THERAPEUTIC DEVELOPMENT FOR CYSTIC FIBROSIS

by

Kristina Thomas

BSE Chemical Engineering, University of Michigan, 2004

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This thesis was presented

by

Kristina Thomas

It was defended on

July 13, 2011

and approved by

Robert S. Parker, Ph.D., Associate Professor, Department of Chemical and Petroleum Engineering

Robert M. Enick, Bayer Professor, Ph.D., Department of Chemical and Petroleum Engineering

Steven R. Little, Assistant Professor, Ph.D., Department of Chemical and Petroleum Engineering

Thesis Advisor: Timothy Corcoran, Ph.D., Assistant Professor, School of Medicine

THERAPEUTIC DEVELOPMENTFOR CYSTIC FIBROSIS

Kristina Thomas, M.S.

University of Pittsburgh, 2011

This thesis considers the aspects of therapeutics development for Cystic Fibrosis (CF). The studies are directed at the development of a new therapeutic outcome measurement for evaluating the performance of CF medications. This imaging-based outcome measures the absorption of a small-molecular radiopharmaceutical, Diethylene triamine pentaacetic acid (DTPA) from the airways as a surrogate measure of liquid absorption. Airway liquid hyper-absorption is a key aspect of CF lung disease that would be expected to correct rapidly after administration of a successful therapy. In vivo pilot studies of this technique have been previously performed at our center [1]. Here we report the results of in vitro studies performed to better define the mechanism underpinning our method and define its utility and limitations.

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1.0 BACKGROUND AND SIGNIFICANCE

1.1 AIRWAY PHYSIOLOGY & ANATOMY

The lungs are the site of gas exchange during respiration. The nose and mouth are connected to the trachea, which branches into two bronchi that enter the left and right lungs. Successive generations of airways, increasing in number while decreasing in size, branch from the bronchi peripherally to provide a path for air to enter and exit the lungs. The terminal branches of the bronchi are called the bronchioles. Each of these divides into 2-11 alveolar ducts. From each alveolar duct, 5-6 alveolar sacs are formed. There are approximately 300 million alveoli in each lung which are covered with capillary sheet and are the site of gas exchange [2].

The epithelia of the large airways are covered with cilia, which are hair-like projections that move in a synchronous manner, and a thin layer of mucus. The mucus and cilia are part of the body's innate immune system and act as a first defense against inhaled pathogens to prevent infections. The mucus traps pathogens and the cilia propel them out of the respiratory tract. This process is called mucociliary clearance (MCC). Pathogens smaller than 0.6nm will be able to evade MCC and can be absorbed via the bronchial epithelia into the bloodstream[3].

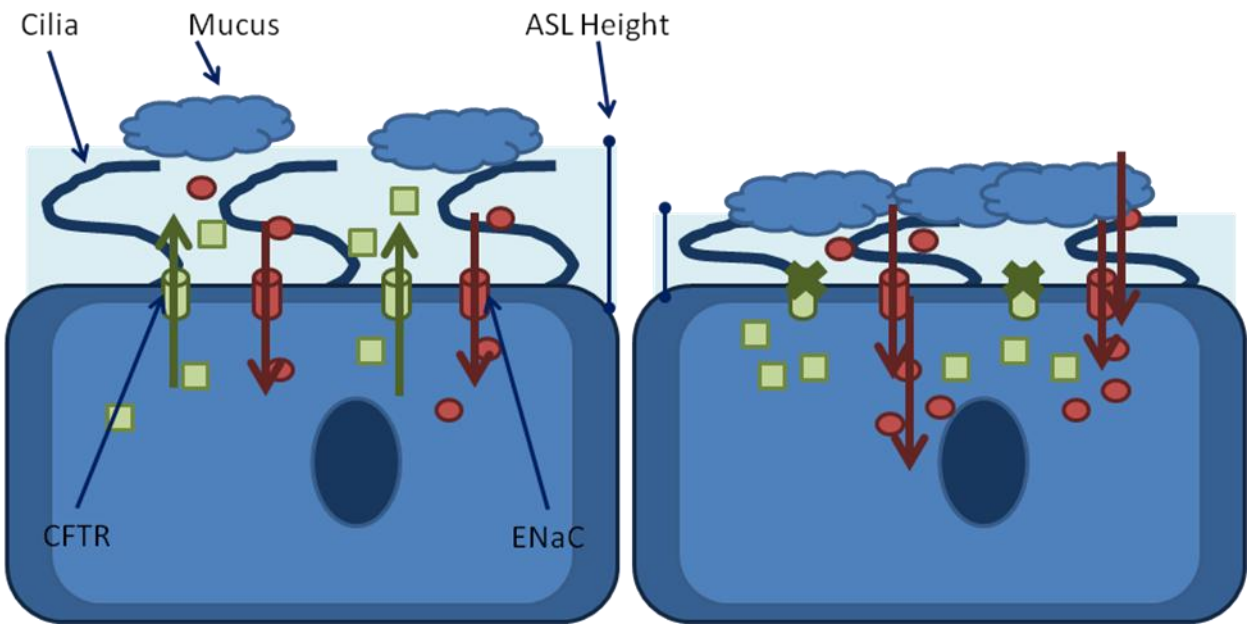

Figure 1 Comparison of a healthy epithelial cell on the left versus a CF epithelial cell on the right. The green protein channels signify the CFTR protein with the green squares representing chloride ions being secreted. The red protein channels represent the ENaC protein with the red dots representing sodium ions being absorbed. In the healthy epithelial cell on the left, there is a balance of ions being secreted and absorbed creating an isotonic ASL. On the right the CFTR channel is defective, causing an imbalance of ions in the ASL which causes the ASL to hyperabsorb creating a layer of thick immobile mucus.

A thin watery layer of liquid called the periciliary layer (PCL) bathes the cilia and has approximately the same height. The mucus lies on top of the cilia and PCL. The tonicity of the PCL layer is maintained by ion channels that control sodium absorption and chloride secretion [4-5]. Sodium is absorbed via the epithelial sodium channel (ENaC). Chloride is secreted via both the cystic fibrosis transmembrane regulator (CFTR) and alternate Calcium activated chloride channels (CaCC)[6]. The CFTR ion channel is associated with the basic defect of CF, and is part of the family of channels called ATP binding cassette transporters (ABC transporter)[7]. The balance of sodium and chloride ions maintains the osmotic homeostasis in the airway surface liquid layer (ASL). If tonicity is disrupted, as would occur with evaporation or liquid volume addition to the airway, the ASL will either absorb or expand via transcellular and paracellular pathways to regain the isotonic layer height of the PCL as shown in Figure 1.

There are multiple liquid transport routes through the airway epithelium. In transcellular transport, liquid is moved through the cell down the osmotic gradient via a family of water protein channels imbedded in the lipid bilayer membrane of cells called aquaporins (AQPs). A

variety of aquaporins are found throughout cells in the body. Aquaporins AQP1, AQP3, AQP4 and AQP5 are found exclusively in the lungs and airways [8]. Paracellular transport involves the passage of liquid through the intercellular gaps. This process is driven by the osmotic gradient [9]. Tight junctions hinder this pathway by connecting neighboring cells together to create a virtually impassible barrier. Not all epithelia contain tight junctions. Epithelia that are considered to be 'leaky' are missing or have weak tight junctions and fluid is able to pass through the intercellular gaps.

Solute transport also occurs via transcellular and paracellular mechanisms. The transcellular protein channels that are used to transport fluids such as AQPs cannot be used for solute movement. Protein channels in the cell bilayer allow for the transport of specific molecules. If the solute does not have a protein channel then transcellular transport is not possible. Unless the solute is able to passively diffuse through the lipid bilayer of the cells. Diffusion through the lipid bilayer of the cells depends on the solute's polarity and molecular size. Paracellular transport of solutes is the same as the mechanism for liquids. Phenomena such as solvent drag improve solutes ability to transport across the epithelia. Solvent drag occurs when the flow of a fluid forces solvents to follow the direction of the fluid. In the case of cellular transport if the direction of fluid flow is towards the epithelia from the ASL, it can transport solute to the epithelia where it can undergo transcellular or paracellular mechanisms depending on the solute particle.

1.2 CYSTIC FIBROSIS LUNG DISEASE

1.2.1 ASL Volume Regulation

Cystic fibrosis (CF) is an autosomal recessive disease that affects the lungs, liver, intestine, sinuses, skin, and reproductive systems. It is related to a single defective gene, the CFTR gene, which is responsible for encoding the CFTR protein in the epithelial cells. It can

mutate, rendering the CFTR protein ineffective to varying degrees depending on the severity of the mutation [10].

A defect classification system has been devised to group CFTR mutations depending on the effect of the mutation of production and transport of the CFTR protein (Class I-V) [11]. The main mutations found in CF patients are: defective protein production (Class I), abnormal intracellular trafficking (Class II), defective chloride transport (Class III), reduction in chloride transport (Class IV) and reduced protein expression of protein but normal function (Class V) [12]. The most common CFTR mutation is a Class II defect involving the deletion of the amino acid phenylalanine at position 508 on the CFTR gene. This defect known as ΔF508 accounts for 70% of mutant alleles and causes the most common form of cystic fibrosis [12].

As previously described, CFTR is an epithelial ion channel responsible for the secretion of chloride ions while ENaC is responsible for the absorption of sodium ions. Together CFTR and ENaC maintain the isomolarity of the ASL. By regulating the concentration of chloride and sodium ions on the luminal surface of the airway epithelium, CFTR and ENaC influence the flux of water across the epithelium. When the CFTR is ineffective, chloride ions are not secreted resulting in an imbalance of ions in the ASL causing it to become hypotonic. Sodium hyper-absorption through ENaC has also been reported on the CF airway epithelium [5, 13-14], further contributing to airway hypotonicity. To compensate for the hypotonic ASL, the PCL is absorbed until a homeostatic balance is reached. This results in a loss of water from the mucus that lines the airways causing it to become hardened and dehydrated. Therefore, dehydrated mucus in the airways is a symptom of CF.

Dehydrated mucus in the airways increases the proclivity for chronic lung infections [15]. Mucus clearance is a key host defense for the lungs that prevents opportunistic infections. CF

patients have a higher risk for bacterial infections by opportunistic pathogens such as Pseudomonas aeruginosa [16]. The height of the PCL in a healthy airway is approximately the height of the extended cilia ~7 μm [17]. In the case of CF patients, the PCL is hyper absorbed to ~4μm. This liquid height collapses the cilia, preventing them from playing their normal role in mucus clearance leaving a layer of thick immobile mucus as well as exposing the cilia of the lung epithelia [5].

1.2.2 Cystic Fibrosis Therapeutics

A variety of therapies are available for the treatment of CF. Focusing on the chronic lung infections linked to cystic fibrosis, there are two approaches in treating CF, prophylactic and symptom based treatment. Symptom based therapy fights the opportunistic infections as they occur, while prophylactic therapy seeks to prevent or reduce the number of infections by treating the underlying pathophysiology of CF.

The treatment of disease progression is still the mainstay of available therapies in CF. Due to the fact that CF patients tend to have chronic infections, symptom based therapies are often used as prophylactic therapies too. For example, antibiotics typically used to treat a lung infection would be prescribed for the current infection and the patient would continue on it to prevent further infections. A common example is the inhalation of the aminoglycoside antibiotic tobramycin, which is frequently used to treat bacterial infections such as P. aeruginosa [18].

Table 1 Pathophysiology of CF with the comparison of biomarkers and therapies. The center column shows the progression of CF from the basic elements to serious conditions of the disease. The right column shows where current treatments are targeting the pathophysiology and the left column shows how which stage Outcome Measures measure the effectiveness of the therapy.

Outcome measures or Biomarkers	**Pathophysiology**	**Therapies**
	Defective CFTR	Gene therapy
Nasal Potential Difference	Improper ion fluxes	sodium channel blockers, alt chloride agonists
Absorptive or 2 isotope clearance scan	Improper fluid fluxes	Hypertonic saline
	Thinned, viscous ASL	
Mucociliary scans	Defective clearance	
Serum cytokines, Exhl Br Condensate, Exhl Gas	Infection, Inflammation	Antibiotics, Anti-inflammatory
CT scan	Mucus plugging, airway damage	Dnase
Pulmonary function	Loss of pulmonary function	
Exacerbations, QOL	Loss of QOL, mortality	

Recent therapeutic development efforts in CF have been directed at the most basic elements of CF lung disease as shown in Table 1. This includes the development of gene therapy techniques to correct the CFTR gene, CFTR "correctors" which repair specific intracellular processes associated with CFTR production and transport, CFTR "potentiators" which assist the function of defective CFTR that is available at the epithelial surface, channel modulators which

correct sodium and chloride flux at the epithelium, and osmotics which drive water transport towards the airway lumen to rehydrate the ASL [19].

Current treatments for CF include therapies that modify the ASL such as inhaled hypertonic saline [20-21]. Hypertonic saline reverses the osmotic gradient at the epithelium, and increases the liquid content of the ASL to the levels similar to those seen in healthy subjects. Along with osmotic therapies there are 2 different CFTR modulating drugs are in phase 3 testing, VX-770 from Vertex Pharmaceutical and Ataluren (formerly known as PTC124) from PTC Therapeutics [22-24].

A crucial research area in CF therapy development is outcomes development. Most current clinical methods used to determine the efficacy of CF therapies require months of treatment and observation before measureable changes can be detected [25]. New methods for determining the efficacy of therapies designed to treat the most basic aspects of CF lung disease would increase the speed with which therapies could be made available to patients [26]. As recent therapies focus on rehydrating the ASL, an outcome measure that has the ability to gauge liquid absorption in airways would be a significant improvement.

This project involves the development of an imaging based outcome measure for use in testing new CF therapies. Specifically in vitro studies were performed in airway cell cultures to help determine the suitability of a radiopharmaceutical for measuring airway liquid absorption.

2.0 OUTCOME MEASUREMENT DEVELOPMENT: TWO ISOTOPE IN VITRO STUDIES

2.1 BACKGROUND

2.1.1 In vivo study – Previous work

We have previously performed a small clinical study using an aerosol based imaging method. Our previous work used a 2 isotope model to measure the absorptive clearance rate of DTPA between CF and control subjects to see if it can be used as a possible early outcome measure [1]. The use of two different isotopes, TC99m-Sulfur Colloid (SC) and In111-DTPA, allowed us to measure the clearance based on MCC and absorption. The TC99m-SC isotope can only be cleared via MCC and the In111-DTPA isotope can be cleared via both MCC and absorptive pathways, therefore by subtracting the clearance rate of TC99m-SC from the clearance rate of the In111-DTPA, the absorptive clearance of the DTPA can be calculated. In our 2 isotope pilot study we found that there was a difference in the absorptive clearance of DTPA in CF and nonCF patients as shown in Figure 2.

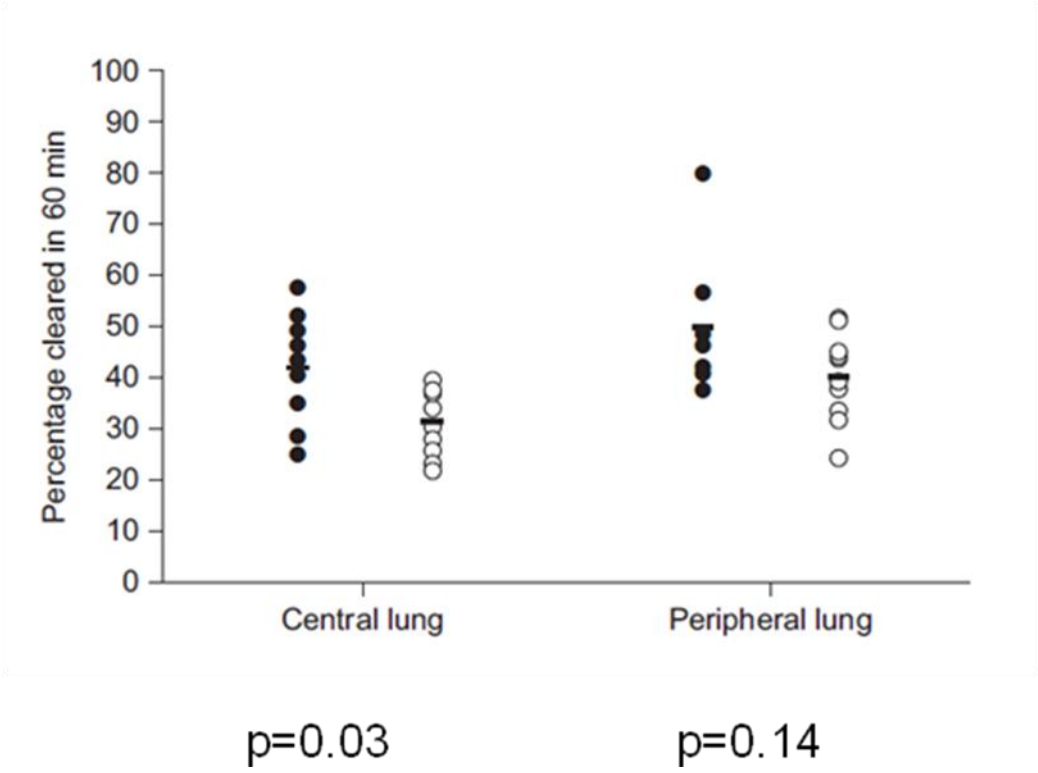

Figure 2 Absorptive clearance in central and peripheral lung zones. The dark circles are the CF cases n=9 and the open circles are the nonCF cases n=10.

2.1.2 DTPA Transport

We have considered the possibility of using a small molecule radiopharmaceutical as a surrogate for measuring liquid absorption in the airways. One option with a significant safety history in the lung is diethylene triamine pentaacetic acid (DTPA). There are two FDA approved radiolabeled forms of DTPA available, Technetium 99m-DTPA (Tc-DTPA) and Indium 111-DTPA (In-DTPA). DTPA is a small negatively charged hydrophilic molecule with a molecular weight 393.35 g/mol. Liquid transport through the epithelium occurs through both transcellular and paracellular routes. Paracellular transport has long been the assumed only pathway for DTPA transport through the epithelium since transcellular route are generally impermeable to

small solute [27-29]. We hypothesized that DTPA absorption might be influenced via a solvent drag mechanism, by the paracellular component of airway liquid absorption, and might provide a gauge of the paracellular absorption rate. As previously described CF lung disease is associated with liquid hyper-absorption. In vitro studies have demonstrated that liquid added to the epithelial surface of a CF airway is absorbed more rapidly than in a normal airway. Rectification of the basic defect of CF lung disease should result in rapid correction of this effect. Though the exact balance between the transcellular and paracellular components of liquid absorption in the CF airway is unknown, we hypothesized that therapeutic correction would result in detectable changes in paracellular transport that would be proportional to changes in overall liquid absorption in the airway. Studies in the intestine have previously demonstrated relationships between paracellular liquid transport and the absorption rate of small solutes [30].

An alternative hypothesis is that the inflammatory disease damages the bronchial epithelia creating sites of increased permeability via epithelial denuding or eroding of the epithelial and by cytokines reducing tight junction permeability [31-34]. The inflammation causes the epithelia to become ‘leaky’ and increases the DTPA transport through the tight junctions. Studies have shown that smokers show increased clearance of DTPA [33]. It was also found that asthma patients had increased DTPA clearance[35].

We hypothesize that DTPA transport across the epithelia is due to a phenomenon known as solvent drag [36]. Normally when particles and solvent are present in a system, the movement of the solvent causes the particles to flow along with the solvent. In the case of CF, the hyperabsorption of the ASL causes water to move from the apical surface towards the basolateral surface, thus dragging the hydrophilic DTPA molecule towards the epithelia where it is assumed to be absorbed via paracellular pathways.

As previously described the CF airway hyper-absorbs liquid due to the errant transport of sodium and chloride ions associated with mutations in the CFTR gene [13, 16, 37]. This hyper-absorption could cause an increase in DTPA absorption and explains the difference in absorptions rates observed in our previous study involving 2 isotopes [1].

2.2 SPECIFIC AIMS

Outcome measures are critical in determining drug efficacy in a timely manner. In the case of cystic fibrosis, early stage outcome measures are crucial as many new therapies target the basic elements of the disease while most available outcome measures quantify effects of later stage disease progression. One of the main goals of these new therapies is to treat the hyperabsorption of liquid in the airways-a key basic element of CF lung disease. We have previously performed clinical studies of a potential radiopharmaceutical surrogate for measuring liquid absorption in the airways [1].

2.2.1 Aim 1: To explore the feasibility of DTPA absorption as a clinical outcome measure for testing CF therapies using human bronchial epithelia cells (HBEs) to mimic the functional imaging technique we have piloted in our CF patients.

2.2.1.1 Hypothesis 1: DTPA absorption will be increased in primary human bronchial epithelial cultures derived from explanted cystic fibrosis lungs when compared to non-CF lungs. *To test this hypothesis we measured the absorption of radiolabeled DTPA in CF and non-CF HBE's over 24 hours.*

2.2.1.2 Hypothesis 2: DTPA absorption will be driven by osmotic gradients in a manner similar to liquid absorption. *To test this hypothesis varying concentrations of an osmolar agent, mannitol, were added to the apical or basolateral surfaces of CF and non-CF HBEs and the absorption of radiolabeled DTPA was measured over 24 hours.*

2.2.1.3 Hypothesis 3: Human bronchial epithelial cells treated with pro-inflammatory cytokines will demonstrate increased rates of DTPA absorption in association with increased tight junction diameter. *To test this hypothesis cytokines were added to the basolateral surface of CF and non-CF HBEs and the absorption of radiolabeled DTPA was measured for 24 hours.*

2.2.1.4 Hypothesis 4: DTPA absorption rate can be directly related to liquid absorption rate. *To test this hypothesis DTPA clearance was measured using radioisotope techniques and water absorption was measured utilizing dye concentration-based techniques.*

2.3 RESEARCH DESIGN:

2.3.1 Aim 1

The Human Bronchial epithelial cell model:

HBE cell cultures are a high fidelity model of the luminal surface of an airway. These cultures exhibit normal airway physiology including ion conductance, liquid transport, mucus generation, and ciliary motion. CF cell lines exhibit the ion and liquid transport defects seen in CF patients. The cell lines are not immortalized and their availability is dependent on recent transplant activity. Contemporary controls are utilized throughout the experiments. Controls designated as 'non-CF' have been generated from lungs with disease states not thought to substantially impact normal airway physiology as illustrated in culture. The specific cell lines used in these experiments along with the associated disease conditions resulting in transplantation are shown in Table 2.

Table 2: Cell lines with corresponding conditions leading to transplant

Cell Line	Disease
HBE 655	Idiopathic pulmonary fibrosis (IPF)
HBE 675	Immotile cilia
HBE 665	Emphysema
HBE 666	Emphysema
CF 150	Cystic Fibrosis ΔF508/D1152H (Class IV)
CF 153	Cystic Fibrosis ΔF508/ΔF508 (Class II)

Primary HBE cells were harvested from lungs removed at the time of lung transplant from CF and other advanced lung diseases according to a University of Pittsburgh Institutional Review Board approved protocol. As previously described [38] airway sections from these lungs were digested in a protease solution to detach the epithelial cells. The epithelial cells were then resuspended in a 1:1 bronchial epithelial growth media/keratinocyte-serum free medium (BEGM/K-FSM) and seeded onto sterile tissue flasks precoated with human placental collagen for 5-6 days. The cells were then seeded onto 1.13 cm^2 collagen-coated transwell filters (0.4μm pore size, Corning-Costar Transwell Collagen T-cols, Acton, MA) at an approximate density of 2 x 10^6 /cm^2. The cells were grown on the filters for 4 weeks; once a confluent monolayer was reached the HBE filters were ready for experiments as shown in **Error! Reference source not found.**. The apical surface of the HBE filters were washed twice the night before the experiment with 100μL 1xPBS and aspirated. This was done to remove any uneven build up of mucus on the apical surface.

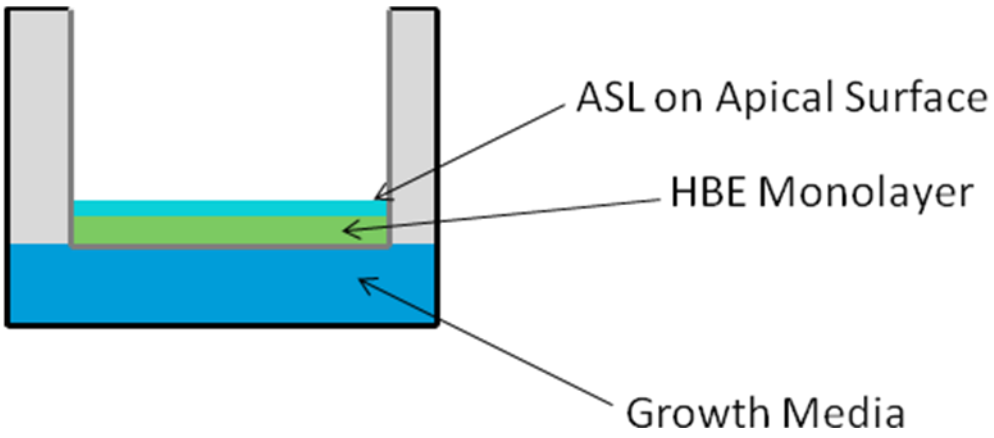

Figure 3 Schematic of HBE culture

The filters were then submerged in DMEM, 550 µL basolateral surface, 250 µL on the apical in preparation for measuring the potential difference across the filter. The filters were submerged for approximately 15minutes. The filters were measured using a Millipore* Millicell-ERS Volt-Ohm Meter (Fisher Scientific #MERS00001). Both trans-epithelial electrical resistance and voltage were recorded. Each filter was measured 3 times before the DMEM was aspirated and the cells were returned to 0.5 % UNC/USG media overnight. The average values obtained is shown in Figure 3 for the mannitol experiments. Measuring potential difference is a quantifiable approach for determining confluence in filters [39].

In vitro measurements of DTPA absorption

A major element of the current experiments is the measurement of DTPA absorption rate in the HBE cell cultures. In most of these studies small volumes (10 µL) of Tc-DTPA in PBS were added to the apical (luminal) surface of the HBE cells. The filter containing the cells and the ASL can be removed from the growth medial allowing for independent measurements of retained radioactivity (ALS, cells, and filter) and absorbed radioactivity (media). Filters were

placed in 300 μL 0.5 % UNC/USG media and 10 μL of 1xPBS Tc-99m DTPA was added to the apical surface. At t=0,4,8,12 hours 50 μL of fluorinert FC-40 (F9755sigma) was added to the apical surface of all the filters regardless of condition tested. Radioactive counts associated with Tc99m-DTPA counts were measured in both the filter and media at t=0, 2, 4,8,12 and 24 hours. Radioactive counts were corrected for background and decay and normalized by starting counts. The retention curves of radioactivity vs. time for each filter were plotted and fit to exponential curves. These curves were evaluated at 24 hours and then averaged. Two-factor ANOVAs were used to determine significance between cases.

2.3.1.1 Hypothesis 1

To compare the DTPA absorption rate between the nonCF vs. CF HBEs a total of 30 filters from 4 nonCF lines (HBE 655, HBE 665, HBE666, HBE 675) and a total of 24 filters from 2 CF cell lines (CF150, CF153) were compared using the technique described in Aim 1 above.

2.3.1.2 Hypothesis 2

Mannitol is sugar alcohol used as an osmolar agent in medicinal therapies. Adding mannitol to the apical surface of HBEs will increase the osmolarity of the ASL creating an osmotic gradient that would favor liquid transport from the epithelium into the ASL.[40]. The addition of mannitol to the basolateral media will create an osmotic gradient favoring liquid absorption by the epithelium. The effects of both apical and basolateral mannitol addition on DTPA absorption are considered here. The general protocols described above were followed with the following modifications. For the apical mannitol case, the filters were placed in 300 μL

0.5 % UNC/USG media and 10 μL of 300mM Mannitol Tc-99m DTPA was added to the apical surface. Two basolateral cases are considered. A volume of 10 μL of 1xPBS Tc-99m DTPA was added to the apical surface and the filters were placed in either 300 μL 150mM Mannitol 0.5 % UNC/USG media or 300 μL 300mM Mannitol 0.5 % UNC/USG media. A total of 30 filters from 4 nonCF lines (HBE 655, HBE 665, HBE666, HBE 675) and a total of 24 filters from 2 CF cell lines (CF150, CF153) were compared.

Table 3 Explanation of Mannitol experiment: the 4 cases and the conditions for each

Case	Apical Conditions	Basolateral Conditions
Control	10 μL of 1xPBS Tc-99m DTPA	300 μL 0.5 % UNC/USG media
Apical Mannitol	10 μL of 300mM Mannitol Tc-99m DTPA	300 μL 0.5 % UNC/USG media
150mM Basolateral Mannitol	10 μL of 1xPBS Tc-99m DTPA	300 μL 150 mM Mannitol 0.5 % UNC/USG media
300mM Basolateral Mannitol	10 μL of 1xPBS Tc-99m DTPA	300 μL 300mM Mannitol 0.5 % UNC/USG media

2.3.1.3 Hypothesis 3

Cytokines are found at sites of inflammation on the bronchial epithelia. They have been shown to increase tight junction permeability in previous studies with airway cultures. [31]. In this work the addition of specific cytokine combinations causes a decrease in the resistances across the epithelia indicating 'loose' tight junctions. Higher levels of proinflammatory cytokines tumor necrosis factor α (TNF α), interleukin (IL)-8, and IL-1β as well as the soluble intercellular adhesion molecule (ICAM)-1 have been measured in airways of CF patients [31]. Increased tight junction permeability is associated with the combination of TNF α and INF-γ. It has been proposed that lung inflammation causes an increase DTPA clearance making Tc99m-

DTPA aerosol is a good marker to judge the permeability of the lung epithelia[34]. By comparing the effects of proinflammatory cytokines has to DTPA clearance, we can determine if proinflammatory cytokines cause DTPA clearance or if there is already a baseline DTPA clearance. As previously stated an alternative hypothesis predicts DTPA clearance is due to the presence of proinflammatory cytokines.

In order to see the effect of cytokine exposure on DTPA absorption, the airway cells were exposed to a mixture of TNF α (10ng/ml) and INF-γ (100ng/ml) on the basolateral surface for 48 hours [31]. In the control case no cytokines were added to the 300 μL 0.5 % UNC/USG media. After 48 hours, the radioisotope part of the experiment as explained in Aim 1 was performed. A total of 24 filters from 1 nonCF line (HBE666) were compared.

2.3.1.4 Hypothesis 4

A dye based method was utilized to directly compare DTPA absorption to liquid absorption. Texas Red Dextran (TRD) is a large molecule (10 kDa) non-absorbable dye. When TRD is added to the ASL, liquid absorption will be reflected in changes in TRD concentration which can be assessed using spectrofluorometry., A 50 μL mixture of 0.25 μg/ ml Texas Red Dextran (TRD and TcDTPA(concentration 0.01 mCi/ml?) in 1x PBS was added apically to each filter. Each filter was used for a single time point measurement. At each time point (t=0, 2, 4,8,12 and 24 hours) radioactive counts were measured in the filter and then a 5 μL sample was removed from the apical surface and placed in 96 well UV spec plate. The sample was diluted to 200ul with the addition of 195 μL 1xPBS. The concentration of TRD was measured using a UV spectrometer. Prior to the experiment a standard curve was plotted using the UV spectrometer

absorption values for known concentrations of TRD. The measured absorption from the UV spectrometer was used to calculate the concentration of the TRD from the standard curve.

With addition of Texas Red Dextran (10kD TRD), we can monitor the rate of water absorption in the ASL and compare it to the absorption of DTPA in the ASL. Since TRD is too large to diffuse or absorb through the HBE epithelia, monitoring the concentration of TRD in the ASL would correlate to water transport across the epithelia.

2.4 RESULTS

The model used a single exponential to explain the flow and decay of Tc-DTPA during the course of the experiment[28]. Equation 1 explains the relationship between the change in mass over time versus the concentration of Tc-DTPA, where Ca is the Airway DTPA concentration and Cp is the blood DTPA concentration. The concentration of Tc-DTPA in the blood can be assumed to be zero due to the large blood volume versus the ASL volume. The constant k is equal to the surface area (S) times the permeability coefficient (P) which can simplify Equation 1 further to lead to Equation 3. Breaking the airway DTPA concentration down in mass (m) over ASL volume (V) and ASL volume to be surface area times ASL thickness (T), equation 3 can be further simplified to equation 6 which when solving for mass leads us to the single exponential model shown in Equation 7.

$$\frac{dm}{dt} = -k(C_a - C_p)$$ **Equation 1**

$$k = SP$$ **Equation 2**

$$\frac{dm}{dt} = -SPC_a$$ **Equation 3**

$$C_a = \frac{m}{V}$$ **Equation 4**

$$V = ST$$ **Equation 5**

$$\frac{dm}{dt} = \frac{-Pm}{T}$$ **Equation 6**

$$m(t) = e^{[(-Pt)/T]}$$ **Equation 7**

2.4.1 Aim 1

2.4.1.1 Hypothesis 1

Figure 2 includes a comparison of DTPA retention vs. time in representative CF and non-CF cell lines. As shown in the figure DTPA retention in the CF cell line CF153 (n=6 filters) is decreased vs. the nonCF cell line HBE675 (n=6 filters) indicating faster clearance. The DTPA retention data fit well to exponential curves with R^2 values of 0.993 and 0.995 for the cell lines HBE 675 and CF 153 respectively.

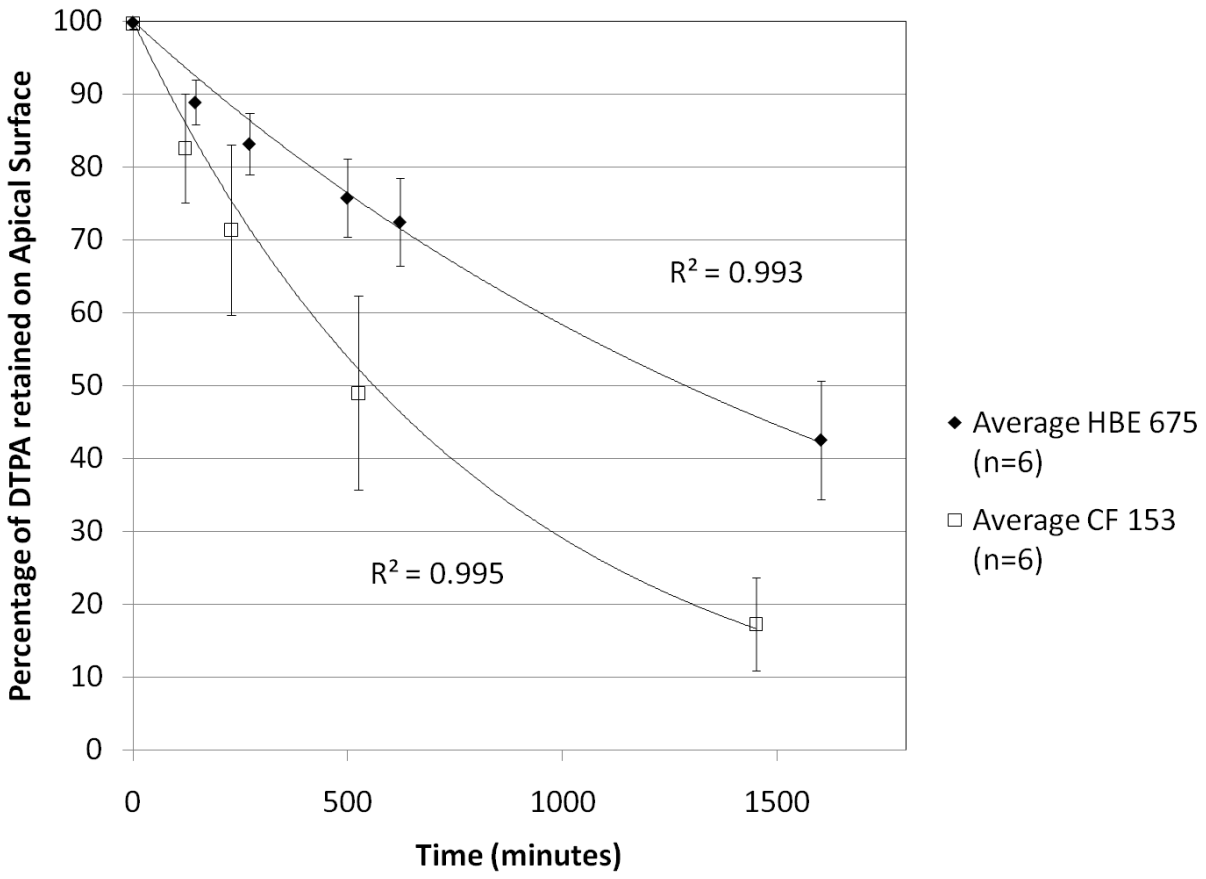

Figure 4 Representative retention curves showing DTPA absorption in CF and nonCF cell lines over 24 hrs (n=6 filters per line). HBE 675 is non-CF cell line (immotile cilia) and CF 153 represents a cystic fibrosis with the genotype ΔF508/ΔF508.

The average DTPA absorption rate at 24 hours for each filter was graphed and the results are shown in Figure 5.

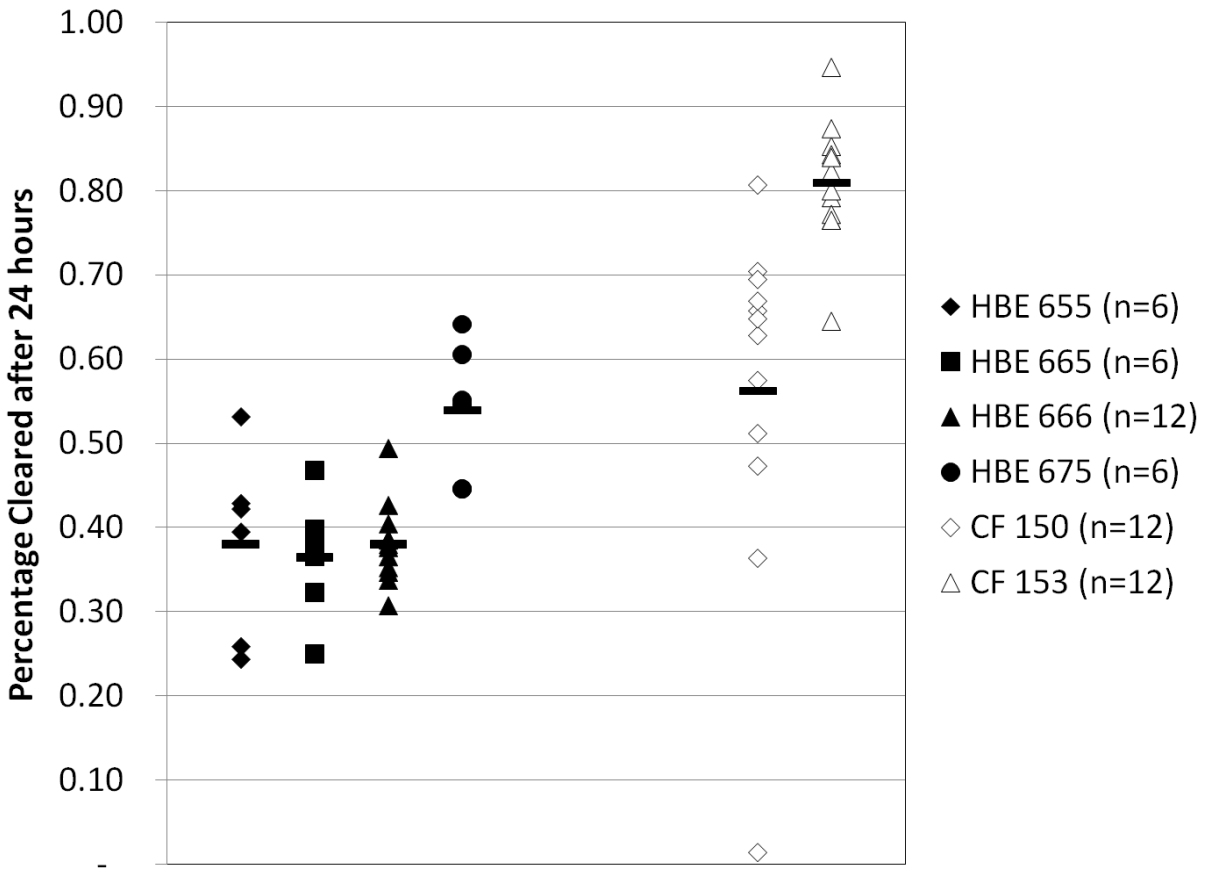

Figure 5 DTPA clearance rates (24 hr) from non-CF (filled) and CF (unfilled) cell lines. HBE 655 is IPF. HBE 675 is immotile cilia, HBE 666 and HBE 675 are emphysema. CF 150 is ΔF508/1152h (class 4 defect; less severe disease) and CF 153 is ΔF508/ΔF508 (class 2 defect; severe disease).

There were significant differences amongst the CF and non-CF lines ($p<0.001$ in both cases). The difference found in the CF cell lines could be related to the difference in mutation. CF 150 is a ΔF508/1152h line. This genotype is a milder mutation than CF 153, which is a ΔF508/ΔF508 line. Comparing the nonCF cell lines with the CF cell lines $p<0.001$.

In summary we observed that CF cell lines cleared the DTPA at a faster rate than the nonCF cell lines.

2.4.1.2 Hypothesis 2

As shown in Figure 6 DTPA absorption rate varied proportionally to osmotic gradients. These gradients were established across the epithelium using different apical and basolateral mannitol concentrations Mannitol added to the apical surface, which would favor liquid transport into the ASL from the epithelium, slows the clearance of DTPA in comparison to the no mannitol control. Whereas mannitol added to the basolateral surface, which would favor liquid absorption, increases the absorption of DTPA as well.

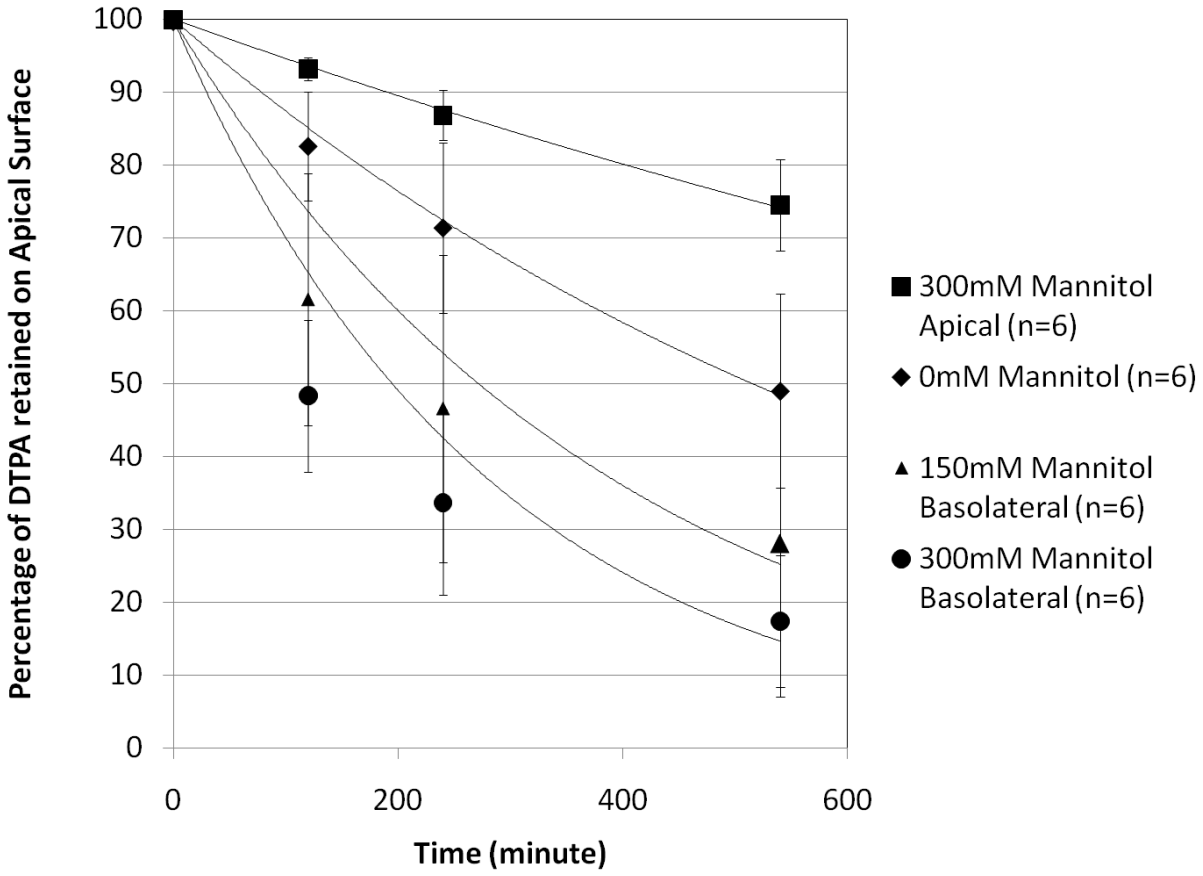

Figure 6 DTPA clearance from apical surface of CF HBE's under the influence of varying osmotic gradients. Retention curves from a single CF cell line are shown (CF 153, n = 12, ΔF508/ΔF508) following an exponential fit.

The mannitol dose to effect relationship was tested with four different cell lines, two nonCF and two CF. Figure 7A considers the average 24hr clearance rate for two nonCF cell lines at the four mannitol concentrations tested.

In both nonCF cell lines a linear mannitol dose to DTPA absorption effect relationship was demonstrated, with R^2 values of 0.76 and 0.86 for HBE 675 and HBE 655 respectively. A similar relationship was seen in the two CF cell line tested (Figure 7b) with R^2 values of 0.907 and 0.951 for CF 153 and CF 150 respectively.

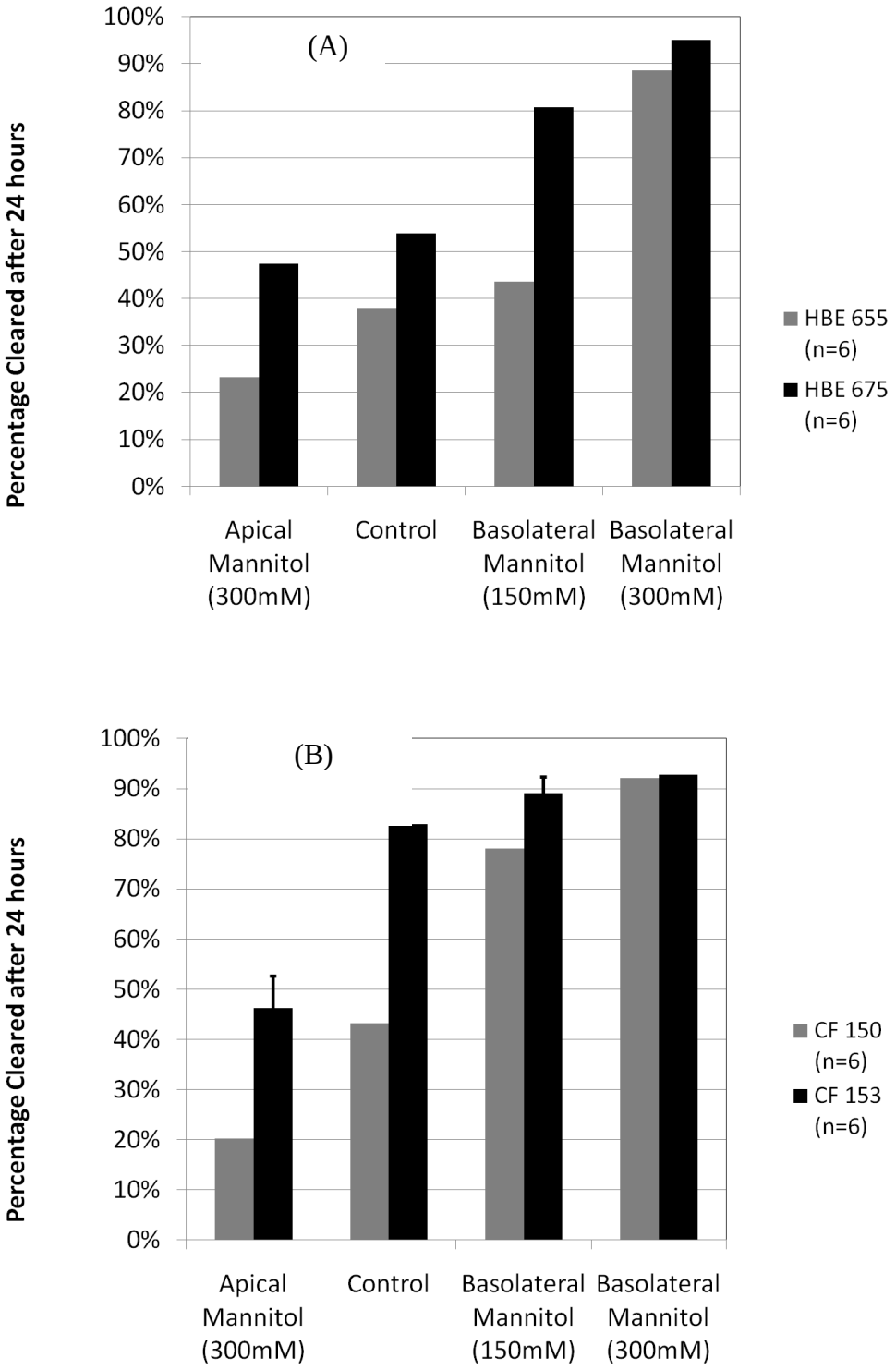

Figure 7 Variation in DTPA absorption rate by osmotic gradient Panel A, includes results from 2 non-CF cell lines. Demonstrating the effect of osmotic on DTPA absorption rate Panel B, includes results from 2 CF lines Figure 5b, on the bottom compares the percent cleared over 24 hours for CF cell lines. HBE 655 is IPF. HBE 675 is immotile cilia. CF 150 is ΔF508/1152h and CF 153 is ΔF508/ΔF508

2.4.1.3 Hypothesis 3

The addition of TNF α (10ng/ml) and INF-γ(100ng/ml) to the basolateral media of the tested cell lines mimicked the effect that pro-inflammatory cytokines would have on the tight junctions in bronchial epithelia. By creating 'inflammed' HBEs we could measure the effect inflammation had on DTPA absorptive clearance. DTPA clearance rate is shown in Figure 8 for cytokine cases and controls. The first set of data expressed DTPA absorptive clearance was significantly increased with the addition of pro-inflammatory cytokines ($p = 0.0104$). The repeated experiment showed similar results with the $p = 0.0102$.

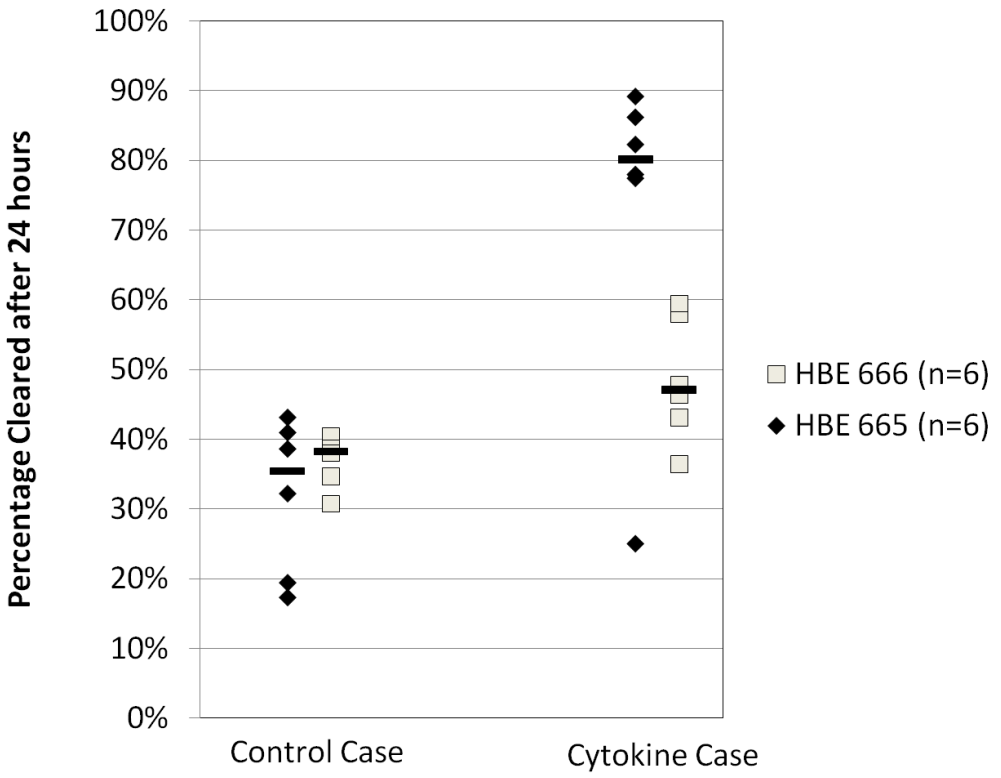

Figure 8 Comparison of DTPA clearance between the control case and the addition of cytokine. Two different sets of HBE filters used on two different test days all from the same cell line.

2.4.1.4 Hypothesis 4

We compared DTPA and liquid absorption in airway cell cultures using a dye based technique. A large fluorescent molecule, Texas Red Dextran (TRD), in a known concentration of 0.25 μg/ ml was added to the apical surface and the control experiment was run. From the measured TRD concentrations a volume retained curve could be plotted. From the retention curves, the percent retained at 24 hours could be calculated and graphed.

To calculate the TRD concentrations from the UV spectrometer data a standard curve was created as shown in **Error! Reference source not found.**. For the experiment each filter was measured only once and the value from the UV spectrometer was recorded. Using the

standard curve the concentrations at each time point was calculated. Based on the concentration the apical volume could be calculated from the starting volume.

Graphing the percentage of apical volume retained at 24 hours against the percentage DTPA absorbed at 24 hours demonstrates a linear relationship between water transport and DTPA transport as shown in Figure 10.

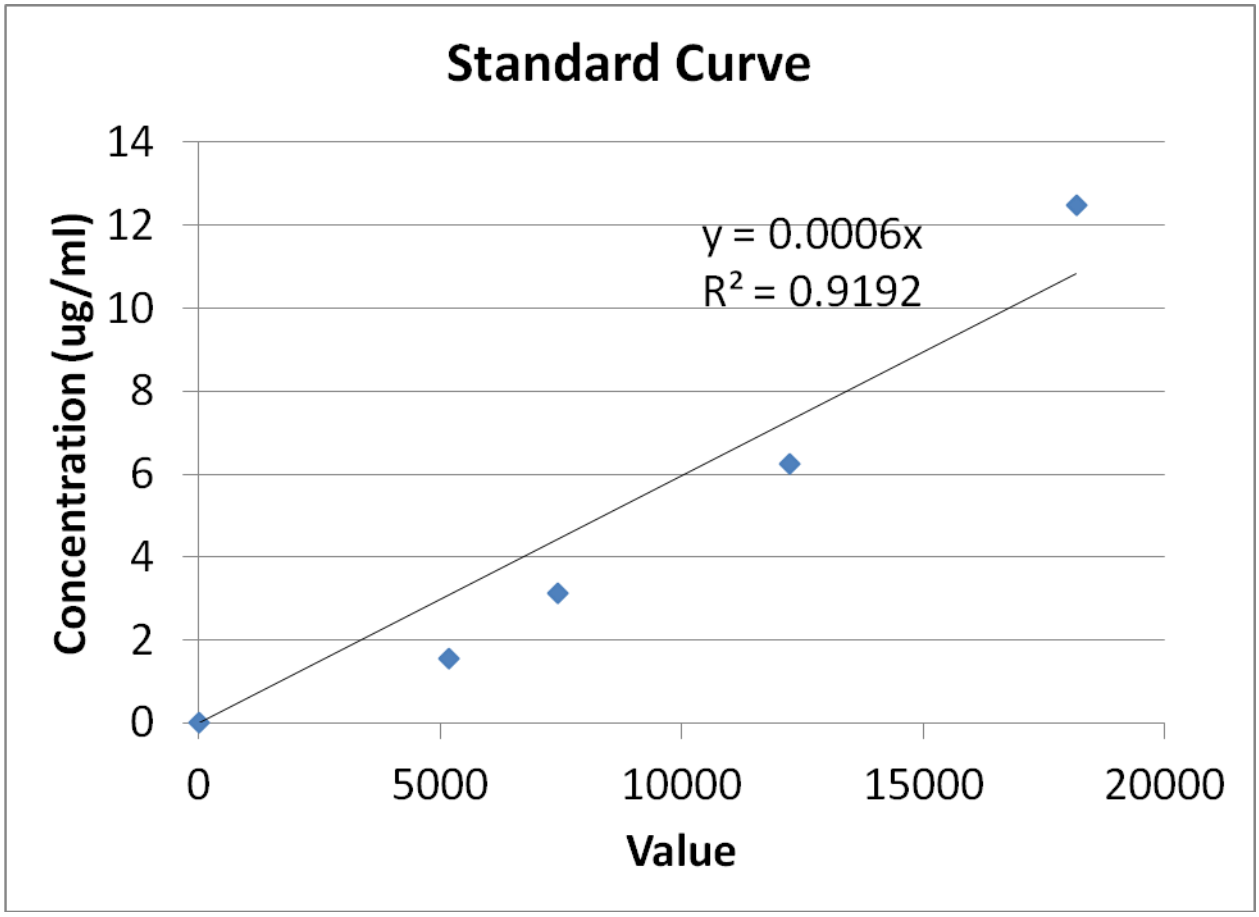

Figure 9 Standard curve used to calculate the concentrations for the TRD experiment.

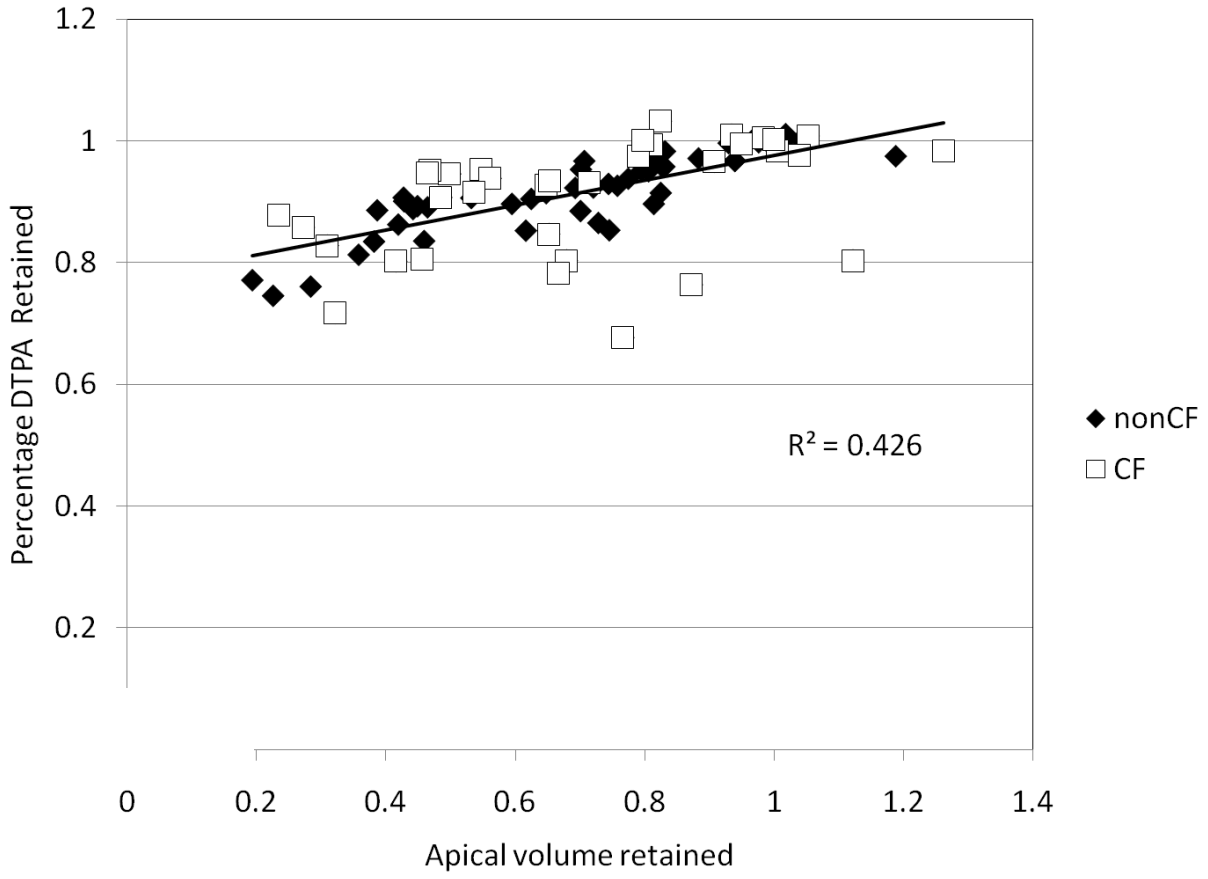

Figure 10 Comparing the percentage of DTPA retained versus the apical volume retained. The dark circles are nonCF HBEs and the open triangles are CF hbes.

2.5 DISCUSSION

These experiments were designed to determine if measuring the DTPA absorptive clearance is a feasible clinical outcome measure. Hypothesis 1 tested if there was a difference in DTPA absorptive clearance in CF and nonCF HBEs when pro inflammatory cytokines were not present. As hypothesized the DTPA absorptive clearance rate in CF HBEs were higher than the

nonCF HBE case. This significant difference in DTPA absorptive clearance rates matches the trends seen in the in vivo trials. These results support the use of DTPA as a clinical outcome measure.

To further understand DTPA clearance feasibility as an outcome measure hypothesis 2 investigated the DTPA transport mechanism by affecting the osmolarity of the ASL. The trends found DTPA absorptive clearance matched the expected fluid flow patterns indicating that solvent drag influenced DTPA absorptive clearance rates. If water transport from the apical surface to the basolateral surface was almost stopped (apical mannitol case hypothesis 2), DTPA absorptive clearance was also stopped. Suggesting a paracellular pathway influenced DTPA clearance.

Using a dye based technique to track the percentage of water absorbed and TC99m to measure the percentage of DTPA absorbed, a linear relationship was discovered between DTPA clearance and water transport (hypothesis 4). As more water moves from the apical surface to basolateral surface, more DTPA is also transported, also showing that DTPA transport is linked to water transport. Suggesting DTPA clearance is influenced by solvent drag. When comparing DTPA clearance in healthy and inflamed HBEs, we saw that inflammation increases DTPA clearance and the healthy HBEs had a baseline clearance (hypothesis 3). From these observations we can conclude DTPA clears via a paracellular pathway and effected by solvent drag.

2.6 SUMMARY

For DTPA absorption to be a viable outcome measure we had to show that DTPA clearance occurs in healthy HBEs along with showing DTPA absorptive clearance is sensitive to detect changes due to a medication. With the results from hypothesis 3, we saw that pro-inflammatory cytokines increases DTPA absorptive clearance, but the healthy HBEs already had a baseline clearance. In the results from hypothesis 2 the DTPA absorptive clearance could detect a change in concentration of Mannitol added to the basolateral surface. Drawing from these results it can be concluded that DTPA absorptive clearance is a viable outcome measure.

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