CFTR Mutation: Evaluation of Uptake Kinetics of Ferret Proximal Tubule Cells and Impact of Potentiators on Renal Excretory Profile

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Abstract

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Pre-clinical testing is an important step in drug development. Generally, in vivo, and in vitro methods are used during pre- clinical stages to test for toxicities and efficacy. In vivo models provide a medium for evaluating pharmacokinetics, pharmacodynamics and margin of safety; however, even with this testing, many drugs may perform well in pre-clinical trials but are discontinued during early phases of clinical trials. This gap is large as many of the drugs developed fall into this category and what may have once looked promising in animal studies and 2D studies do not make it to the patient's bedside. Microphysiological systems may hold the answer to closing this gap. Microphysiological systems offer unique environments that if validated, can serve to reduce the number of animals used in toxicity studies. These models may be able to identify certain drug properties that are not amenable to be used as therapies earlier in the pre-clinical process. While work has been done to create a ferret kidney on a chip, rats and canines are the most used animal species in the drug development phase. Thus, more work should be done to create a model of rat and canine kidney on a chip utilizing proximal tubule cells that are able to effectively identify species specific toxicities as a result of treatment with drug compounds. We hypothesize that MPS systems will allow for rat proximal tubule cells to maintain normal morphology and will effectively model

species -specific nephrotoxicity when exposed to an in vivo relevant toxicant by exhibiting increased kidney injury molecule 1 levels.

Of note, antibiotics such as aminoglycosides, polymyxin B and chemotherapeutics are known to carry the risk of nephrotoxicity. Not only is this nephrotoxicity important in seemingly healthy patients, but also patients with diseases such as Cystic Fibrosis who commonly require treatment of complicated infections with high doses of these antibiotics. While there are many models of cystic fibrosis, the ferret model closely resembles a majority of the pathology that human patients face. With alterations in the CFTR gene and increased cubilin shedding in the urine, we hypothesize that kidneys collected from ferrets with a CFTR mutation will have decreased cubilin/megalin complexes at the apical surface of ferret proximal tubule epithelial cells, while the presence of urinary cubilin will be increased at the initial stage of treatment in human CF patients. Renal uptake of proteins and aminoglycosides will be altered due to this phenotype. While some data has been collected in regard to rat proximal tubule cells in 2D culture and MPS as well as evidence of differential uptake of the protein albumin and gentamicin, an amino glycoside, more data is needed to develop a conclusion in both arenas.

Table of Contents

Chapter 1: Animal Proxies- Microphysiological Systems…………………………………… pg 1-15

INTRODUCTION……………………………………………………………………… pg 2-3

HYPOTHESIS…………………………………………………………………….. pg 4

MATERIALS AND METHODS……………………………………………………….. pg 5-6

RESULTS……………………………………………………………………………. pg 7-8

FIGURES……………………………………………………………………………. pg 9-14

DISCUSSION……………………………………………………………………….. pg 15

Chapter 2: CFTR Animal Models ……………………………………………………… pg 16-28

INTRODUCTION……………………………………………………………………… pg17-18

HYPOTHESIS…………………………………………………………………….. pg19

MATERIALS AND METHODS……………………………………………………….. pg 20-22

RESULTS……………………………………………………………………………. pg 23-24

FIGURES……………………………………………………………………………. pg 25-26

DISCUSSION………………………………………………………………………….pg 27-28

Chapter 3: Dublin TRIKAFTA Study- Urinary Excretory Profiles ………………………….. pg 29-42

INTRODUCTION……………………………………………………………………… pg 30-32

MATERIALS AND METHODS……………………………………………………….. pg 33-37

RESULTS……………………………………………………………………………. pg 38

FIGURES……………………………………………………………………………. pg 39-40

DISCUSSION……………………………………………………………………….pg 41-42

References ……………………………………………………………………………… pg 43-46

Chapter 1: Animal Proxies-Microphysiological Systems

INTRODUCTION

Drug induced nephrotoxicity accounts for 25% of reported serious side effects after drug administration in humans. The mechanisms in which this nephrotoxicity occurs is poorly understood (Dzidic- Krrivic et al. 2024). Typically, toxicities are evaluated in preclinical trials during drug development, as “efficacy, toxicity, and dosing requirements are important factors for the success of these agents.” (Atkins et al. 2020) Specific classes of antibiotics, anti- inflammatory, chemotherapeutics, and antiviral are implicated in acute kidney injury. A large number of resources and time are utilized in drug development and discovery. Despite the number of resources used, it is estimated that “9 out of 10 new drugs fail in Phase I, II, and III clinical trials.” (Mahalmani et al. 2022) In vivo and in vitro models are currently used when conducting pre-clinical studies. Based on a retrospective study, the most common species used in preclinical toxicity studies are rats followed by dogs, then monkeys and lastly, mice; however, the predictive value of these studies when translated to humans is poor (Atkins et al. 2020). This translational gap is known as the “Valley of Death.” (Mahalmani et al. 2023)

Polymyxin B and Cisplatin are known nephrotoxic agents in multiple species. Polymyxin is a drug that undergoes renal elimination and is a polypeptide antibiotic used to treat multidrug resistant infections. Its safety margin overlaps with the concentration needed for the drug to be bactericidal. Cisplatin is a chemotherapeutic in which nephrotoxicity is its dose limiting factor. This drug is transported into renal epithelial cells and injures nuclear and mitochondrial DNA. (Miller et al. 2010) GSK 23240 is a compound that was in development to treat endocrine diseases associated with aging. GSK has been found to exhibit species specific nephrotoxicity as it was found to be non-nephrotoxic in rats but caused tubular necrosis in canines.

Many scientific advances have been employed to try to help bridge the gap of benchtop to bed side. One of these technologies is organ on a chip technology. Organ on a chip models utilize microfluidics to help bolster invitro work.

This technology allows for cells to be cultured in 3D and allow for a microscale environment with improved physiological relevance. The drawbacks of this

technology currently include lack of validation, low – throughput, and specialization or lack of integrating various regions of the kidney. Despite lack of validation, proximal tubule chips have shown the ability of proximal tubule epithelial cells to maintain morphology, polarity, and primary cilia. In addition, albumin uptake has also been demonstrated (Ashammakhi et al. 2018)

Proximal tubule epithelial cells are often the kidney region of focus when evaluating drug toxicities because their role in drug uptake via resorption and transport make them susceptible to acute kidney injury. Clinically, injury is evaluated using values such as creatinine or other biomarkers, or urine output but these measures are not specific to damage to the proximal tubule. A biomarker that is specific to proximal tubule damage is kidney injury molecule 1 (KIM-1). This molecule is shed from tubule cell membranes after acute damage. (Maas et al. 2019) Ichimura et al. 1997 was able to show that Kim-1 is expressed in mow levels in the adult rat kidney and significantly increases with injury. In addition, the isolated rat KIM-1 is 43.8% identical and 59.1% similar to human isolates. Thus, KIM-1 will be used as a measure of proximal tubule injury.

HYPOTHESIS

1. Microphysiological systems utilizing rat proximal tubule cells will effectively model species - specific nephrotoxicity when exposed to an in vivo relevant toxicant by exhibiting increased kidney injury molecule 1 levels.

AIM1: Optimize KPT-MPS culture protocols and assay parameters for toxicology screening.

AIM2: Characterize species -specific toxicity in KPT-MPS and correlation to in vivo outcomes.

AIM3: Confirm assay performance of pre- seeded kidney chips after shipment to the lab.

MATERIALS AND METHODS

Isolation of rat proximal tubule epithelial cells

Rat PTECs were obtained from Innovative Research as well as the Cruzen Lab at the University of Washington. The rat kidneys were transported under refrigerated conditions in PBS ++ and processed for isolation of proximal tubule epithelial cells within 24 h.

Rat kidneys were decapsulated under aseptic conditions. Cortex was dissected from the medulla using a sterile scalpel.

The cortex tissue was finely minced on a sterile Petri dish on ice using a pair of sterile razor blades and transferred to a 50 mL falcon tube with a Dulbecco's phosphate-buffered saline solution (dPBS++) (Gibco, Waltham, MA) containing 0.75 mg/mL of collagenase Type IV (Gibco, Waltham, MA) and 0.75 mg/mL of dispase (Gibco, Waltham, MA). The solution was placed in a 37°C water bath for 10 min followed by incubation for 40 min at 37°C in shaker at 200-220 rpm.

Next, the fragments were vortexed and after sedimentation, the supernatant was collected into a 50 mL falcon tube containing 10 mL of horse serum (Gibco, Waltham, MA) and spun at 200 g for 6 min. The supernatant was aspirated, and the pellet was resuspended in cell media culture, and filtered in a 100 μm pore size strainer to a new 50 mL falcon tube. The process was repeated to wash the cells and the pellet was resuspended and plated into a T25 cell culture flask. Media was changed 24 h later and every 48 h until confluency. Cells were incubated in humidified 95% air / 5% CO2 at 37°C. Following isolation, PTECs were expanded in tissue culture flasks to passages 1 and 2 before all experiments. (Carnavale 2021)

2D Drug Exposure

The cells isolated were labeled by letters and numbers. Cells isolated from the kidney of wild type rat were named RPT#. The numbers following the initial description represent the number of the donor in chronological order. The cells were plated in a T25 flask in 0.5% FBS Cell Biologics Media. The cells were grown until confluent, and microscopy images were acquired at various time

points after plating. After confluency was achieved, cells were trypsinized with 2 mL of trypsin-ethylenediaminetetraacetic acid (EDTA) 0.05% (Gibco, Waltham, MA), resuspended in cell culture media, plated in 48 well plates and imaged prior to the start of treatment. Treatment for cells cultured in the 48 well plate included 25uM PMB- 100uM PMB, 0.25uM Cisplatin- 10 uM Cisplatin and 250uM GSK to 600 uM GSK. Both vehicle controls and no treatment controls were included. All treatments were administered in triplicate.

3D Rat MPS System

Chips and reservoirs were received from Nortis Bio pre-seeded with rat proximal tubule cells. 5 mL of 0.5% FBS Cell Biologics media was added to each reservoir and changed every 3-4 days until the start of experiment. Experiments were started no earlier than Day 5 after seeding and flow rate of the pneumatic pump was set to a standard rate of 1uL/min. After Day 5, treatment was initiated with 100 uM PMB and effluent was collected at 0-hour, 24 hour, and 48 hour timepoints. Representative images were taken pretreatment and at every timepoint that effluent was collected.

Effluent Biomarker Assay

Samples of 3D MPS effluent were submitted to the University of Washington Functional Genomics and Bioinformatics Core Laboratory and assayed using MILLIPLEX Rat Kidney Toxicity Bead Panel 1 and Panel 2. This Panel is able to use fluorophore dye microbeads to evaluate several injury markers: Clusterin, Cystatin C, KIM-1, NGAL and Osteopontin.

RNA Sequencing

For each channel on the chip, the inlet port was closed and the outlet port was opened. A blunt metal tip was attached to the outlet port and a 1ml syringe was used to inject RLT buffer in the upstream injection port. Fluid was collected in 1.5 mL DNA LoBind tubes and placed on ice. All samples were collected and stored at -80 degrees Celsius. Samples were sent to Novogene USA Inc. for RNA sequencing analysis.

RESULTS

Evaluation of Cell Morphology and Expansion in 2D Culture

As shown in Figure 1, we were able to grow a mixed population of renal cells with preferential attachment of epithelial cells. The morphology displayed is typical of epithelial cells with regions of rosettes forming in a monolayer and attached to the T25 flask. On average, flasks were confluent 5 days post plating. There was significant variability in the successful propagation of the rat proximal tubule cells with altered morphology occurring in some of the samples when grown in 2D culture.

Evaluation of Cell Morphology and Expansion in 3D Culture

Cells growing in 3D culture displayed a a homogenous morphology when compared to cells grown in 2D culture. Distinct transitions in cell morphology could be noted after treatment with the nephrotoxic drug, polymyxin B. The number of tubules of good integrity was low; thus, full assessment of tubular response to treatment could not be evaluated. Integrity of the tubules was compromised by blockage of flow or migration of cells into the surrounding matrix leading to abnormal tubular structure and inability to feed the cells. (Figures 2 and 3)

Luminex Assay

Experiment 1

There is a baseline concentration of biomarkers in proximal tubule cells not exposed to various concentrations of PMB or Cisplatin. There were no significant differences between control groups and groups treated with 50uM PMB and 100 uM PMB as well as 5uM cisplatin. In general, Clusterin and cystatin were found in higher concentration than KIM-1, osteopontin, and NGAL(Figure 4).

Experiment 2

Osteopontin could not be detected in these samples. Overall, there was no significant difference in biomarker concentration when comparing cells exposed to 5uM cisplatin with time

matched controls, exposure to 50uM, 100 uM, and250 uM PMB with time matched controls except for the biomarker clusterin. In this case, the 24 hour time matched control had a significantly higher clusterin concentration than effluent from cell exposure to 50 uM PMB for 24 hours. (Figure 5)

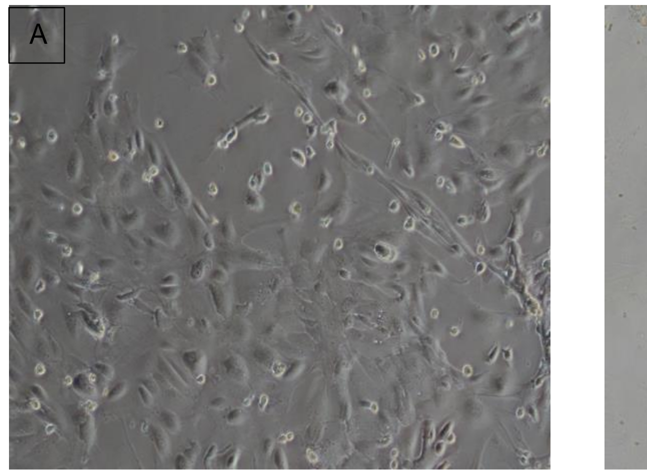
Experiment 3

No significant difference is observed in the concentration of Cystatin C when comparing time matched controls to cells exposed to 100uM PMB for 24 hours and 48 hours. There are no differences in NGAL, Cystantin, osteopontin, and KIM-1 when comparing time matched controls to cells exposed to 50uM PMB and 250uM PMB. The time matched controls have a significantly higher concentration of Clusterin when compared to exposure of cells to 50 uM and 250 uM PMB for 48 hours. NGAL shows a significant increase in concentration when exposed to 100 uM PMB for 24 and 48 hours. (Figure 6)

RNA Seq

Figure 7 shows bioanalyzer results for effluent from MPS RPT chips that were sent to Novogene. A ladder is typically included with each run to serve as an internal standard for each sample. Unfortunately, the bioanalyzer results indicate that no or very minimal RNA was detected. Thus, the samples submitted did not pass quality control to be analyzed.

FIGURES



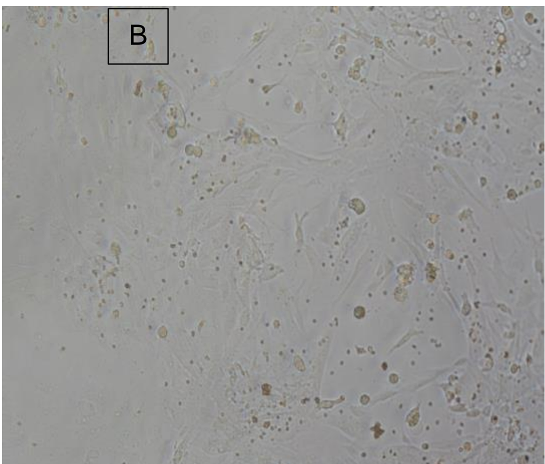


Figure 1: Phase Contrast microscopy at 10x magnification 4 days post plating of Wild type RPT donors at 5 days post isolation. (A) Desired cell morphology for wild type rat proximal tubule epithelial cells. (B) Consistent growth of RPT cells was not achieved and altered morphology could be noted in some of the culture flasks.

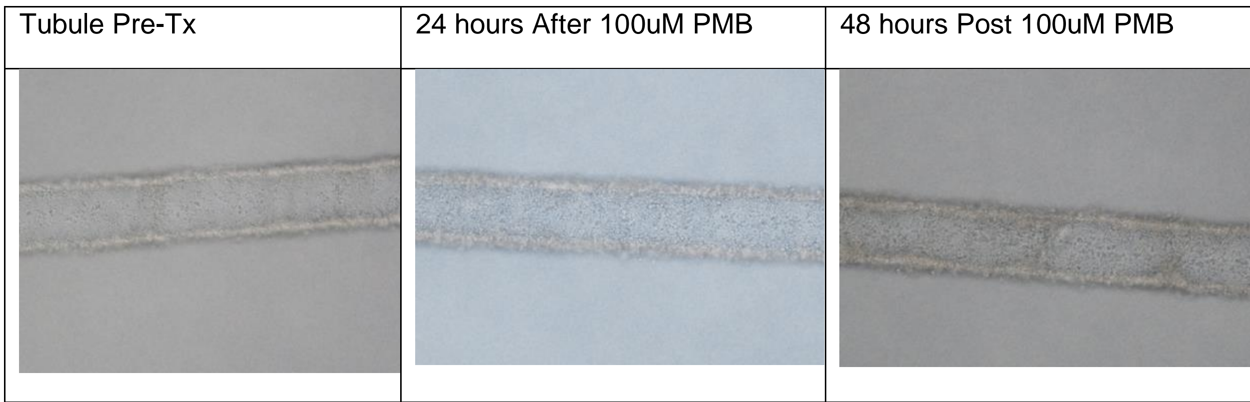


Figure 2: Phase Contrast Microscopy at 10 x magnification of pre-seeded rat proximal tubules in Nortis Chip platform exposed to incubation 100uM PMB for 48 hours. Effluent and images were collected from chip channels the day of treatment, 24 hours after treatment and 48 hours post treatment.

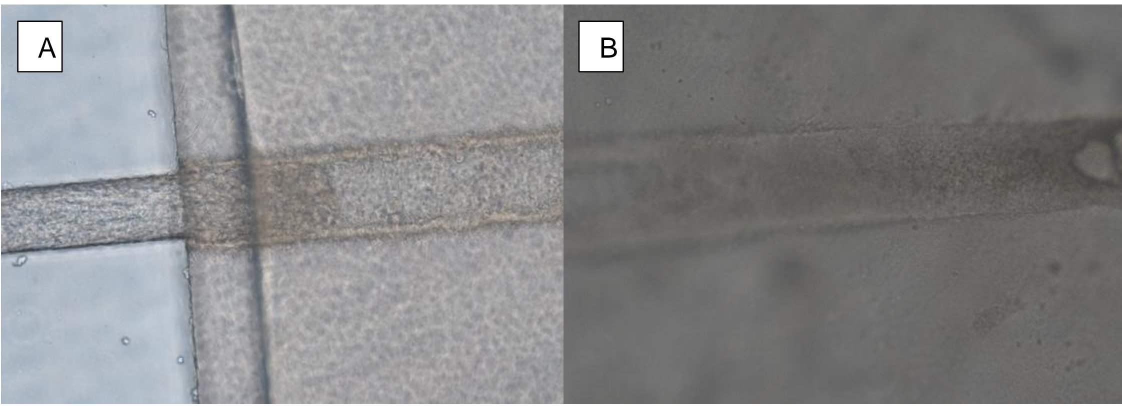


Figure 3: Phase Contrast Microscopy at 10x magnification. Examples of problems encountered in Nortis platform. Pane A shows migration of cells into the extracellular collagen matrix. Panel B shows indication of blockage with sloughing of dead cells toward the center of chip when media flow has been disrupted.

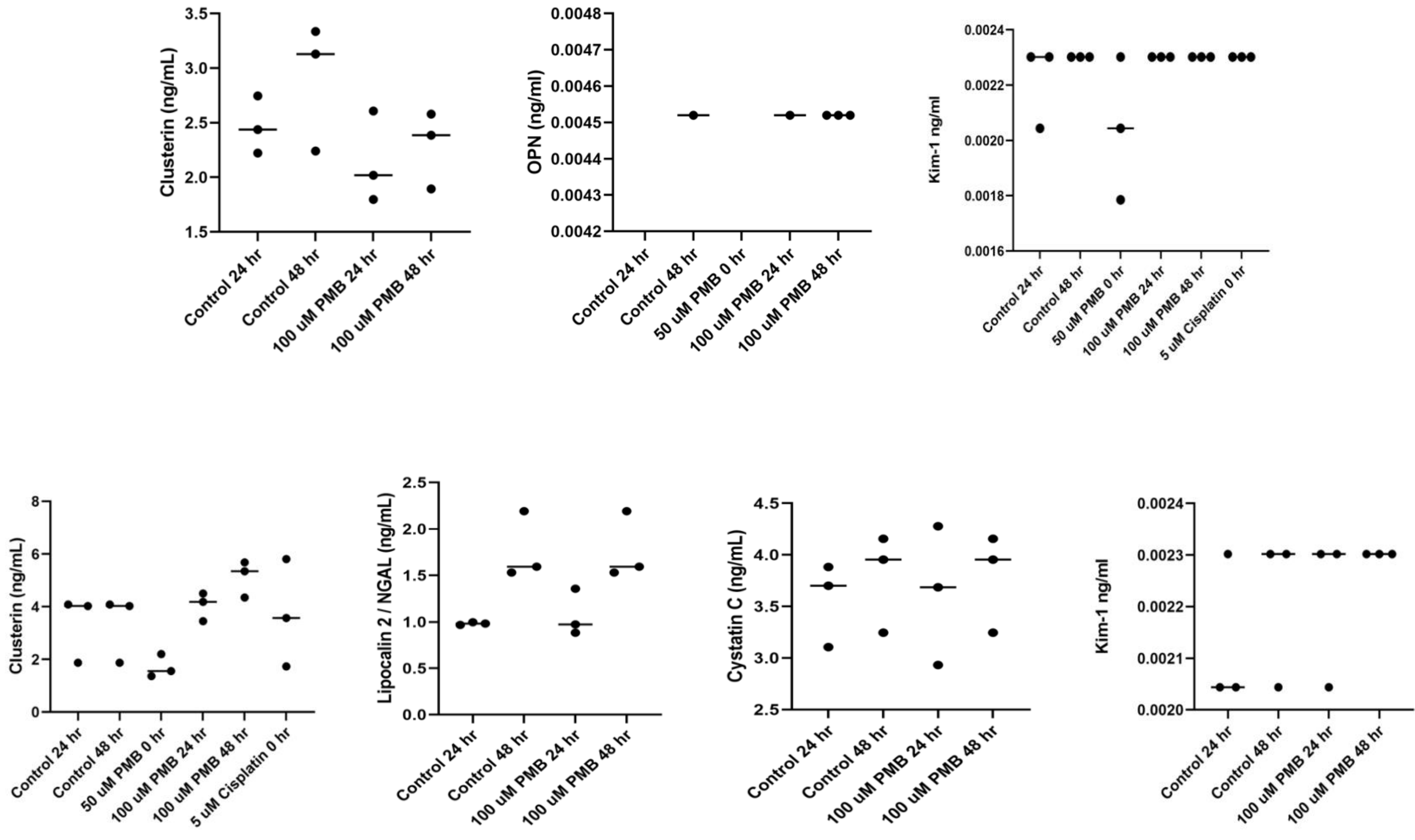
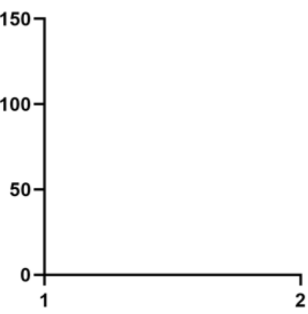


Figure 4: Luminex Assay results. Effluent was collected from rat proximal tubule cells grown in 2D and exposed to various concentrations of PMB and cisplatin at the following time points: 0 hour, 24 hour, 48 hour. Samples were run in triplicate. Biomarkers evaluated were Clusterin, Osteopontin, NGAL, Cystatin C and KIM-1.



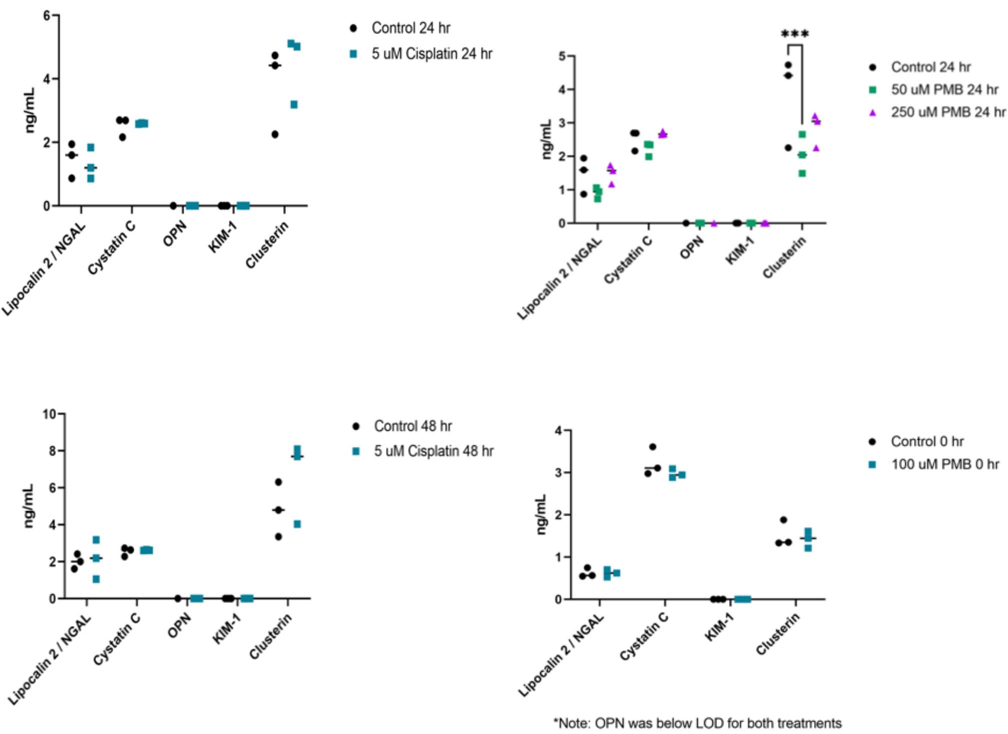


Figure 5: Luminex Assay results. Effluent was collected from rat proximal tubule cells grown in 2D and exposed to various concentrations of PMB and a single concentration of cisplatin at the following time points: 0 hour, 24 hour, 48 hour. Samples were run in triplicate. Biomarkers evaluated were Clusterin, Osteopontin, NGAL, Cystatin C and KIM-1. Because this data was run on a different plate, it cannot be combined with the data in Figure 4.

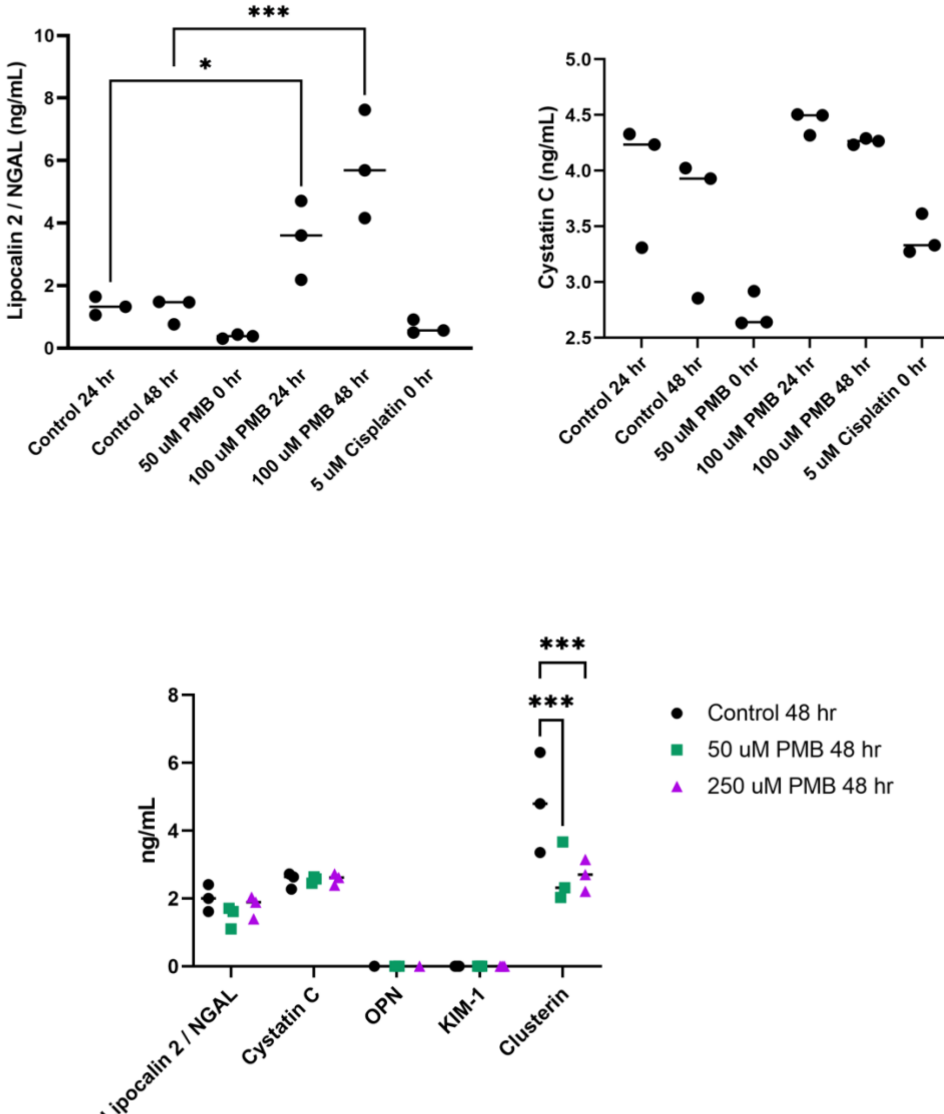


Figure 6: Luminex Assay results. Effluent was collected from rat proximal tubule cells grown in 2D and exposed to various concentrations of PMB and a single concentration of cisplatin at the following time points: 0 hour, 24 hour, 48 hour. Samples were run in triplicate. Biomarkers evaluated were Clusterin, Osteopontin, NGAL, Cystatin C and KIM-1. Because this data was run on a different plate, it cannot be combined with the data in Figure 4 or 5.

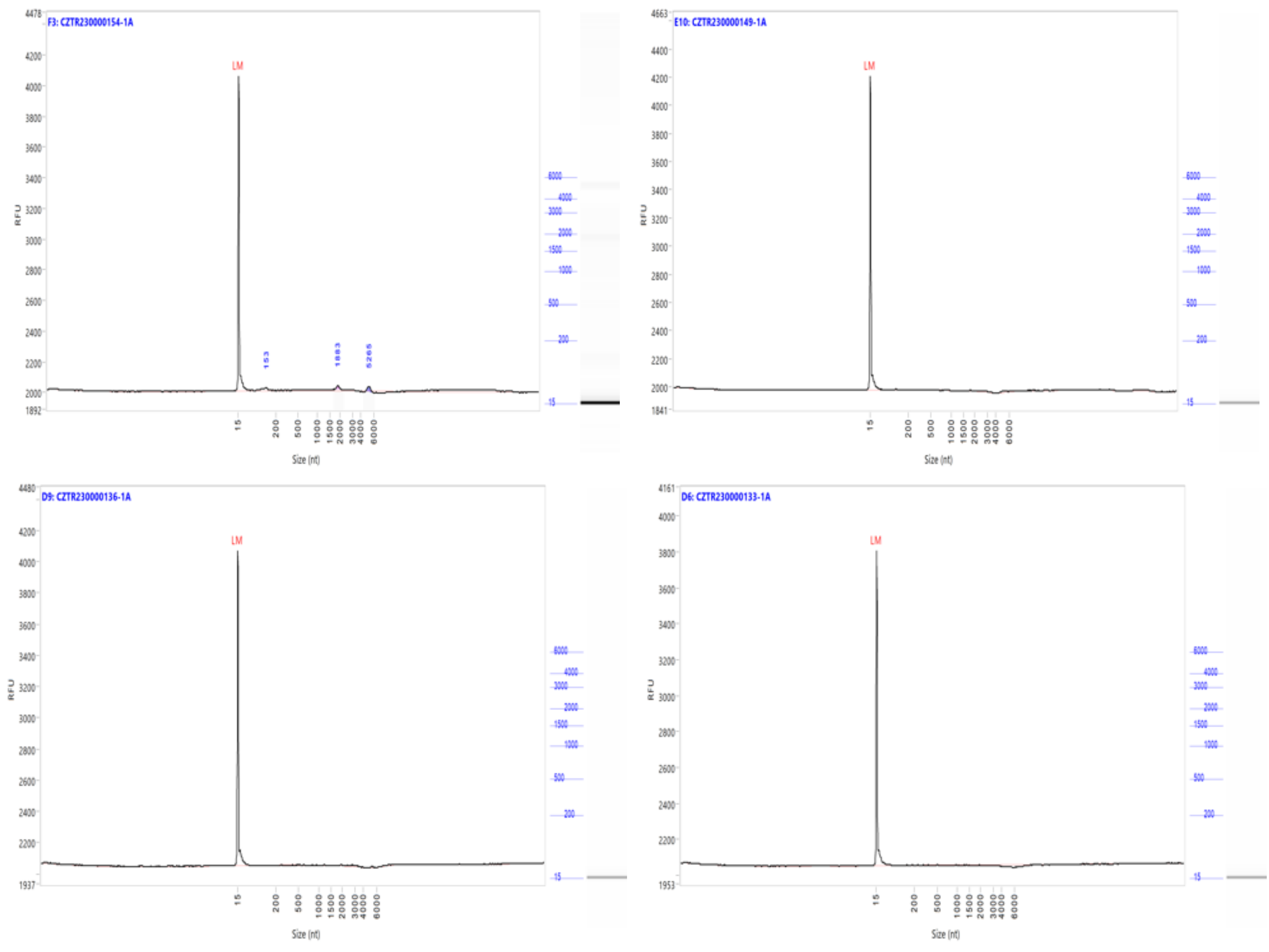


Figure 7: RNA Sequence Data From RPT PTEC Chip Effluent Samples Submitted to Novogene

DISCUSSION

Rat Proximal tubule cells can be successfully isolated to start a primary 2D culture; however, results have been inconsistent. Further study is needed to determine if changes to serum concentration, media makeup or shipping time have any impact on outcomes. Proximal tubules were supported in the Nortis 3D MPS chip. Changes to morphology and the cell level and tubule level indicate that further assessment of the viability of the system is needed. Common problems encountered were clumping of cells at the ends of the tubule or the middle of the tubule which obstructed fluid flow. In addition, expansion outside of the tubule and into the collagen matrix was also seen similar to ferret proximal tubule cells Carnevale 2021.

Effluent from treatments was used to evaluate a urine- like product to determine the state of injury of the proximal tubule cells after known exposure to a nephrotoxic drug. Results were inconsistent and showed no true pattern of injury. Clusterin may have been higher in control cells when compared to treatment groups due to cell death during treatment. Perhaps elucidating this hypothesis with a live/dead or cytotoxicity assay would be helpful in determining if a lower dose is needed. The setup of the Luminex panel limited how samples could be compared, thus it would be helpful to be able to compare 50 uM PMB to 100 uM PMB and biomarker concentrations. In addition, confirmation of cell type may aid us in interpretation of the Luminex panel results. Each sample was run in triplicate; however, more robust testing may produce a clearer picture with regard to cellular response at the biomarker level.

Lastly, RNA seq data proved non qualitative or quantitative when analyzing effluents. Contributing factors may be the abnormality of the tubules in these chips or RNA degradation. Repeat studies need to be performed to tease this out. Luminex data parred with RNA sequencing data would provide a better picture of how the cells respond to insult.

Chapter 2: CFTR Animal Models

INTRODUCTION

Cystic Fibrosis

Cystic fibrosis, a disease first described by Dr. Dorothy Anderson, is the most common autosomal recessive disease affecting the Caucasian population. The incidence of this disease globally is 1 in every 3500 births. Hallmarks of this disease are a result of mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) and generally include pancreatic disease, poor weight gain, gastrointestinal disease, reproductive infertility, and significant lung disease. Lung disease can often be complicated by infection with the following organisms: *Staphylococcus aureus, Pseudomonas aeruginosa, Burkholderia cepacia*, etc. These infections typically require treatment with, but not limited to the aminoglycoside antibiotic class. Respiratory disease is the most severe phenotypic manifestation of the disease and is the most frequent cause of death in cystic fibrosis patients. Since the discovery of the CFTR gene in 1989 paired more recently with a focused effort toward new drug development, the median age of survival has significantly increased by 10- 15 years (Bradburry et al. 2016)

CFTR Mutation

There are many mutations in the CFTR gene responsible for cystic fibrosis. These mutations fall into six general classes and affect the population at various incidences. CFTR protein with no mutation normally moves to the cell surface and allows for the transfer of chloride and water. A Class 1 mutation leads to production of shortened, nonfunctional CFTR and roughly 22 % of cystic fibrosis patients have this mutation. Class 2 mutations result in misfolded proteins that are unable to move to the cell surface. 88 percent of cystic fibrosis patients have this mutation with an example being ∆F508. A class 3 mutation leads to the creation of the protein that is able to move to the apical surface but creates a channel gating defect which depleted ATP binding and downstream hydrolysis of proteins. G551D is an example of a Class 3 mutation and is prevalent in 6% of cystic fibrosis cases. Class 4,5, and 6 mutations occur less frequently and lead to less function, less protein, and less stability, respectively. (Marson et al. 2016, Lopez- Pacheco et al. 2016).

Current Animal Models

The discovery of the CFTR gene paired with the development of CRISPR technology has led to the ability to provide animal models of the disease ranging from zebrafish to rats, mice, pigs, sheep, and ferrets. The amino acid CFTR ortholog of the ferret is 92% identical to that of the human and has shown similar patterns of disease, making it a good model. The ferret model is an induced model utilizing vectors. Although many mutations have been discovered and implemented in these animals, disease study is focused on lung pathology, reproductive dysfunction, intestinal disease and pancreatic disease, a gap still remains in the knowledge of how this disease impacts the kidneys. (Semaniakou et al. 2019)

The Kidney

The kidney is comprised of an organization of functional units called the nephron which consists of proximal tubules, loops of Henle, distal tubules and collecting ducts. CFTR is abundantly expressed in the kidney and is found most prominently in the renal cortex and outer medulla. Based on studies in mice, CFTR is found primarily on the apical surface of proximal tubular cells. CFTR can also be found in intracellular organelles in endocytic pathways. The uptake of aminoglycosides and low molecular weight protein occurs via endocytosis. This endocytosis requires receptors, namely cubilin and megalin.(Souza-Menezes 2009) Cubilin and megalin are large, multiligand glycoproteins and are expressed in epithelial cells of various tissues. (Christensen et al. 2002) The function of these specific glycol proteins has been confirmed using CFTR KO mice. Studies in these mice show excretion of Clara cell secretory protein 16. In addition, there was a significant decrease in renal uptake of radiolabeled macroglobulin and aminoglycosides in KN mice when compared to WT. Lastly, KO mice exhibited a significantly higher excretion of cubilin in the urine; however, no changes to excretion of megalin were observed. (Souza-Menezes et. al 2009)

HYPOTHESIS

We hypothesize that kidneys with a CFTR mutation will have decreased cubilin/megalin complexes at the apical surface of ferret proximal tubule epithelial cells, while the presence of urinary cubilin will be increased at the initial stage of treatment in human CF patients. Renal uptake of proteins and aminoglycosides will be altered due to this phenotype.

AIM 1: Evaluate the cellular uptake kinetics of aminoglycoside dosing in 2D culture of PT cells affected by changes in the CFTR gene.

AIM 2: Determine the relative phenotype of kidneys affected with a mutation in the CFTR gene.

AIM 3: Determine the comparative effectiveness of treatment of human CF patients with CFTR potentiators on the urinary excretory profile.

MATERIALS AND METHODS

Isolation of ferret proximal tubule epithelial cells

Ferret PTECs were obtained from ferret kidney necropsies in the laboratory of Dr. John Engelhardt (University of Iowa), who developed the original CFTR gene edited ferret model and, more recently, has used CRISPR/Cas-9 gene editing to generate a ferret harboring a G551D and ΔF508 CFTR mutations.(32) The ferret kidneys were transported under refrigerated conditions in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Waltham, MA) containing 1% of penicillin-streptomycin (Gibco, Waltham, MA) and processed for isolation of proximal tubule epithelial cells within 24 h.

Ferret kidneys were decapsulated under aseptic conditions. Cortex was dissected from the medulla using a sterile scalpel. fPTECs were isolated accordingly to previously described procedures (30) with some modification.

The cortex tissue was finely minced on a sterile Petri dish on ice using a pair of sterile razor blades and transferred to a 50 mL falcon tube with a Dulbecco's phosphate-buffered saline solution (dPBS++) (Gibco, Waltham, MA) containing 0.75 mg/mL of collagenase Type IV (Gibco, Waltham, MA) and 0.75 mg/mL of dispase (Gibco, Waltham, MA). The solution was placed in a 37°C water bath for 10 min followed by incubation for 40 min at 37°C in shaker at 200-220 rpm.

Next, the fragments were vortexed and after sedimentation, the supernatant was collected into a 50 mL falcon tube containing 10 mL of horse serum (Gibco, Waltham, MA) and spun at 200 g for 6 min. The supernatant was aspirated, and the pellet was resuspended in cell media culture, and filtered in a 100 μm pore size strainer to a new 50 mL falcon tube. The process was repeated to wash the cells and the pellet was resuspended and plated into a T75 cell culture flask. Approximately 3 cm3 of tissue was enough to plate a T75 flask. Media was changed 24 h later and every 48 h until confluency. (30) Cells were incubated in humidified 95% air / 5% CO_2 at 37°C.

Following isolation, fPTECs were expanded in tissue culture flasks to passages 1 and 2 before all experiments. (Carnavale 2021)

2D PTEC Culture

The cells isolated were labeled by letters and numbers. Cells isolated from the kidney of wild type ferret were referred as WT or KO/WT, those isolated from a G551D received the nomenclature of (G551D)# and cells from a ΔF508 ferret were named as ΔF508#. The numbers following the initial description represent the ID system utilized by the University of Iowa. The cells were plated in a T25 flask in DMEM/F12 (Gibco, Waltham MA) cell culture media supplemented with 1% of penicillin-streptomycin (Gibco, Waltham, MA), 1% ITS (Sigma-Aldrich, St. Louis, MO) and 0.1% of hydrocortisone (Sigma-Aldrich, St. Louis, MO). The cells were grown until confluent, and microscopy images were acquired at various time points after plating. After confluency was achieved, cells were trypsinized with 2 mL of trypsin-ethylenediaminetetraacetic acid (EDTA) 0.05% (Gibco, Waltham, MA), resuspended in cell culture media, plated in new T25 flasks or 8 well microscope slides and imaged prior to the start of treatment.

Gentamicin and Albumin Dose Finding Uptake Study

The albumin and gentamicin uptake were performed in 2D with the fPTEC WT, ΔF508/WT. The cells were plated in an 8 well microscope slide with a clear bottom grown until mostly confluent in DMEM/F12 with 10 mg/dL of glucose (Gibco, Waltham MA) with 1% of penicillin-streptomycin (Gibco, Waltham, MA), 1% ITS (Sigma-Aldrich, St. Louis, MO) and 0.1% of hydrocortisone (Sigma-Aldrich, St. Louis, MO) and 0.05% FBS. After wells were at least 50% confluent, cells were incubated for 1 hour, 4hours, or 24 hours with either 40 µg/mL albumin-FTIC (Abcam, Cambridge, MA) or 0.2 ug/ml, 1ug/ml, or 5ug/ml of Gentamicin- Texas Red conjugate (AATBIO, Sunnyvale, CA) or a combination of albumin and gentamicin at the aforementioned concentrations. After incubation, the cells were washed with warm dPBS ++ and 4% formaldehyde fixed and imaged via microscopy.

Gentamicin and Albumin Uptake Study

The albumin and gentamicin uptake were performed in 2D with the fPTEC WT, ΔF508/WT, ΔF508, G551D, and G551D/WT. The cells were plated in an 8 well microscope slide with a clear bottom grown until mostly confluent in DMEM/F12 with 10 mg/dL of glucose (Gibco, Waltham MA) with 1% of penicillin-streptomycin (Gibco, Waltham, MA), 1% ITS (Sigma-Aldrich, St. Louis, MO) and 0.1% of hydrocortisone (Sigma-Aldrich, St. Louis, MO) and 0.05% FBS. After wells were at least 50% confluent, cells in 7/16 wells were incubated for 30 minutes with 1 uM recombinant human receptor associated protein (RAP) which is an endocytic inhibitor (US Biological, Salem, MA). (Chapron et al. 2018) After incubation with RAP was complete, all wells were incubated for 1 hour with either 40ug/ml of albumin-FITC or 1ug/mL of gentamicin- texas red, or a combination of gentamicin and albumin. After incubation, the cells were washed with warm dPBS++ and fixed with 4% formaldehyde and imaged via microscopy.

Cell Fixation

The cells were washed with warn DPBS++. A 4% formaldehyde solution was then added to PBS for 10-15 minutes at room temperature. After 10-15 minutes, the cells were washed three times with DPBS ++ and either imaged or stored at 4 degrees Celsius and protected from light.

RESULTS

Morphology

Df508 homozygous and G551D homozygous CFTR ferret cells show a distinct changes in morphology when grown in low glucose DMEM media. DF508 cells and GK cells both displayed more elongated fibrotic morphology and took longer, overall, to become confluent. DF508 and G551D heterozygous cells maintained wildtype morphology and followed WT confluency timelines. On average, DF/DF and GK/GK flasks did not become confluent even one week after being plated whether from primary isolation or from previously isolated cells thawed from cryofreezing as seen in Figure 2.1.

Uptake of Protein and Aminoglycosides Characterized by Donor Genotype

After running preliminary uptake study utilizing FITC labeled albumin and Texas Red labeled gentamicin (images not shown), ideal dose and exposure time were determined and used for this experiment. The purpose of this uptake study was to determine variations in uptake due to the lack of cubulin/megalin receptors. Strongest signals in uptake of albumin and Gentamicin occurred in wild type cells in which albumin was found to have both intranuclear and cytoplasmic distribution. Pre incubation of cells with RAP did not affect uptake of albumin in wild type cells while evaluation of its effect on gentamicin uptake could not be determined. Furthermore, uptake of albumin was not impacted when G551D cells and dF508 cells were pre incubated with albumin. Gentamicin uptake in G551D cells does not appear to be altered when preincubated with RAP, while gentamicin uptake in dF508 cells may be slightly decreased when preincubated with RAP.(Figure 2.1)

dF508 cells show general decreased uptake of albumin in cells while maintaining nuclear and cytoplasmic distribution, but are more tightly nuclear bound. Gentamicin also has less uptake and when uptake is present, is peri nuclear. When albumin and gentamicin are incubated together, there is generally less gentamicin signal appreciated. G551D cells follow the same general pattern of uptake as dF508; however, more gentamicin and albumin nuclear uptake is noted relative to that in dF508. Pre incubation with RAP in G551D cells still leads to cytoplasmic and well defined nuclear

albumin uptake and minimal gentamicin uptake; however, when gentamicin and albumin are incubated together, staining is greater for albumin in all cell types. (Figure 2.2)

FIGURES

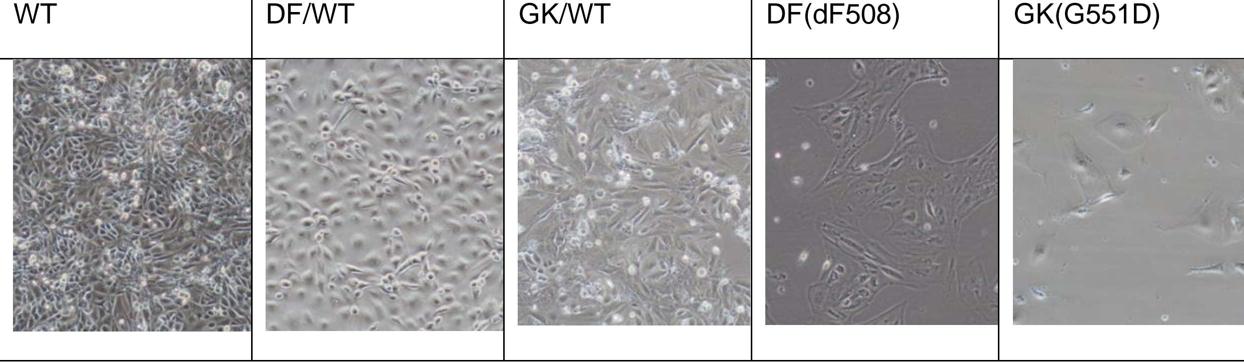


Figure 2.1: Phase Contrast Microscopy at 10x magnification. Primary isolates of renal proximal tubule cells from ferret donors of the following genotypes of interest: WT, GK, dF508.

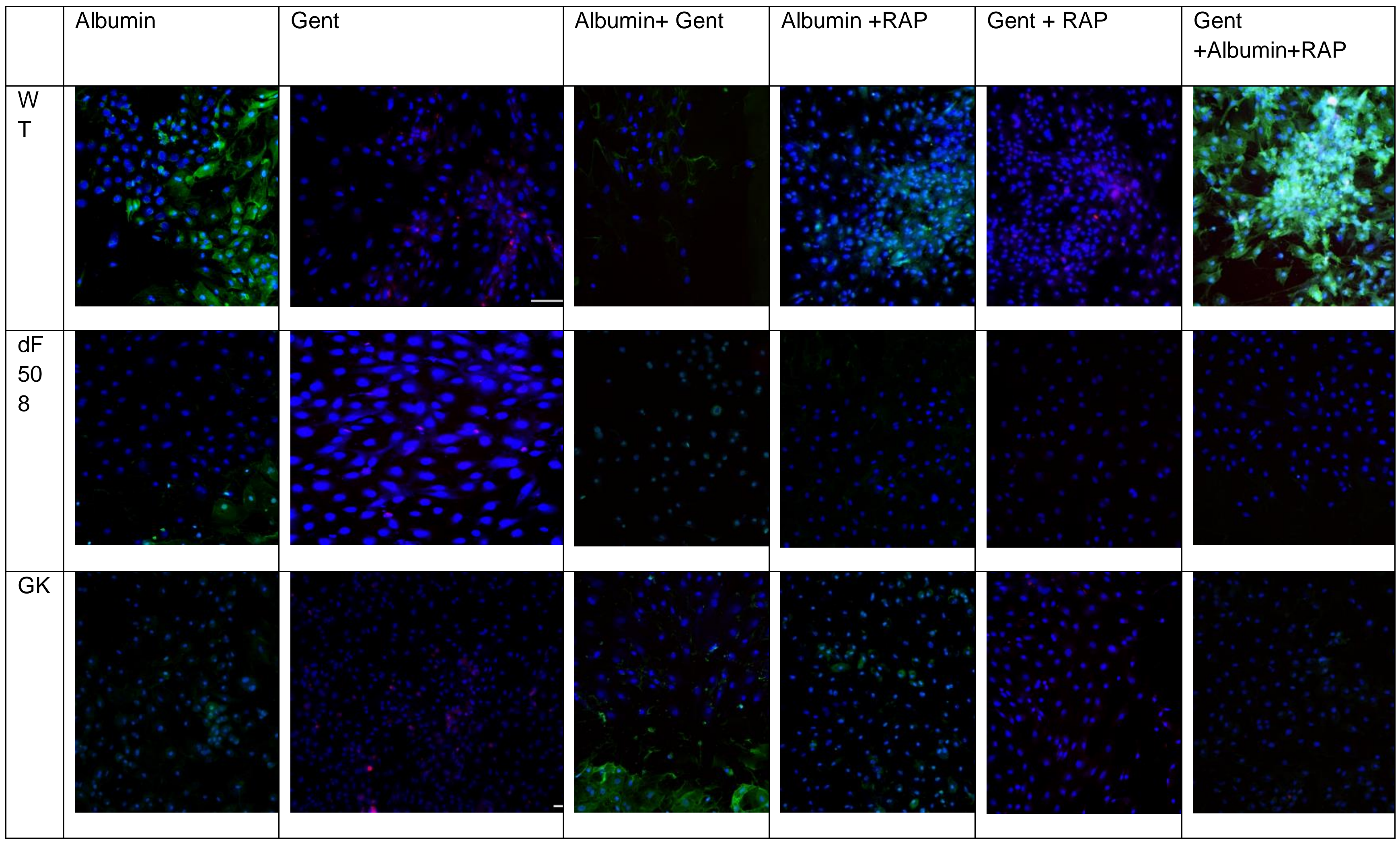


Figure 2.2: Confocal Microscopy at 10x magnification. Immunofluorescent tagged albumin (FITC) and gentamicin (Texas Red) were incubated with cells of each genotype of interest and imaged to visualize uptake. DAPI staining was added to identify the nucleus of each cell.

DISCUSSION

Gentamicin and albumin uptake are both mediated by cubilin and megalin on the apical surface of the proximal tubule. This uptake is what leads to accumulation of these products and kidney injury/ toxicity. Although there are many variables that can contribute to the development of kidney injury, kidney disease has been a growing concern amongst the population at large. With the increase in lifespan of patients affected with cystic fibrosis due to intervention with drugs called potentiators, new questions will arise regarding current dosing practices for these patients. Typically, cystic fibrosis patients will require a higher dose of antibiotics or other drugs with renal reabsorption due to the CFTR mutation. With new drug interventions, the question becomes what dosages clinicians should be using as the lethal dose limit may decrease. Further work will need to be done looking at cells in 2D culture as well as evaluating more urine samples of patients started on these potentiators such as Ivacaftor.

Confirmation of cell type should be completed by staining for specific membrane receptors. In addition, immunohistochemical staining for presence of cubilin/ megalin receptors is needed to confirm the relative abundance in WT ferrets when compared to affected CFTR ferrets. When paired with uptake image, a more complete picture may be formed with regard to the effect of receptor loss or function as a function of genotype on uptake properties and may serve as a predictor for the potential development of drug associated nephrotoxicity in cystic fibrosis patients.

When incubating gentamicin and albumin together, the signal associated with gentamicin uptake is decreased. This may indicate preferential binding of albumin to cubilin / megalin receptors for uptake in comparison to gentamicin. Timed studies as well as order of addition of drug solutions may help elucidate this phenomenon. Furthermore, optimization of cell culture media and techniques to support consistent growth and improved morphology of various cell genotypes.

Lastly, future directions include a proof-of-concept study in regard to genotype and the effect of pre incubation with RAP. Although dF508 and G551D cells performed as expected, the wild type cells did not follow the same paradigm. Because RAP is a molecular chaperon it will bind to

receptors such as the cubulin/megalin complexes on the surface of the cells (Lee et al. 2007).RAP serves as a megalin inhibitor. In cells that have a CFTR mutation these complexes should be decreased and or/ absent. Thus, RAP would have limited action in these cells with limited effect on uptake characteristics but should minimize uptake in WT cells that have functioning cubulin/megalin complexes. Relative abundance should be established first using IHC. After establishing relative abundance, optimization of concentration and incubation time with RAP should be optimized for ferret kidney cells.

Even with the investigation of the uptake characteristics and IHC determining abundance of cubulin/megalin complexes in CF ferrets, a major question remains: how many functional complexes are needed to influence uptake properties in patients as this may influence dosing regimens for known nephrotoxic drugs as new CFTR modulators are being investigated in clinical trials as they aim for FDA approval for use in human cystic fibrosis patients.

Chapter 3: Dublin TRIKAFTA Study-Urinary Excretory Profiles

INTRODUCTION

Cystic fibrosis (CF) was first described by Dorothy Anderson in the 1930s. CF was initially described as a disease of the pancreas. Since its initial description and dedicated study toward the disease, median survival times have drastically increased upwards of 30 years. Key factors in this increase include two critical time points paired with improved treatment regimens for secondary disease processes: the discovery of the CFTR gene and the effective production of new, more targeted pharmaceuticals. The first of these targeted pharmaceuticals was Ivacaftor.

In terms of targeted therapies, two main methodologies have been followed: developing a drug that targets the genetic abnormality or developing a drug that corrects the protein abnormality. The success of these therapies has been bolstered by the sequencing of the human genome which has led to a more individualized treatment of CF patients as more than 1900 mutations have been identified in CFTR. These mutations have been categorized as 6 primary classes and corresponds to the mechanisms of disease. Mechanisms and prevalence are provided in Table 3.1.

CF patients can have multiple mutations spanning multiple classes. Subsequently identifying a single therapy that is successful in restoring CFTR function can be challenging. Ivacaftor is considered a CFTR potentiator and was developed by Vertex Pharmaceuticals. Also known as VX-770, Ivacaftor is used in patients with at least one G551D mutation and is approved for use by the U.S. Food and Drug Administration. G551D falls into the Category of Class III mechanisms which is a comparatively small subset of patients with CF. This potentiator improves the gating of CFTR channels on the cell surface. Clinical trials demonstrated thst Ivacaftor reduced sweat chloride levels but did not result in improved lung function. Thus, evaluation of combination treatments was necessary. A combination of Ivacaftor and Lumacaftor is also FDA approved for patients with dF508 mutation. This drug combination has seen some setbacks as decreased efficacy has been observed and may be explained by the use of a potentiator and corrector in direct combination. (Bradburry 2016)

Table 3.1. General classification of CFTR mutations and associated protein defects and prevalence amon cystic fibrosis patients. Adapted from Guilbault et al. 2007.

Mutation Class	Protein Defect	Prevalence
I	production	22%
II	trafficking	88%
III	Channel gating	6%
IV	Channel conductance	6%
V	Low mRNA	5%
VI	Decreased stability	5%

In 2019, a new drug combination was granted FDA approval. Named TRIKAFTA, this drug combines the pharmaceutical properties of three individual drugs: elexacaftor, tezacaftor and ivacaftor. This is only the third FDA approved drug and is a targeted approach to individuals with a dF508 mutation, which has a historically high prevalence within the CF populations. Success of this drug combination is also measured by changes in lung function and decreased chloride levels in sweat paired with a better quality of life. Tezacaftor is the most recent modulator and enhances CFTR processing and trafficking; chloride transport is also improved. Elexacaftor is unlike other previously discussed correctors. Typically, patients resistant to other modulators or have a genetic profile the leads to lack of protein production are prescribed this medication. Ultimately, this drug, when used in combination with other potentiators (TRIKAFTA) , leads to improvement of comorbidities, lung function, nutrition and quality of life. This total body improvement is likely because TRIKAFTA targets all epithelial cells that line tubular organs. (Zaher et al. 2021)

11 CF mouse models have been characterized to date. In evaluating phenotypic changes of these mice in relation to changes in the expression of receptors in the kidney and impact on the excretory profile of the urine. For example, CFTR knockout mice have shown significant increases in cubulin excreted in the urine while no changes were observed in the presence of megalin. It has been hypothesized that instability of cubulin and its low molecular weight ligands at the brush border leads to increased shedding into the urine. (Jouret 2009)This proteinuria is observed in human CF patients as well.

MATERIALS AND METHODS

Inclusion criteria for participants: 18 years or older. Medical documentation of a cystic fibrosis diagnosis with the F508del CFTR mutation on at least 1 allele. Prior chronic Pa or Sa in the sputum as defined by > 50% of prior sputum cultures with each organism in the year prior to enrollment. Be able to expectorate sputum. Be clinically stable at the time of initiation of elexacaftor / tezacaftor / ivacaftor. Written informed consent obtained from subject or subject's legal representative. Ability to communicate with the Investigator and comply with the requirements of the protocol. If female – a negative pregnancy test on day one before treatment is administered. Willing to use adequate contraception for the duration of the study and for 1 month following the study; acceptable forms of contraception: hormonal birth control, intrauterine device, barrier method plus a spermicidal agent or abstinence from Visit 2 through Visit 7 unless surgically sterilized or postmenopausal. Patients will be taking inhaled tobramycin therapy month-on/month-off.

Exclusion criteria for participants: Use of elexacaftor / tezacaftor / ivacaftor within 6 months prior to Visit 1. Any upper or lower respiratory symptoms requiring treatment with any form of antibiotics within the 2 weeks prior to Visit 1. History of solid organ transplantation. Presence of a condition or abnormality that in the opinion of the investigator would compromise the safety of the patient or the quality of the data. History of massive hemoptysis (>240 mL) within 72 hours of Visit 3. Inability to produce sputum. Females who have a positive pregnancy test at Visit 2, and or are lactating. Not practicing (or willing to practice) a medically acceptable form of contraception.

Drugs administered: Each morning the participants took elexacaftor 100 mg, tezacaftor 50 mg, and ivacaftor 75mg per dose; In the afternoon they took ivacaftor at 75mg per dose.

Collection of urine: Urine was collected from the participants at time points: day 0 (before treatment), 2 days, 7 days, 365 days.

Urine processing for storage: Place the 50 mL conical tubes filled with urine in the centrifuge bucket and seal the lid. Centrifuge at 1500g x 10 minutes at room temperature. Wait for an extra two minutes after centrifuge spin completes. Transfer tube (maintaining bucket seal) to the BSC, and then aliquot urine into tubes sequentially. Swirl the left-over urine and urine cell pellet. Pipette 1 mL of urine with cell pellet into one 4.5 ml cryovial prefilled with 2mL of Zymo Research's DNA/RNA Shield™ (Zymo). Swirl a final time to ensure the mixture is homogenized and then freeze the vials at -80°C.

Protein extraction and digestion: A one mL urine sample was processed through an Amicon Ultra-0.5 Centrifugal Filter 100 kDa MWCO (EMD Millipore, Burlington, MA), following manufacturer's recommended protocols. The protein retained in the filter (> 100 kD) was eluted and resuspended using 50 µL of 100 mM ABC. The total protein was estimated using the BCA method and the concentration normalized to 1 mg/mL or less. For each individual sample, 20-80 µg total protein was combined with 30 µL of ABC (100 mM), 10 µL of DTT (250 mM), and 20 µL of BSA (0.02 mg/mL). The mixture was incubated at 95 °C with gentle shaking (300 rpm) for denaturation and reduction. After 10 min, the sample was allowed to cool to room temperature, and was then

treated with 10 μL of IAA (500 mM) in darkness for 30 min. Ice-cold acetone (1 mL) was added, mixed by vortex, and stored at -80 °C for 1 hour. The sample was then centrifuged at 16,000g at 4 °C for 15 minutes. The supernatant was discarded, and the pellet rinsed with cold methanol (0.5 mL), followed by another centrifugation under identical conditions for 10 minutes. After removing the supernatant, the pellet was air-dried for 30 min at room temperature. The dried protein was reconstituted in 60 μL of 50 mM ABC and digested with a freshly prepared trypsin solution (20 μL total volume in 50 mM ABC buffer) at a protein-to-trypsin ratio of 25:1, at 37 °C for 16 hours with gentle shaking (300 rpm). The reaction was stopped with 5 μL of 5% formic acid and 15 μL of internal standard (stable isotope labeled or heavy peptide) added to 15 μL of sample digest.

LC-MS/MS conditions: The digested urine samples were analyzed by targeted proteomics utilizing MRM scan. The analysis was carried out using an M-class Waters UPLC system coupled with Waters Xevo TQ-XS μLC-MS/MS. The peptides were separated on ACQUITY UPLC® HSS T3 column (1.8 μm, 1.0 x100 mm) connected with VanGuard™ ACQUITY UPLC® HSS T3 pre-column (1.8 μm, 2.1 x 5 mm). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a flow rate of 50 μL/min. The following gradient was utilized: 0-3 min: constant 5%B, 3-8 min: increase from 5 to 13%B, 8-20 min: increase from 13 to 45%B, 20-21.5 min: increase from 45 to 80%B, 21.5-22.5 min: constant 80%B, 22.5-23 min: return to 5%B, and 23-28 min: constant 5%B. The column temperature and injection volume were 40 °C and 1 μL, respectively. The optimized MRM transitions with cone voltage

and collision energy for selected peptides of cubilin and bovine serum albumin are provided in Table 3.2.

Table 3.2: Optimized MS/MS parameters used for the quantification of selected proteins in urine samples.

Protein	Peptide	Light/Heavy	Precursor ion	Precursor charge	Product ion	Product charge	Cone voltage	Collision energy
Cubilin	ITFTFFR	Light	466.2554	2	570.3035	1	30	16
	ITFTFFR	Light	466.2554	2	717.3719	1	30	16
	ITFTFFR	Light	466.2554	2	818.4196	1	30	16
	ITFTFF**R**	Heavy	471.2596	2	580.3117	1	30	16
	ITFTFF**R**	Heavy	471.2596	2	727.3801	1	30	16
	ITFTFF**R**	Heavy	471.2596	2	828.4278	1	30	16
Bovine serum albumin	AEFVEVTK	Light	461.7477	2	476.2715	1	35	16
	AEFVEVTK	Light	461.7477	2	575.3399	1	35	16
	AEFVEVTK	Light	461.7477	2	722.4083	1	35	16
	AEFVEVT**K**	Heavy	465.7548	2	484.2857	1	35	16
	AEFVEVT**K**	Heavy	465.7548	2	583.3541	1	35	16
	AEFVEVT**K**	Heavy	465.7548	2	730.4225	1	35	16
Bovine serum albumin	LVNELTEFAK	Light	582.319	2	218.1499	1	35	20
	LVNELTEFAK	Light	582.319	2	595.3086	1	35	20
	LVNELTEFAK	Light	582.319	2	951.4782	1	35	20
	LVNELTEFA**K**	Heavy	586.326	2	226.1641	1	35	20
	LVNELTEFA**K**	Heavy	586.326	2	603.3228	1	35	20
	LVNELTEFA**K**	Heavy	586.326	2	959.4924	1	35	20

Data analysis: Peptides were integrated and the area under the curve was quantified using Skyline 23.1.0.455. The area of the multiple fragments of the light and heavy forms of surrogate peptides (Table 1) for both cubilin and BSA were averaged and the area ratio of light to heavy peptide was calculated. The amount of cubilin protein (pmol) in each sample was calculated using the heavy peptide as single-point calibrator by multiplying the area ratio (light/heavy) by the amount (pmol) of heavy peptide injected and correcting for the total protein (mg). The consistency of trypsin digestion was monitored and corrected by BSA area ratio. Finally, the pmol cubilin protein was

normalized to both amount (mg) of the total urinary protein and volume (mL) of urine to allow for comparison across samples.

Three adult samples were analyzed for method validation and quality control. The amount of cubilin in pmol, normalized to total mg urinary protein in both the adult and pediatric populations are shown below.

Data analysis for time point comparisons: The data was normalized to creatinine. Creatinine was measured in mg/dL, the units were then converted to mg/mL. To normalize to creatinine, creatinine was divided by cubilin pmol / urine mL. Leaving the results as creatinine / urine (mg/mL). The next calculation is (cubilin pmol / urine ml) / (creatinine mg / urine ml) giving data values in cubilin pmol / creatinine mg.

RESULTS

Urine profiles were completed for 16 patients via the analysis of cubulin levels. The urine from these patients was collected at various time points during treatment with TRIKAFTA. Each patient served as their own internal control. Due to lab mix-up when processing the samples, all data points are not available for analysis for 9/16 patients. When comparing day 0 to Day 365, 5 out of 6 patients had a decrease in cubulin in the urine when normalized to total protein in the urine. 1 out of 6 patients showed an increase in urinary cubulin despite treatment for 1 year with TRIKAFTA as illustrated in Figure 3.1. Despite a general downward trend with regard to cubulin detected in the urine, the difference is not statistically significant (Figure 3.3).

Patients prescribed TRIKAFTA have been documented to have improved function across multiple organ systems (Zaher et al. 2021). This includes a potential decrease in the excretion of low molecular weight proteins. Thus, cubulin normalized to the total protein in the urine may not provide a complete assessment of the excretory profile. We proceeded to normalize cubulin to creatinine levels in the urine as shown in Figure 3.2. When normalized to creatinine, cubulin levels were decreased by the one year mark in 3 out of 4 patients. Seven days after the initiation of treatment, TRIKAFTA was found to either hold steady or decrease cubulin levels in 5 out of 7 patients; however, the difference is not statistically significant. (Figure 3.3)

FIGURES

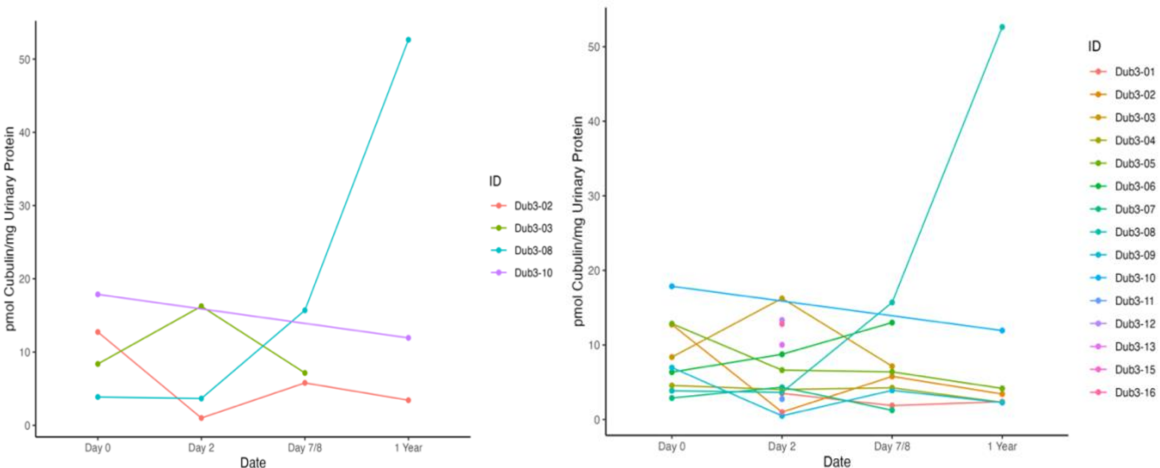


Figure 3.1: MS/MS quantification of the protein cubilin normalized by total protein concentration in the urine of 16 patients enrolled in TRIKAFTA study in Dublin over the course of 1 year.

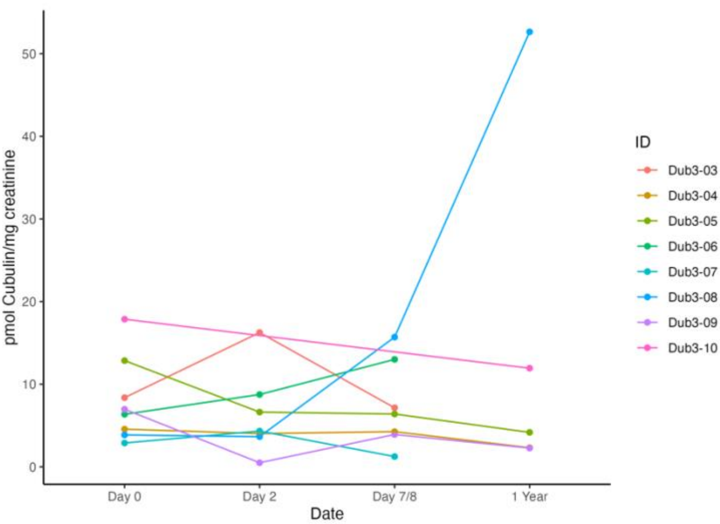


Figure 3.2: MS/MS quantification of the protein cubilin normalized by creatinine concentration in the urine of 10/16 patients enrolled in TRIKAFTA study in Dublin over the course of 1year.

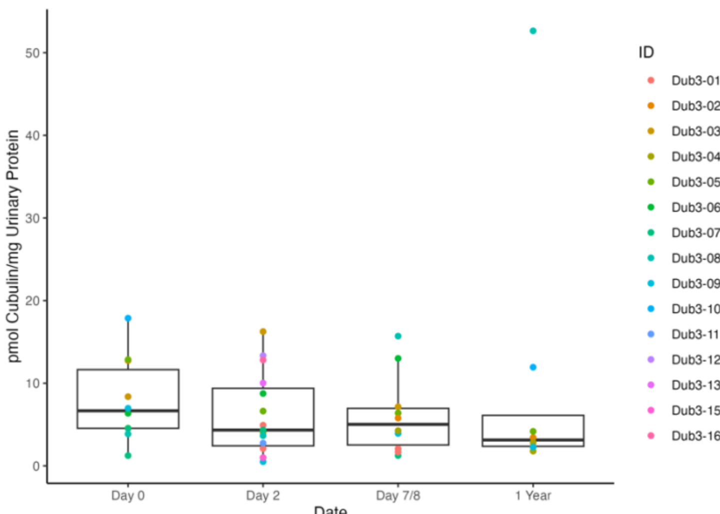


Figure 3.3: Boxplot of MS/MS data for patients enrolled in TRIKAFTA study. Nonsignificant downward trend is shown from Day 0 to Day 365 when normalized with total protein in the urine.

DISCUSSION

A general trend downward in most patients enrolled in the TRIKAFTA study that had urine submitted to the lab for analysis at the time point of 365 days is evident; however, the interesting trends are not statistically significant. Further urine samples will need to be processed at various timepoints for all patients to further elucidate this data. In addition to additional urine samples, further information can be correlated to response to treatment with TRIKAFTA. These include: sex of the patient, ethnicity, history of treatment with aminoglycosides outside of the two week exclusion criteria, additional urine properties such as specific gravity and filtration rate, and evaluation of levels of the CFTR modulator in the system at time of urine collection.

The sex of the patient is important to this study and distinction may help elucidate trends in resistance or response to treatment. The information may also influence our current standard of normalizing values. Mean creatinine levels have been shown to differ between various ethnicities as well as men and women (Jones et al 1998). In addition, Brumley et al. 2014 highlights gender differences in outcomes of patients with cystic fibrosis as the median age of survival is a few year less for women when compared to men.

Another exclusion criteria of this study included the use of aminoglycosides two weeks prior to the start of study. History of the need for aminoglycosides during the study will also need to be included. It may be important to have data regarding previous aminoglycoside use and/or baseline kidney function or biomarkers of kidney injury such as kidney injury molecule-1 prior to enrolling with study as further investigation of patients resistant to TRIKAFTA is warranted. Aminoglycosides have been implicated in many cases of acute kidney injury with up to 20-30% of children exposed to aminoglycosides subject to the formation of renal insult (McWilliam et al. 2017).

Overall, with more data collected, we hope to see a strong negative correlation between duration of treatment and cubulin levels in the urine as the combined effect of ivacaftor, elexacaftor and ivacaftor work to improve CFTR activity by modulating the amount of CFTR as well as its functionality at the cells surface. Once established, cubulin may serve as a proxy biomarker for

success of treatment, but more data is needed to determine whether this is an appropriate determinant of outcomes associated with TRIKAFTA treatment.

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