reverse transcriptase and integrase- into the cell, where the RNA molecule must produce as DNA copy from its RNA molecule before it can be integrated into the genetic material of the host. In the first successful gene therapy trials for SCID-XI, 5 out of 20 patients treated developed leukemia- 2 years post initial treatment- due to vector integration upstream of a proto-oncogene, with this insertion increasing its rate of transcription [110-114]. One of these patients has consequently died due to the

gene therapy treatment. Although this death was very unfortunate, the trial also gave a normal life to the other patients, who would not have had this if it was not for the gene therapy. However, it emphasizes the cost half of cost: benefit analysis for gene therapy, and makes it obvious that any means by which the risk can be reduced is of great importance. Insertional mutagenesis of this sort may be expected as a consequence of treatment with any integrating vector.

1.6.2   Barriers to Treatment of CF

1.6.2.1   Physical Barriers to gene delivery

Gene delivery for CF is currently targeted to the lungs due to their major involvement in the morbidity and mortality associated with the disease. The lungs are designed to clear inhaled pathogens, and therefore many inherent barriers have to be overcome for the gene transfer vector to gain access to the airway epithelium cells which are the targets for CF gene therapy. The respiratory epithelium has a series of barriers that present a challenge for the administration of gene transfer agents whether they are based on viruses or on non-viral delivery systems. These barriers consist of: (i) a well defined mucus layer that can readily bind inhaled vectors and remove them *via* normal mucus clearance mechanisms, (ii) a glycocalyx covering the apical surface, microvilli and bases of the cilia that can bind vectors and prevent them binding to cell surface receptors. The glycocalyx is comprised of many carbohydrate-bearing structures including glycoproteins, glycolipids and proteoglycans [115]. These surface barriers are complemented by tight junctions found between epithelial cells. These junctions are moderately 'leaky' to ions but do not allow larger solutes or particles, such as a virus, to pass through [116]. Tight junctions also prevent virus particles reaching the basolateral receptors required for attachment and infection of many viruses, including VSV-G pseudotyped lentivirus, to enable gene transfer (Figure 1-6).

The barrier function of tight junctions can be transiently disrupted so that vectors can access the basolateral surface of target cells and also the niche of progenitor like basal cells that lie beneath the epithelial layer. Opening of tight junctions can be achieved by the use of Ca^{2+} chelating agents such as EGTA [117, 118], non-ionic detergents such as polidocanol [119]and lysophosphatidylcholine (LPC) [93], and antibodies able to block the functions of proteins involved in the tight junction complex [120]. Modification of a 60-kDa membrane protein- Occludin- found in the tight junctions has also been shown to increase epithelial permeability [121].

The use of recombinant viral vectors, pseudotyped with glycoproteins from other enveloped viruses that have been shown to infect the apical surface of airway cells, also provides a means to bypass the tight junction barrier to virus infection [122].

Mucociliary clearance is thought to decrease the contact time with the gene transfer vector to the epithelia, such that the contact time may be too short to achieve significant uptake of the vector [123]. Methods of negating mucociliary clearance in normal airway epithelia have been described by Sinn *et al* 2005, who used the viscoelastic gel methylcellulose, which provides increased epithelial residence time, and acts to slow mucociliary clearance. Vectors formulated in 1% methylcellulose were able to increase the level of gene transfer achieved with adenovirus vectors and a feline derived lentivirus construct in murine nasal airways. Methylcellulose was also seen to decrease the mucociliary transport rate to 14% of normal, which suggests that decreasing mucociliary clearance rates increases vector residence time, allowing more vector particles to be integrated into target cells [123].
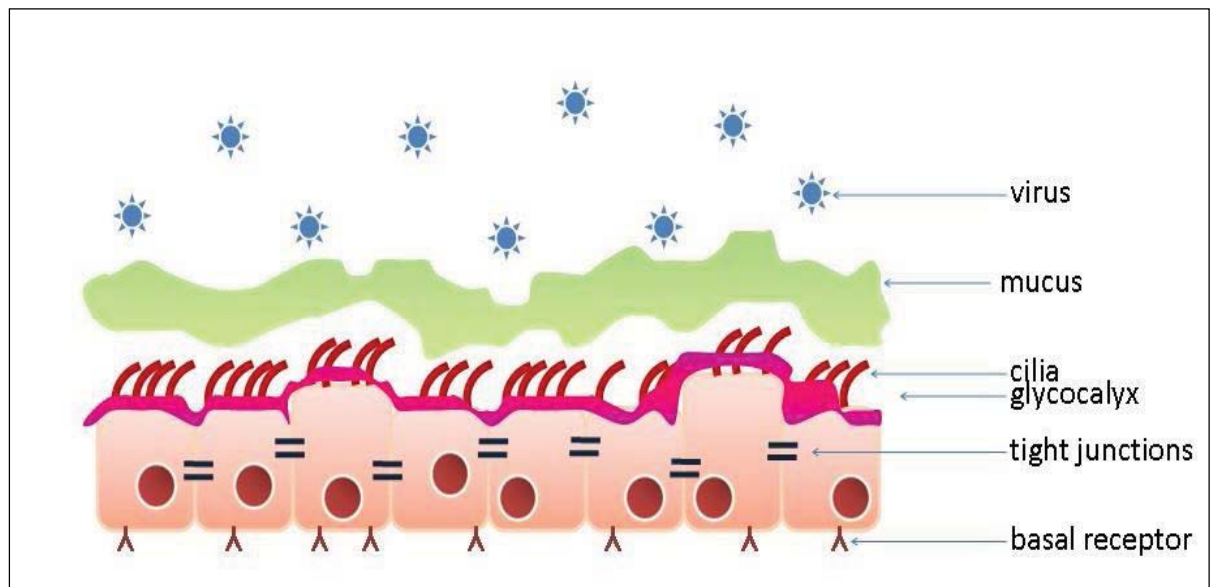
Figure 1-6: Barriers to viral gene therapy

Barriers to viral gene therapy include mucus, the glycocalyx, cilia involved in mucociliary clearance and tight junctions, all of which prevent viral vectors reaching their relevant receptors.

1.6.2.2   Immune Response

The immune response has developed and evolved to remove foreign particles from the body. This defence mechanism constrains successful use of gene replacement therapy with both viral and non viral vectors. Controlling immune responses for gene transfer will be important for the safety of the patient, for the efficacy of the therapy and the ability to repeat treatment effectively.

Stimulation of adaptive immune responses requires a foreign antigen and concomitant cues from the innate immune system. Cells of the innate immune system include dendritic cells which are activated by microbes through pathogen recognition receptors (PRR's) that recognise pathogen-associated molecular patterns. These molecular patterns are common in microbes, but absent in healthy mammals.

PRR's can be expressed on the cell surface as in some of the Toll-like receptors (TLR's), mannase, glycan and scavenger receptors, where as others reside in the cytoplasm. PRR's also react to endogenous molecules including cell stress signals such as heat shock proteins and material released from necrotizing tissue [124].

1.6.2.2.1   B-cell responses to viral gene transfer agents

B-cells are stimulated by soluble cell-surface-expressed antigens on engagement of the B-cell receptor. Additional signals are provided by CD4+ T-helper cells, which in turn require stimulation by antigens expressed in the form of peptides by major histocompatibility complex class II molecules on mature dendritic cells. Upon activation, B-cells undergo Ig gene rearrangement and hypermutation which results in the production of antibodies of increased affinity and different isotypes.

Most viral vectors result in CD4+ T-cell responses- predominantly the Th1 type, with Th1 cells promoting a switch to immunoglobulin G2a (IgG2a). Once stimulated, B-cells replicate and differentiate into antibody producing plasma or long lived memory cells. A second encounter with the antigen (vector) results in activation of memory B cells, which mount an enhanced and accelerated antibody response [124].

1.6.2.2.2 CD8+ T-cell responses to viral vectors

T-cells can be divided into two groups- CD4+ and CD8+. CD4+ cells have regulatory functions, whereas CD8+ clear virus infected cells through direct lysis. The latter is the main problem associated with viral gene therapy. CD8+ is stimulated by antigens expressed in the form of peptides on MHC class I molecules on mature dendritic cells. Processing of antigens generally involves proteolysis by a proteasome complex in the cytoplasm of proteins synthesized *de novo* [125].

The resulting peptides are transported by a peptide transporter into the endoplasmic reticulum where they can associate with MHC class I molecules, which are then translocated to the cell surface.

CD8+ T-cell responses to the transgene product are induced by endogenously synthesized transgene products, whereas CD8+ T-cell responses to some viral vectors such as AAV that are fully "gutless" and therefore fail to synthesise viral gene products, are induced upon cross presentation. Cross-presentation is a process in which proteins or particles that are not synthesised in dendritic cells are taken up by pino or phagocytosis can also be degraded and presented by MHC class I molecules [126]. CD4+ and CD8+ T-cells are positive for CD25 and can also be described as T-regulatory (T-reg) cells.

Our immune system has evolved to eliminate all pathogens including viruses and does not distinguish between harmful replicating viruses and beneficial viral vectors- vectors carrying therapeutic genes.

The main hindrance for permanent gene transfer is the response by CD8+ T-cells, which can readily eliminate target cells expressing antigens of the gene therapy vehicle or transgene product. Permanent gene therapy needs to focus on the pathways that prevent or eludes the activation of, or the effector functions of this T-cell subset [125].

Table 1-3: Immune profiles of commonly used gene transfer vectors

Gene transfer vector	Immune response	Suitability for gene transfer
Adenovirus	Induce innate immunity and dendritic cell maturation, induce T and B-cell responses to antigens of the Ad virus and the transgene product [127], [128],[129]	Not suitable for permanent gene transfer
Adeno-Associated virus	Induce antibodies and T-cells to the viral capsid antigens, can induce B- and T-cell responses to the transgene product in experimental animals, AAV-induced T-cell responses to the transgene product are functionally impaired [130], [131]	Highly suitable for gene transfer
Herpes Simplex Virus	Induce innate immunity and dendritic cell activation, induce T and B cell responses to antigens of HSV and the transgene product; responses might be blunted owing to HSV's ability to subvert immune responses [132]	Vectors are hightly immunogenic and unlikely suitable for sustained gene transfer
Lentivirus	Induces innate immunity and adaptive immune responses, immune responses are likely to be exacerbated upon pseudotyping with VSV glycoprotein [133], [134]	Vector is moderately immunogenic, may be suitable for permanent gene transfer

1.6.2.2.3   The innate immune system

Innate immunity is the first line of defence against invading pathogens through recognition of conserved microbial structures or products known as pathogen-associated molecular patterns (PAMPs) by a set of receptors called pattern recognition receptors (PRRs) noted earlier [135]. The best studied PRRs are Toll-like receptors which are expressed on various immune cells including macrophages and dendritic cells. Toll-like receptors are transmembrane proteins characterised by the presence of extracellular leucine-rich repeats and a cytoplasmic signalling domain [136]. Thirteen Toll-like receptors have been identified in mammals, each with a unique set of PAMPs that are distinct in their chemical structure [135]. On recognition of viral PAMPs, PRRs trigger a series of signalling cascades leading to induction of antiviral genes and inflammatory cytokines which results in direct killing of the invading virus as well as promotion of the initiation of adaptive immune responses [137].

The type I interferons IFN-α and IFN-β, are key cytokines produced in response to viral infection that play an essential role in both the innate and adaptive immune responses [138] [139].

1.6.2.2.3.1   Inhibitors of the innate anti-retroviral immune response

As described previously, infection of a virus, including HIV-1, invokes an immune response. HIV-1 infection involves integrase protein (INI1), (a nuclear integrase different from HIV-1 integrase) and a protein known as PML. These are both found in the nucleus of cells, where they enhance HIV-1 infection. The innate anti-retroviral immune response involves relocation of INI1 and PML to the cytoplasm, which decreases the efficiency of HIV-1 infection. Leptomycin B (LMB) has been found to interrupt this relocation of INI1 and PML effectively inhibiting the innate anti-retroviral response [140].

LMB is a secondary metabolite produced by the *Streptomyces* sp. Strain ATS 1287. It has attracted attention due to its antifungal [141-143] and anti-tumour effects [144]. LMB has also been found to be active at low nanomolar (nM) concentrations [145].

Table 1-4: Approaches to overcome immune barriers to permanent gene therapy

Approach	Pathway	Advantage	Disadvantage
Rapamycin	Induces T-reg production	Inhibition of immune activation and functions	Total immunosuppression
mycophenolate mofetil (MMF) + rapamycin	Inhibits proliferation of immunocytes	Short term treatment is likely to succeed with AAV transfer	Total immunosuppression
MMF + rapamycin + daclizumab	Reduces T-reg production	-	Favours induction of immune responses to the transgene product
CTLA-4 Ig	Reduces co-stimulation	Inhibition of induction of T and B cells	Potential for total immunosuppression
Antibodies to co-stimulatory ligands	Reduces co-stimulation, may induce T-reg's	Inhibition of immune activation functions	Potential for total immunosuppression
Inhibition of co-stimulation *via* vector expressed antagonists	Reduces local co-stimulation	Selective inhibition of immune activation at the site of vector administration	Unlikely to prevent cross-presentation
Adoptive transfer of Tregs or inducible regulatory T cells	Increases T-reg or TR1 frequencies	Inhibition of immune activation and function	Potential for total immunosuppression
Vector modifications	Elimination of B- or T-cell epitopes or masking of epitopes	Allows efficient transduction in the presence of vector-neutralising antibodies	Will not prevent T-cell responses in an outbred population; may affect vector tropism
MicroRNA	Inhibits expression in specific cell types	Inhibition of expression in hematopoietic cells can prevent induction of immune responses	Unlikely to prevent cross-presentation

Adapted from [125]

1.6.2.2.4 *The Proteasome and Lysosome as targets for increasing gene expression*

The proteasome is a multi-subunit complex that plays a major role in the selective degradation of intracellular proteins- these are proteins that are misfolded, damaged, proteins involved in signal transduction, transcription factors and processing of foreign proteins for antigen presentation [146-148]. The proteasome structure consists of a cylindrical central core, the 20S particle, composed of 4 stacked rings of 7 proteins in each ring. The 2 outer rings are identical to each other and are called α-rings; the inner β-rings are also identical to each other and contain the catalytic proteinase functions on their inner central channel facing surfaces. The 20S core is bound symmetrically to 2 copies of a regulatory 19S particle. The role of the regulatory complex is to allow for recognition of substrates destined for degradation as well as ATPases required to hydrolyse ATP that provides energy for the unwinding of proteins before degradation. Unwound proteins are fed down the central channel in the 20S core and undergo progressive degradation by threonine residues in the beta-rings. The 20S core and the two 19S regulatory units are collectively known as the 26S proteasome [149]. See Figure 1-7.

Proteasomes recognise proteins and degrade them due to the polyubiquitin attached to their lysine side chains. Non ubiquitinated proteins can also be degraded by the same proteasome. Peptide DNA condensates composed of mainly polylysine sequences may also be recognised as foreign and processed by this pathway resulting in degradation [150].

One method of enhancing both non-viral and viral mediated gene transfer is by the inhibition of proteasome function. Premature degradation or metabolism of plasmid DNA limits the level and duration of transient gene expression by non-viral gene delivery systems [151]. There are several natural and synthetic compounds that act as proteasome inhibitors [149].

__Naturally occurring__

- − Lactacystin
- − Eponemycin
- − Epoxomycin
- − Aclacinomycin

__Synthetic:__

- − Calpain inhibitor I
- − PS-519 (synthetic compound similar to lactacystin)
- − Aldehyde inhibitors (CEP-1612, MG132)
- − Benzamide (CVT-634)
- − Boronic acid inhibitors (PS-341)
- − Vinyl sulfone tripeptides
- − HIV-1 protease inhibitor (Ritonavir)
- − Doxorubicin HCl

Proteasome inhibitors have been shown to be effective in cancer models, angiogenesis, asthma, ischemia, multiple sclerosis, rheumatoid arthritis and psoriasis. A number of pre-clinical and early clinical studies have been performed to test the usefulness of proteasome inhibition for the treatment of Alzheimer disease, amyotrophic lateral sclerosis, autoimmune thyroid disease, cachexia, Crohn disease, Hepatitis B, inflammatory bowel disease, sepsis, systemic lupus and transplant rejection and its related immunology [149]. A broader application of proteasome inhibitors is their use to increase the level of gene transfer seen in a viral or non-viral gene delivery event. Recently, proteasomal processing has been implicated in the intracellular trafficking and metabolism of viral vectors used for gene delivery.

Proteasome inhibitors have been shown to increase AAV-mediated transduction *in vitro* and *in vivo*. The mechanism of how this occurs remains unclear, however, it does appear that it is not solely related to the inhibition of vector degradation through the ubiquitin-proteasome degradation pathway [152].

Possible mechanisms for increased gene transfer when proteasome inhibitors are incorporated into therapy are:

- Prevention of virus degradation

- Improved nuclear uptake of virus/ gene transfer agent

- Improved genome uncoating

- Enhanced capsid processing

- Improved second strand synthesis

- Reduced degradation of the recombinant protein

The effect of proteasome inhibitors on non-viral gene transfer have been described in studies by Kim JL *et al* who showed that proteasome inhibitors increased non-viral peptide-mediated gene transfer and Kim KI *et al*, who showed Doxorubicin to increase lipid-mediated gene transfer. The proteasome inhibitor Z-LLL (MG132) has also been shown to increase the efficiency of AAV-mediated gene delivery [153].

Lysosomes are intracellular organelles that are membrane enclosed compartments filled with hydrolytic enzymes that are used for the controlled intracellular digestion of macromolecules. Lysosomes contain approximately 40 types of hydrolytic enzymes which include proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases and sulfatases, all of which are acid hydrolases. For optimal activity, these enzymes require an acidic environment, which the lysosome provides; lysosomes maintain a pH of 5 in their interior. Like all other intracellular organelles, the lysosome not only contains a unique collection of enzymes, but also has a unique surrounding membrane. Transport proteins in this membrane allow the final products of digestion of macromolecules- amino acids, sugars and nucleotides- to be transported to the cytosol where they can be excreted or re-utilised by the cell. A H^+ pump in the lysosome membrane uses the energy of ATP hydrolysis to pump H^+ into the lysosome, maintaining the lumen at its acidic pH.
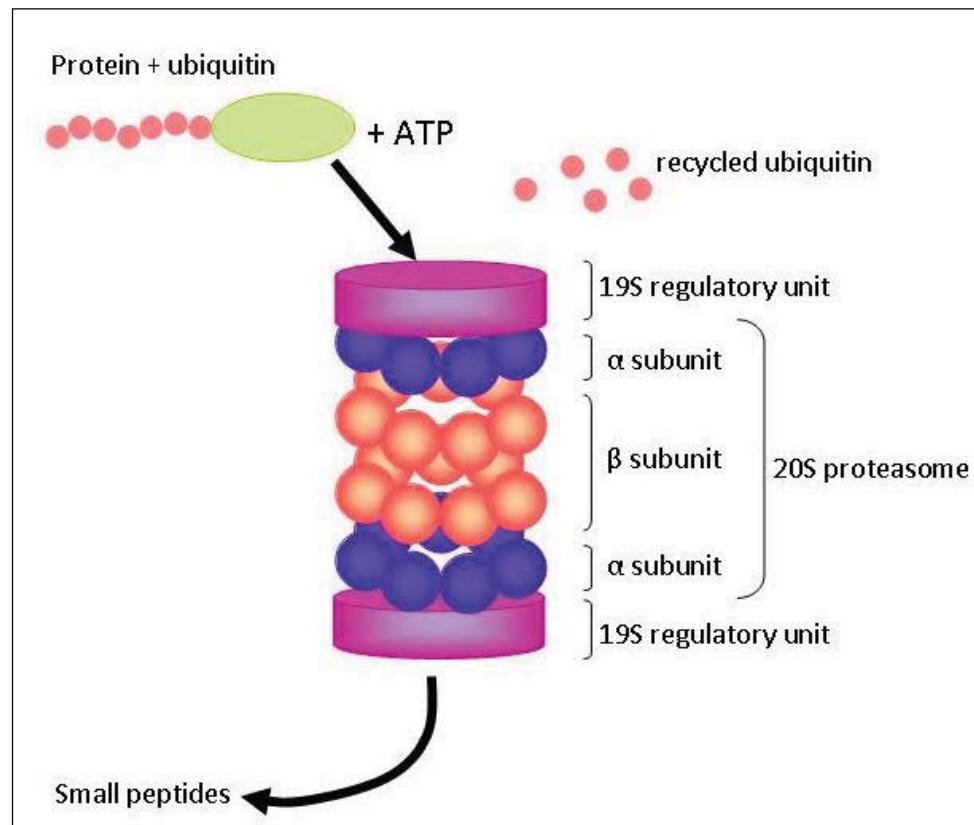
Figure 1-7: Structure of the 26S proteasome

The 26S proteasome consists of two 19S regulatory units and a 20S proteasome consisting of two α and two β subunits. The proteasome acts to breakdown ubiquitinated proteins into small peptides.

The best studied path to degradation in lysosomes is macromolecules taken up from extracellular fluid by endocytosis. Endocytosed molecules are initially delivered in vesicles to small, irregularly shaped intracellular organelles called early endosomes. Some of these ingested molecules are selectively retrieved and recycled to the plasma membrane while others pass on into late endosomes. It is here that endocytosed materials first meet the lysosomal hydrolases which are delivered to the endosome from the Golgi apparatus. The interior of the late endosomes is mildly acidic (pH=6) and this is where the hydrolytic digestion of the endocytosed molecules begins. Mature lysosomes form from the late endosomes accompanied by a further decrease in internal pH. Lysosomes are thought to be produced by a gradual maturation process, during which endosomal membrane proteins are selectively retrieved from the developing lysosome by transport vesicles that deliver these proteins back to endosomes or the trans Golgi network.

The second pathway to degradation in lysosomes is used in cell types for the disposal of obsolete parts of the cell itself in a process called autophagy. The process is believed to begin with enclosure of an organelle by membranes of unknown origin creating an autophagosome, which then fuses with a lysosome. The process is highly regulated and selected cell components can be marked for lysosomal destruction during cell remodelling.

Phagocytosis is the third pathway that brings material to the lysosomes for degradation.

The lysosome was long believed to be the only site for protein breakdown within cells. However, studies of lysosomal inhibitors established that lysosomes play a minor role in degradation of cytosolic proteins. It is however involved primarily in the breakdown of membrane-associated proteins or extracellular proteins taken up by endocytosis which may include virus or viral components [154].

1.6.2.2.4.1 Doxorubicin

Doxorubicin inhibits proteasome function, and is a non-peptide inhibitor with discrete selectivity for the chymotrypsin-like proteolytic activity of the 20S proteasome [155]. Doxorubicin is also cytotoxic by virtue of it inducing DNA strand breaks by interacting with topoisermerase II [156] [157] and this effect of doxorubicin can induce cell cycle arrest or apoptosis.

As doxorubicin is a widely used cancer chemotherapy drug, toxicity has been widely studied [158] [159]. Cardio-toxicity is the limiting factor in the use of doxorubicin in humans. The total lifelong dose influences the cumulative cardio-toxicity of the compound [160].

In a study by Kim KI *et al* 2007, iodide uptake was increased when doxorubicin was administered to human sodium/iodide symporter (hNIS) gene transfected ARO (anaplastic thyroid cancer) cells. Adenovirus carrying either luciferase or hNIS was used to transfect the cells, and doxorubicin was tested in a concentration range from 1- 15 µg/mL. The cells treated with doxorubicin showed increased iodide uptake (i.e. gene transfer) without loss of cell viability. Iodide uptake was seen to increase in a dose dependant manner 21.9-fold, with the highest increase seen at 3 ug/mL. Similar results were seen with the use of a Luciferase vector. Higher concentrations of doxorubicin did not increase the level of gene transfer observed [161].

1.6.2.2.4.2 Z-LLL (MG132)

Z-leu-leu-leu (Z-LLL or MG132) is a potent and reversible inhibitor of the chymotryptic-like activity of the proteasome. It has been shown *in vitro* and *in vivo* to enhance transduction of human polarised epithelial cells and mouse lung tissue with rAAV-2 [162]. The exact mechanism by which rAAV transduction is enhanced has not been elucidated, however, general observations suggest proteasome

inhibitors act to enhance capsid processing and evaluation of viral DNA suggests that the inhibitors don't act by simply preventing enzymatic degradation of internalized rAAV virions [162].

The effect of this proteasome inhibitor has been tested on HIV-1 transduction *in vitro*. HIV-1 transduction of HeLa CD4+ cells was much increased in the presence of Z-LLL. HIV-1 modified with the VSV-G envelope protein transduction was also increased in the presence of Z-LLL [163]. Again, the exact mechanism of how HIV-1 transduction is enhanced by Z-LLL has not been determined, however several observations give clues as to possible methods of action. The presence of ubiquitinated material in HIV particles indicates that at least a fraction of incoming virions are potential targets for ubiquitin-dependant proteasomal degradation. Alternatively, viral proteins may serve as a target for ubiquitin-independent degradation by the 20S proteasome complex as seen with misfolded or normal cellular proteins [163].

1.6.2.2.4.3   Bafilomycin A1

Bafilomycin A1 (Baf A1) is a member of a group of macrolide antibiotics that at low concentrations specifically inhibit the vacuolar H+ ATPase. Baf A1 is known to neutralise acidic organelles such as endosomes and inhibits the endocytic pathways from the early endosome. At high concentrations (1 µM) it is seen to inhibit retrograde transport from the Golgi to the endoplasmic reticulum.

It has recently been shown that treatment of cells with vacuolar H+ ATPase inhibitors at concentrations that completely block VSV infection markedly increases the infectivity of HIV-1. It therefore appears that not only can the endocytic pathway of HIV-1 entry be infectious under these conditions, but also the normal acidification of endosomes results in extensive inactivation of HIV-1.

Wei *et al* 2005 reports Baf A1 to increase HIV-1 infectivity due to a block of virus degradation upon viral entry to the cell *via* endocytosis. The largest enhancement of infectivity was 20- to 50-fold. The effect was seen to be not saturable by the amount of input virus and their least infectious HIV-1 isolate had the greatest response to the Baf A1 lysosomal inhibitor [164].

When both a proteasome (lactacystin) and Lysosome inhibitor (Baf A1) were tested, an additive effect was seen with some HIV-1 isolates tested, whereas a synergistic effect resulting in a 400-fold enhancement of infection was seen for the least infectious HIV-1 isolate. When another proteasome inhibitor MG132 (described above) was used in conjunction with Baf A1 a synergistic increase of 95-fold was seen. These synergistic effects of proteasome and Lysosome inhibitors suggest that they may act sequentially [165].

Fredericksen *et al* 2002 showed pre-treating cells with Baf A1 ensured maximal inhibition of lysosomal acidification prior to virus infection and therefore minimized the likelihood of lysosomal degradation of infectious virus [166].

1.6.2.3   CF specific barriers

The products of inflammation in CF bronchoalveolar lavage fluid, such as neutrophil elastase and neutrophil α-defensins are inhibitory to AAV transduction, however, these effects can be overcome *via* the use of antiproteinases such as α-1-antitrypsin [167]. Pre-existing immunity to wild-type viruses such as adenovirus can also hinder the ability for the gene to transduce target cells. Anti-adenovirus antibodies found in the sputum of CF patients have been shown to inhibit adenovirus vector mediated transduction [168].

The thickened, dehydrated mucus layer found in CF airways also acts as a barrier to gene transfer agents, binding inhaled vectors, preventing access to the target cells [115].

1.7   Animal Models

Animal models are important in the understanding of many genetic diseases, including CF. While cultured epithelial cells allow many important studies to be performed these models lack the structural complexity of intact organs. Three years following the cloning of the CFTR gene, the first CF mouse model was reported [169]. To date there are 11 mouse models of CF [170]. Initial mouse models of CF were created with mutations that resulted in a complete loss of function, with other mouse models being developed to address specific mutations found in CF. Mouse models have been developed for the most common mutations found in the CF population- the ΔF508, G480C and G551D mutations.

Intestinal disease is the most prominent feature in CF mouse models, with symptoms comparable to CF in humans. Mouse models demonstrate a wide range of intestinal phenotypes with some models having severe intestinal obstruction resulting in 95% mortality. A colony of CF mice with such severe intestinal obstruction and such a high mortality rate is difficult to manage and is not generally useful for experimental purposes unless modifications to their husbandry (such as the use of agents that maintain gut transit-permitting relatively normal survival) are made.

Pancreatic insufficiency is a major problem in many CF patients; however, it has not been convincingly demonstrated in CF mouse models.

As lung disease is the most common cause of mortality in CF patients, a mouse model displaying the same characteristics as those present in a human would be most beneficial. Although there have been a variety of pulmonary abnormalities reported in the existing CF mouse models, they are complex and

do not fully replicate the tissue abnormalities of lung disease in CF individuals [170]. This may be due to the mouse lung containing other chloride channels that compensate for the knocked out CFTR gene, or perhaps because mouse airways express only relatively small amounts of CFTR, so a knockout has little effect [171] [172]. However, it was found that the mouse nasal airways mimicked the physiology of CF lung disease, with studies clearly demonstrating the basic ion-channel defect in the mouse nasal passage. In addition, the CF mouse nasal airway accurately represents the human (conducting) respiratory tract in that it consists of cells of the type that need to be targeted in CF – cells of the respiratory epithelium, and also displays the dysfunction of CFTR and ion channels that are characteristic of CF [170]. The murine nasal airway is therefore widely used to analyse gene transfer in a relevant and intact biological system. However, the more recent study by Griesenbach *et al* [173] has shown that the murine nasal airways may not be the most representative model for CFTR functional assays due to confounding factors from CFTR present in olfactory epithelium.

CF mouse models previously used for gene therapy research, including lentiviral mediated gene therapy, include the *cftr*^{tm1Unc} mouse [93], which has an inframe stop at exon 10 [169]. In these studies, virus was delivered into the nasal passage of the mouse, and gene transfer could be analysed *via* transepithelial potential differences to measure correction of the ion channel defect [93]. This model exhibits severe intestinal obstruction, which can be managed by the inclusion of colonlytely in drinking water. Later, Zhou *et al*, developed a gut corrected mouse from the *cftr*^{tm1Unc} mouse model. This was achieved by the inclusion of human CFTR under control of the rat intestinal fatty acid binding protein (FABp) resulting in expression of CFTR in the gut, but not in the airway or other tissues. This mouse model is described in detail in chapter 5. Although the CFTR knockout mouse models have been widely used to test therapies for CF, a model that replicates the human lung disease remains highly sought after, as have larger animal models of CF.

There has been success with the creation of a mouse model that replicates the CF-like lung disease through over expression of the ENaC channel, leading to excess sodium absorption [174]. These animals show key abnormalities of cystic fibrosis including airway obstruction with dehydrated mucus and inflammation leading to lung destruction. These animals should be useful for evaluating therapeutic interventions to treat CF lung disease, but not for those related to restoring CFTR function such as gene therapy.

To date there have been few successful attempts to produce a model of CF in a larger animal. Recently Rogers *et al* 2008 published the development of a CF pig model. Pigs were chosen due to their anatomy, biochemistry, physiology, size, lifespan and genetics more closely resembling humans than the mouse models [175, 176].

Homologous recombination in fibroblasts of outbred domestic pigs to disrupt the CFTR gene and somatic cell nuclear transfer were used to generate CFTR$^{+/-}$ pigs [177]. At sexual maturity female CFTR$^{+/-}$ pigs were bred to CFTR$^{+/-}$ males, with 6 litters producing 64 piglets. Genotyping confirmed 18 CFTR$^{+/+}$, 26 CFTR$^{+/-}$ and 20 CFTR$^{-/-}$ animals. All pigs looked normal at birth and their genotype could not be determined by their appearance, which is similar to the case in humans with CF. The earliest manifestation of disease was meconium ileus leading to intestinal obstruction by 24-40 hours of age. Surgery was carried out in the same manner as would be done for a CF patient experiencing the same condition. Once the piglets grew, pancreatic insufficiency was seen- again, in the same way as observed in humans [178].

The lungs of neonatal CFTR$^{-/-}$ piglets appeared similar to their wild type litter mates, with no evidence of inflammation or plugging of the submucosal gland ducts [178]. Tracking the lungs of CFTR$^{-/-}$ piglets as they are exposed to environmental challenges may provide further opportunities to use the pig

model as a better representation of the CF disease observed in humans. Overall, the CF pig appears to be an extremely promising model for CF research.

Serial nuclear transfer has been recently used to produce a CFTR gene-deficient model in the domestic ferret, using recombinant adeno-associated virus-mediated gene targeting in fibroblasts, followed by nuclear transfer cloning [179]. Using this cloning technique, 8 healthy CFTR gene-disrupted male ferrets were obtained from 11 recipient jills.

The ferret is a potentially good model for studying CFTR lung biology as the ferret lung has marked similarities to humans in terms of lung physiology, airway morphology and cell types. Secondly, the expression of CFTR in ferret airway epithelium and submucosal glands is identical to that in humans and thirdly, the amino acid identity between the ferret and human nucleotide binding domain 1 of CFTR is 97%, which is as high as in non-human primates (96%) and is significantly higher than that of rodents (80% in rats and mice). Lastly, the ferret has a gestation period of 42 days and reaches sexual maturity at 6 months of age, which has obvious advantages over larger species for animal modeling in terms of colony maintenance and production of affected animals [180].

As yet it is not known whether either the CF pig or ferret model will demonstrate the lung disease associated with human CF patients. However, as ferrets are a preferred model for other devastating human lung diseases such as H5N1 influenza and the SARS virus, the ability to generate genetically engineered ferrets may also be of significant utility to pandemic viral disease research [179].

1.8 Methods for analysing CFTR gene transfer

Analysis of successful transfer of the CFTR gene can be done in a number of ways, including using the differences seen in ion transport between CF affected and non-affected individuals as a measure of success.

Active ion transport by the respiratory epithelium is thought to play an important role in the regulation of the volume and composition of airway surface liquid and ultimately contributes to the efficiency of mucociliary clearance and cough clearance. *In vitro* airway epithelial tissues can be freshly excised and studied in Ussing chambers. When the TPD is electrically clamped to zero, basal patterns of ion transport can be directly determined by isotopic measurements of the transepithelial flux of specific electrolytes, including Na^+ and Cl^- ions. The net flux of ions cannot be measured *in vivo*, but the interpretation of bioelectric changes can be inferred from principles developed from studies of human tissues and basic principles of ion transport and membrane physiology. The measurement of Cl^- conductance can be assessed by establishing a gradient for Cl^- ions to exit the luminal/apical membrane of the airway cell by perfusing the surface with a Cl^- free solution, whereby Cl^- ions are replaced by an impermeate anion, such as gluconate. The TPD is measured between a fluid filled exploring cannular positioned in the airway surface and an agar filled reference bridge in the subcutaneous space [181].

The presence of any physiological function of CFTR that has been delivered to airway epithelia *via* a gene therapy vector can be determined with the measurement of TPD *in vivo*. This technique is technically challenging, as it requires the positioning of a cannula in the nasal airways, which is done by a simple depth measure, as the inside of the airways cannot be seen and much practice is required to obtain the correct positioning to measure the bioelectric signal. Griesenbach *et al* 2009 [173] suggest that the CF knockout mouse model may not be a representative model in which to assess gene transfer efficiency. They report corrective gene transfer in human airway epithelia in clinical trial, but using the

same vector system, did not achieve the same correction in the mouse nose [173]. Grubb *et al* 2009 have also speculated that olfactory cells, which express higher levels of CFTR than respiratory cells, contribute significantly to ion transport in the nasal epithelium of mice, therefore, altering the potential difference measured in the mouse nasal airways [182]. However, some studies, such as that reported by Limberis *et al* 2002, have successfully shown correction of TPD following a gene transfer event, in this case with a lentivirus containing the CFTR gene [93].

A number of other assays can also be used to assess the effectiveness of CFTR gene transfer. In a study by Griesenbach *et al* 2009, TPD, Periciliary liquid (PCL) height and bacterial adherence were measured for correction of the CF defect in CF-knockout mice following delivery of CFTR complexed to the cationic lipid GL67A. Periciliary liquid height is central to CF pathophysiology, and has been shown to be reduced in human primary airway epithelium air-liquid interface cultures and in the nose of CF mice. In this study no significant differences in PCL height were seen after transfection with CFTR cDNA compared to the untreated controls. Pseudomonas aeruginosa adherence to ciliated epithelial cells from CF patients and CF knockout mice is increased. The Griesenbach study also assessed whether the number of bacteria binding to the cilia of CF mice was reduced following gene transfer. Once again, no significant difference was seen when comparing bacterial adherence in treated and untreated mice [173].

Another method of determining the presence of CFTR is by immunohistochemistry. This can be used to quantify CFTR expression on a cell-by-cell basis. However, the availability of CFTR antibodies that have been shown to work well is a limiting factor to immunohistochemical detection of CFTR. Griesenbach *et al* 2009 report no detection of CFTR after using a variety of antibodies on a control cell line expressing CFTR [182]. To date CFTR immunohistochemistry has not been convincing in providing a sensitive assay for detection of CFTR in the mouse respiratory epithelium.

1.8.1   Aptamers

Aptamers are single stranded nucleic acid molecules that offer an alternative to traditional monoclonal antibodies. As these molecules are made to bind to a specific target without the use of animals they offer a rapid technology to produce molecules with high affinity and specificity to the desired protein- and are not limited by the requirement for immune recognition [183]. The attempted development of aptamers to the CFTR gene is described in chapter 6.

1.9 Specific Aims of Research

As it is not known how much gene transfer is required by a gene transfer vector to achieve therapeutically beneficial outcomes, a number of methods to optimise gene transfer to the murine nasal airways were investigated with both marker (LacZ, Luciferase) and therapeutic (CFTR) genes.

The use of proteasome inhibitors Doxorubicin and Z-LLL and the lysosome inhibitor Bafilomycin A1 were investigated for their potential beneficial effects on the level of gene transfer achieved. It was proposed from *in vitro* data collected from other groups where these inhibitors were seen to increase gene transfer in cell culture that these effects may translate to the enhancement of gene transfer *in vivo*. The use of these inhibitors in conjunction with our lentivirus gene transfer protocol were investigated for their effects on the level of gene transfer achieved *in vivo*.

Another compound investigated was Leptomycin B, an inhibitor of the innate anti-retroviral immune response. Leptomycin B was incorporated into the gene therapy protocol and tested for any enhancement of gene transfer compared to the standard protocol. Leptomycin B was tested for its effects on both LacZ gene transfer and also CFTR gene transfer in CFTR-knockout mice.

Animal models of disease are essential for translating marker gene research to therapeutic gene transfer. The current mouse model of CF exhibits intestinal and gut disease due to the knockout of the CFTR gene, requiring modification of the diet to avoid premature death. A mouse model with the CFTR gene knocked out, but the human CFTR "knocked in" under control of the fatty acid binding protein promoter-specific to the gut was obtained and breed for use with our lentivirus gene therapy protocol.

Methods of visualising CFTR gene transfer have been limited to a select range of antibodies which are hard to obtain, and some of which have low specificity and low binding abilities. Another method was sought to visualise CFTR gene transfer in a method comparable to that of LacZ gene transfer. The

attempted development of aptamers- small specific 3-dimensional DNA molecules were developed *via* SELEX technology. These molecules were derived from a random sequence library to find sequences that bind specifically to the CFTR gene. These aptamers hold great potential as tools for the visualisation of the CFTR transgene in our gene transfer system.

Real time PCR is another important technique for gaining a quantitative value of the number of transgene copies incorporated from a gene transfer event per cell. Assays to the gag gene found in our lentivirus and a house-keeping gene found in mouse cell lines (mouse transferrin) had previously been developed for use in determining viral titre of viruses containing therapeutic genes, such as CFTR that cannot be stained or undergo FACS analysis-as can be carried out with LacZ and EYFP viruses. Current protocols for the titre assay of LacZ virus involved assaying on CHOK-1 cells and staining for the presence of LacZ. As a better method of comparison between viruses titred by staining assay and real time PCR, a real time PCR assay with a house-keeping gene found in CHOK-1 cells was developed.

As the development of more complex lentivirus was made, incorporating up to three transgenes into one virus preparation, a series of real time PCR assays were designed to each of the transgenes to allow a titre to be determined for each transgenes.

As previously mentioned, it is not known the level of gene transfer or gene expression required for therapeutically beneficial outcomes for CF gene therapy. Investigations into the type of promoter driving transgene expression were made. Testing of high ubiquitous expression (EF1α), low ubiquitous expression (pgk) and ciliated cell specific expression (K18) promoters was carried out to determine whether high level ubiquitous expression gave the best therapeutic outcomes, or whether low level or ciliated cell specific (thought to mimic the pattern of expression exhibited by CFTR) gave better therapeutic outcomes.

Finally, to move gene therapy into the clinic and for it to be beneficial to patients, aerosolisation of the lentivirus needs to be considered as the best method of delivering the gene therapy agents to the area of interest-the lungs. Initial studies into the aerosolisation of lentivirus expressing the LacZ transgene were carried out using a rat model.

2 Materials and Methodology

2.1 Materials

2.1.1 Reagents, Chemicals, Kits and Plasticware

Acetic acid	Ajax fine chemicals (Sevenhills, NSW, Australia) Cat # A2335, 2.5 L
Agar	Becton, Dickson and Co. (Detroit, Mich., USA) Cat # 214 010
Agarose, DNA grade	Progen Industries Ltd. (Durra, Qld, Australia) Cat # 206-0011
Amiloride	Sigma Chemical Co. (St Louis, MO, USA) Cat # A7410
Antisedan (atipamezole hydrochloride)	Lyppard Australia Ltd. (Berverly, SA, Australia)
Bafilomycin A1	Sigma Chemical Co. (St Louis, MO, USA) Cat # B1793
Blood and cell culture gDNA Midi kit	Qiagen (Hilden, Germany) Cat # 13343
Boric Acid	AnalaR (BDH chemical supplies, Poole, England) Cat # 10658.8W
Bromophenol Blue	BDH Chemicals Ltd. (Poole, Dorset, England) Cat # 200152E
Calcium Chloride	AnalaR (BDH chemical supplies, Poole, England) Cat # 19970.4Y
Chloramphenicol	Sigma Chemical Co. (St Louis, MO, USA) Cat # C-0387)
Chloroform	AnalaR (BDH Chemical supplies, Poole, England) Cat # 10077.6B
D-Gluconic Acid sodium salt	Sigma Chemical Co. (St Louis, MO, USA) Cat # G9005-500G
Deoxyribonucleotides	Boehringer Mannheim (Mannheim, Germany) Cat # 1581-295
Dimethylformamide	Sigma Chemical Co. (St Louis, MO, USA) Cat # D4551
Domitor (medetomidine hydrochloride)	Lyppard Australia Ltd. (Berverly SA, Australia)
Doxorubicin HCl	Sigma Chemical Co. (St Louis, MO, USA) Cat # D1515
EDTA (Ethylenediaminetetraacetic acid)	Asia Pacific Specialty Chemicals Ltd (Sevenhills, NSW, Australia) Cat # 663-500G
97-100% Ethanol	Ajax Fine Chemicals (Sevenhills, NSW, Australia) Cat # 1046-2.5L
Ethidium Bromide	Ameresco (Solon, Ohio, USA) Cat # J844-10TABS

FastStart High Fidelity PCR system	Roche Applied Sciences (Indianapolis, IN, USA) Cat # 03553426001
Formaldehyde	Ajax Fine Chemicals (Auburn, NSW, Australia) Cat # 230
G418 Sulphate (Geneticin)	Invitrogen Corporation (Carisband, CA, USA) Cat # 11811-031
Gel Extraction DNA purification kit	AdBiotech (Adelaide, South Australia, Australia) Cat # GEK-1
Genopure Plasmid MidiKit	Roche Applied Sciences (Indianapolis, IN, USA) Cat # 3143414
Glutaraldehyde	Sigma Chemical Co. (St Louis, MO, USA) Cat # G2657
Glycerol	BDH (Merck) (Victoria, Australia) Cat # 10118.6M
High Pure Plasmid Kit	Roche Applied Sciences (Indianapolis, IN, USA) Cat # 11754785001
HIV-1 p24 ELISA Kit	Perkin Elmer (Boston, MA, USA) Cat # NEK050A
HotStaTaq DNA Polymerase	Qiagen (Hilden, Germany) Cat # 203205
Hydrochloric Acid (concentrated)	BDH (Merck) (Victoria, Australia) Cat # 1012583
Isopropanol	Ajax Fine Chemicals (Auburn, NSW, Australia) Cat # 425-2.5L
Ketamine	Parnell Laboratories Australia Pty. Ltd. (Alexandria, NSW, Australia)
Leptomycin B	Sigma Chemical Co. (St Louis, MO, USA) Cat # L2913
Lithium Chloride	BDH (Merck) (Victoria, Australia) Cat # 10374
Lysophosphatidylcholine (LPC)	Sigma Chemical Co. (St Louis, MO, USA) Cat # L4129
Magnesium Chloride	BDH (Merck) (Victoria, Australia) Cat # 10149.4V
Megaprep Plasmid Kit	Qiagen (Hilden, Germany) Cat # 12181
Methanol	Merck Pty Ltd. (Victoria, Australia) Cat # 4.10023.2500
Midiprep Plasmid Kit	Qiagen (Hilden, Germany) Cat # 12143
Miniprep Plasmid Kit	Qiagen (Hilden, Germany) Cat # 12125
Normal goat serum	Vector Laboratories, (Burlingame, CA, USA), Cat # S-1000
Paraformaldehyde	Sigma Chemical Co. (St Louis, MO, USA) Cat # P6148
PCR DNA purification kit	AdBiotech (Adelaide, South Australia, Australia) Cat # PCK-1

Phenol	Sigma Chemical Co. (St Louis, MO, USA) Cat # P1037
Polybrene (Hexadimethrin bromide)	Sigma Chemical Co. (St Louis, MO, USA) Cat # 52495
Potassium Ferricyanide	Sigma Chemical Co. (St Louis, MO, USA) Cat # P8131
Potassium Ferrocyanide	Sigma Chemical Co. (St Louis, MO, USA) Cat # P3289
Proteinase K	Qiagen (Hilden, Germany) Cat # 19131
pUC/HpaII DNA Molecular weight marker	Geneworks (Thebarton, South Australia, Australia) Cat # DMW-P1
Safranin O	ProSciTech (Thuringowa, QLD, Australia) Cat # C138
Sodium Acetate	BDH (Merck) (Victoria, Australia) Cat # 10236
Sodium Chloride	BDH (Merck) (Victoria, Australia) Cat # 10241.AP
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co. (St Louis, MO, USA) Cat # L-5750
Sodium Hydroxide	Asia Pacific Specialty Chemicals Ltd (Sevenhills, NSW, Australia) Cat # 482-2.5
SPPI/EcoRI Molecular weight marker	Geneworks (Thebarton, South Australia, Australia) Cat # DMW-S1
T4 DNA ligase	Roche Applied Sciences (Indianapolis, IN, USA) Cat # 716359
Tris base	Roche Applied Sciences (Indianapolis, IN, USA) Cat # 1814273
Triton X-100	BDH (Merck) (Victoria, Australia) Cat # 30632
Tryptone	Becton, Dickson and Co. (Detroit Mich., USA) Cat # 211705
Tween 20	BDH Chemicals Ltd. (Poole, Dorset, England) Cat # 663 6843
ULTRAhyb	Ambion (Austin, Texas, USA) Cat # 8670
VectaShield	Vector Laboratories, (Burlingame, CA, USA) Cat # H-1200
Viagen DirectPCR® DNA extraction	Viagen Biotech Inc. (Los Angeles, CA, USA), Cat # 101-T
Wizard SV Genomic DNA Isolation Kit	Promega (Madison, WI, USA) Cat # A2361
Yeast Extract	Becton, Dickson and Co. (Detroit, Mich., USA) Cat # 212 750
Z-LLL	Sigma Chemical Co. (St Louis, MO, USA) Cat # C2211

2.1.2 Buffers and Solutions

5 x agarose gel loading buffer	25% (v/v) glycerol, 50 mM EDTA pH 8.0, 0.025% (w/v) Bromophenol blue
Aptamer binding/wash buffer	20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$
2 x Aptamer elution buffer	80 mM Tris-HCl pH 8.0, 20 mM EDTA, 7 M Urea, 0.02% (v/v) Tween 20
Basal (salt solution)	Krebs Buffered Ringers, 135 mM NaCl
Carnoy's fixative	60% (v/v) Absolute ethanol, 30% (v/v) chloroform, 10% (v/v) glacial acetic acid
DeCal	7% (v/v) HCl, 1.5% (w/v) EDTA
2 x HeBS	0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na_2HPO_4, pH 7.04
Krebs Buffered Ringers	2.4 mM KH_2PO_4, 0.6 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$ (hexahydrate), 10 mM Hepes
LiCl Lysis buffer	2.5 M LiCl, 50 mM Tris-HCl pH 8.0, 62.5 mM EDTA, 4% (v/v) Triton X-100
Low Chloride (low salt solution)	Krebs Buffered Ringers, 135 mM Na-Gluconate
0.07% Safranin O	0.07% (w/v) Safranin O
20x SSC	3 M NaCl, 0.3 M Tri-sodium Citrate, pH 7.0
Paraformaldehyde fixative	2% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde
Pre X-gal	5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$
1 x TBE	89 mM Tris-base, 89 mM Boric Acid, 2 mM EDTA, pH 8.3
1 x TE	10 mMTris-HCl pH 7.5, 0.1 mM EDTA
X-gal	2% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside dissolved in dimethylformamide

2.1.3   Bacterial Strains and Media

E. coli DH10β   F-*mcr*A Δ(*mrr-hsd-mcr*BC)φ80*dlac*X74*end*A1 *deo*RΔ(*ara, leu*)7697
                             *ara*D139 *gal*U *gal*K *nup*G *rps*L λ-

Luria broth (LB)   1% (*w/v*) tryptone, 0.5% (*w/v*) yeast extract, 1% (*w/v*) NaCl, 0.1 mM
                             NaOH

LB agar   LB, 1.5% (*w/v*) agar

2 x TY   1.6% (*w/v*) tryptone, 1% (*w/v*) yeast extract, 0.5% (*w/v*) NaCl

All media was autoclaved at 121 °C for 20 minutes

2.1.4   Cell lines and cell culture products

2.1.4.1   Cell lines

A549   American Type Culture Collection (ATCC) CCL 185

293T   ATCC, CRL 11268

NIH3T3   ATCC, CRL 1658

CHOK-1   ATCC, CCL 61

2.1.4.2   Materials

Dulbecco's Modified Eagles Medium   SAFC Biosciences (Lenexa, KS, USA) Cat #51441C
(DMEM) with 4500 mg/L Dextrose and
4.0 mM L-Glutamine without Sodium
Pyruvate

Hams Nutrient Mixture F12 Media   SAFC Biosciences (Lenexa, KS, USA) Cat #51655

Foetal Calf Serum (FCS)   JRH Biosciences (Lenexa, KS, USA) Cat # 12103-500M

L-Glutamine solution 200 mM   SAFC Biosciences (Lenexa, KS, USA) Cat # 59202C

Penicillin G 5000 U/mL, Streptomycin   JRH Biosciences (Lenexa, KS, USA) Cat # 59620
Sulphate 5000 µg/mL

Phosphate Buffered Saline (PBS)   SAFC Biosciences (Lenexa, KS, USA) Cat #
without calcium and magnesium   59321C

OptiPro ^{TM} SFM	Invitrogen Corporation (Carisband, CA, USA) Cat # 12309-019
Trypsin/EDTA solution (0.12% (w/v) Trypsin, 0.2% (w/v) EDTA)	SAFC Biosciences (Lenexa, KS, USA) Cat # 59430C

All disposable cell culture plasticwares were supplied by Costar (Corning, NY, USA), NUNC (Nalgene Nunc International, Rochester, NY, USA), Sarstedt (Numbrecht, Germany) and Greiner Labortechnik (Solingen, Germany)

2.1.5   Real Time PCR plastics and reagents

ABI PRISM ^{TM} Optical Adhesive Cover	Applied Biosystems (Foster City, CA, USA) Cat # 4311971
ABI PRISM ^{TM} Optical Caps	Applied Biosystems (Foster City, CA, USA) Cat # 4323032
ABI PRISM ^{TM} Optical Tubes	Applied Biosystems (Foster City, CA, USA) Cat # 4316567
MicroAmp® Optical 96 well reaction plates	Applied Biosystems (Foster City, CA, USA) Cat # N8010560
TaqMan MGB Probe (50,000 pmol)	Applied Biosystems (Foster City, CA, USA) Cat # 4316032
20 x TaqMan Universal Master Mix	Applied Biosystems (foster City, CA, USA) Cat # 4304437

2.1.5.1   Real time PCR primer and probe sequences

Gag forward primer	AGCTAGAACGATTCGCAGTTGAT
Gag reverse primer	CCAGTATTTGTCTACAGCCTTCTGA
Gag probe	6FAM-CCTGGCCTGTTAGAAAC-NFQ
Mouse transferrin forward primer	AAGCAGCCAAATTAGCATGTTGAC
Mouse transferrin reverse primer	GGTCTGATTCTCTGTTTAGCTGACA
Mouse transferrin probe	6FAM-CTGGCCTGAGCTCCT-NFQ

Human transferrin forward primer   GCCCTGCCTGCCTACA

Human transferrin reverse primer   CAGGTTGTGCTTCTGACTCACT

Human transferrin probe   6FAM-CCACCTCGATGCCCC-NFQ

2.2 Molecular Biology Methods

2.2.1 Agarose Gel Electrophoresis

Electrophoretic separation of DNA fragments was performed in a Kodak Biomax QS710 horizontal gel apparatus. All DNA samples were made to 1 x DNA loading buffer and loaded on a 1-4 % (w/v) agarose gel in 1 x TBE, and containing 1 μg / mL ethidium bromide. Gels were run at between 80 and 110 volts until the front of the bromophenol blue dye had migrated 2/3 to 3/4 of the way through the gel. Gels were photographed under ultra violet light using UVIPhotoMW version 11.01 for Windows.

2.2.2 Preparation of Competent E.coli

2.2.2.1 Electrocompetent DH10ß

DH10ß cells were streaked onto an LB agar plate. A single colony was picked and a 10 mL culture grown overnight in 2 x TY at 37°C in a orbital shaker. This overnight culture was then subcultured into 500 mLs 2 x TY broth and incubated at 37°C with aeration until the OD_{550} was approximately 0.8. The bacteria were pelleted *via* centrifugation at 6000 rpm, 0°C for 10 minutes and resuspended in 300 mLs of ice cold 10 % (*v/v*) glycerol. This process was repeated twice. The cells were then pelleted and resuspended in 1/250th of the original culture volume. Cells were then snap frozen in liquid nitrogen in 100 μL aliquots and stored at -70°C until required.

2.2.3 Transformation of *E.coli*

2.2.3.1 Electroporation

One µL of sample-such as a ligation, was electroporated at 1.5 kV into 25 µL of DH10ß *E.coli* cells using the Electro Cell Manipulator (BTX electronic genetics). The cells were then quickly transferred into 1 mL of 2 x TY broth and incubated for 1 hour at 37 °C with aeration. 330 µL was then plated on agar containing the appropriate selective antibiotic. The plates were then incubated at 37°C overnight to allow colonies to grow.

2.2.4 Plasmid Purification

2.2.4.1 Small Scale

A single colony was picked from an agar plate and placed into 1 mL of 2 x TY broth containing the appropriate antibiotic. Cultures were grown overnight at 37 °C with aeration. Half a millilitre of culture was removed into an eppendorf tube and cells were pelleted *via* centrifugation at 13,000 rpm for 2 minutes. The supernatant was aspirated and the pellet resuspended in 200 µL of LiCl Lysis Buffer. The DNA was then purified *via* phenol/chloroform extraction. The top phase (aqueous phase) was transferred into a fresh tube containing a 3/5th volume of isopropanol and mixed. The DNA was then precipitated via centrifugation at 13,000 rpm for 10 minutes, the supernatant aspirated, the pellet washed in 70% ethanol, re-centrifuged at 13,000 rpm for 5 minutes, re-aspirated and the pellet resuspended in 20 µL of 0.5 x TE buffer.

2.2.4.2 Large Scale

Medium and large scale plasmid purification was carried out using Qiagen Miniprep, Midiprep, Megaprep and Gigaprep kits as per manufacturer's instructions.

2.2.5 Restriction enzyme digest of plasmid DNA

The vector was digested using the appropriate enzyme(s) in the appropriate 10 x buffer as recommended by the manufacturer. Digestion was carried out until completion; approximately 1-2 hours at $37^°$C.

2.2.6 Calf Intestinal Phosphatase treatment

Vectors digested for vector preparation had terminal phosphates removed *via* incubation at $37^°$C with 1.5 µL CIP for 30 minutes to 1 hour. The vector was then phenol chloroform extracted and ethanol precipitated.

2.2.7 Phenol chloroform extraction

An equal volume of phenol/chloroform was added to the sample. This mixture was mixed by vortexing for 15 seconds and centrifugation at 13,000 rpm for 5 minutes. The aqueous (top) phase was then transferred into a clean tube.

2.2.8 Ethanol precipitation

The DNA was precipitated *via* the addition of 1/10th volume of 3 M Sodium Acetate pH 5.2 and 2.5 volumes of 100% Ethanol and incubated at room temperature for 1 hour. DNA was recovered *via* centrifugation at 13,000 rpm for 1 hour, the supernatant aspirated and the pellet resuspended in 0.5 x TE buffer.

2.2.9   Ligations

DNA inserts and vectors were quantified by agarose gel electrophoresis, and 30-50 ng of vector and ≈100 ng of insert were used in a ligation (20 uL) using Roche T4 DNA ligase with 1 x ligation buffer.

2.2.10 Blunt end ligations

Vector DNA (~500 ng) digested with the appropriate enzymes was prepared for blunt end ligation *via* incubation at room temperature for 30 minutes with 1 μL of dNTP's and 1 μL Klenow fragment to fill in 5' and 3' overhangs. Ligations were carried out using 50 ng of DNA and ≈100 ng of insert using Roche T4 DNA ligase with 1 x ligation buffer.

2.2.11 Polymerase Chain Reaction

PCRs were setup to a total volume of 20 μL to 100 μL using the Roche Expand high fidelity system. PCR mix was made to 1 x including buffer containing $MgCl_2$, 100 μM primers, 10 mM dNTPs, and high fidelity Taq. PCR reactions were generally run under the following conditions:

95 °C	10 minutes	
95 °C	20 seconds	
57 °C	20 seconds	"x" cycles
68 °C	2 minutes	

2.2.12 PCR purification

PCR reactions were purified using the AdBiotech PCR purification kit, as per the manufacturers' instruction.

2.2.13 Agarose Gel Extraction of DNA fragments

DNA fragments were extracted from agarose gels using the AdBiotech Gel Extraction kit, as per the manufacturers' instruction.

2.2.14 Creation of Real Time PCR standards

A small scale virus preparation of a lentivirus expressing a neomycin resistance gene was assayed on CHOK-1 cells with a low MOI. Cells were grown from a 24 well plate to 100 mm round dishes and media was supplemented with G418 (to select cells that are expressing the neomycin resistance gene). When distinct colonies of cells were present, individual colonies were isolated and grown into a T75 flask until confluent. Cells were harvested and 2×10^7 cells were spun down and gDNA extracted using the Qiagen Blood and Cell Culture DNA midi extraction kit. Genomic DNA standards were stored at -20 °C.

2.2.15 Southern Blot analysis of Real Time PCR standards

Ten micrograms of gDNA was subject to overnight restriction enzyme digestion in a 50 μL reaction. The reaction was run on a 300 mL 1.2% *w/v* agarose gel at 50 V overnight. Transfer of restricted gDNA was carried out using an assembly consisting of a nitrocellulose membrane sandwiched between the agarose gel and two pieces of Whatman filter paper all cut to the size of the agarose gel, and weighed down by paper towelling and a weight in a tank of 0.4 M NaOH. Setup was left for 24 hours to allow the transfer to occur, with towels changed every 4-5 hours. Following transfer, the membrane was washed in 2x SSC buffer. The membrane was dried between 2 sheets of filter paper and then microwaved to fix the DNA to the membrane.

The membrane was rehydrated in 4x SSC and transferred to a glass canister with 20 mLs of Ambion blocking reagent for 1 hour at 42°C. Following incubation, the radio-labelled probe was added to the membrane and was again incubated at 42°C for 15 hours.

The labelled membrane was removed and washed twice in 2x SSC + 0.1% SDS at 60°C for 15 minutes. The membrane was again washed twice using 0.1x SSC + 0.1% SDS for 15 minutes. Finally, membrane was washed with 4x SSC and blot dried between filter paper. Membrane was sealed in a plastic bag and imaged using the IMAGER © software.

2.2.16 Aptamer isolation- Initial Screening

Negative selection cells (WEHI-3BD or CHOK-1) were harvested and washed in 3 x 1mL of Binding/Washing buffer *via* centrifugation and resuspension.

Five micrograms of aptamer library (see chapter 6) was diluted into 500 μL of Binding/Washing buffer, heat denatured at 90°C for 10 minutes and cooled to 4°C for 15 minutes. Samples were then brought back to room temperature and incubated for 5 minutes. The negative selection cells were resuspended in the aptamer library and incubated at room temperature with continuous agitation for 10 minutes. Cells were separated from the supernatant *via* centrifugation, with the supernatant (depleted library) kept for further use. Positive selection cells (WEHI or CHOK-1 cells expressing CFTR) were resuspended in the depleted library at room temperature for 30 minutes with constant agitation. Cells were washed with 5 x 500 μL of Binding/Washing buffer *via* centrifugation/resuspension. Cells were then lysed in 100 μL of 1% v/v Triton X-100 at 0°C for 5 minutes. Nuclei were removed *via* centrifugation at 3000 rpm and the supernatant decanted. Elution buffer was added to the supernatant and aptamer DNA precipitated with 5 μg tRNA, 0.4 M NaCl and 3 volumes of EtOH overnight.

Aptamer DNA was pelleted *via* centrifugation at 13,000rpm for 60 minutes and resuspended in 20 μL of 0.5x TE buffer.

Aptamers were amplified in a 2 x 100 μL reaction using the Qiagen HotStarTaq system. PCR mix was made to 1 x including buffer containing $MgCl_2$, 100 μM primers- Apt Forward-FITC and Apt Reverse-HEGL A_{20}, 10 mM dNTPs, and HotStarTaq. PCR reactions were run under the following conditions:

94°C   15 minutes

94°C   20 seconds
64°C   30 seconds   } x 40 cycles
72°C   30 seconds

PCR reactions were pooled and precipitated with 0.3 M NaOAc and 3 volumes of EtOH. Precipitated aptamers were run on a 10% *w/v* denaturing acrylamide gel until the BPB reached the end of the gel. The fluorescent band was cut from the gel and eluted in 0.3M NaOAc, 2mM EDTA at 80°C for 45 minutes. DNA was precipitated with 3 volumes of EtOH and pelleted *via* centrifugation.

2.2.17 Aptamer Isolation- Subsequent Screens

Subsequent screening of aptamers was carried out essentially as described in §1.1.17. The negative selection was removed and the positive selection cells used in its place.

2.3   Cell Culture Techniques

2.3.1   Cell maintenance and Subculturing

Cells were generally subcultured when confluent. Media was removed from the cells by aspiration, and the cell monolayer rinsed with a 10 % (*v/v*) dilution of trypsin solution in 1 x PBS, to remove traces of

media. The trypsin was aspirated from the cells and the cells were then incubated with 1-3 mLs of 10 % trypsin until the cells were seen to be detaching from the culture dish when observed under the microscope. The cells were then spritzed into a single cell suspension. Cells were then split to the required ratio, generally 1:2 to 1:10, into a fresh flask containing the appropriate amount of growth medium.

2.3.2 Large Scale Virus Production

Confluent 293T cells were subcultured 1:6 into 4 x T75 flasks and incubated for 2 days until confluent. The 4 flasks were then subcultured 1:2 into 8 x T75 flasks. The following day cells were subcultured into 18 x 150 mm dishes. Twenty four hours later cells were harvested with 7 mLs of Trypsin and 7 mLs of media to spritz/plate. The cells were pooled into 2 x 1 L bottles containing another 200 mLs of media, the cell count determined using a haemocytometer and adjusted to 4.5 x 10^5 cells per mL. One hundred and five millilitres of the cell suspension / plate was dispensed into 20 x 245 mm plates. The following day, the 293T cells were transfected with the following plasmids: pHIV-1MPSvLacZnls (or other relevant vector), pcDNA3Tat101ml, pHCMRevmlwhvpre, pHCMVgagpolmllstwhv and pHCMV-G. The DNA mix was made to 250 mM $CaCl_2$.

Plasmid	mount added per plate
Vector (pHIV-1MPSvLacZnls)	158 µg
pHCMVRevmlwhvpre	3.16 µg
pcDNA3Tat101ml	3.16 µg
pHCMVgagpolmllstwhv	15.8 µg
pHCMV-G	7.9 µg
2.5M $CaCl_2$	320 µL

Transfection was carried out *via* calcium phosphate coprecipitation using an equal volume of 2 x HeBS. The DNA was rapidly (over 5-10 seconds) added to the HeBS while vortexing, which was continued for a further 20-25 seconds. The precipitate was then incubated for a further 90 seconds before being gently dripped onto the plates [103]. The plates were then incubated for 8 hours then the media was changed with 170 mLs of pre-warmed 37 °C OptiPro serum free media supplemented with glutamine and Pen Strep. Cells were incubated for 2 days and the virus containing medium then harvested.

2.3.3 Large scale virus ultrafiltration

Filters used for ultrafiltration were equilibrated to 1 x PBS before virus filtration. The virus supernatant was clarified by centrifugation at 2800 rpm for 5 minutes at 20°C to remove cell debris. The supernatant was pooled and filtered through an Amersham 0.45 μm hollow fibre cartridge (CFP-4-E-4MA) to remove large proteins, cell debris and bacteria.

The filtered virus was then concentrated approximately 1000-fold using an Amersham Quickstand system with a 750 kDa cut-off cartridge (UFP-750-E-4x2MA) and a Master Flex peristaltic pump. The concentrated virus was filtered through a 0.8 μm filter (Millipore Millex-AA) and virus pelleted by ultracentrifugation at 20,000 rpm, 4 °C for 90 minutes in a SW40 rotor, or for later preparations, in a SW32 rotor. The supernatant was discarded and the virus pellet was gently resuspended in an appropriate volume of 1 x PBS. The resuspended virus was pooled, and aliquoted into sterile screw capped Eppendorf tubes and frozen at -70°C until required.

2.3.4 Small Scale Virus production

A confluent T75 flask of 293T cells, grown to confluency, was subcultured 1:2 into a new T75 flask. Twenty-four hours later, the cells were subcultured 1:5 into 5 x T75 flasks, and grown for 2 days. The 5 flasks of cells were harvested essentially as described above and the cell count adjusted to 0.375×10^6

cells/mL and then plated on 6 x 245 mm cell culture dishes using 105 mLs/plate. The cells were grown

for 24 hours (until confluent) and were then transfected with the following plasmid mixes:

Plasmid	Amount required per plate
Vector	158 µg
pcDNA3Tat101ml	3.16 µg
pHCMVRevmlwhvpre	3.16 µg
pHCMVgagpolmllstwhv	15.8 µg
pHCMV-G	7.9 µg
2.5M CaCl$_2$	320 µL

Plasmids were transfected *via* calcium phosphate coprecipitation as described above in 2.3.2 using 3.2

mL/plate. Eight hours post transfection, the media was aspirated from the cells and replaced with 31

mLs of pre-warmed OptiPro SFM, and cells incubated for 2 days. Virus was harvested 2 days post

transfection as described in 2.3.3 but using a small scale 750 kDa hollow fibre cartridge and the Watson

Marlow peristaltic pump, and virus pelleted *via* centrifugation using a SW-32 rotor, with virus

resuspended in an appropriate volume of 1 x PBS.

2.3.5 Determination of Viral Titre

2.3.5.1 LacZ assay (for titre of LacZ vectors)

A T75 flask of CHOK-1 cells was harvested and counted using a haemocytometer. After counting, cells

were plated at a concentration of 250,000 cells per well in a 24 well cell culture plate. Cells were

incubated for 3 hours. Following incubation, virus, typically equivalent to 0.005 µL of the final virus

preparation, 1 µL of virus supernatant and 1 µL of post-750 kDa filtration virus was added onto the

cells in the presence of 4 µg / mL polybrene. Cells were incubated for 24 hours, and the media then

exchanged with 0.5 mLs per well of pre-warmed media. Three days post transfection, cells were

washed with 1 x PBS. PBS was aspirated from the wells and the cells fixed with 0.01% Glutaraldehyde in PBS (Sigma, G2657) for 10 minutes. Following fixation, cells were washed 3 times in 1 mM $MgCl_2$ in PBS, with the second wash being for 10 minutes with gentle agitation. A solution of 1 x X-gal in Pre X-gal was prepared, and mixed by inversion. To each of the wells, 250 µL of the X-gal / Pre-X-gal solution was added and incubated at 37 °C for 5 hours. Once staining could be seen, the staining solution was removed *via* aspiration and the cells washed twice with 1 x PBS, and then stored in 80 % (v/v) glycerol. Viral titre was determined using light microscopy to count the number of stained cells in a given area (with clusters of cells counted as one cell). Viral titre was determined by counting the number of blue cells, multiplying by the area of the well and then adjusting for the volume of virus assayed, to give the number of transducing units of virus per mL.

2.3.5.2   Real time PCR

Real time PCR was required to determine viral titres for virus containing therapeutic transgenes, as these do not contain a "marker" that can be easily measured *via* an alternative method.

2.3.5.2.1   Sample preparation

The desired cells (CHOK-1) were transfected with virus as described in 2.3.5.1, except the cells were split 1:4 on day 3 of the assay into 24 well plates and incubated for 48 hours at 37 °C/5 % CO_2. Cells were subcultured for 4 weeks to ensure dilution of the plasmid DNA signal. After the cells were harvested and spun down, the cells were washed 2 times in PBS and the cell pellet stored at -20 °C until required. The gDNA was isolated from the cells using the Wizard SV Genomic DNA isolation Kit and stored at -20°C until required.

2.3.5.2.2   *Virus titre assay*

The gDNA was assayed for the presence of the desired gene in either MicroAmpR Optical 96 well Reaction Plates with an ABI PRISM™ Optical Adhesive Cover or ABI PRISM™ Optical tubes with ABI PRISM™ optical caps. Each reaction contained 2-5 µL gDNA, 1 x TaqMan Universal PCR Master Mix, 0.9 µM of each primer and 0.25 µM of probe in a total volume of 20 µL. Each assay also included standards and no template controls in triplicate for each gene analysed. The assays were run on an Applied Biosystems 7300 Real Time PCR machine under the following conditions: 50 °C for 2 minutes, 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The fluorescence of each reaction was read at the end of each cycle. The data was outputted as an amplification plot. Using the standards as a guide, the threshold bar was placed in the linear part of the plot and the cycle threshold value (Ct) for each sample determined. The following formulas were then used to determine the titre of the virus initially added to the cells.

ΔCt = Ct gag – Ct transferrin (control gene)

ΔΔCt = ΔCt of sample – average ΔCt of standard

Copy number per cell = 1/2ΔΔCt

Titre = copy number per cell x number of cells initially plated x 1000 volume assayed in µL

2.3.6   Assay for Replication Competent Virus (RCR-p24 assay)

293T cells were plated at a cell density of 5×10^5 in duplicate wells of a 12 well plate in 1 mL of media. Four hours following plating, the media was aspirated and replaced with 1 mL of fresh medium containing 4 µg / mL polybrene. To three wells >10^7 A549 transducing units of recombinant virus was added, three wells were used as controls. Twenty-four hours later, media was aspirated and replaced with 1 mL of pre-warmed (37 °C) media. Media was aspirated and cells were subcultured 1:10 every three days with a 450 µL sample taken on days 6, 12, 18 and 24. These samples were stored at -80 °C.

When all samples had been collected, samples were then clarified *via* microcentrifugation at 2000 rpm. The supernatant was decanted and assayed undiluted for p24 via p24 ELISA.

2.3.7   p24 ELISA

The p24 assay was carried out using the PerkinElmer HIV-1 p24 ELISA kit. All reagents were equilibrated to room temperature. The plate wash concentrate was diluted 1:20 in distilled water to make a final volume of 600 mL. Standards were prepared from diluting the positive control in PBS/1 % (v/v) FCS to concentrations of 800, 400, 200, 100, 50, 25, 12.5 and 6.25 pg/mL p24. 200 µL of the dilutions were added to the antibody-coated microplate wells following the addition of 20 µL of Triton X-100 to all wells. 200 µL of PBS/1 % FCS was added to three designated as negative controls. 200 µL of appropriately diluted virus samples were added to the remaining wells. All samples were mixed *via* pipetting up and down. The plate was sealed and incubated for 2 hours at 37 °C. The plate was then washed six times with 300 µL/ well of diluted (1x) wash buffer. The plate was gently shaken and the contents of the well aspirated. 100 µL of detector antibody was added to all wells. The plate was again sealed and kept at 37 °C for 1 hour. Following incubation, the plate was washed six times with wash buffer. A 1:100 dilution of Streptavidin-HRP concentrate : Streptavidin-HRP diluent was made and 100 µL of this dilution added to each well. The plate was sealed and left for 30 minutes at room temperature. The plate was then washed with 300 µL/ well of wash buffer six times. 100 µL of OPD substrate solution was added to all wells. The plate is sealed and left to incubate at room temperature in the dark for 5-15 minutes, until the colour was visible in wells containing the 12.5 and 6.25 pg/mL standards. The reaction was stopped by the addition of 100 µL of stop solution to all wells. The plate was read at 490 nm and the concentration of p24 in the virus dilutions determined by construction of a standard curve.

2.3.8 Virus assay: Measuring the effect of Leptomycin B on virus titre

CHOK-1 cells were plated at a cell density of 2.5×10^5 in a 24 well plate and incubated for 3 hours.

Leptomycin B was prepared at 3 concentrations dissolved in 1 x PBS, and each concentration incubated with 20 µL of a 1/10 dilution of LV-SV40EYFP at 37°C. Samples of 3 µL were removed at 5, 10, 15, 20 and 60 minutes into 6 mLs of F12 media. Following sampling, 0.5 mLs of the samples were assayed on the plated CHOK-1 cells. Cells were maintained for 4 days and then analysed by FACscan.

2.3.9 Virus assay: Measuring the effect of proteasome and lysosome inhibitors on virus titre

The assay was carried out essentially as described in section 2.3.8, with 3 test concentrations of Z-LLL, Bafilomycin A1 and Doxorubicin.

Assays were analysed *via* FACScan.

2.3.10 FACscan Analysis

Live cell populations were gated by side and forward scatter, and expression of EYFP was analysed through the FITC channel on a Becton Dickinson FACScan machine. Results were analysed using Cell-quest software version 3.0.1 Becton Dickinson. Analysis of untransduced CHOK-1 cells (n=3) was used to provide a background control, with the FITC signal being adjusted to give less than 1% positive cells (Fl* >10 units). The number of EYFP positive cells minus the average background controls provided the percentage of positive cells in the experimental sample. Viral titre was determined by multiplying the corrected percentage of positive cells by the number of CHOK-1 cells plated and corrected for the amount of virus assayed.

2.3.11 Genomic DNA extraction

The extraction of genomic DNA (gDNA) from cells grown from cell culture or animal tissue was performed as per manufacturers' instruction using the Promega SV Wizard Genomic DNA kit.

2.4 Animal Techniques

All animal experimentation was undertaken with ethics approval from the Women's and Children's Hospital Animal Ethics committee.

2.4.1 Instillation of Lentiviral vector

C57Bl/6 mice of 6-8 weeks of age were anesthetised with a mixture Domitor (75 µg/g body weight) and Ketamine (100 µg/g body weight) given *via* intraperitoneal injection. Mice were kept in a 35 °C environment to prevent loss of body temperature due to the anaesthesia. For instillation of the pre-treatment and lentivirus, mice were suspended by their front incisors and their body supported from underneath. Four µL of the appropriate pre-treatment solution (0.3 or 1 % (*w/v*) LPC) was then delivered as a single bolus dose into the right nostril using a gel loading pipette tip. One hour after the pre-treatment solution was delivered, a 20 µL dose of lentivirus was administered slowly (over 30 seconds to 1 minute) in 2 x 10 µL aliquots, again to the right nostril. Mice were kept under observation for 5 minutes before being placed back into the 35 °C incubator where they were allowed to come out of anaesthesia. Mice were weighed daily for 7 days post-treatment and weekly thereafter (if required). Anaesthesia was reversed with Antisedan (0.2 µg/g body weight) delivered intramuscularly into the hind leg.

2.4.2   Assessment of Gene Transfer

2.4.2.1   Processing of mouse heads

Mice were sacrificed at specified time points by CO_2 overdose. A cardiac puncture was performed by inserting a 21G x 1-½" needle upwards to pierce the skin just below the sternum up through the diaphragm. The needle tip was advanced until the tip was approximately level with the mouses' front paws. The syringe was withdrawn to remove approximately 0.5 mL of blood *via* the heart. The head of the mouse was then removed with bone cutting scissors from just above the shoulders, and the fur on the head removed. A small amount of soft cartilage was removed from the nose. Fixation of nasal airway tissue was achieved using a bent, blunt needle, attached to a 2 mL syringe containing paraformaldehyde fixative solution. The solution was flushed through the trachea and allowed to escape *via* the nose tip.

2.4.2.2   X-gal staining of Murine heads

The entire head was then submerged and fixed for 2 hrs in a solution of 2 % (*w/v*) paraformaldehyde / 0.5 % (*v/v*) glutaraldehyde in PBS on ice, flushing the solution through the trachea to the nose tip every 30 minutes. Tissues were then rinsed twice with 1 mM $MgCl_2$ in PBS on ice for 15 minutes. Tissues were stained with a 1:20 dilution of X-gal : pre X-gal. This was flushed through the trachea every hour for 4 hours. Tissues were rinsed in 0.9 % (*w/v*) NaCl for 15 minutes and then fixed in 10% neutral buffered formalin (NBF) for 22 hours. Following NBF fixation the tissues were placed into decalcifying fluid for 22 hours. Decalcified tissues were then rinsed clean with running water for 30 minutes before being stored until further processing in 70 % (*v/v*) ethanol.

2.4.2.3   Sectioning of Mouse heads

The jaw of the mouse was removed, beginning at the mouth on one side of the head cutting along the jaw line towards the ear. This was repeated on the other side of the head. A final cut was used at the back of the head to completely remove the jaw, exposing the upper palate. The right side of the head (this being the treated-nostril side) was marked with a line of black ink, and the inked area set by brief immersion in Carnoy's fixative. The head was turned upside down, and using a disposable microtome blade, incisions were made in several standard locations: before the front incisors, behind the front incisors, between the 2nd and 3rd ridge of the upper hard palate, before the last ridge of the hard palate and finally, approximately level with the ears. The 5 sections were placed into a histology cassette with the last cut side facing upwards and the right side of the head facing the front of the cassette. Histology cassettes were lined with foam pads, soaked in 70 % (*v/v*) ethanol, and sealed with the appropriate cover. The cassette was then returned to the specimen pot containing fresh 70 % (*v/v*) ethanol. The gross sections were first examined under a dissecting microscope to observe gene transfer, before being sent to the Histopathology Department (WCH, Child, Youth and Women's Health Service) for embedding in paraffin wax. Following embedding, the blocks were sent to Adelaide Microscopy (University of Adelaide) for cutting and staining with Haematoxylin and Eosin (for anatomical and morphological differentiation), and Safranin O (as a counterstain for the blue stain of cells expressing LacZ). Numbers and types of LacZ positive cells were determined using the latter sections observed under 400 x magnification light microscopy.

2.4.2.4   Transepithelial Potential Difference measurements

Mice were anesthetised with a mixture of Domitor (75 µg/g body weight) and Ketamine (100 µg/g body weight), and suspended by their dorsal incisors with their body supported. An agar-filled needle bridge was placed subcutaneously in the mouse abdomen as a reference electrode. A heat-drawn PE10 cannula marked at 2.5 mm and 5 mm was inserted in the treated nostril and connected to a

perfusion/recording apparatus (consisting of a dual syringe pump (WPI SP2201Z Syringe Pump), a WPI Isomillivoltmeter and a laptop running DataStudio (Version 1.9.8r2, Pasco Scientific, CA, USA). The syringe pump was loaded with three 1 mL Hamilton glass syringes (Series 1000 gastight syringes, Hamilton Company, NV, USA) containing either the basal, basal plus amiloride or low Cl⁻ plus amiloride solutions and was connected to the tubing system. The cannula was inserted to a depth of 2.5 mm to 3 mm to ensure the reading was taken from respiratory epithelial region of the nasal cavity. The basal solution was infused at a rate of 1 μL/min and the transepithelial potential difference (TPD) was recorded in DataStudio. For a reading to be accepted, a minimum steady state plateau of five minutes was required for each solution. The basal solution was infused until a stable TPD value was recorded before the solution was switched to basal plus amiloride. Amiloride was included to block the function of the sodium channel so changes in airway TPD could be ascribed to the function of the chloride channel. Again, the basal plus amiloride solution was infused until a stable value was recorded. The infusion was then switched to the low Cl⁻ plus amiloride solution until a new stable TPD value was obtained. At the end of each TPD recording, the reference electrode and the cannula were placed into 0.9% NaCl solution to assess any drift in electric potential of the recording system compared to the same assessment made prior to recording from an animal. If the drift value was greater than 2 mV the reading was discarded.

During the testing, mice were monitored for respiratory distress and excess fluid in the nostril was removed by wicking with fine twists of tissue. Wicking events were noted on the traces, as they could induce sudden changes in TPD that required notation and explanation. Once TPD readings were complete, mice were administered with Antisedan (0.2 μg/g body weight) and allowed to recover in a 35 °C air chamber.

Individual ΔTPD values were blinded and assessed by experienced TPD technicians to remove the possibility of bias from printouts of the DataStudio. The ΔTPD values were calculated by subtracting the TPD value recorded under basal plus amiloride conditions from the TPD value recorded under low Cl^- plus amiloride conditions. A diagrammatic setup of the TPD apparatus is shown in Figure 2-1.
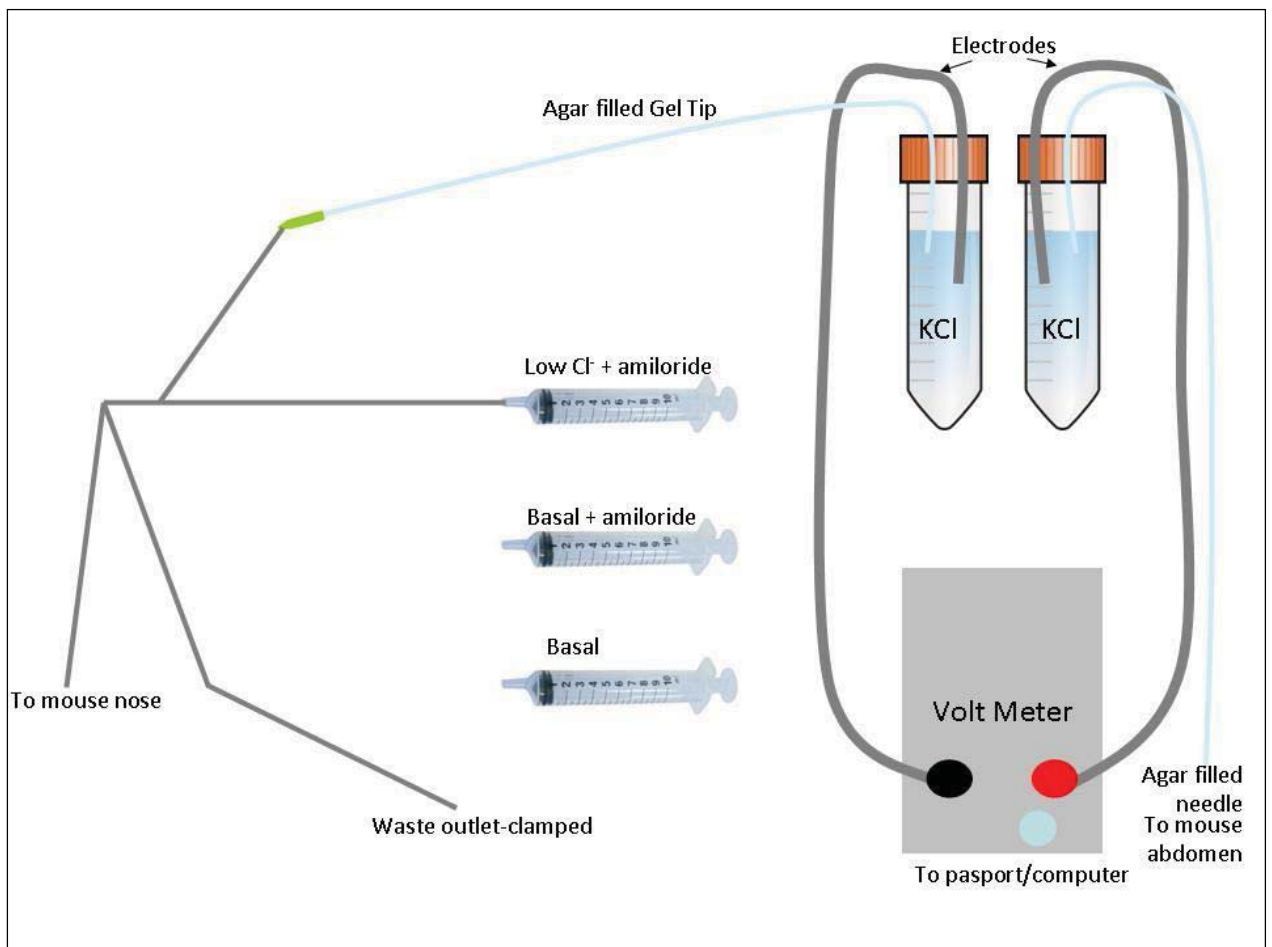
Figure 2-1: Diagrammatic view of TPD set up

TPD apparatus setup as described in 2.4.2.4.

2.4.2.5   Live animal Imaging using the Xenogen IVIS 1000 system

Mice treated with lentivirus expressing the Luciferase marker gene were transported to Adelaide Microscopy using the appropriate transport procedures as outlined by the OGTR. Mice were anaesthetised with a mixture of Domitor (75 µg/g body weight) and Ketamine (100 µg/g body weight), and were then instilled intranasally with 0.75 mg of D-Luciferin. Ten minutes following Luciferin instillation, mice were placed into the Xenogen imaging machine, in the supine position. Luminescent imaging was carried out with medium binning, f-stop open and a subject height of 1.5 cm (representing the height of the animal from the imaging platform) for 4 minutes. Data (photons/sec/cm^2/sr) was analysed using the Living Image software v2.50.1 2005, Xenogen Corporation. Luminescence was quantified as Flux (photons/second) in a contour area defined by the program.

2.4.2.6   Removal of mouse nasal septum

Mice were sacrificed as outlined in section 2.4.2. Following removal of the head and fur, incisions were made by placing the point of the scissors inside one nostril of the mouse and cutting up towards the brain. Once a cut of approximately 0.8 cm was made, the same was carried out through the other nostril. A cut from the tip of the nostril (the top of the septum) was then made to remove the bony flap above the nose, and expose the nasal septum. The septum was then cut away from the head *via* an incision at the back of the septum (cutting down towards the mouth) and at the front, following the septum along the base to completely remove it from the head. Septum was then placed in an eppendorf tube to be used for embedding in OCT or extraction of gDNA.

2.4.2.7   Embedding nasal septum in OCT

Mouse nasal septums were placed treated side facing up into a square plastic mould. Tissue was covered in OCT and placed into a beaker containing supercooled isopentane. Isopentane was

supercooled by sitting in a flask of liquid nitrogen. Tissue was frozen into the OCT and wrapped in aluminium foil and kept at -80 °C until required.

2.4.3   Genotyping PCR

Genomic DNA from Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mice was used for genotyping PCR's using the following cycling conditions:

2.4.3.1   CFTR genotyping PCR

1 x   94° C   3 minutes

           94° C   30 seconds

40 x   56° C   45 seconds

           72° C   45 seconds

1 x   72° C   2 minutes

           10° C   ∞

2.4.3.2   FABP Genotyping PCR

1 x   94° C   3 minutes

           94° C   30 seconds

35 x   55° C   45 seconds

           72° C   45 seconds

1x   72° C   2 minutes

           10° C   ∞

2.5 Statistical Analysis

All Statistical analyses, where appropriate, were performed using SigmaStat Version 3.0 for Windows.

Analysis was performed using Analysis of Variance (ANOVA) for multiple test groups and the students

t-test for comparison of two groups. Results were considered significant when $p < 0.05$.

When stated, the 'n=' value is the number of samples in each individual group.

3 Proteasome and Lysosome Inhibitors to enhance gene transfer

3.1   Proteasomes and Lysosomes

The effectiveness of gene therapy can be limited by the amount of gene transfer achieved. The efficiency of gene transfer by viral vectors can be influenced by many factors. These can include the physical barriers presented by the site of delivery- in the CF airways this includes the mucous barrier, beating cilia and the glycocalyx. It can also include systems within each target cell that degrade foreign proteins- in which lysosomes and proteasomes play an important role. It is thought that enveloped viruses gain access to a target cells cytoplasm by two possible mechanisms, firstly, *via* direct fusion with the plasma membrane and secondly *via* endocytosis induced by a low pH to facilitate fusion with endosomal or lysosomal membranes [165].

Inhibitors of vacuolar H+-ATP have been used directly to demonstrate infection by VSV and other viruses *via* the endocytosis pathway [166, 184, 185]. Once inside the cell cytoplasm, foreign proteins, such as viral particles, may be susceptible to degradation by the proteasomes.

This chapter describes *in vitro* and *in vivo* testing of proteasomal and lysosomal inhibitors, Z-LLL (MG132)- an inhibitor of the proteasome, Bafilomycin A1, an inhibitor of the lysosome and Doxorubicin, which has discrete selectivity for the 20S proteasome with regard to the effects of these compounds on gene transfer.

3.2   Testing Inhibitors in vitro

The inhibitors Z-LLL (MG-132), Bafilomycin A1 and Doxorubicin HCl, , were tested *in vitro* to study the effect of the inhibitors and their solvents on the viability of the lentivirus used for gene delivery following co-incubation of the inhibitor with the lentivirus over a one hour time period. This was important as the initial approach to the study was to deliver the compounds locally by mixing with both the vector and/or the LPC pre-treatment. The concentrations chosen for investigation were based

around experiments described by Wei *et al* [165] utilising high and low concentrations of the inhibitors. Results for all inhibitors are shown in Figure 3-1.

Z-LLL was initially dissolved in DMSO at a concentration of 10 mg/mL. Test concentrations were made by serial dilution of the concentrated Z-LLL in PBS. Final concentrations of 10 µM, 100 µM 300 µM and 1 mM were tested as described in 2.3.9. As serial dilutions of the compound were made in PBS, the concentration of the solute DMSO was also decreased. A dilution of DMSO equivalent to the highest Z-LLL concentration was also tested for its effect on LV viability. Three out of the four concentrations of Z-LLL tested were seen to decrease viral titre following co-incubation for 60 minutes. Titres decreased by 28% for 10 uM, 44% for 300 uM and 30% for 1 mM. However, in each case the decrease in titre seen at the 60 minute time point was not significantly different from the starting titre of the virus. (Figure 3-1a) p = 0.445 (ANOVA). The carrier solution tested (DMSO) did not have a statistically significant effect on virus viability, however, a trend towards decreasing virus titre was again seen. As the effects on vector viability were modest, all concentrations of Z-LLL were deemed suitable for *in vivo* evaluation.

Bafilomycin A1 was initially dissolved in DMSO at 0.1 mg/mL and was tested at concentrations 0.1 µM, 1 µM and 10 µM, again using serial dilutions in PBS and testing using the same co-incubation study as described in 2.3.9. The highest concentration of Bafilomycin A1 tested, 10 µM, was seen to decrease titre 2.7 fold, or 64%, by 20 minutes following co-incubation. However, the decrease in titre was not significant, although the large variation in the results should be noted (p=0.333 t-test, Figure 3-1b). All concentrations of Bafilomycin A1 were deemed to be acceptable for *in vivo* experiments as virus titre was not compromised to a point where it would not be possible to see a change in the level of gene transfer compared to our standard gene delivery protocol. The effects on virus titre were also seen to be time dependent, as effects were only seen after the 10 minute test time point, by which time there is likely to be significant dilution of the solutions *via* mixing with nasal fluids when used *in vivo*.

Doxorubicin HCl was dissolved in sterile water at 50 mg/mL, diluted in PBS, and tested at concentrations of 10 μM and 1 mM for the effect of the inhibitor on LV titre following co-incubation. Results from virus treated with 10 uM Doxorubicin showed that this concentration did not have a significant effect on viral titre over the 60 minute testing period (Figure 3-1c). The effect of the highest concentration 1 mM however, is more difficult to determine as the Doxorubicin inhibited the growth of the cells used to assay LV titre; it was noted that the cells exposed to the 1 mM/LV sample did not grow following sub-culture. This renders the results for the 1 mM co-incubation study technically doubtful as the measurement used to analyse titre is FACS analysis of EYFP expression from the LV following transduction of CHO-K1 cells. This obviously relies on having a population of viable cells to analyse, and on integration of the vector. Although the cells appeared viable on days one and two post addition of the sample, following subculture there were far less viable cells and these did not appear to be growing at a normal rate. In addition, the time to reach confluency was much greater than what was usually seen and there were not enough cells for analysis until approximately 10 days following subculture as opposed to the 5 days for all other assays done in this way. It is difficult to determine from this assay if the cells that survived the subculturing process were a representative population of cells for the assay. In this instance, it is unlikely that the cells that remained in culture were a good representative population, due to the extreme loss of cell viability that occurred during the assay.

Figure 3-1: Effect of inhibitors on lentivirus titre

a) The effect of Z-LLL on virus titre over 60 minutes, p=0.445

b) The effect of Bafilomycin A1 on virus titre over 60 minutes, p=0.333

c) The effect of Doxorubicin HCl on virus titre over 60 minutes

d) The effect of concentrated DMSO on virus titre over 60 minutes, p=0.063

n = 3 replicates of each experiment, error bars represent SEM

Effect of Z-LLL on lentivirus titre

a)

Effect of Bafilomycin A1 on lentivirus titre

b)

Effect of Doxorubicin on lentivirus titre

c)

d)

Effect of Concentrated DMSO on lentivirus titre
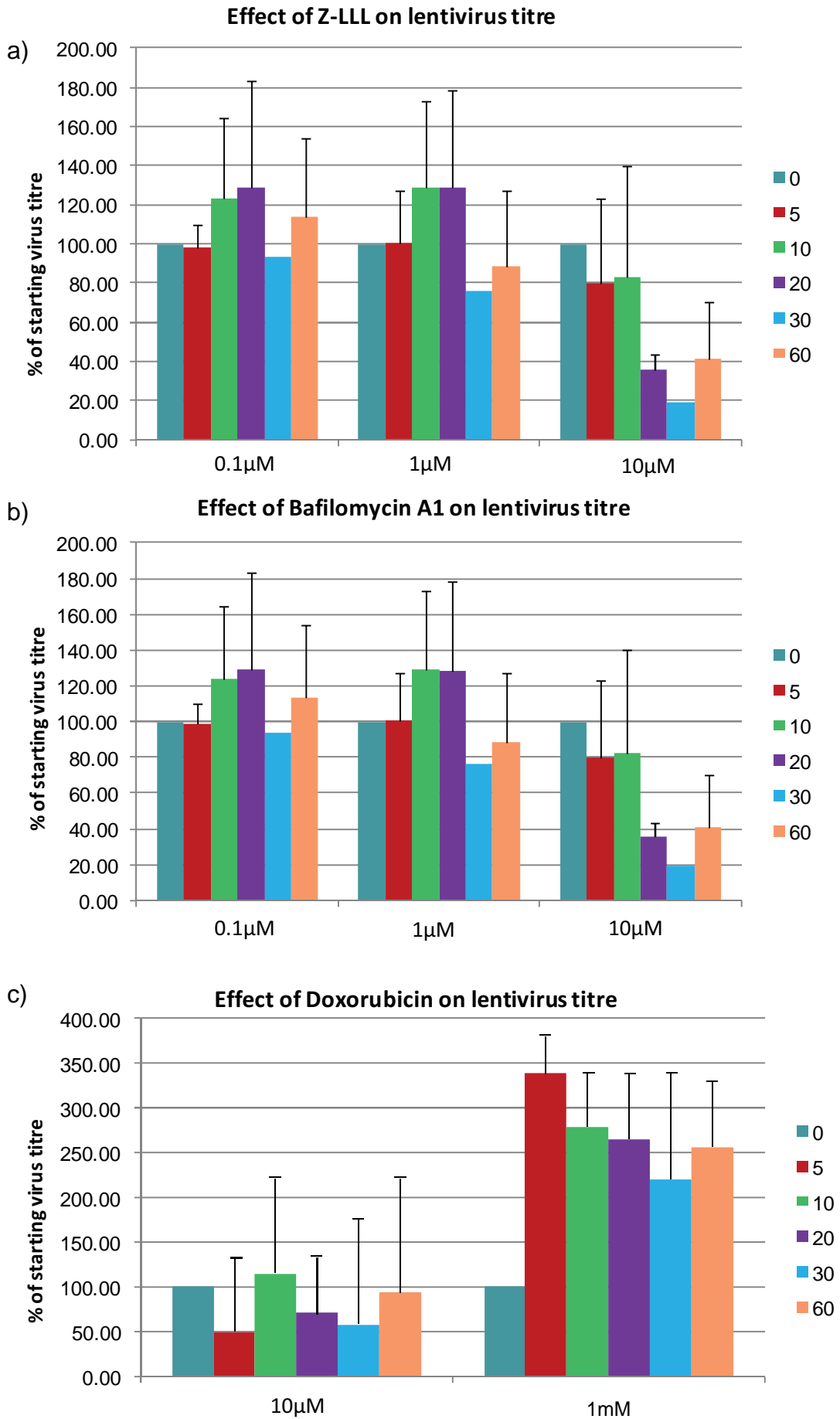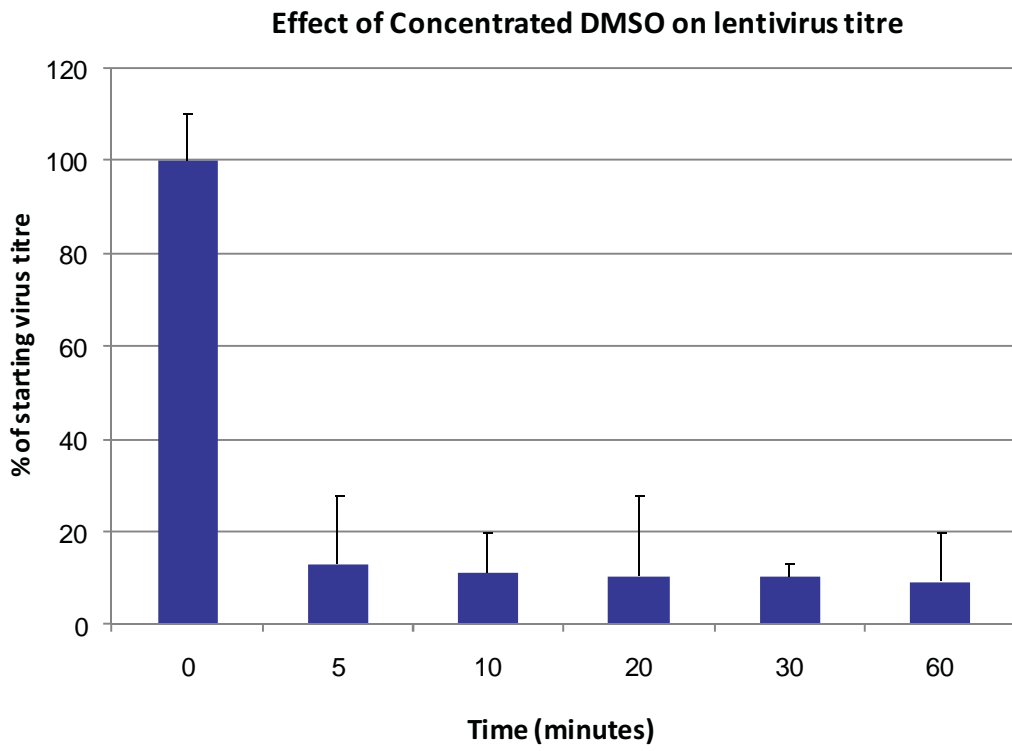
Following the co-incubation studies it was determined that all the tested concentrations of the three different inhibitors were suitable for use *in vivo* as they had no or a relatively small effect on viral titre.

3.3   Testing Inhibitors In Vivo

Following the *in vitro* testing of Z-LLL, Bafilomycin A1 and Doxorubicin which showed their effect on viral titre were within tolerable limits, they were assessed for their effect on gene transfer in the murine nasal airway.

Working stocks of each inhibitor were made in PBS immediately before being diluted to their final test concentrations in both LPC and LV-LacZ. Working concentrations of Bafilomycin A1 were 0.01 mg/mL, Z-LLL, 0.2 mg/mL and Doxorubicin HCl 0.5 mg/mL. This decreased the possible effect that may be attributed to the carrier solute DMSO for Z-LLL and Bafilomycin A1. The dilution (1:100) of the inhibitors in PBS before use meant the final concentration of solute was lower than that used in the *in vitro* experiments. This was carried out to dampen any negative effects the solute had on the virus titre and the consequential gene transfer.

Each inhibitor was delivered to the murine nasal airways *via* the inhalation instillation method described in 2.4.1. The inhibitors were delivered in both the LPC dose and Lentivirus dose (as determined by Leptomycin B studies 4.4).

Animals were monitored post treatment daily for one week.

One week post inhibitor/virus delivery, animals were sacrificed as described in 2.4.2 and processed for β-galactosidase activity (LacZ). Histology was analysed as described in section 2.4.2.3. Cell counts from mice treated with either Z-LLL, Bafilomycin A1 or Doxorubicin all showed no evidence of gene transfer

– no cells expressing β-galactosidase were observed. Prior to this experiment being carried out, 3 normal mice were instilled with the same LV-LacZ virus and protocol to ensure the virus was suitable to be used for *in vivo* experiments, LacZ activity was seen at the appropriate levels (over 200 blue cells in all three test mice) to continue with further experiments. This therefore rules out a problem with the virus for the lack of gene transfer seen here. What was observed was a disordered, damaged and re-growing epithelium. The treated side appeared significantly different to the non-treated side, and appeared somewhat inflamed when compared to the non-treated side. Representative slides were analysed by Dr John Finnie (Senior Veterinary Pathologist at the Institute of Medical and Veterinary Science (IMVS)) who confirmed the epithelia lining the nasal septum showed signs of damage, cell regrowth and disorganisation caused by the treatment delivered. Severity of the damage appeared to be dose dependant with the lower concentration inhibitors showing less damage than that seen by the high concentrations. Figure 3-2 shows the damage seen in sections taken from mice treated with the inhibitors and sacrificed one week post instillation.

Damage was also inhibitor dependent, with Doxorubicin showing greater damage than that seen in mice treated with Z-LLL or Bafilomycin A1.

It was also noted that all mice in these groups were housed together initially before being put into separate treatment groups. To rule out that all the mice had contracted a cold or infection that may have impacted on results, a repeat experiment was carried out testing the inhibitors *in vivo*, with only the lowest concentration of each inhibitor tested, with mice housed in separate cages.

Mice treated with Doxorubicin were seen to be showing signs of distress following the first dose of Doxorubicin in the 0.3% LPC pre-treatment. These mice were sacrificed on advice from the animal house manager and processed as per 2.4.2. Results from histopathology showed similar damage to that seen in the previous experiment.

The result of this experiment was the same as was observed in the first. With all three compounds, sections of mouse nasal tissue showed damaged, disordered epithelia with no evidence of gene transfer.

As the inhibitors did not show enhancement of gene transfer, and significant side effects when delivered in topical fashion, that is *via* nasal delivery in conjunction with the LPC pre-treatment and the virus dose, systemic administration *via* I.P injection of the inhibitors was assessed as an alternative route of delivery.

Mice (n=3/group) were given an I.P injection of either Bafilomycin A1 at 50 µg/kg, Z-LLL at 1 mg/kg or a solution containing both Bafilomycin A1 and Z-LLL at 50 µg/kg and 1 mg/kg respectively or a saline control injection one hour prior to standard nasal dosing 2.4.1. The dose of Z-LLL was determined from studies published by Jennings, K *et al* and Orlowski, RZ *et al* and for Bafilomycin A1, a study by Keeling *et al* [186-188].

Mice were sacrificed one week later as per section 2.4.2. Sections of murine nasal epithelium were observed under 400 x magnification. Gene transfer was observed in mice treated with Bafilomycin, Bafilomycin + Z-LLL and saline, however, no gene transfer was observed in mice treated I.P with Z-LLL only.

Figure 3-2: Damage caused by instillation of inhibitors *in vivo*

S= septum, right side of septum indicates treated side, left side of septum indicated untreated control tissue

   a) 100 x magnification of murine nasal tissue, Z-LLL treated
   b) 400 x magnification of squared area on a)
   c) 200 x magnification of murine nasal tissue, Bafilomycin A1 treated
   d) 40 x magnification of murine nasal tissue, Bafilomycin A1 treated
   e) 100 x magnification of murine nasal tissue, Doxorubicin HCl treated
   f) 400 x magnification of squared area in e)

Areas highlighted by black outline show areas of inflammation and tissue damage caused by treatment with test inhibitors.
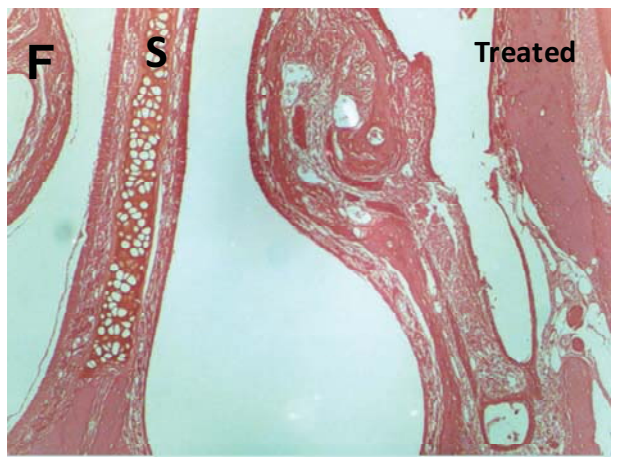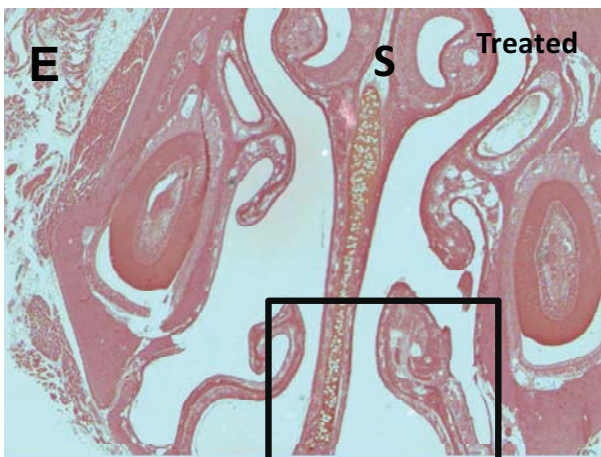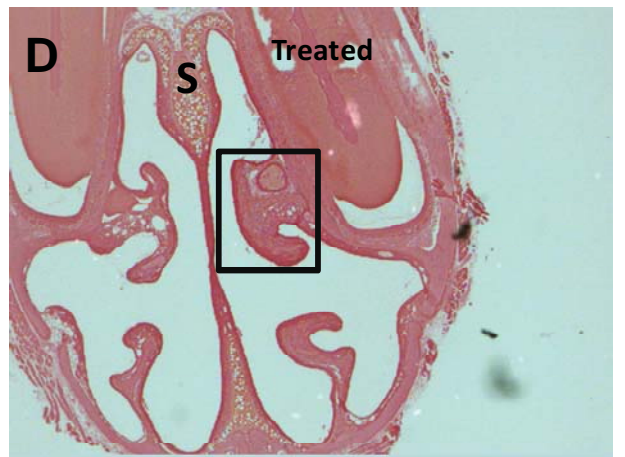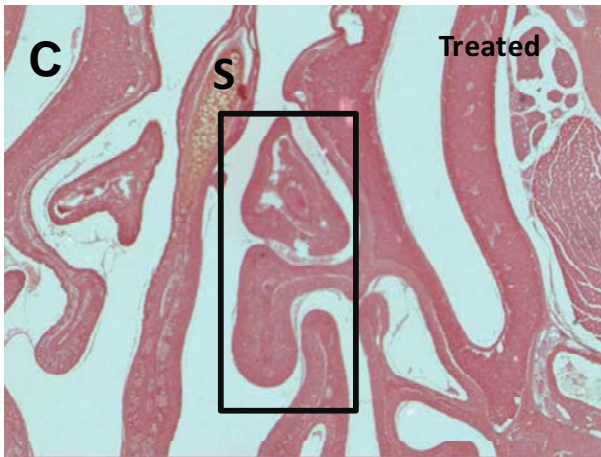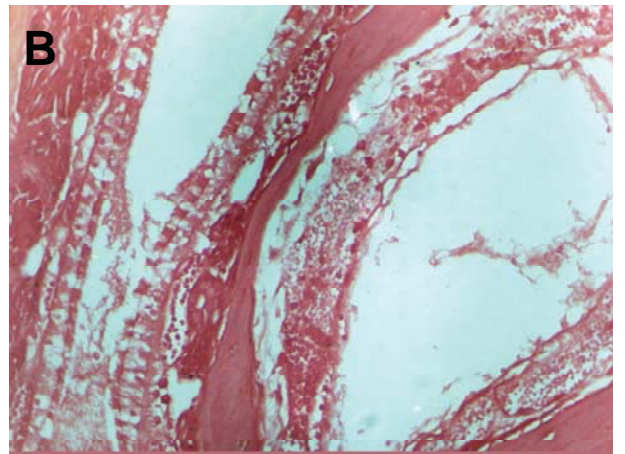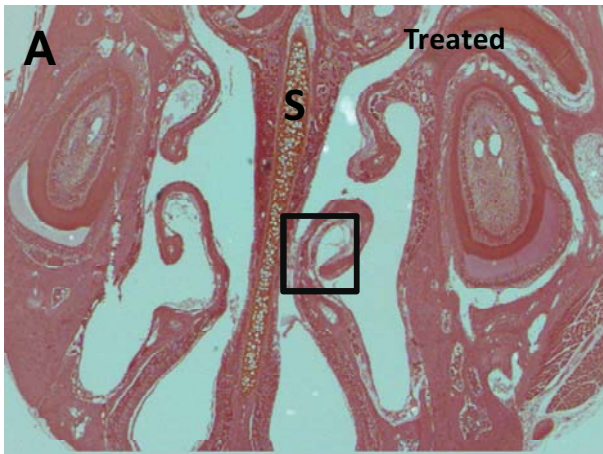
The amount of gene transfer observed was low in all mice (Figure 3-3) and the differences seen in gene transfer were not significant (ANOVA, p=0.351). This lack of significance was due to the large intra-group variability and the small variation between the different treatment groups. In each treatment group there were mice that were seen to respond to the treatment protocol and others that showed low or no gene transfer, however, the group treated with Z-LLL only did not show any gene transfer. It was interesting to note that the group that received Bafilomycin A1 only showed no difference in the level of gene transfer observed when compared to the group that received Bafilomycin A1 + Z-LLL. The group treated with saline I.P were seen to double the level of gene transfer achieved compared to all other treatment groups. Given the negative effects seen, it was felt that it was not worth pursuing this approach, despite the poor statistical quality of the data. When compared to the previous experiment where inhibitors were delivered topically, there were no morphological changes evidenced when inhibitors were delivered I.P.

3.4   Discussion

The use of proteasome and Lysosome inhibitors to enhance gene transfer has been reported by a number of groups. Yan *et al* showed increased transduction of up to 2000 fold greater when a combination of Z-LLL and Doxorubicin were delivered directly onto polarized airway epithelia following rAAV-2 and rAAV-5 transduction of the airway epithelia *in vitro*. It was also shown that Bafilomycin A1 at test concentrations of 1 to 100 nM could increase HIV-1 infectivity up to 50 fold *via* pre-treatment of cultured HeLa Magi cells with the inhibitor [166]. Wei *et al* also showed Bafilomycin A1 treatment to increase infectivity of HIV-1 derived viruses to be increased between 5 and 28 fold in cell culture using P4-R5 Magi cells. Previously the largest increase in infectivity attributed to treatment with Bafilomycin A1 was 2.7 fold increase reported by Schaeffer *et al*, utilizing a Magi cell culture system and Bafilomycin A1 concentrations of 10 µM, 25 µM and 20 mM. However, longer treatment times and

higher concentrations were used in the Wei *et al* study and may have attributed to the larger increases in infectivity that were noted.

The results obtained from the experiments described above, show that gene transfer was not enhanced when the proteasomal and lysosomal inhibitors were applied topically by incorporating them directly into our nasal dosing protocol. Results showed quite the opposite, gene transfer was not only decreased, but inhibited all together when inhibitors were incorporated into the nasal dosing protocol. In addition, it was shown 45 minutes after contact with the inhibitor Doxorubicin, nasal airway epithelia suffered significant damage, with cells being seen to be sloughed from the airway surfaces.

Mice that received a dose of Z-LLL and Bafilomycin A1 in both LPC and LV-LacZ and were sacrificed one week post treatment also showed signs of damage in the airway epithelia. Airways were filled with proteinacious fluid released from the treated cells. Airways also showed that the epithelia was damaged, and had begun re-growing in the week post treatment. It is important to note that all mice were monitored for body weight and other signs of distress such as coat condition (over/under grooming), changes in eyes, breathing, movement, alertness/sleeping, nose and normal vocalization and movement upon handling and while undisturbed in the cage. No changes in any of these monitored areas were noted and mice were steadily gaining weight, indicating that the mice were not overtly distressed by the treatment.
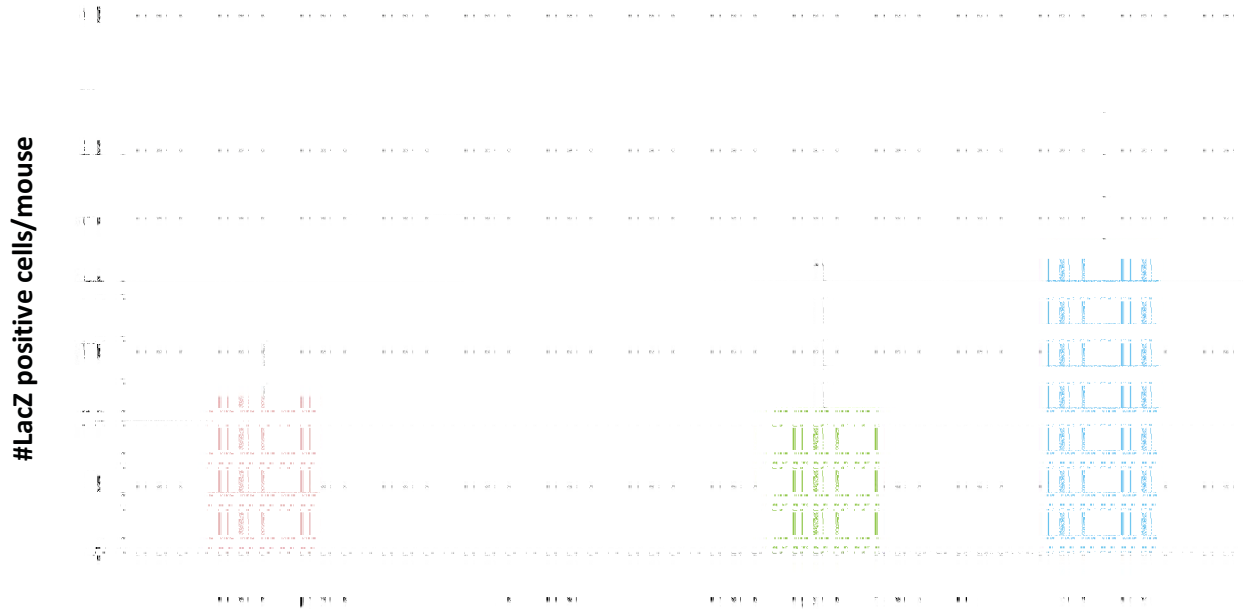
Figure 3-3: Effect of I.P inhibitor injection on nasal gene transfer

Inhibitors delivered *via* I.P injection prior to nasal gene transfer showed no enhancement of gene

transfer compared to mice given saline I.P prior to gene transfer, n = 3, p=0.351

It is not known why the intra nasal delivery of the inhibitors was not conducive for gene transfer. It is possible that the small amount of damage caused by the pre-treatment LPC (which is used to open tight cell to cell junctions to allow entry of the LV to the basolateral surface of the cells) may have allowed the inhibitor access across the epithelium, allowing the inhibitor to cause more substantial damage to the epithelia- such as the sloughing of cells that was observed, than might otherwise have been the case.

It is interesting to note that in a similar study published by Griesenbach *et al* 2009, Doxorubicin was tested as a potential enhancer of cationic gene transfer using GL67A complexed with a plasmid containing a luciferase gene for lung gene transfer. Doxorubicin was delivered either as a bolus I.P injection immediately before gene transfer or topically administered *via* sniffing the solution in through the nose. The dose ranged from 0-100 mg/kg I.P and 125-500 ug (equivalent to between 6-25 mg/kg) for the nasal inhalation method, which are higher than the concentrations used in this study. During this study, it was noted that that 25 mg/kg was the highest dose of Doxorubicin that could be administered topically due to toxicity. Gene transfer was assessed 24 hour post gene transfer by bioluminescent imaging. The high concentration of Doxorubicin HCl- 100 mg/kg – did not increase gene transfer compared to a dose of 50 mg/kg. Mice treated with 100 mg/kg also showed signs of toxicity with 1/8 mice dying before the 24 hour assessment of gene transfer and 4/7 remaining mice showing signs of hunching and piloerection that were distinguishable from other mice. The I.P injection of Dox was seen to increase gene transfer significantly, 20-fold over gene transfer without Dox. However, when Dox was applied topically, no increase in gene transfer was noted. Lung homogenates were made to assess the level of luciferase, however, no histological analysis was carried out, which, given the results presented here, may have given insight into the results in mice treated topically with Doxorubicin [152]. These results, along with those described previously in this chapter, reinforce the narrow efficacy-toxicity window that Doxorubicin exhibits. It is not likely that Doxorubicin would be used clinically in CF patients to enhance gene transfer.

It is also possible that gene transfer did occur, but the subsequent damage meant that transduced cells were lost. If this occurred, the same conclusion can be drawn, as this is not clinically useful for CF patients.

The results of the I.P inhibitor delivery presented in this study showed that neither Bafilomycin A1 nor Z-LLL, when used separately or together, enhanced the level of gene transfer observed. Results of I.P delivery of Z-LLL followed by our standard gene delivery showed no gene transfer was present in the nasal epithelia. Pre-treatment with Bafilomycin A1 showed that gene transfer had occurred with an average of 12 LacZ positive cells observed per mouse. When both inhibitors were delivered I.P, it is interesting to note that there was a slight decrease in the amount of gene transfer observed compared to mice pre-treated only with Bafilomycin A1. This suggests that the effect of the Bafilomycin A1 may override the negative effect of the Z-LLL. Compared to the control group that received an I.P injection of saline, there was a non significant decrease in gene transfer in the inhibitor groups that showed gene transfer. As the mechanisms of how these inhibitors have increased viral gene transfer previously have not been entirely elucidated it is hard to conclude why these inhibitors have not enhanced gene transfer with the lentivirus used in this study.

It should also be considered that the studies showing enhancement of gene transfer using viruses derived from HIV-1 have only been shown in cell culture and not in a live animal [165, 166]. As with many systems, the results achieved in cell culture are not replicated when transferred into a living system such as a mouse.

3.5 Conclusions

These experiments showed there to be no beneficial effect from any of the three inhibitors- Bafilomycin A1, Z-LLL and Doxorubicin HCl- on the level of gene transfer observed. The method of

inhibitor delivery did have an effect on the level of gene transfer seen, however these effects were generally negative, with significant damage seen when the inhibitors were delivered topically. No damage to airway epithelia was observed when inhibitors were delivered *via* i.p injection, however there was no increase in gene transfer that warranted pursuing these experiments further.

This set of experiments shows the difficulty in translating data from *in vitro* cell culture systems to practical *in vivo* experiments. The complex biological nature of *in vivo* systems is evident here as the results achieved in these experiments could not be predicted from the *in vitro* data collected by other groups using the same group of inhibitors in cell culture to enhance gene transduction.