CYCLIC AMP AND CFTR MODULATION IN THE AIRWAYS

CYCLIC AMP AND CFTR MODULATION IN HUMAN AIRWAY EPITHELIAL CELLS IN THE CONTEXT OF LUNG HEALTH AND DISEASE

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LAY ABSTRACT

Cystic fibrosis (CF) is the most common genetic condition affecting Canadian newborns, caused by inheritance of mutations in the CF transmembrane conductance regulator (CFTR) gene. These mutations result in respiratory issues, including breathlessness, excess mucus, and susceptibility to infections, causing lung damage and premature death. Despite progress in CF drug development, some patients remain unresponsive to existing drug combinations, highlighting the need for new combinations to improve the quality of life for all CF patients. CFTR function is also compromised in other respiratory diseases like chronic obstructive pulmonary disease, a lung disease that shares many characteristics with CF and is mainly caused by tobacco smoke exposure. This Ph.D. thesis explores the effectiveness of a new drug strategy targeting proteins interacting with CFTR. By investigating drugs to complement existing treatments, we aim to improve CFTR function. This research offers a promising strategy to improve treatment for CF and other respiratory diseases.

ABSTRACT

Cystic fibrosis (CF) is the most common genetic disease affecting Canadian newborns (1 in 3,850) and is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. This gene encodes for CFTR, a phosphorylation-dependent ion channel localized at the apical membrane. Phosphorylation of CFTR by the cyclic adenosine monophosphate (cAMP)-dependent enzyme protein kinase A activates its activity, facilitating the transport of chloride and bicarbonate ions across the epithelial membrane. CFTR contributes to ion and airway surface liquid regulation, crucial for maintaining host defenses.

The inheritance of CFTR mutations leads to a variety of respiratory complications, including impaired mucociliary clearance, excessive mucus production, persistent airway infections, and heightened inflammation, ultimately causing lung damage. While there is currently no cure for CF, the development of CFTR modulators, targeting the defective CFTR protein directly, has significantly improved the quality of life for many CF patients. Despite these advancements, many patients remain unresponsive to current treatment options.

It has been well-established that combination therapies outperform monotherapies, emphasizing the need for alternative or complementary therapeutic strategies for CF management. Furthermore, CFTR dysfunction extends beyond CF and has been implicated in other respiratory diseases, such as chronic obstructive pulmonary disease, which is primarily linked to tobacco smoke exposure.

This Ph.D. thesis explores a complementary therapeutic approach, targeting proteins within the CFTR-containing macromolecular signaling complex to elevate intracellular cAMP levels, thereby enhancing CFTR function. We hypothesized that synergistic use of cAMP modulators, alongside CFTR modulators, will serve as an effective therapeutic strategy for CF and other respiratory diseases. Collectively, our studies highlight the potential of cAMP and CFTR modulation as a therapeutic strategy for improving the treatment of CF and other respiratory diseases, warranting further investigation, offering insights for future studies, and contributes to the ongoing pursuit of improved combination treatments.

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
ABC	ATP binding cassette
ABCC4	ATP binding cassette transporter C4
AC	Adenylyl cyclase
AKAP	A-kinase anchoring protein
ALI	Air-liquid interface
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ASL	Airway surface liquid
ATP	Adenosine triphosphate
AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CHDP	Cationic host defense peptide
COPD	Chronic obstructive pulmonary disease
DEP	Diesel exhaust particles
DMSO	Dimethyl sulfoxide
EC_{50}	Half-maximal effective concentration
E_{max}	Maximal effective concentration
ENaC	Epithelial sodium channel
FEV_1	Forced expiratory volume in 1 second
FSK	Forskolin
GPCR	G protein-coupled receptor
H2DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide

HBEC	Human bronchial epithelial cell
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Half-maximal inhibitory concentration
IL	Interleukin
Inh-172	CFTR Inhibitor-172
ISO	Isoproterenol
LDH	Lactate dehydrogenase
MCC	Mucociliary clearance
MRP4	Multi-drug resistance protein-4
NHERF1	Sodium hydrogen exchanger regulatory factor 1
PAH	Polycyclic aromatic hydrocarbon
PBEC	Primary bronchial epithelial cell
PDE	Phosphodiesterase
PDZK1	PDZ domain-containing scaffolding protein 1
PKA	Protein kinase A
PM	Particulate matter
RF	Roflumilast
Shank2	SH3 and multiple ankyrin repeat domains 2
SLC26A4	Pendrin
TEER	Transepithelial electrical resistance
TNF	Tumor necrosis factor
TSE	Tobacco smoke extract
VX-121	Vanzacaftor
VX-445	Elexacaftor
VX-561	Deutivacaftor
VX-661	Tezacaftor
VX-770	Ivacaftor
VX-809	Lumacaftor

PREFACE

This Ph.D. thesis dissertation follows the *"sandwich"* thesis format in accordance with the guidelines set by the School of Graduate Studies at McMaster University. Comprising five chapters, the dissertation is structured to present a comprehensive overview of the research undertaken for the Ph.D. degree. Chapter 1 serves as an introduction, presenting fundamental concepts that unify the three primary bodies of work pursued throughout the Ph.D. This chapter outlines the overarching theme and objectives of the thesis. Chapters 2 to 4 features three distinct yet interconnected research studies, each either previously published or in preparation for submission to peer-reviewed scientific journals. At the beginning of each chapter, the specific contributions of the authors to the corresponding research study are outlined. The dissertation concludes with Chapter 5, which covers a comprehensive discussion of the overall implications of the research conducted. This final chapter synthesizes the findings and explores their broader significance within the field of study.

CHAPTER 1:

Introduction

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Interactions between ABCC4/MRP4 and ABCC7/CFTR in human airway epithelial cells in lung health and disease

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Section Overview:

This section serves as the prelude to the upcoming chapters, introducing fundamental concepts and providing framework for the research explored in the following sections (Chapters 2 to 4).

Interactions between ABCC4/MRP4 and ABCC7/CFTR in human airway epithelial cells in lung health and disease

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1.1 Airway Epithelium and Host Defenses

1.1.1 Airway Epithelium

Following birth and development, the lung relies on both innate and adaptive immune responses orchestrated by the airway epithelium, which acts as the first line of defense against inhaled environmental insults and pathogens that could potentially cause pulmonary injury[1,2]. Comprising various cell types, including basal cells, ciliated cells, mucus-producing goblet cells, and secretory club cells, the cellular composition of the airway epithelium varies along the airway tree, influenced by its specific location within the lungs[3–5]. In respiratory diseases like chronic obstructive pulmonary disease (COPD) and asthma, exposure to environmental insults induce inflammation, leading to alterations in cellular composition characterized by goblet cell hyperplasia, resulting in airway obstruction and recurrent infections[4,6–9].

The airway epithelium is involved in multiple host defense mechanisms, including barrier function, mucociliary clearance (MCC), secretion of cationic host defense peptides (CHDPs), and the production of soluble mediators, including cytokines and chemokines, to mediate adaptive immune responses[4,6]. The formation of tight and adherens junctions within the airway epithelium establishes a tight protective barrier, however, exposure to inhaled insults can compromise this barrier, increasing susceptibility to pathogens and potentially becoming life-threatening[4,6].

Therefore, understanding the role of the airway epithelium in regulating host defenses is important for advancing our understanding of various respiratory diseases, which will contribute to development of therapeutics for disease management.

1.1.2 Innate Host Defenses

The airway epithelium functions as a physical barrier to the environment and a regulator of innate host defenses, including MCC and antimicrobial activity via CHDPs [4,10]. MCC, a major innate host defense mechanism, protects the airways by capturing harmful environmental insults in the mucus and expelling them from the lungs[10,11]. The effectiveness of this process is highly dependent on ciliary function, along with hydration of the airway surface[10–14].

Motile cilia, present on airway epithelial cells at the apical membrane, contribute to mucociliary clearance via their coordinated and synchronized movement[15]. Airway epithelial cells typically have over 200 motile cilia present on their surface[3,15]. Impaired ciliary function, leading to disrupted MCC, is associated with several respiratory complications, such as persistent wet coughs, recurrent respiratory infections, and chronic airway inflammation[3,15]. Ciliary function is dependent on several factors, including ciliary beat frequency, ciliary pattern, and airway surface liquid (ASL) composition, volume, and pH[5,16]. It has been demonstrated that ciliary function can be regulated by various factors, including cyclic adenosine monophosphate (cAMP) levels[12,16,17]. In airway epithelial cells, it has been shown that increases in cAMP levels leads to an increase in ciliary beat frequency[18,19].

The ASL plays a crucial role in the protection of the airways, contributing to the binding, trapping, bacterial killing, and clearance of particles and pathogens from the lungs[10,20]. The ASL consists of two distinct layers: the periciliary layer, which surrounds the cilia, and the mucus layer, situated directly above it[10]. The mucus layer is responsible

for trapping the inhaled insults for removal by MCC[10]. The ASL is tightly regulated by various ion transporters, including cystic fibrosis transmembrane conductance regulator (CFTR) and epithelial sodium channel (ENaC)[13,21,22]. In cystic fibrosis (CF), loss of CFTR function leads to dysregulated bicarbonate transport, resulting in acidification of the airways, resulting in impaired MCC and reduced antimicrobial activity[13,20–25].

Within the ASL, there is also the presence of CHDPs, which play a role in protecting the airways against inhaled pathogens. In addition to their antimicrobial activity, they also serve as chemoattractants, contributing to the modulation of immune responses[4,26]. The two best families of CHDPs characterized in mammals are cathelicidins and defensins[26–28]. While some CHDPs are produced constitutively, others are produced in response to stimuli, such as infection or injury[28]. It has been suggested that impaired antimicrobial activity is a major contributing factor to CF lung disease, resulting in recurrent infections and increased airway inflammation[23,25].

Altogether, the airway epithelium has several host defense mechanisms for protecting the airways from potential damage. Impairment of these host defense mechanisms, whether due to environmental factors or genetic factors, as seen in CF, can result in several respiratory complications such as airway obstruction and increased susceptibility to pathogens, ultimately becoming life-threatening.

1.2 CFTR Function in Lung Health and Disease

1.2.1 CFTR Overview

CFTR, also known as ABCC7, is a member of the ATP binding cassette (ABC) transporter family – a group of membrane proteins that facilitate the transport of substrates across membranes via ATP hydrolysis[29]. Encoded at the locus 7q31.2 and spanning 1480 amino acids in length (Uniprot.org), CFTR is the primary ABC transporter associated with lung pathology. Currently, there are over 2000 genetic variants described, resulting in promotor, missense, nonsense, frameshifts, splices, deletions, and insertions abnormalities (www.genet.sickkids.on.ca). Of these genetic variants, roughly 200 variants lead to a wide range of biological and functional defects in subjects with CF[30–32].

The molecular structure of dephosphorylated ATP-free human CFTR in the closed conformation, recently solved by cryo-electron microscopy, is comprised of two homologous halves, each consisting of 6-alpha-helix transmembrane domains connected to a cytoplasmic nucleotide-binding domain[30,33,34]. The two transporter halves are connected by a cytoplasmic regulatory "R" domain unique to CFTR, located between the transmembrane domain and nucleotide-binding domain complex, and is distinct from other ABC transporter family members[30,33,34]. The "R" domain, once phosphorylated by cAMP-dependent protein kinase A (PKA), moves itself away from the nucleotide-binding domains, allowing for the nucleotide-binding domains to dimerize which leads to increased open channel probability of the ion channel[34]. Therefore, activation of CFTR is influenced by molecules and pathways that can regulate cAMP levels, including beta-2 (β_2) adrenergic receptor agonists and phosphodiesterase-4 inhibitors[35].

In healthy individuals, CFTR is a phosphorylation-regulated ion channel responsible for conducting chloride and bicarbonate ions across the apical membrane of

epithelial cells lining the primary conducting airways and submucosal glands, contributing to ion and fluid homeostasis[32,36,37]. Studies examining CFTR expression patterns in the proximal airways, via localization and expression analyses, emphasize the critical role of CFTR in the regulating ASL and MCC[38–41]. Additionally, CFTR also regulates the function of other ion channels and transporters, including ENaC, to regulate ion and fluid homeostasis[42]. Moreover, CFTR is a crucial component of a multi-protein macromolecular signaling complex, interacting either directly or indirectly, with PDZ domain-containing scaffolding proteins, β_2 adrenergic receptors, cAMP-efflux transporters, and various other proteins at the apical membrane[43–46]. Thus, the inheritance of variants in the CFTR sequence can lead to several health complications, including pulmonary disease, gastrointestinal and reproductive complications, and endocrine disorders[30].

Traditionally, CFTR variants have been divided into 6 classes[47]. Class I consists of protein synthesis defects that result from frameshift, splice-site, or nonsense mutations that lead to the introduction of pre-mature codons, resulting in severe reduction or absence of CFTR expression. Class II involves protein trafficking defects comprised of protein misfolding, abnormal trafficking, and premature degradation in the endoplasmic reticulum, ultimately leading to a reduction of CFTR protein reaching the apical membrane. Class III are gating defects resulting from impaired regulation of the channel, while Class IV are ion conductance defects that result from changes in the pore shape, via amino acid residue substitutions, reducing the ion channel conductance. Class V are reduced quantity defects that result from promoter or splice abnormalities, altering the quantity of CFTR protein. Class VI includes reduced stability defects caused by either a reduction in conformational

stability or increased internalization signals, resulting in accelerated degradation at the cell surface via endocytic activity, without affecting biosynthesis or channel function. While CFTR mutation sites have been identified for each of the classes above, this classification system has recently been re-interpreted to consider evidence supporting CFTR variants with complex phenotypes that cannot be adequately classified into a single class, thus contributing to the design and development of novel therapeutic approaches for CF subjects[32].

1.2.2 Cystic Fibrosis

Cystic fibrosis is an autosomal recessive disease, characterized by the inheritance of genetic variations in the CFTR gene, resulting in the presentation of lung pathology and disease early and throughout life[48]. It is the most common genetic disease in young Canadians, affecting an estimated 1 in 3,850 newborns each year[49]. The concept of CF as a genetic recessive disorder was first proposed by Lowe et al. in 1949[50]. However, the groundbreaking discovery of the CFTR gene and its link to CF was not made until 1989 by Tsui, Riordan, and Collins et al.[48].

Individuals who inherit a combination of alleles from over 2000 CFTR variants experience defects in ion transport, resulting in characteristic CF pathologies[30–32,36,48,51]. The inheritance of CFTR variants leads to impaired epithelial fluid transport, resulting in increased mucus viscosity, chronic airway infections, and modulated immunity[10,20,52].

In CF, lung disease is believed to be initiated by impaired host defenses due dysfunctional CTFR, predisposing the airways to bacterial infections and triggering

inflammatory responses[52–54]. The inheritance of CFTR variants leads to dysregulated epithelial fluid transport, impacting multiple host defenses, leading to the observed consequences in CF[55,56]. The loss of CFTR function leads to dysregulated ASL, influencing its composition, volume, and pH[20,24,25,57–60]. These alterations affect mucus biology and acidify the airways, leading to airway obstruction and reduced antimicrobial activity[20,25,60,61]. This, in turn, leads to impairment of MCC, fostering increased bacteria attachment and proliferation, resulting in inflammation in the lungs[52–54]. Altogether, the simultaneous disruption of these host defenses significantly contributes to CF lung disease pathogenesis and understanding these underlying causes will be important for the development of novel therapeutics for CF management.

1.2.3 Acquired CFTR Dysfunction

In addition to genetic variations leading to CFTR deficiencies, acquired CFTR deficiencies have also been implicated in respiratory diseases such as COPD and chronic bronchitis. The primary risk factor for the development of these diseases is tobacco smoke exposure. Notably, exposures to environmental air pollutants, including tobacco smoke, ozone, reactive oxygen nitrogen species, and diesel particulate matter, have been reported to cause acquired CFTR dysfunction[62–67].

Despite their distinct pathophysiological mechanisms, CF and COPD, specifically the chronic bronchitis phenotype, share numerous phenotypic features, including airflow obstruction, recurrent infections, increased airway inflammation, excess mucus production, and dysregulated MCC[68–70]. Several studies have indicated that tobacco smoke is associated

with reduced CFTR protein expression and ion channel activity, independent of CFTR variants, suggesting its potential contribution to the pathology of chronic bronchitis in COPD[62,63,71,72]. Furthermore, tobacco smoke indirectly affects CFTR function by modulating ABCC4 function, a cAMP-efflux transporter within the CFTR-containing macromolecular signaling complex in the airway epithelium[73]. Beyond CFTR, tobacco smoke negatively impacts ciliary function by reducing ciliary beat frequency and the number of ciliated cells[19,74,75]. Moreover, tobacco smoking is associated with an increased risk for respiratory infections and increased inflammation[76].

Regarding other environmental air pollutants and their impact on CFTR function, although less studied than tobacco smoke, emerging evidence has suggested that air pollutants, like diesel particulate matter, can reduce CFTR expression and decrease ASL secretion[65–67].

Given the shared respiratory symptoms and potential dysregulation of CFTR function in both CF and COPD, therapeutic strategies aimed at enhancing CFTR function would not only be beneficial for CF, but also hold promise for treating other respiratory diseases, such as COPD.

1.3 CF Therapeutic Landscape

1.3.1 CFTR Modulators

While there is currently no cure for CF, advancements in our understanding of CFTR variant structure and function relationships have contributed to the development of therapeutics known as CFTR modulators. These modulators can correct misfolded proteins

and potentiate channel activities, thus managing the pathologies associated with this once fatal disease. To date, the United States Federal Drug Administration (FDA) has approved of four CFTR modulator therapies for the treatment of CFTR deficiencies, however, these treatments can be financially burdensome for patients, with annual costs exceeding $300,000[77].

The two main types of CFTR modulators are potentiators and correctors[32]. Potentiators directly target the CFTR protein to increase the open gating probability, thus leading to an increase in chloride and bicarbonate secretion[78]. Potentiators are particularly beneficial for Class III and IV CFTR variants with gating and ion conductance defects, notably the G551D-variant. Presently, there is only one FDA-approved potentiator, ivacaftor (VX-770), which has led to improved lung function in G551D-CFTR subjects demonstrated by an increase in forced expiratory volume in 1 s[79]. Correctors, on the other hand, target protein trafficking defects by assisting with the folding and trafficking of CFTR to the apical membrane. To date, there are three FDA-approved correctors called lumacaftor (VX-809), tezacaftor (VX-661), and elexacaftor (VX-445)[80–85]. The combination of VX-770 and VX-809 has also led to improved lung function and a reduction in pulmonary exacerbations in F508del-variant subjects[86]. Currently, there are on-going studies for novel CFTR modulators aiming to restore CFTR function, including novel potentiator deutivacaftor (VX-561), an altered form of VX-770, and novel corrector vanzacaftor (VX-121), which was recently demonstrated to be safe and efficacious in patients, resulting in improvements in lung function[87].

1.3.2 Alternative CF Therapeutics

Apart from CFTR modulators, there are several other therapeutics that are currently under development or used for CF management. These include antibiotics to suppress recurrent infections, mucolytics to hydrate mucus and enhance MCC, anti-inflammatory drugs to modify or dampen immune responses, and inhaled bronchodilators to widen the airways[88–90].

Another therapeutic approach for CF treatment that is currently under development is gene editing. In contrast to the use of small molecules, which are only effective in a subset of CF subjects and require lifelong management, the use of CRISPR-based approaches, which can lead to permanent solutions, has also garnered interest. The application of CRISPR-based editing and modified CRISPR-based editors has been assessed in several studies and has shown to be effective in restoring CFTR function in several variants, including F508del mutations and select nonsense mutations, demonstrating the feasibility of CRISPR-based therapeutic approaches for potential clinical applications[91,92]. In addition to gene editing, there is also mRNA and gene therapy, which aims to deliver either correct genetic instructions or a functional copy of the CFTR gene. However, with the current existing limitations, including potential detrimental off-target effects and delivery, further research is needed before genetic therapies become a viable approach. Several genetic therapies, including 4D-710, ARCT-032, SPL84, and VX-522 mRNA (NCT05248230, NCT05712538, NCT05668741), are currently undergoing phase one studies, indicative of the ongoing research efforts[93].

1.4 cAMP-Mediated CFTR Regulation

1.4.1 cAMP Regulatory Mechanisms

Cyclic AMP, a second messenger discovered in the late 1950s by Sutherland and Rall, plays a critical role in various cellular pathways and biological processes[94,95]. In the lungs, cAMP regulates airway smooth muscle relaxation, the release of inflammatory mediators, immune cell function, ion transport, and MCC[96,97]. For this reason, intracellular levels of cAMP are tightly regulated by various mechanisms, including G protein-coupled receptors (GPCRs), adenylyl cyclases (ACs), phosphodiesterases (PDEs), and cAMP-efflux transporters[96]. A primary target of cAMP is PKA, which is activated upon cAMP binding to its regulatory domains, leading to the dissociation of its catalytic subunits, which then goes on to phosphorylate various target proteins, such as CFTR[98,99].

Adenylyl cyclases are key players in cAMP regulation, upon stimulation, these enzymes catalyze the conversion of ATP to cAMP, thereby initiating cAMP signaling[100,101]. In mammals, there are currently 10 known AC isoforms, with AC1 to AC9 being transmembrane proteins associated with GPCRs, and AC10 as a soluble isoform[100,101]. Cryo-electron microscopy studies have elucidated the molecular structure of AC, revealing common features across different isoforms, including two cytoplasmic catalytic domains and a pair of 6-transmembrane bundles anchoring AC to the membrane[100,102,103]. The activity of ACs is regulated by GPCRs. When GPCRs bind to their appropriate ligands, they undergo a conformational change, leading to the dissociation of the G_s alpha subunit from its associated heterotrimeric G protein, subsequently activating AC[101]. An important GPCR involved in CFTR activation is the β_2 adrenergic receptor[104]. Multiple AC isoforms

have been identified in the lungs, with AC2, AC4, AC6, AC8, and AC9 being predominantly expressed[101]. However, AC10 expression has also been demonstrated in human airway epithelial cells, contributing to ciliary beat frequency regulation[105]. Additionally, studies have also suggested the involvement of AC1 in CFTR activation, with localization experiments in primary human bronchial epithelial cells revealing co-localization of AC1 with CFTR at the apical membrane[106].

Unlike ACs, phosphodiesterases are the enzymes responsible for terminating cAMP signaling by hydrolyzing cAMP to AMP[107]. To date, there are 11 distinct PDE gene families recognized in mammalians, PDE1 to PDE11[107,108]. Each family is characterized by its substrate specificity, primary molecular structure, catalytic properties, and subcellular localization[107,108]. Moreover, each family typically consists of multiple isoforms and splice variants[107,108]. Notably, PDE4, PDE7, and PDE8 are specific to cAMP whereas PDE1, PDE2, PDE3, PDE10, and PDE11 exhibit dual specificity for both cAMP and cyclic GMP[107,108]. The remaining PDE families, PDE5, PDE6, and PDE9 are specific to cyclic GMP[107,108]. Common to all PDEs is a conserved catalytic domain, along with regulatory domains. The catalytic domain is comprised of two main regions responsible for its enzymatic activity, a deep hydrophobic pocket containing two binding sites for zinc and magnesium, and a glutamine-containing region that facilitates the interaction with the specific cyclic nucleotide[108,109]. While several PDE families have been detected in the lung, there is a specific focus on PDE-4, due to its predominant expression in human airway epithelial cells and its specificity for cAMP[107]. Furthermore, studies have implicated PDE-

4 in the regulation of CFTR activity through its anchoring to the CFTR-containing macromolecular complex via the PDZ domain-containing protein Shank2[110,111].

In addition to regulation by GPCRs, ACs, and PDEs, intracellular cAMP levels are also influenced by cAMP-efflux transporters, including ABCC4, a member of the ABC transporter family. ABCC4, expressed throughout the respiratory mucosa, facilitates the transport of various endogenous substrates, including prostaglandins, leukotrienes, uric acid, and cyclic nucleotides[112–119]. To date, no ABCC4 structures have been resolved, although a homology model has been proposed in two states in association with membrane lipid bilayers[120,121]. The initial demonstration of ABCC4 expression in the human lung was provided by complementary gene expression and proteomic profiling in lung epithelial cell lines and whole human lung tissue[122,123]. Furthermore, immunohistochemical staining patterns revealed ABCC4 expression is restricted to proximal airway epithelial cells, with decreasing expression down the airway tree[118]. The first functional demonstration of ABCC4 in the human lung epithelium was the transport of prostaglandins[116]. In this study, ABCC4 gene and protein expression was demonstrated in primary human airway epithelial cells and mechanistically linked to the cyclooxygenase generation of prostaglandins, signaling through the EP4 receptor, activating AC activity to increase cAMP levels, regulating CFTR function[116].

1.4.2 CFTR-Containing Macromolecular Signaling Complex

The importance of compartmentalized cAMP signaling in relation to CFTR is emphasized by the creation of microenvironments. These microenvironments, resulting

from cAMP compartmentalization, influence the function of neighbouring proteins[46]. Additionally, emerging studies have unveiled interactions, either directly or indirectly, between CFTR and its diverse interacting partners, forming the CFTR-containing macromolecular signaling complex[45]. This complex has been suggested to play an important role in human airway epithelial cells, contributing to the regulation of innate immune responses and ion transport mechanisms.

The CFTR-containing macromolecular signaling complex is comprised of various proteins, including PDZ domain-containing scaffolding proteins, A-kinase anchoring proteins (AKAPs), β_2 adrenergic receptors, ACs, PKA, PDEs, cAMP-efflux transporter ABCC4, and many others[45]. PDZ domain-containing scaffolding proteins, such as NHERF1, Shank2, and PDZK1, which bind to the C-terminal end of CFTR, have been demonstrated to co-localize with CFTR to the apical membrane[44,45,110,111,124–126]. These proteins, together with AKAPs that recruit PKA to its substrates for effective phosphorylation, have been suggested to facilitate multiple interactions between CFTR and other proteins within the macromolecular signaling complex.

β_2 adrenergic receptors play a major role in compartmentalized cAMP signaling. Activation of β_2 adrenergic receptors by β_2 agonists results in a localized increase in cAMP levels via ACs, triggering downstream activation of CFTR via cAMP-dependent phosphorylation by PKA[43,104]. Studies have demonstrated the co-localization and co-immunoprecipitation of β_2 adrenergic receptors with CFTR[43,127]. This protein-protein interaction has been shown to be mediated by NHERF1[43]. Moreover, the loss of NHERF1 results in a decrease in CFTR activity, due to the physical uncoupling of β_2 adrenergic

receptor and CFTR[43]. NHERF1 has also been implicated in the interaction between ezrin, an AKAP, to CFTR[43,126]. Additionally, Shank2, involved in the anchoring of PDE-4 to the CFTR-containing macromolecular signaling complex, competes with NHERF1 for CFTR-binding[110].

ABCC4 and CFTR are physically linked via PDZK1[44]. This phenomenon was first observed in gut epithelium models investigating cAMP-efflux by ABCC4 and its downstream effects on CFTR ion secretion and mucosal surface hydration[44]. The direct interaction was demonstrated via immunoprecipitation and observed using confocal fluorescence microscopy. Additionally, ABCC4 and CFTR are functionally coupled via cAMP-dependent activation of PKA[46,112]. ABCC4 inhibition leads to the rise in localized intracellular cAMP levels, activating PKA, subsequently phosphorylating CFTR, leading to increased channel activity. This functional coupling has subsequently been confirmed in primary human airway epithelial cells, where inhibiting ABCC4 leads to increased CFTR activity[44,128].

Due to the important role of the CFTR-containing macromolecular signaling complex in regulating both innate immune responses and ion transport mechanisms, there has been an interest in developing novel therapeutic interventions to specifically target this complex, which relies on cAMP signaling compartmentalization.

1.4.3 cAMP Modulating Agents

The discovery of CFTR modulators has revolutionized CF management, leading to improvements in lung function and overall quality of life. However, the current FDA-

approved CFTR modulators are restricted to a specific subset of CF patients, and their effectiveness varies due to phenotypic differences in individuals sharing the same CFTR variants. This emphasizes the need for the development of combinatorial therapies aligned with precision medicine approaches. In addition to CFTR modulators, potential therapeutic approaches include cAMP modulating molecules, such as β_2 agonists, PDE-4 inhibitors, ABCC4 inhibitors, and other molecules targeting proteins within the CFTR-containing macromolecular signaling complex[44–46,72,96]. Exploring cAMP modulation as a therapeutic approach will not only broaden the therapeutic landscape, but also holds promise for addressing CFTR deficiencies.

The use of β_2 agonists has already found clinical application in the management of respiratory diseases like COPD and asthma, due to their bronchodilator effects[129,130]. These agonists can be classified as either short-acting or long-acting based on their onset of action and duration. Examples of clinically approved β_2 agonists include albuterol and formoterol[130]. By acting as ligands to β_2 adrenergic receptors, they initiate cAMP signaling, leading to downstream activation of ACs, resulting in a rise in intracellular cAMP levels. In addition to β_2 agonists, forskolin, a direct activator of AC, is also capable of raising intracellular cAMP levels. Several research studies in airway epithelial cells have demonstrated the ability of these molecules to induce CFTR function[36,43,131].

There has been a growing interest in developing selective PDE inhibitors due to their involvement in cAMP signaling, particularly PDE-4 inhibitors, to prevent cAMP breakdown. Selective PDE-4 inhibitors have been a focus due to their predominant expression in the lungs, inflammatory and immune cells, along with evidence

demonstrating their altered expression, in response to tobacco smoke, in various lung diseases[75,132,133]. To date, there have been several selective PDE-4 inhibitors developed, including rolipram, cilomast, and roflumilast, which have demonstrated anti-inflammatory effects within the lungs, reducing exacerbations in patients[107,134,135]. Of these, roflumilast is the only clinically approved selective PDE-4 inhibitor for the treatment of severe COPD[129,134,136,137]. Despite some off-target effects and limited therapeutic window, thus limiting their clinical effectiveness, roflumilast has shown promising results in studies, including improvement in ciliary function and potentiation of CFTR activity, offering potential benefits in airway hydration and MCC[19,72,138–144].

To date, there are no therapeutics selectively targeting ABCC4 developed for respiratory diseases. The potential importance of regulating intracellular cAMP levels via ABCC4 inhibition for respiratory diseases is justified by the use of β_2 agonists or selective PDE-4 inhibitors, alone or in combination with glucocorticoids, to manage chronic lung disease progression and exacerbations in COPD and asthma[145–148]. The therapeutic benefits of combining cAMP elevating agents and glucocorticoids stems from their ability to enhance the transcription of anti-inflammatory and protective genes, such as dual specificity protein phosphatase 1 and regulator of G-protein signaling[118,149–151]. Inhibition of ABCC4 could be used as an adjunct therapy to increase intracellular cAMP levels, thereby potentiating both glucocorticoid signaling and CFTR activity[46]. In the absence of ABCC4, the benefits of increasing intracellular cAMP levels by activating ACs or inhibiting PDEs may be minimized due to increased extracellular transport via concentration-dependent ABCC4 mechanisms[46]. Elevating intracellular cAMP levels may,

in turn, regulate ABCC4 expression in a feedback loop, with exchange proteins directly activated by cAMP proteins activated by elevated cAMP levels[152]. Additionally, inhibitors of ABCC4 may also regulate PDE-4 expression[153]. The most selective ABCC4 inhibitors developed to date are Ceefourin-1 and Ceefourin-2, although these have not been validated *in vivo*[154]. In contrast, non-selective inhibitors originally designed for alternate targets, such as MK-571 (cysteinyl leukotriene receptor antagonist), have been exploited[113,154,155].

The discovery and approval of these cAMP modulating drugs, coupled with existing FDA-approved combinatorial therapies such as Orkambi (VX-809/VX-770), Symdeko (VX-661/VX-770), and Trikafta (VX-445/VX-661/VX-770), which have demonstrated clinical benefits for a subset of CF patients, creates an opportunity for personalized combinatorial therapies. These approaches have the potential to target multiple CFTR defects at once, with broad applications for other respiratory diseases impacted by acquired CFTR dysfunction. Furthermore, the effectiveness of combinatorial therapies can potentially be enhanced by incorporating cAMP modulating agents. The use of cAMP modulating agents would lead to a localized rise in intracellular cAMP levels, further potentiating CFTR activity. Beyond its role in CFTR potentiation, elevated intracellular cAMP levels have also been shown to improve ciliary function.

1.5 Central Hypothesis and Research Objectives

1.5.1 Project Rationale

There is a critical need for the development of effective therapeutic strategies for the management of CF, the most common autosomal recessive disease affecting Canadian

newborns. CF, caused by mutations in the CFTR gene, leads to impairment of innate host defenses, such as MCC and antimicrobial activity, due to dysfunctional ion transport of chloride and bicarbonate ions across the epithelia. While there have been great strides in CFTR-targeted therapies, leading to improvements in lung function and overall quality of life, combinatorial therapeutic approaches are necessary given the varying patient responses. Since CFTR activity is dependent on phosphorylation by cAMP-dependent enzyme PKA, cAMP modulation as an add-on therapeutic has the potential to enhance CFTR functionality. Furthermore, CFTR dysfunction has also been implicated in other respiratory diseases, caused by environmental insults, suggesting that therapeutics used for CF management could be repurposed and have broad applications for other respiratory diseases. This Ph.D. thesis dissertation will explore the effects of cAMP and CFTR modulation in human airway epithelial cells (summarized in **Figure 1**).

1.5.2 Central Hypothesis

The central hypothesis of this Ph.D. thesis dissertation is as follows: The use of cAMP modulating agents, to elevate intracellular cAMP levels, will serve as an effective add-on therapeutic strategy alongside CFTR modulators for the management of CF. This therapeutic approach holds promise for managing other respiratory diseases that exhibit similar pathological features. Moreover, it is anticipated that this therapeutic approach will improve downstream consequences commonly associated with CFTR dysfunction.

1.5.3 Research Objectives

To test the central hypothesis, three research objectives were developed. **Chapter 2** investigates the efficacy of cAMP modulation, achieved through pharmacological inhibition of ABCC4 and PDE-4, as an add-on therapeutic strategy to VX-770 for enhancing CFTR activity. Building on these findings, **Chapter 3** assesses the effect of environmental air pollutants on CFTR expression and function, exploring the potential link to acquired CFTR dysfunction and the rescue using cAMP and CFTR modulating drugs. In **Chapter 4**, the focus shifts to the impact of CFTR potentiation via cAMP and CFTR modulators on host defenses, specifically examining ASL pH. Collectively, these studies are interconnected by the overarching theme of cAMP and CFTR modulation in airway epithelial cells, providing insights into their effects within the broader context of lung health and disease.

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Figures

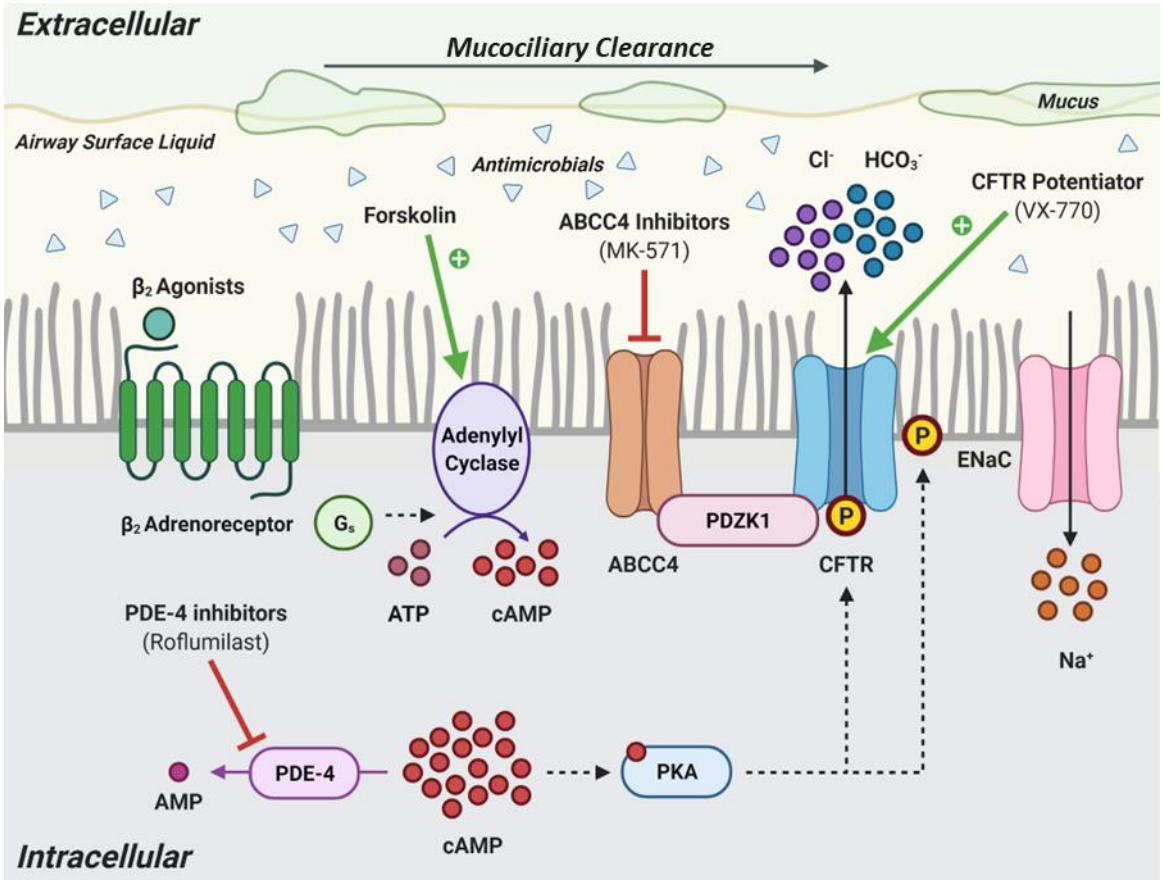

Figure 1: The CFTR-containing macromolecular signaling complex in human airway epithelial cells. In healthy conditions, the CFTR-containing macromolecular complex plays an important role in the airway epithelium by regulating innate immune responses and ion transport, impacting airway surface liquid, mucociliary clearance, and bacterial killing. In diseased conditions, compromises to the macromolecular complex disrupts ion homeostasis, leading to dysregulated airway surface liquid, mucociliary clearance, and mucus accumulation, increasing the risk of airway infections and inflammation. Several small molecule therapeutics and investigational drugs have been developed to address the defects that occur in disease conditions to alleviate these associated consequences. These include ABCC4 inhibitors (MK-571) - which prevent cAMP-efflux; PDE-4 inhibitors (Roflumilast) - which prevent cAMP breakdown; β_2 Agonists - which leads to the downstream activation of adenylyl cyclase to increase cAMP production; and CFTR potentiator (VX-770) - which increases open gating probability. ABCC4: ATP binding cassette C4; CFTR: cystic fibrosis transmembrane conductance regulator; ENaC: epithelial sodium channel; PDE-4: phosphodiesterase-4; PDZK1: PDZ domain-containing scaffolding protein 1; PKA: protein kinase A.

CHAPTER 2:

Modulation of cAMP metabolism for CFTR potentiation in human airway epithelial cells

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Author Contributions:

J.P.N. performed *in vitro* CFTR membrane potential assay experiments, data analyses, figure generation, and literature review; contributed to the manuscript conception; and drafted the manuscript. M.B. performed *in vitro* CFTR membrane potential assay experiments. R.D.H performed *in vitro* extracellular cAMP assay experiments, data analyses, and figure generation. N.T. performed western blotting and figure generation. M.D.I. contributed to the manuscript conception. J.A.H. (Principal Investigator and Corresponding Author) was responsible for the oversight of the entire study (data collection, analyses, drafting, finalization) and supervision of trainees.

Study Overview:

We explore the potential of cAMP modulation, via ABCC4 and PDE-4 inhibition, as an add-on therapy to CFTR modulators for the treatment of CF. Through experiments performed on Calu-3 cells, we show that the combination of cAMP modulation with CFTR modulator VX-770 enhances CFTR activity compared to VX-770 alone, suggesting cAMP modulation could be a promising strategy for optimizing CF disease management.

Modulation of cAMP metabolism for CFTR potentiation in human airway epithelial cells

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Abstract

Cystic fibrosis (CF) is a genetic disease characterized by CF transmembrane conductance regulator (CFTR) dysfunction. With over 2000 CFTR variants identified, in addition to known patient to patient variability, there is a need for personalized treatment. The discovery of CFTR modulators has shown efficacy in certain CF populations, however, there are still CF populations without valid therapeutic options. With evidence suggesting that single drug therapeutics are insufficient for optimal management of CF disease, there has been an increased pursuit of combinatorial therapies. Our aim was to test cyclic AMP (cAMP) modulation, through ATP Binding Cassette Transporter C4 (ABCC4) and phosphodiesterase-4 (PDE-4) inhibition, as a potential add-on therapeutic to a clinically approved CFTR modulator, VX-770, as a method for increasing CFTR activity. Human airway epithelial cells (Calu-3) were used to test the efficacy of cAMP modulation by ABCC4 and PDE-4 inhibition through a series of concentration-response studies. Our results showed that cAMP modulation, in combination with VX-770, led to an increase in CFTR activity via an increase in sensitivity when compared to treatment of VX-770 alone. Our study suggests that cAMP modulation has potential to be pursued as an add-on therapy for the optimal management of CF disease.

Introduction

Cystic fibrosis (CF) is a recessive genetic disease characterized by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, a phosphorylation-regulated ion channel responsible for conducting chloride and bicarbonate ions across epithelial cell membranes[1–4]. CFTR is localized at the apical membrane of epithelial cells lining the primary conducting airways and submucosal glands, contributing to the regulation of airway surface liquid volume and composition[5]. In CF, disease-causing mutations in CFTR lead to dysregulated ASL volume and composition in the lung, which is associated with increased mucus viscosity, increased susceptibility to pathogens, and modulated lung immunity[6–9].

There are over 2000 known CFTR variants identified that are associated with a wide range of biological and functional consequences (www.genet.sickkids.on.ca). These variants have traditionally been classified into six classes based on their phenotype, including impairments to protein maturation, protein gating, and ion conductance[10–12]. However, evidence has suggested that this traditional classification is outdated since there exists CFTR variants with complex phenotypes that cannot be adequately classified into a single class[13]. Moreover, there has been evidence demonstrating that there are phenotypic differences between CF patients carrying the same genotypic CFTR variant[14]. This emphasizes the need for combinational therapies that will target several biological and functional defects.

Recent advancements in the molecular understanding of the functional impacts of specific CFTR variants has revolutionized drug development. As a result of discovery

research focused on the function of specific CFTR variants, the Federal Drug Administration has approved four CFTR modulators[15–18]. The first approved CFTR modulator was ivacaftor (VX-770), a CFTR potentiator that directly binds to CFTR, increasing channel opening and chloride conductance[19]. In G551D-CFTR variants, VX-770 has demonstrated significant increases in predicted forced expiratory volume in 1 second (FEV_1) from baseline at various doses and treatment durations[20,21]. VX-770 was subsequently assessed in combination with the CFTR corrector lumacaftor (VX-809), which increases the amount of CFTR reaching the cell surface. This combination showed mild improvements in FEV_1 (2.6 - 4 percent points) and decreases in pulmonary exacerbations (30 - 39%) in the F508del homozygous population, which represents the global majority of CF patients[22–24]. Additional approved CFTR modulators include CFTR correctors tezacaftor (VX-661) and elexacaftor (VX-445)[25–28]. Ongoing combinatorial studies with VX-770, novel potentiators (such as VX-561 – which is an altered form of VX-770, ABBV-3067, and PTI-808), novel correctors (including VX-661, VX-445, VX-659, VX-121, ABBV-2222, and PTI-801) and the amplifier PTI-428, which increases the amount of CFTR produced, suggest that a single drug therapy will not be sufficient for optimal management of CF disease (NCT03912233, NCT03969888, NCT03500263)[25–30]. Furthermore, while combinatorial CFTR modulator therapies have shown efficacy in previously poorly managed CF populations, many CFTR variants have not been studied individually. In addition, these CFTR modulator therapies directly target CFTR defects and do not take into account mechanisms implicated in CFTR activity, such as phosphorylation of the regulatory domain of CFTR[31–33]. As such, new drugs and drug combinations that

align with precision medicine approaches should be developed to ensure that there are valid therapeutic options for everyone with CF.

While understanding the genetic variation in CF patients is important for developing combinatorial therapies, there are many factors that can influence CFTR potentiation, including cyclic AMP (cAMP) metabolism. CFTR is phosphorylated by the cAMP-dependent enzyme protein kinase A (PKA)[31,32]. Following a rise in intracellular cAMP, PKA is activated and can phosphorylate CFTR, increasing open channel probability[31–33]. There are multiple mechanisms contributing to cAMP regulation, including cAMP-efflux transporters, phosphodiesterases (PDE), and β_2 adrenergic receptors[14,34–39]. A key cAMP transporter is ATP Binding Cassette Transporter C4 (ABCC4), formerly known as multi-drug resistance protein-4[34,40]. ABCC4 is functionally and physically coupled with CFTR through the scaffolding protein PDZK1[34]. Inhibition of ABCC4 with MK-571 was first shown to be able to attenuate cAMP transport and potentiate CFTR activity in gut epithelial cells, however, this has been later demonstrated in airway epithelial cells[14,34,41,42]. In the context of CF, we have demonstrated that pharmacological inhibition of ABCC4 in human airway epithelial cells is able to potentiate CFTR in G551D-variants above and beyond VX-770[14]. Aside from ABCC4 inhibition, there has also been evidence demonstrating that pharmacological inhibition of PDE-4 was able to potentiate CFTR activity through the elevation of cAMP levels[37,38,43]. The interaction of ABCC4 and PDE-4 inhibition to increase cAMP levels to augment CFTR potentiation remains to be explored.

Currently, there are several investigational or clinically available therapeutics that have potential applications in influencing CFTR activity through cAMP elevation. MK-571

has been previously used as an ABCC4 inhibitor to prevent cAMP-efflux, thereby increasing CFTR activity in human airway epithelial cells[14,41,42]. Originally designed as a cysteinyl leukotriene receptor antagonist, MK-571 is also exploited as a research compound due to its off-target effects, including ABCC4 and PDE-4 inhibition, and has been used safely in humans[44]. Conversely, Ceefourin-1 and 2 have been reported as ABCC4 inhibitors with demonstrated *in vitro* efficacy and selectivity[45]. Roflumilast is a clinically available selective PDE-4 inhibitor with anti-inflammatory effects that is commonly administered to chronic obstructive pulmonary disease (COPD) patients suffering from chronic bronchitis[46–49]. Similar to Roflumilast, Rolipram is also known to be a selective PDE-4 inhibitor. Originally it was investigated as a potential antidepressant drug, however, it is not used clinically due to adverse side-effects and its small therapeutic window[50–52].

Due to our previous demonstration that pharmacological inhibition of ABCC4, in the presence of CFTR modulator VX-770, leads to CFTR potentiation beyond VX-770 alone and evidence demonstrating the use of PDE-4 inhibitors is also able to potentiate CFTR activity, we hypothesized that cAMP modulation with ABCC4 and PDE-4 inhibitors, in the presence of VX-770, will potentiate CFTR activity. In order to begin defining the efficacy of cAMP modulation by ABCC4 and PDE-4 inhibitors on CFTR activity, we pursued a series of concentration-response studies on Calu-3 human airway epithelial cells with wild-type CFTR[53–55]. We demonstrate that combinatorial additions of CFTR modulator VX-770 with either an ABCC4 or PDE-4 inhibitor led to CFTR potentiation via an increase in sensitivity. Our results suggest that combinatorial additions of VX-770 with ABCC4 or PDE-4 inhibitors may increase sensitivity and efficacy of VX-770 alone.

Results

In vitro extracellular cAMP assay analysis of ABCC4 inhibitor compounds in human airway epithelial cells

An *in vitro* extracellular cAMP assay of two commercially available ABCC4 inhibitors, MK-571 and Ceefourin-1 (**Fig. 1a and b**), was performed in human airway epithelial Calu-3 cells and human bronchial epithelial cells (HBEC6-KT, **Supplementary Fig. 1**) to evaluate their efficacy in decreasing extracellular cAMP levels. Concentration-dependent ABCC4 inhibition using MK-571 and Ceefourin-1 (**Fig. 1**) caused a decrease in extracellular cAMP levels in both cell lines. The half-maximal inhibitory concentration (IC_{50}) values of MK-571 and Ceefourin-1 were found to be 2.6 μM and 0.7 μM respectively, defining a role for ABCC4-mediated cAMP transport in human airway epithelial cells that may be leveraged for CFTR potentiation.

Detection of ABCC4 and CFTR in human airway epithelial cell lines

The expression and function of ABCC4 has been confirmed in HBEC6-KT cells (**Supplementary Fig. 1**), although CFTR expression and function remain to be defined[41,42]. In contrast, human airway epithelial Calu-3 cells are well-documented for CFTR expression and function but not for ABCC4[53–55].

ABCC4 and CFTR protein expression levels were therefore probed via immunoblot in HBEC6-KT and Calu-3 cell lines, with total protein staining used as a loading control (**Fig. 2**). Due to the reported time-dependent differentiation and polarization of Calu-3 cells, protein was extracted at four different time points (0, 7, 14, and 21 days post-

confluency)[53,56–58]. ABCC4 was present in both HBEC6-KT and Calu-3 cells at all time points, as indicated by the bands at 150 kDa. CFTR was present at all time points in Calu-3 cells, but no CFTR was detected in HBEC6-KT cells. Due to the co-expression of ABCC4 and CFTR, Calu-3 cells were used for subsequent studies exploring the interrelationships between ABCC4, cAMP modulation, and CFTR.

Receptor-dependent and -independent activation of CFTR activity – Impact of VX-770

A concentration-response analysis was performed with forskolin (FSK) and isoproterenol (ISO), which are G protein-coupled receptor-independent and -dependent cAMP inducers, respectively (**Fig. 3**). The half-maximal effective concentration (EC_{50}) values for FSK and ISO were determined to be 0.19 μM and 0.07 μM respectively, with both compounds demonstrating the ability to induce CFTR activity as measured by a validated membrane potential sensitive assay[14].

Concentration-response curves with CFTR modulator VX-770, using both receptor-independent FSK and receptor-dependent ISO cAMP inducers (**Fig. 4a and e**), demonstrated an upward and leftward shift in the curve, indicating an increase in CFTR activity. Area under the curve (AUC) analysis showed a significant increase with the addition of VX-770 with either cAMP inducer (**Fig. 4b and f**; $^{**}P \leq 0.01$ and $^{****}P \leq 0.0001$). VX-770 increased the sensitivity of FSK-induced CFTR activity as measured by EC_{50} analysis (**Fig. 4c**; $^{**}P \leq 0.01$). However, the maximal effective

concentration (E_{max}) remained unchanged (**Fig. 4d**). Despite changes in AUC, no changes in EC_{50} or E_{max} were observed with the combination of VX-770 and ISO (**Fig. 4g and h**).

Collectively, these results demonstrate that both receptor-dependent and receptor-independent mechanisms of cAMP elevation can induce CFTR activity in Calu-3 cells, which is further potentiated by VX-770.

Effect of ABCC4 and PDE-4 inhibition on CFTR activity

Following our quantification of CFTR activity with the clinically approved CFTR potentiator VX-770, we next investigated the effect of ABCC4 inhibition and PDE-4 inhibition in Calu-3 cells.

For pharmacological inhibition of ABCC4, we used MK-571 and Ceefourin-1 (**see Fig. 1**) with FSK and ISO (**Fig. 5a-d**). No combination of ABCC4 inhibitor and cAMP elevating agent changed CFTR activity in Calu-3 cells when examining AUC, EC_{50}, E_{max}, and baseline values (**Fig. 5e-h**).

For pharmacological inhibition of PDE-4, we used Roflumilast and Rolipram with FSK and ISO (**Fig. 6a-d**). In contrast to ABCC4 inhibition, the concentration-response curves of PDE-4 inhibitors showed leftward shifts with ISO (**Fig. 6c and d**). Similarly, AUC analysis revealed significant increases with the addition of either Roflumilast or Rolipram with ISO, but not FSK (**Fig. 6e**; $*P \leq 0.05$). EC_{50}, E_{max}, and baseline values for PDE-4 inhibitors with either cAMP inducer were not different (**Fig. 6f-h**). In addition to Roflumilast and Rolipram, pharmacological inhibition using non-specific PDE inhibitor IBMX was also tested (**Supplementary Fig. 2**). Significant increases in AUC

(**Supplementary Fig. 2c and i**; **P≤0.05; **P≤0.01*) were observed with either cAMP inducer. Additionally, significant increases in baseline values for IBMX were revealed with ISO only (**Supplementary Fig. 2l**; **P≤0.05*).

Effect of cAMP modulation with VX-770 on CFTR activity

Due to our observations that ABCC4 and PDE-4 inhibition alone showed minor potentiation of CFTR activity relative to VX-770, we next investigated whether a combinatorial approach could be more efficacious[13].

Concentration-response curves of VX-770 + MK-571 or Roflumilast combinations with FSK stimulation led to an upward shift of the curve at all concentrations, indicating an increase in CFTR activity (**Fig. 7a and b**). VX-770 + MK-571 or Roflumilast combinations with ISO also led to shifts in the curves, but only at lower concentrations (**Fig. 7c and d**). For the AUC analysis of each combination, increases were observed with FSK but not ISO (**Fig. 7e**; ***P≤0.01*, and ****P≤0.001*). EC_{50} and E_{max} analysis for each combination with both cAMP inducers showed no difference (**Fig. 7f and g**).

The consequences of these pharmacological intervention combinations with FSK, in addition to Roflumilast + MK-571 + VX-770 combinations, were also tested in primary bronchial epithelial cells (PBECs), from two independent donors, grown under air-liquid interface culture conditions (**Supplementary Fig. 3**). In one donor sample (male, age 47), significant increases in AUC and max peak analysis, beyond VX-770 alone, were observed. For AUC, Roflumilast ± VX-770, Roflumilast + MK-571, and Roflumilast + MK-571 + VX-770 combinations were significantly greater than VX-770 alone (**Supplementary Fig.**

3c; ***P≤0.001; ****P≤0.0001*). Similarly, the max peak achieved with Roflumilast ± VX-770, MK-571 + VX-770, Roflumilast + MK-571, and Roflumilast + MK-571 + VX-770 were significantly greater than VX-770 alone (**Supplementary Fig. 3d**; **P≤0.05; ****P≤0.0001*). These effects were not observed in the other donor sample (male, age not provided) (**Supplementary Fig. 3g and h**).

To compare our series of cAMP modulation interventions with and without VX-770 (**Fig. 4-7**), fold-change comparisons of AUC, EC_{50}, and E_{max}, relative to control conditions, were performed for all experiments (**Fig. 8**). Fold-change analysis of AUC with FSK stimulation showed that VX-770 was superior to either ABCC4 (MK-571) or PDE-4 (Roflumilast) inhibition alone, and that combinatorial approaches did not potentiate CFTR AUC values (**Fig. 8a**, ***P≤0.01* and ****P≤0.001*). ISO stimulation resulted in similar trends that were not significant (**Fig. 8d**). Fold-change EC_{50} analysis between VX-770 alone and VX-770 with ABCC4 or PDE-4 inhibition showed a significant decrease with ISO for both combinatorial approaches, observations that were absent with FSK stimulation (**Fig. 8b and e**, **P≤0.05*). Lastly, fold-change E_{max} analysis performed with either FSK or ISO showed no changes for any combinatorial approach (**Fig. 8c and f**).

Discussion

There is an increased need for combinatorial therapies for CF subjects to optimally treat all subjects at a personalized level[13,25,27,29]. CFTR potentiation with VX-770 represents a high-water mark for increased chloride conductance in CF patients. In the present study, we quantified the capacity of combinatorial approaches that modulate intracellular cAMP levels to activate CFTR. Specifically, we inhibited ABCC4, a cAMP-efflux pathway, and PDE-4, a cAMP metabolism pathway. Using Calu-3 cells, we demonstrate that interventions targeting intracellular cAMP are unable to potentiate CFTR to levels observed with VX-770. Combinatorial approaches of cAMP modulation with VX-770 suggest that increases in CFTR activity, as measured by AUC and EC_{50} values, are possible and warrant further exploration in primary human airway epithelial cells expressing loss of function CFTR variants[14].

The addition of VX-770 to Calu-3 cells induced shifts in the curve with both FSK and ISO, providing context for the amount of CFTR potentiation possible in Calu-3 cells with a clinically approved CFTR potentiator. While there was a significant change to EC_{50} with FSK, no changes were seen with ISO, suggesting that mechanisms governing cAMP elevations may influence downstream analyses of CFTR activities[59–63]. For both cAMP inducers, the E_{max} values were not significant and while there were no significant changes to baseline with FSK, there was a significant increase in baseline with ISO (data not shown). Collectively, this suggests not only is the mechanism of action in elevating cAMP levels affecting sensitivity, but also efficacy[59–63]. Collectively, these results suggest that it is possible to potentiate CFTR activity in Calu-3 cells by modulating cAMP levels.

Combinatorial approaches have been suggested as the future for modulating CFTR activity and may include direct or indirect approaches[13]. Modulation of cAMP levels functions as an indirect mechanism to modulate CFTR and could potentially be combined with direct CFTR modulator approaches. We therefore decided to investigate ABCC4 and PDE-4 inhibitors, which differentially modulate cAMP levels by blocking extracellular transport and intracellular breakdown, respectively.

It has been previously demonstrated that pharmacological inhibition of ABCC4 by MK-571, in combination with VX-770, was able to improve CFTR activity in primary nasal epithelial cells from CF patients heterozygous for the G551D-variant[14]. In addition, in non-CF PBECs, it has been demonstrated that the administration of Roflumilast led to CFTR activation[38]. However, in this same study, the addition of Roflumilast to Calu-3 cells did not potentiate CFTR activity beyond the max stimulation that resulted from the addition of FSK[38].

In our concentration-response analysis of ABCC4 and PDE-4 inhibitors in Calu-3 cells, only mild CFTR potentiation was observed alone or in combination with VX-770. Our results for ABCC4 inhibition contrast with our previous demonstration that the ABCC4 inhibitor MK-571 potentiates CFTR activity in primary human airway epithelial cells expressing the G551D-variant[14]. Conversely, our results for PDE-4 inhibitors align with previous findings in PBECs that were exposed to whole cigarette smoke, in which Roflumilast in combination with VX-770 demonstrated an increase in short-circuit current, suggesting this combination leads to an increase in CFTR activity[38]. We highlight that it may be possible that wild-type CFTR expressed in Calu-3 cells is maximally active and

difficult to further potentiate with cAMP modulating agents. Our data showing shifts in the left of the curve (increased sensitivity of CFTR activity) without changes in maximal response support this concept. Specifically, the addition of VX-770 with either cAMP inducer to Calu-3 cells led to an upward shift in the curve and a significant increase in AUC, but no significant changes to E_{max}. However, VX-770 with FSK showed a significant decrease in EC_{50}, and VX-770 with ISO showed a significant increase in baseline values. When VX-770 was combined with an ABCC4 or PDE-4 inhibitor and cAMP inducer FSK, there were significant increases in AUC, indicating an increase in CFTR activity, but again, no change in E_{max}. To determine whether the combination of ABCC4 and PDE-4 inhibitors with VX-770 had an additive effect beyond VX-770 alone, a fold-change analysis for AUC, EC_{50}, and E_{max} values from the independent experiments was performed for indirect comparisons. Combinatorial additions of VX-770 with ABCC4 or PDE-4 inhibitor, in the presence of cAMP inducer ISO, led to a decrease in fold EC_{50} values compared to VX-770 alone, suggesting that ABCC4 and PDE-4 inhibitors may increase the sensitivity to VX-770. In addition, with cAMP inducer FSK, VX-770 with Roflumilast had a significant increase in fold baseline values, suggesting that there may also be an increase in efficacy. With these fold-change comparison analyses in mind, further investigation of these combinatorial treatments should be performed.

Our interrogation of the role of ABCC4 in airway epithelial cell modulation of cAMP levels is grounded in reports from primary human airway epithelial cells and cell lines, while examining CFTR activity in Calu-3 cells has a strong foundation[35,41,42,53,54]. To determine the optimal cell system to perform ABCC4 interventions with outcomes of CFTR

activity, we interrogated both HBEC-6KT and Calu-3 cells for ABCC4 and CFTR protein expression levels. To our surprise, while ABCC4 is present in both cell lines, CFTR is only present in Calu-3 cells, leading to the selection of Calu-3 cells as the model of study. Our observation highlights the importance of basal expression analysis for CFTR protein in any human airway epithelial cells or lines prior to performing functional experiments.

Elevations in intracellular cAMP can be induced via multiple G protein-coupled receptor-dependent (β-adrenergic receptor and adenosine receptor agonists – ISO and adenosine respectively) and independent mechanisms (direct activation of the enzyme adenylyl cyclase – FSK)[64–68]. The compartmentalization of cAMP signaling may be important for downstream signaling pathways within the cell – with specific implications for CFTR activity[34,69]. To determine whether the mechanism of cAMP production impacts CFTR activity, Calu-3 cells were treated with FSK and ISO at varying concentrations. Sigmoidal concentration-response curves were observed for both cAMP elevating agents, with the maximal degree of change in CFTR activity being greater with ISO. This observation suggests that the compartmentalization of cAMP to the plasma membrane may more effectively couple protein kinase A activation to CFTR phosphorylation, which could be masked by global cytosolic increases in cAMP induced by FSK[34,69].

In our previous studies, we did not perform a complete concentration-response analysis for the leukotriene receptor antagonist MK-571, which has off-target ABCC4 inhibition effects, alongside the more selective ABCC4 inhibitor Ceefourin-1[14,42,45]. Therefore, we wanted to explore both in greater detail. In our concentration-response analysis experiments using an *in vitro* extracellular cAMP assay platform, both

commercially available Ceefourin-1 and MK-571 were able to decrease extracellular cAMP levels. Therefore, both were used in downstream *in vitro* CFTR membrane potential assays to investigate the consequences of ABCC4 inhibition on CFTR function. Since both compounds showed similar results in the *in vitro* CFTR membrane potential assay, and MK-571, in combination with VX-770, was previously demonstrated to potentiate CFTR activity beyond VX-770 alone, we selected MK-571 for combinational studies[14].

While our findings suggest that cAMP modulation has the potential to be used as an add-on therapy with existing therapeutics, limitations of the study should be noted. Although Calu-3 cells expressed both ABCC4 and CFTR, it might not have been an appropriate model of study due to its wild-type CFTR expression. Potentiation of CFTR activity by ABCC4 or PDE-4 inhibitors in the literature were performed in primary cells, suggesting that a potential add-on combinatorial therapy may only show an effect in primary cell cultures, especially ones with CFTR defects. This emphasizes that further investigation of ABCC4 and PDE-4 inhibitors as a potential add-on combinatorial therapy should be performed in primary human airway epithelial cells from a large subset of CF subjects. Such studies should cover a wide range of CFTR variants with compromised CFTR expression or function to determine which patient populations could benefit from cAMP modulation therapy. In addition, further investigations of cAMP modulation with other drugs that modulate CFTR, such as VX-770, VX-809, VX-661, VX-445, and genistein, should be tested as it may be more efficacious.

Materials and Methods

Reagents

The human bronchial epithelial cell (HBEC6-KT) line was cultured in Keratinocyte Serum-Free Medium 1X (Gibco) according to the manufacturers directions and supplemented with P/S (VWR) at 1X[70]. Human airway epithelial cell line Calu-3 (ATCC HTB-55) were cultured in Minimum Essential Medium Alpha Medium (Corning) and supplemented with Premium Grade FBS (VWR) at 10%, HEPES (Corning) at 1X, and P/S (VWR) at 1X. The following chemical reagents were dissolved in DMSO, cAMP elevating agents Forskolin (Cayman Chemical) and Isoproterenol (Cayman Chemical), ABCC4 inhibitors MK-571 (Cayman Chemical) and Ceefourin 1 (Abcam), PDE inhibitors Roflumilast (Cayman Chemical) and Rolipram (Cayman Chemical), and CFTR Inhibitor CFTRinh-172 (Selleck Chemicals).

Human airway epithelial cell lines

Two human airway epithelial cell lines were used for *in vitro* experiments. HBEC6-KT cells, provided by Dr. John Minna and Dr. Darryl Knight, are derived from healthy non-smoking individuals and immortalized through human telomerase reverse transcriptase and cyclin-dependent kinase 4 expression. These cells were used for *in vitro* concentration-response analysis of ABCC4 inhibitors using an extracellular cAMP assay[41,42,70–72]. Calu-3 cells, derived from the metastatic site of lung adenocarcinoma tissue, were used for *in vitro* analysis of CFTR function.

ABCC4 and CFTR protein expression analysis

HBEC6-KT and Calu-3 cells were lysed using RIPA Lysis Buffer containing Protease Inhibitor Cocktail powder (Sigma-Aldrich) for 60-90 minutes at 4°C on a rocker. Lysates were centrifuged at 16,000xg for 15 minutes, and the supernatants were collected for downstream immunoblot analysis of ABCC4 and CFTR. Protein quantification was performed using a BCA protein assay. Twenty micrograms of protein were loaded on 4-15% gradient TGX Stain-Free Protein Gels and transferred to Immun-Blot LF PVDF membranes (Bio-Rad). The membranes were blocked with 1X TBS with 0.05% Tween 20 and 5% skim milk powder for 2 h at 25°C. The membranes were then incubated with primary antibody ABCC4/MRP4 (1:40, Abcam, AB15602) or CFTR (1:5000, UNC-Chapel Hill, AB596) overnight. The membranes were then washed in 1X TBS with 0.05% Tween 20 and incubated with HRP-linked anti-rat secondary antibody (1:3000, Cell Signaling Technology, 7077S) or anti-mouse secondary antibody (1:3000, Cell Signaling Technology, 7076S) for 2 h at 25°C. A chemiluminescence image of the blot was taken using the Bio-Rad Image Lab software. Full western blot images have been included and can be found in the supplementary information (**Supplementary Fig. 4**).

***In vitro* extracellular cAMP assay**

Calu-3 and HBEC6-KT cells were cultured as described previously[41,42,71,72]. Cells were pretreated with IBMX (20 μM) for 2 h prior to experimental conditions. After the pre-treatment of IBMX, cells were treated with ABCC4 inhibitors (0.01-100 μM) or DMSO for 30 min. Following these exposures, the cells were treated with forskolin (10 μM) for 6 h

and cell culture supernatants were collected for analysis of extracellular cAMP. The negative control was IBMX alone and the positive control was IBMX and forskolin.

***In vitro CFTR* membrane potential assay**

An *in vitro* CFTR membrane potential assay was performed on Calu-3 cells and primary bronchial epithelial cells, from two independent donors, cultured at 37°C as previously described[14]. All human samples were collected from consented individuals under ethics approval granted by Hamilton Integrated Research Ethics Board (Project Number 5099-T).

Calu-3 cells were grown for 21 days on 96-well plates, while primary HBECs were cultured for 14 days under air-liquid interface conditions on collagen-coated (Sigma-Aldrich) 6.5 mm Transwell Inserts (Corning) after reaching 100% confluency. All cells were washed with HBSS prior to experimental conditions. After washing with HBSS, cells were loaded with BLUE Membrane Potential Dye (Molecular Devices, #R8042) dissolved in 37°C chloride-free buffer (NMDG Gluconate Buffer – 150 mM NMDG-gluconate, 3 mM potassium gluconate, 10mM HEPES, pH 7.35, 300 mOsm). Immediately after dye loading, CFTR activity measurements were taken. CFTR activity measurements are fluorescent readouts for detecting ion channel activity. CFTR inhibition was performed with CFTRinh-172 (10 μM) for all experiments to attribute changes in fluorescence as CFTR channel activity. This assay has been validated using proof of concept studies where results obtained from this assay were able to recapitulate findings obtained from direct measurements of ion channel activity using Ussing chambers[14]. At the end of this assay,

raw data was exported for statistical analysis. Tracings demonstrating the variation in CFTR Activity as a function of time, for key experiments, have been included in the supplementary information (**Supplementary Fig. 5**).

For Calu-3 cells, measurements were taken during baseline (40 min), cAMP elevation (30 min), and CFTR inhibition (20 min). cAMP elevation was performed using forskolin or isoproterenol (0.0005-50 μM). For assays with ABCC4 or PDE-4 inhibitors, a pre-incubation with the inhibitors (30 min) in the plate reader was performed prior to cAMP elevation. CFTR modulator VX-770 was used at 1 μM. CFTR activity was determined by dividing a single membrane potential peak measurement after the ABCC4 or PDE-4 inhibition and cAMP elevation additions to the stabilized baseline over DMSO control.

For PBECs, measurements were taken during baseline (8 min), drug additions (40 min), and CFTR inhibitions (18 min). cAMP elevation was performed using forskolin (10 μM). The same concentrations used in Calu-3 cells of ABCC4 and PDE4 inhibitors and VX-770 were used for PBECs, but pre-incubation omitted. Instead, all drug combinations were added once at the beginning of the drug addition step. CFTR activity was determined by averaging six independent measurements within a single Transwell insert. Measurements were normalized to the averaged baseline over DMSO control.

Statistical analysis

For the *in vitro* extracellular cAMP assay, values were normalized to the positive control (IBMX and forskolin) and the half-maximal inhibitory concentration (IC_{50}) was determined. SD was calculated using data from biological replicates (n=4-5).

For *in vitro* CFTR membrane potential assays, in Calu-3 cells, values were normalized to the averaged baseline over averaged vehicle (DMSO) control of all biological replicates. SD was calculated using data from biological replicates (n=4-8). An area under the curve (AUC), half-maximal effective concentration (EC_{50}), maximal effective concentration (E_{max}), and baseline analysis was performed for each individual replicate then averaged together. The AUC analysis is an aggregate measure of sensitivity and maximal response, allowing for the observation of the total net responsiveness of the cells. The AUC analysis was done by normalizing the individual replicates to the bottom most value of the averaged vehicle control. In PBECs, six independent measurements were taken within a single Transwell insert. The value is the average of at least six measurements. These values were normalized to the averaged baseline over the averaged vehicle (DMSO) control. SD was calculated by treating the six measurements as independent, with additional technical replicates (n=6-18). An AUC and max peak analysis were performed for each measurement, then averaged together. The max peak analysis was the max CFTR activity achieved during the drug addition step.

To compare the independent experiments performed, a fold-change analysis using individual AUC, EC_{50}, and E_{max}, determined from their respective concentration-response curves, was performed and was normalized to the averaged DMSO vehicle control. A one-way ANOVA with subsequent post-hoc test or a paired *t*-test was performed. Statistical analysis was performed using GraphPad Prism 6.

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Figures

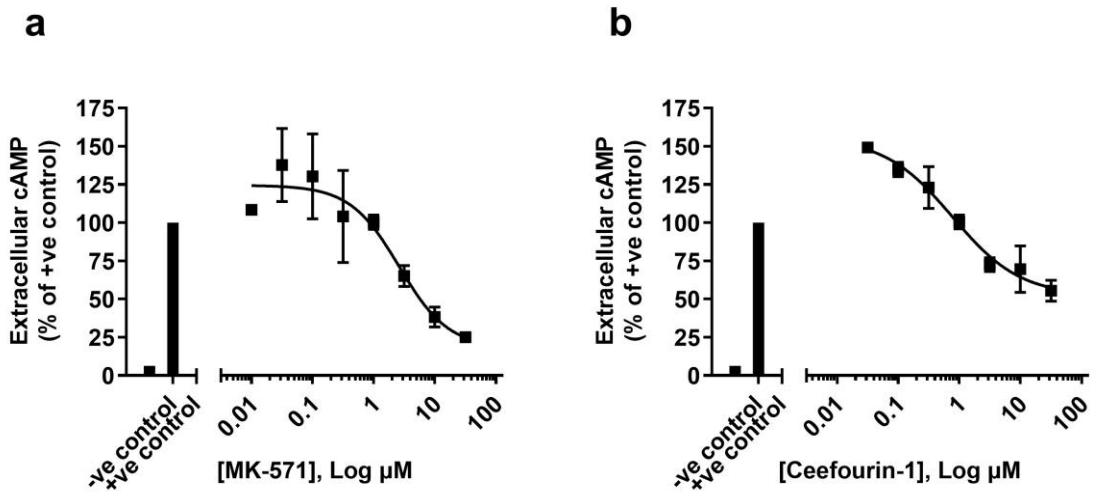

Figure 1: Concentration-response analysis of compounds with ABCC4 inhibition properties on extracellular cAMP from human airway epithelial cells. Human airway epithelial (Calu-3) cells were pre-treated with IBMX (20 µM), exposed to (**a**) MK-571, (**b**) Ceefourin-1, or DMSO vehicle control, and then treated with forskolin (10 µM). Cell culture supernatants were assessed for cAMP levels 24h post-treatment. Each concentration-response curve was normalized to positive control (IBMX + Forskolin) with data presented as means ± standard deviations (n=3, MK-571; n=3, Ceefourin-1).

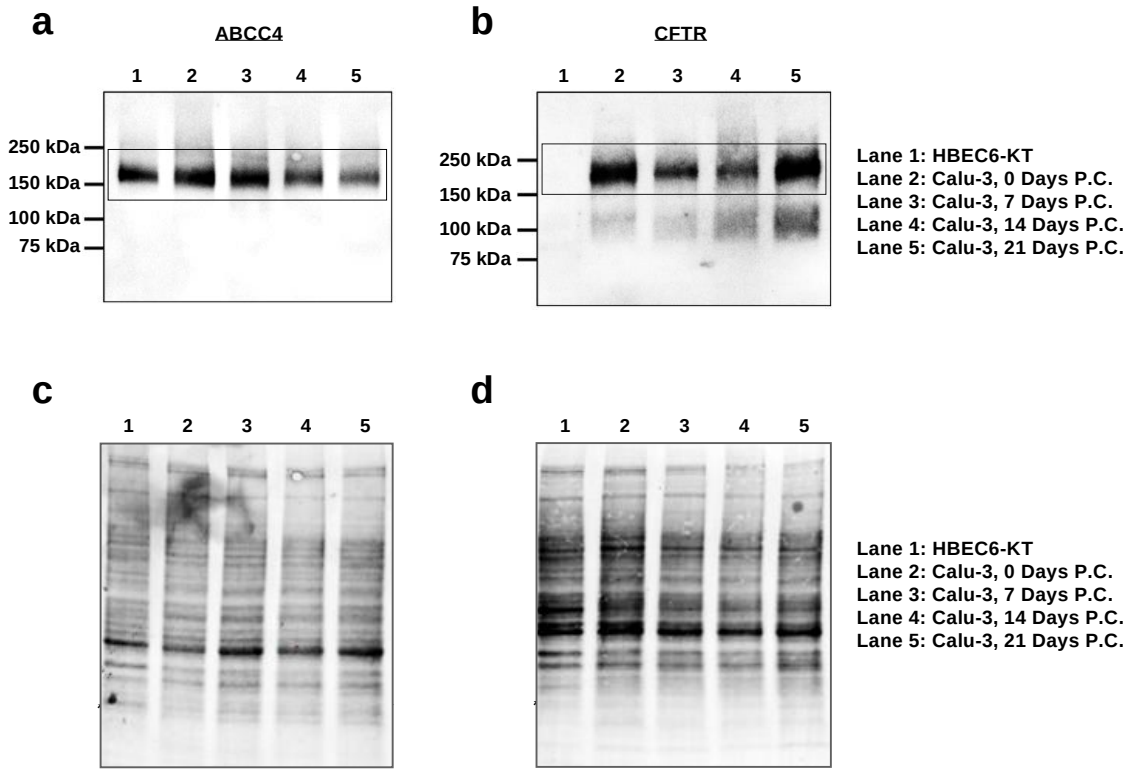

Figure 2: Characterization of ABCC4 and CFTR protein expression in two human airway epithelial cell lines. (**a**) ABCC4 and (**b**) CFTR in both HBEC6-KT and Calu-3 cells. For Calu-3 cells, extracts were retrieved at several different time points post-confluency (P.C.). (**a**) ABCC4 was detected in both HBEC6-KT and Calu-3 cells and (**b**) CFTR was only detected in Calu-3 cells. A total protein stain was performed as the loading control for (**c**) ABCC4 and (**d**) CFTR blots. Full-length blots are presented in Supplementary Fig. 4.

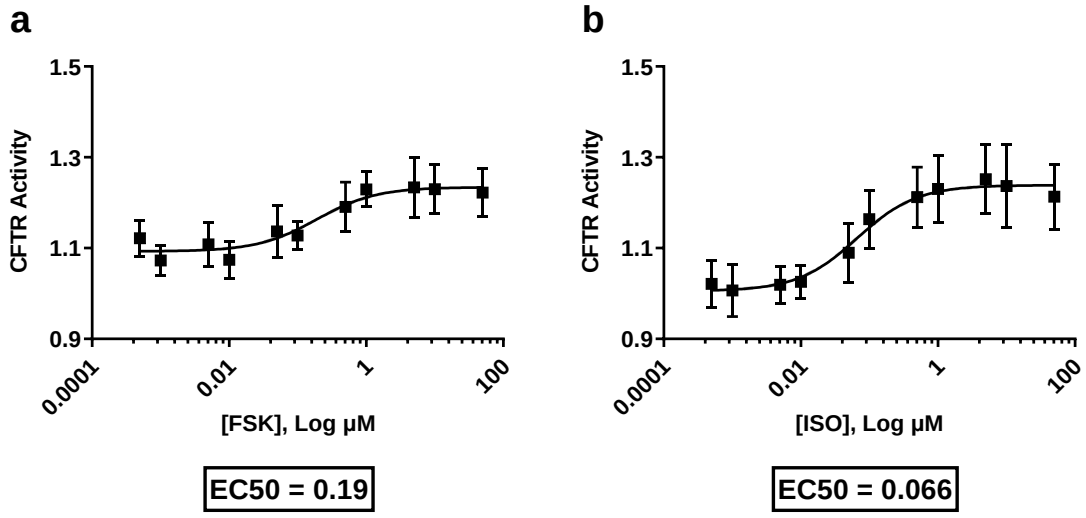

Figure 3: **Concentration-response analysis of two different cAMP elevating agents on CFTR activity.** Calu-3 cells were exposed to G protein-coupled receptor-independent cAMP inducer (**a**) forskolin and G protein-coupled receptor-dependent cAMP inducer (**b**) isoproterenol at increasing concentrations while measuring CFTR activity using an *in vitro* CFTR membrane potential assay. EC_{50} values were analysed and reported. Each concentration-response curve was normalized to baseline over DMSO vehicle control with data presented as ± standard deviations (n=8).

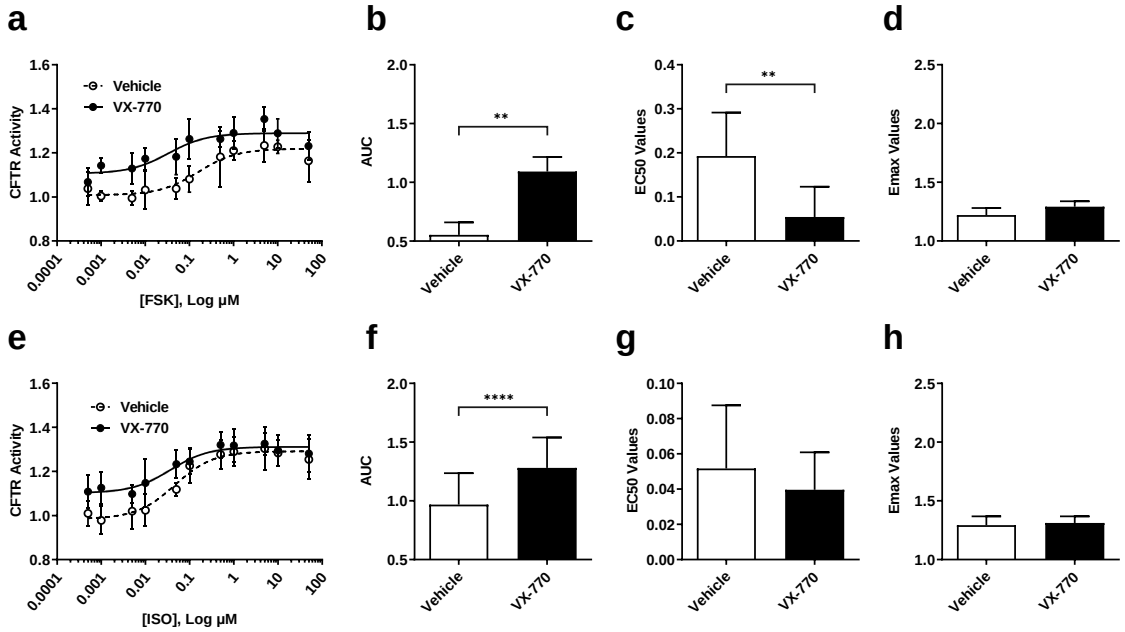

Figure 4: Consequences of CFTR modulator VX-770 on CFTR function using receptor-independent (forskolin) and receptor-dependent (isoproterenol) cAMP inducers. Calu-3 cells were stimulated with VX-770 (1 µM) in the presence of cAMP inducer (**a**) forskolin and (**e**) isoproterenol. Analysis of concentration-response curves was performed for (**b** and **f**) AUC, (**c** and **g**) EC_{50}, and (**d** and **h**) E_{max}. The concentration-response curve was normalized to baseline over DMSO vehicle control. All data presented as ± standard deviations (n=4, FSK; n=8, ISO). A paired *t*-test was used for statistical analysis. ***P≤0.01*; ******P≤0.0001*

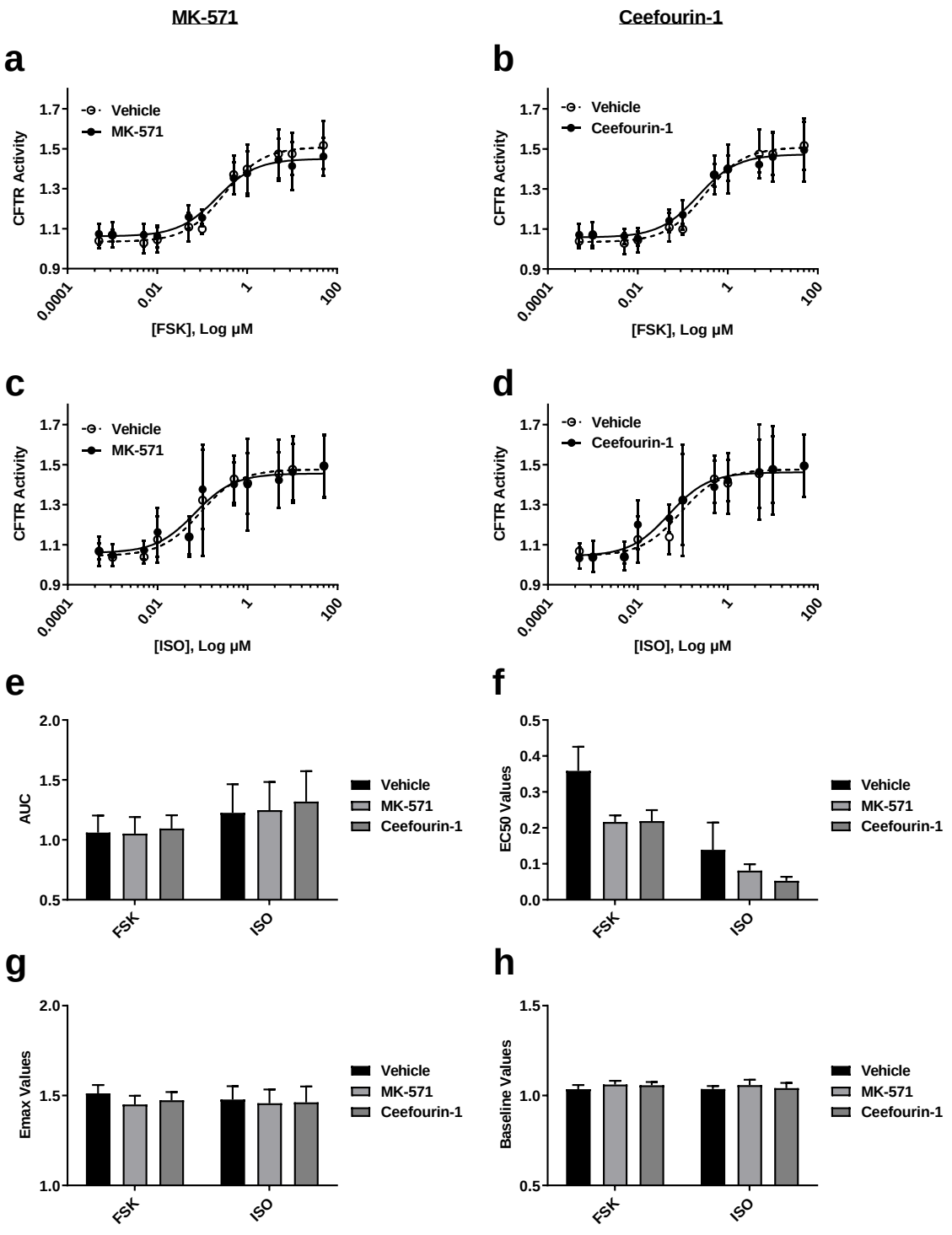

Figure 5: *Figure legend continued on the following page.*

Figure 5: **Consequences of ABCC4 inhibition on CFTR function using receptor-independent (forskolin) and receptor-dependent (isoproterenol) cAMP inducers.** ABCC4 was inhibited with MK-571 and Ceefourin-1 using submaximal concentrations determined from extracellular cAMP assays. Forskolin-stimulated (**a-b**) CFTR activity in the presence of (**a**) MK-571 (1.8 μM) and (**b**) Ceefourin-1 (4.8 μM). Isoproterenol-stimulated (**c-d**) CFTR activity in the presence of (**c**) MK-571 and (**d**) Ceefourin-1. Analysis of concentration-response curves was performed for (**e**) AUC, (**f**) EC_{50}, (**g**) E_{max}, and (**h**) Baseline. Each concentration-response curve was normalized to baseline over DMSO vehicle control. All data presented as means ± standard deviations (n=5, FSK; n=4, ISO). A one-way ANOVA with subsequent post-hoc test was used for statistical analysis.

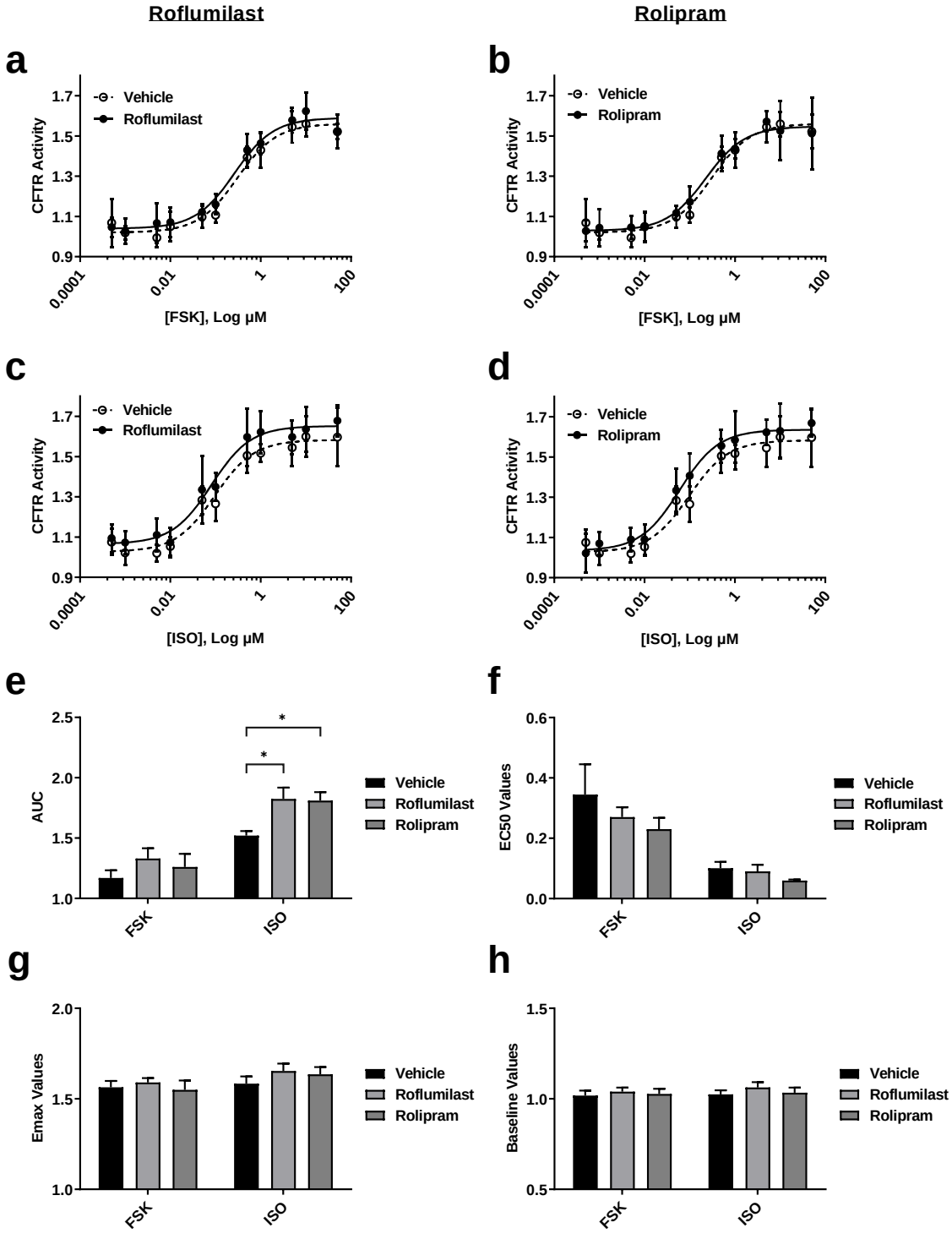

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Figure 6: Consequences of PDE-4 inhibition on CFTR function using receptor-independent (forskolin) and receptor-dependent (isoproterenol) cAMP inducers. PDE-4 was inhibited with Roflumilast and Rolipram at submaximal concentrations. Forskolin-stimulated (**a-b**) CFTR activity in the presence of (**a**) Roflumilast (1 μM) and (**b**) Rolipram (10 μM). Isoproterenol-stimulated (**c-d**) CFTR activity in the presence of (**c**) Roflumilast and (**d**) Rolipram. Analysis of concentration-response curves was performed for (**e**) AUC, (**f**) EC_{50}, (**g**) E_{max}, and (**h**) Baseline. Each concentration-response curve was normalized to baseline over DMSO vehicle control. All data presented as means ± standard deviations (n=5). A one-way ANOVA with subsequent post-hoc test was used for statistical analysis. *$P \leq 0.05$

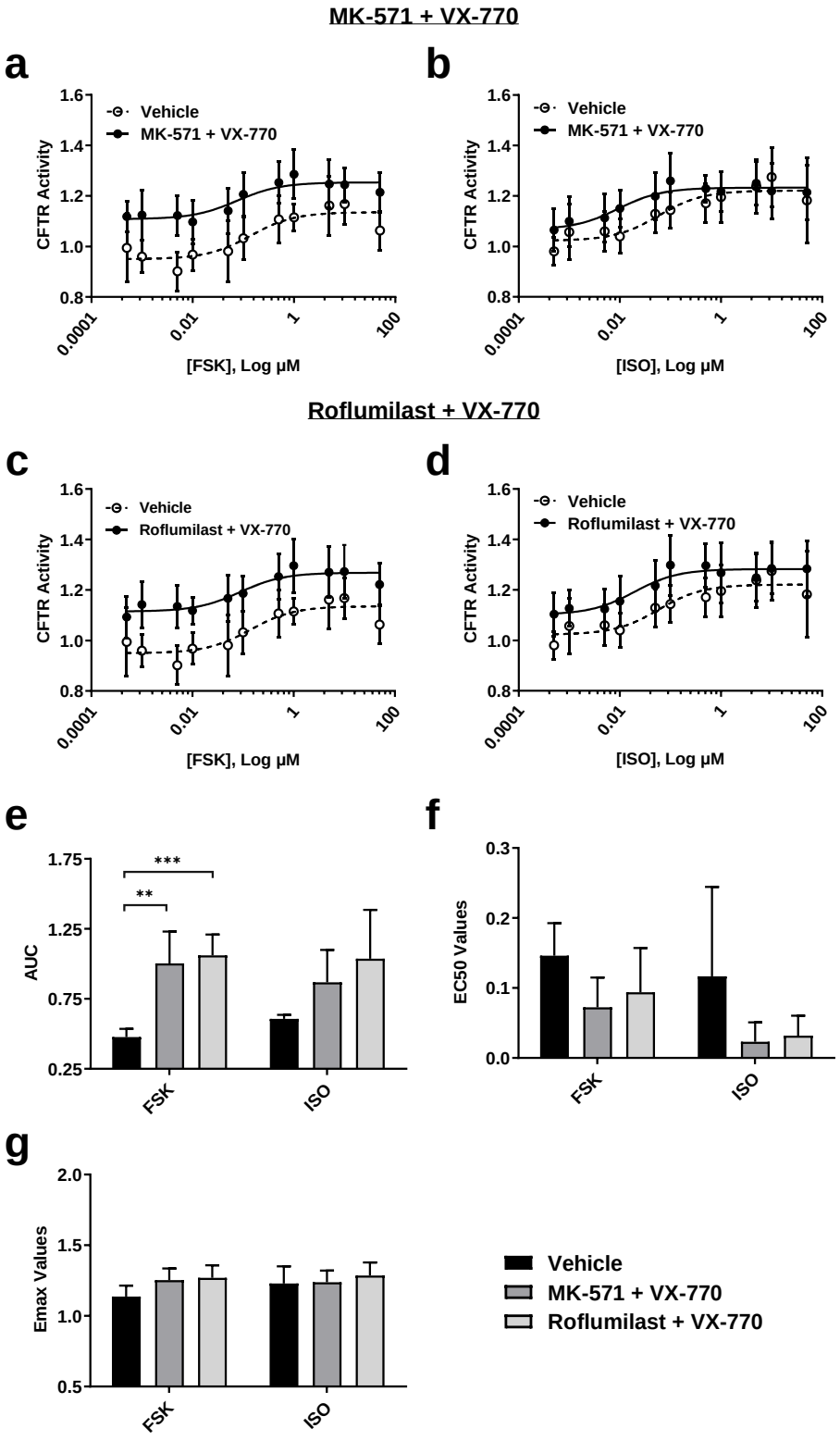

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Figure 7: Consequences of ABCC4 and PDE-4 inhibition on CFTR function in the presence of CFTR modulator VX-770 using receptor-independent (forskolin) and receptor-dependent (isoproterenol) cAMP inducers. ABCC4 and PDE4 were inhibited with MK-571 (1.8 μM) and Roflumilast (1 μM) respectively prior to VX-770 (1 μM) stimulation. Forskolin-stimulated (**a-b**) CFTR activity in the presence of (**a**) MK-571 + VX-770 and (**b**) Roflumilast + VX-770. Isoproterenol-stimulated (**c-d**) CFTR activity in the presence of (**c**) MK-571 + VX-770 and (**d**) Roflumilast + VX-770. Analysis of concentration-response curves was performed for (**e**) AUC, (**f**) EC_{50}, and (**g**) E_{max}. Each concentration-response curve was normalized to baseline over DMSO vehicle control. All data presented as means ± standard deviations (n=6-7, MK-571 + VX-770 and Roflumilast + VX-770). A one-way ANOVA with subsequent post-hoc test was used for statistical analysis. ***P≤0.01*; ****P*≤0.001

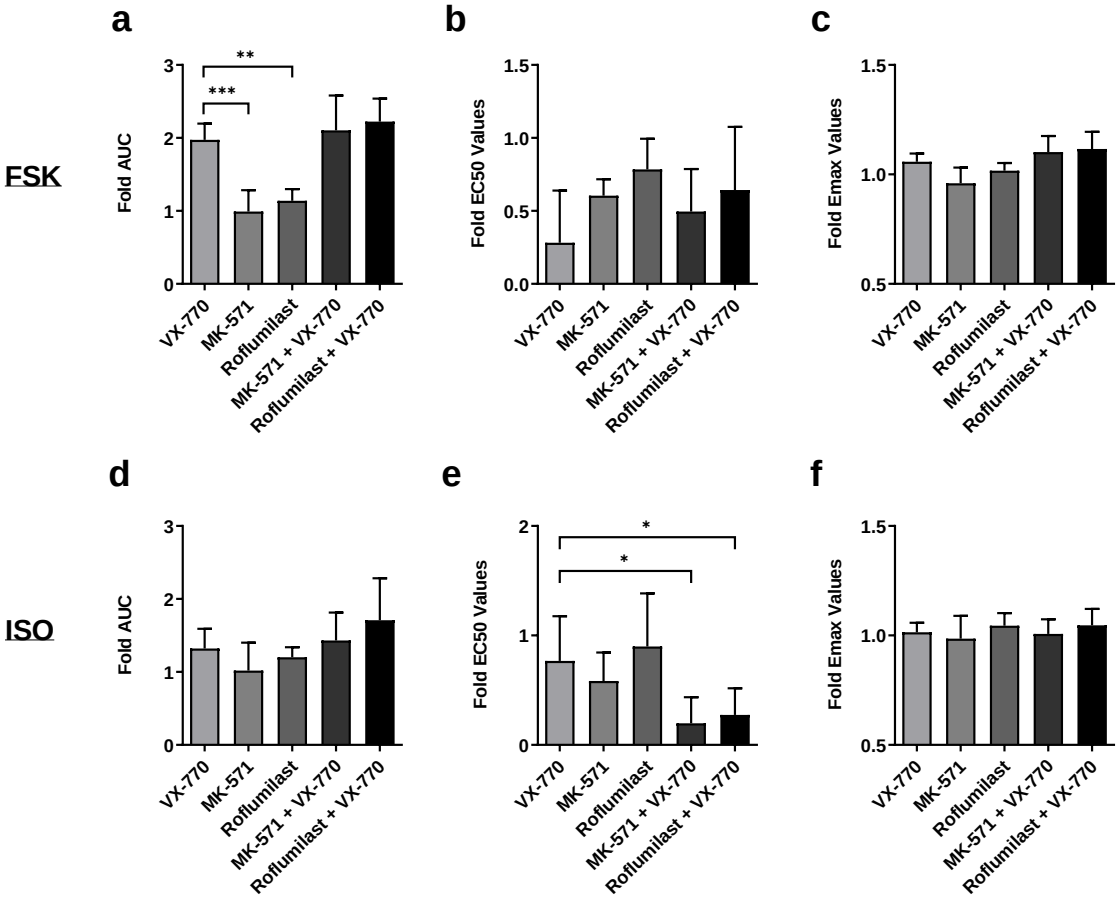
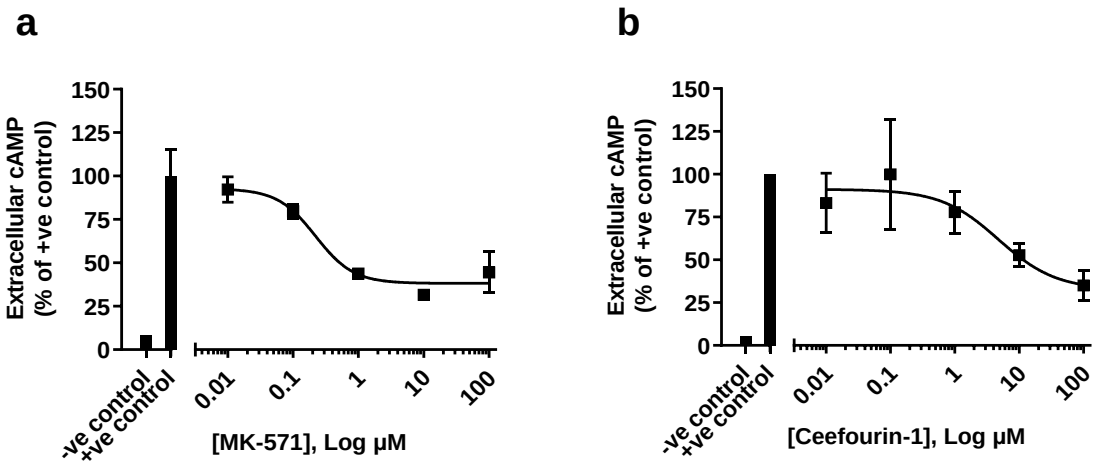


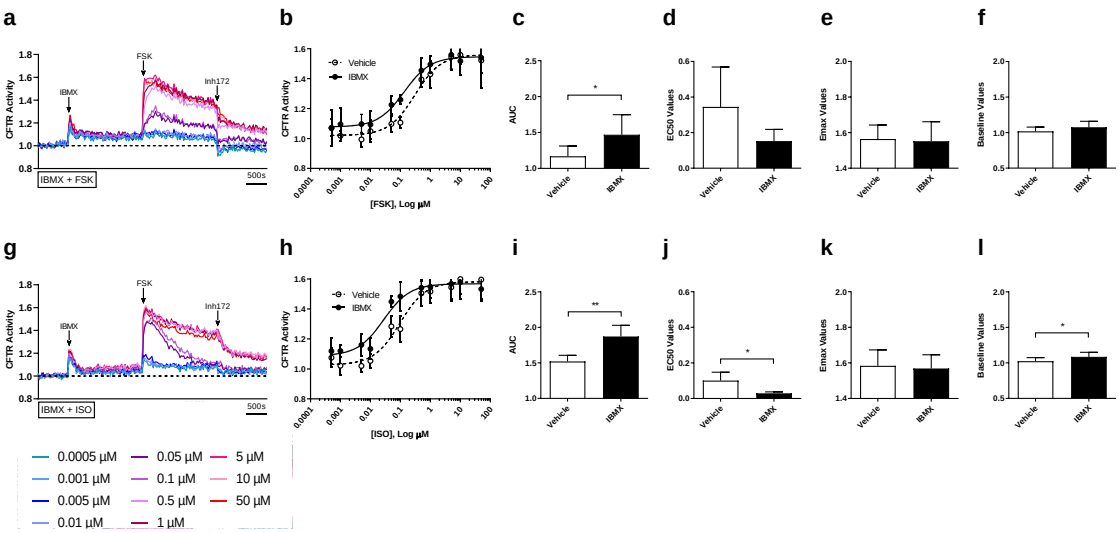
Figure 8: Fold-change comparisons between CFTR modulator VX-770, ABCC4 inhibition, and PDE-4 inhibition. A comparison between CFTR modulator VX-770, ABCC4 inhibitor MK-571, PDE-4 Inhibitor Roflumilast, and VX-770 ± MK-571 or Roflumilast was performed using the values determined from their concentration-response curves in the presence of forskolin (**a-c**) or isoproterenol (**d-f**). Each treatment was normalized to its vehicle control to allow for comparison. A fold-change of (**a** and **d**) AUC, (**b** and **e**) EC_{50}, and (**c** and **f**) E_{max} are depicted. All data presented as means ± standard deviations (n=4-8, VX-770; n=4-5, MK-571; n=5, Roflumilast; n=6-7, MK-571 + VX-770 and Roflumilast + VX-770). A one-way ANOVA with subsequent post-hoc test was used for statistical analysis. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Supplementary Information

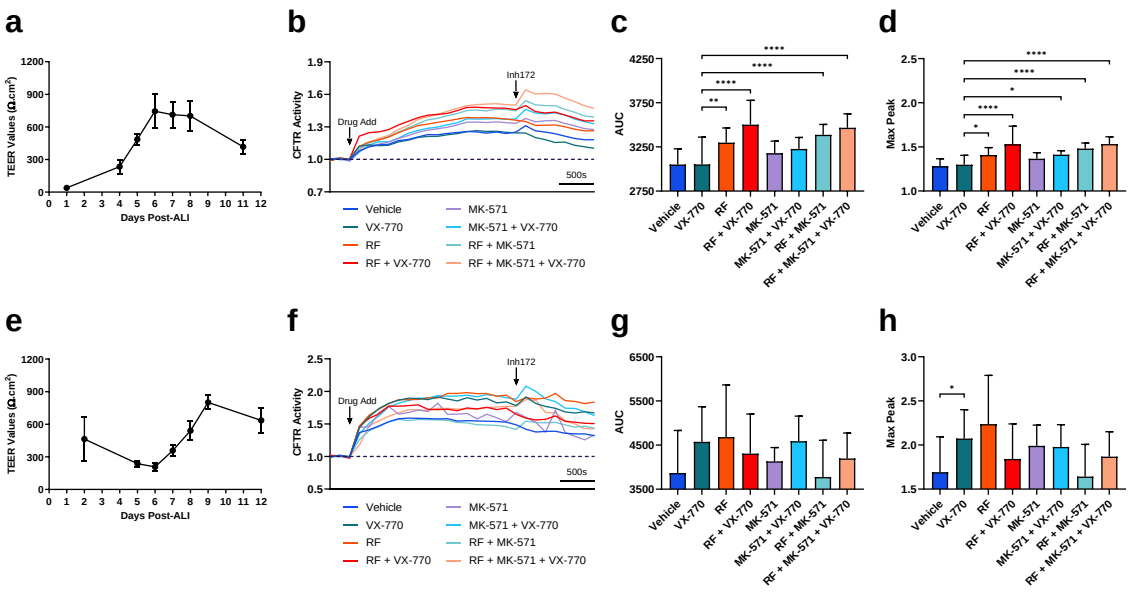
Supplementary Figures



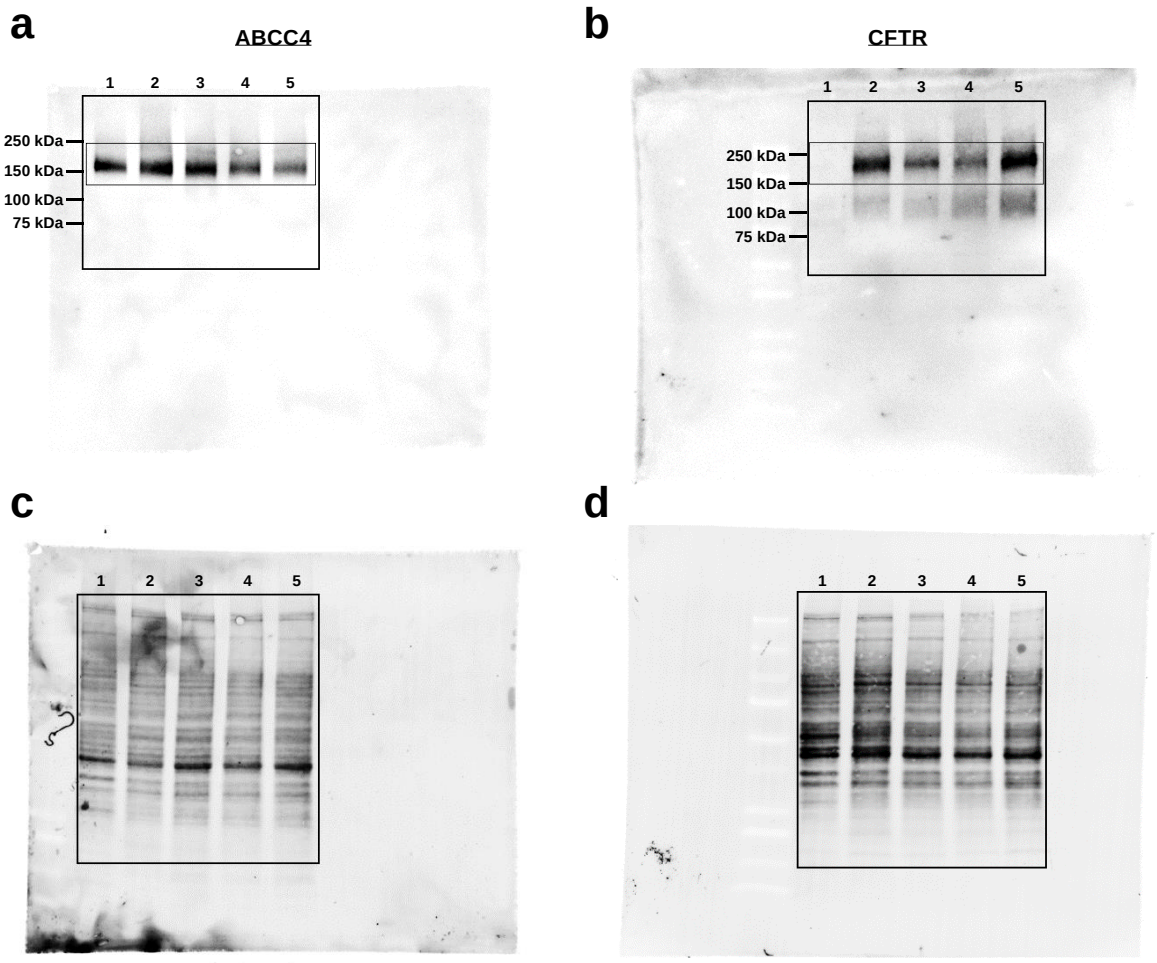
Supplementary Figure 1: Concentration-response analysis of compounds with ABCC4 inhibition properties on extracellular cAMP from human airway epithelial cells. Human airway epithelial (HBEC6-KT) cells were pre-treated with IBMX (20 µM), exposed to (**a**) MK-571, (**b**) Ceefourin-1, or DMSO vehicle control, and then treated with forskolin (10 µM). Cell culture supernatants were assessed for cAMP levels 24h post-treatment. The half-maximal inhibitory concentration (IC_{50}) values of MK-571 and Ceefourin-1 were found to be 0.2 µM and 4.8 µM respectively. Each concentration-response curve was normalized to positive control (IBMX + Forskolin) with data presented as means ± standard deviations (n=4, MK-571; n=5, Ceefourin-1).



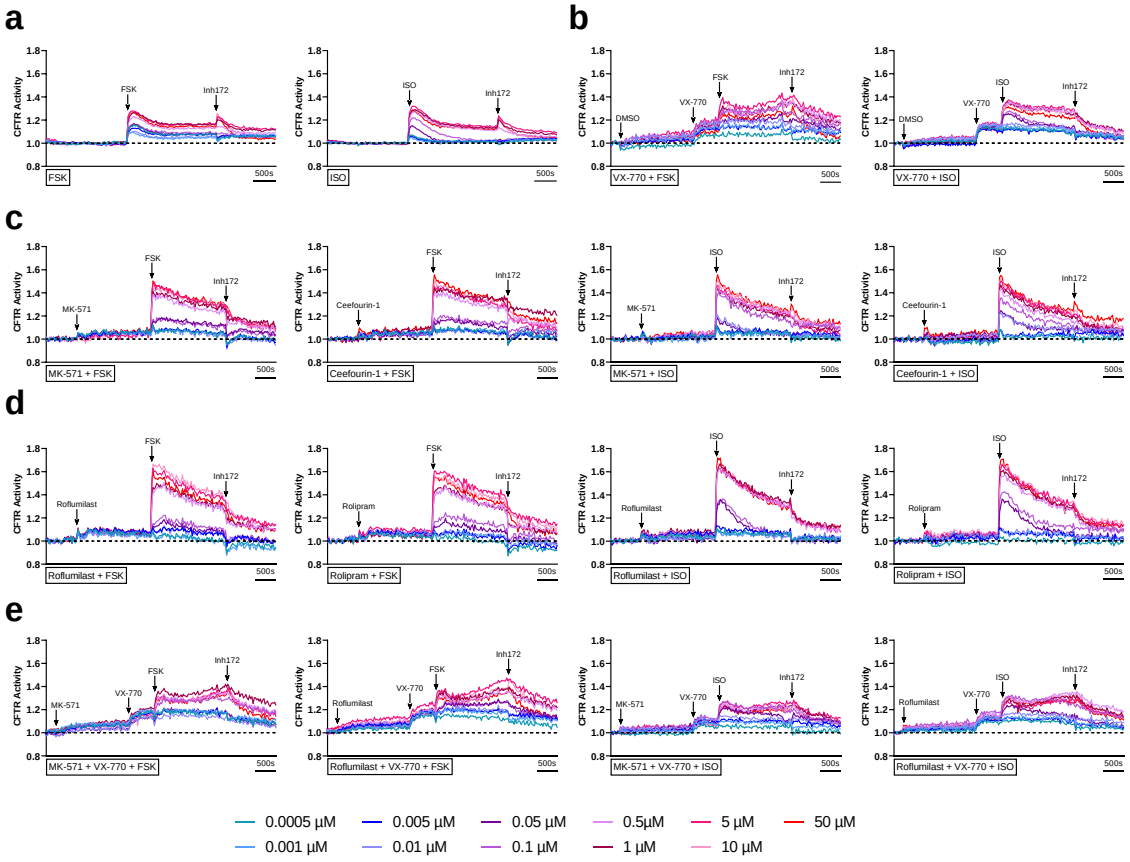
Supplementary Figure 2: Consequences of non-specific PDE inhibitor IBMX on CFTR function using receptor-independent (forskolin) and receptor-dependent (isoproterenol) cAMP inducers. Calu-3 cells were stimulated with IBMX (100 µM) in the presence of cAMP inducers forskolin (FSK) and isoproterenol (ISO). Max peaks obtained after the addition of the cAMP elevating agent from the (**a** and **g**) time course analysis was used to generate the (**b** and **h**) concentration-response curves. Analysis of the concentration-response curves were performed for (**c** and **i**) AUC, (**d** and **j**) EC_{50}, (**e** and **k**) E_{max}, and (**f** and **l**) baseline. The time course and concentration-response curve were normalized to baseline over DMSO vehicle control. All data presented as ± standard deviations (n=5). A paired *t*-test was used for statistical analysis. *$P \leq 0.05$; **$P \leq 0.01$



Supplementary Figure 3: Consequences of pharmacological interventions of CFTR modulator VX-770, ABCC4 inhibition, and PDE-4 inhibition on CFTR activity using a receptor-independent cAMP inducer. Primary bronchial epithelial cells from two donors were treated with combinations of VX-770 (1 μM), MK-571 (1.8 μM), and Roflumilast (RF – 1 μM) in the presence of cAMP inducer forskolin (10 μM). (**a** and **e**) Transepithelial electrical resistance (TEER) measurements were taken for each donor (n=24 and 12, respectively). (**b** and **f**) A time course analysis was generated and analysed for (**c** and **g**) AUC and (**d** and **h**) max peak (n=6-18 technical replicates). All data presented as ± standard deviations. A one-way ANOVA with subsequent post-hoc test was used for statistical analysis. *$P \leq 0.05$; **$P \leq 0.001$; ****$P \leq 0.0001$



Supplementary Figure 4: Full western blot images used in Fig. 2. (**a**) ABCC4 and (**b**) CFTR blots. (**c**) ABCC4 and (**d**) CFTR total protein stain blots.



Supplementary Figure 5: Experimental time course analysis for experiments in Fig. 3-7. (**a**) cAMP elevating agents forskolin (FSK) and isoproterenol (ISO) alone or with (**b**) CFTR modulator VX-770 (1 µM), (**c**) ABCC4 inhibitor MK-571 (1.8 µM) or Ceefourin-1 (4.8 µM), (**d**) PDE-4 inhibitors Roflumilast (1 µM) or Rolipram (10 µM), or a combination of ABCC4 inhibitor MK-571 or PDE-4 inhibitor Roflumilast with VX-770 treatment.

CHAPTER 3:

Effects of environmental air pollutants on CFTR expression and function in human airway epithelial cells

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Author Contributions:

J.P.N. performed *in vitro* CFTR membrane potential assay experiments, data analyses, figure generation, and literature review; contributed to the manuscript conception; drafted and edited the manuscript. R.D.H. performed cell viability assays, *in vitro* oxidative stress assays, data analyses, figure generation; contributed to the manuscript conception and edited the manuscript. Q.T.C. performed western blotting, data analysis, and edited the manuscript. N.T. performed western blotting, microscopy imaging, and edited the manuscript. C.C. contributed to the manuscript conception and edited the manuscript. J.A.H. (Principal Investigator and Corresponding Author) oversaw the entire study (data collection and analysis, manuscript drafting and finalization, and trainee supervision).

Study Overview:

In this study, we investigate the impact of environmental air pollutants, specifically tobacco smoke extract, urban particulate matter, and diesel exhaust particles, on CFTR expression and function in Calu-3 cells. We aim to understand whether these pollutants lead to acquired CFTR dysfunction, and whether pharmacological interventions using cAMP and CFTR modulators can rescue CFTR function. Our findings reveal that both tobacco smoke extract and diesel exhaust particles induce acquired CFTR dysfunction, and that pharmacological intervention can rescue CFTR function in diesel exhaust particles-exposed cells, suggesting potential therapeutic applications beyond CF management. This study aligns with the thesis topic as it explores the role of CFTR, a cAMP-dependent ion channel, in the context of environmental air pollutants and its impact on lung health. Furthermore, this study provides valuable insights into the broader application of CFTR modulators for the management of respiratory diseases influenced by environmental air pollutants, thus extending beyond CF.

Effects of environmental air pollutants on CFTR expression and function in human airway epithelial cells

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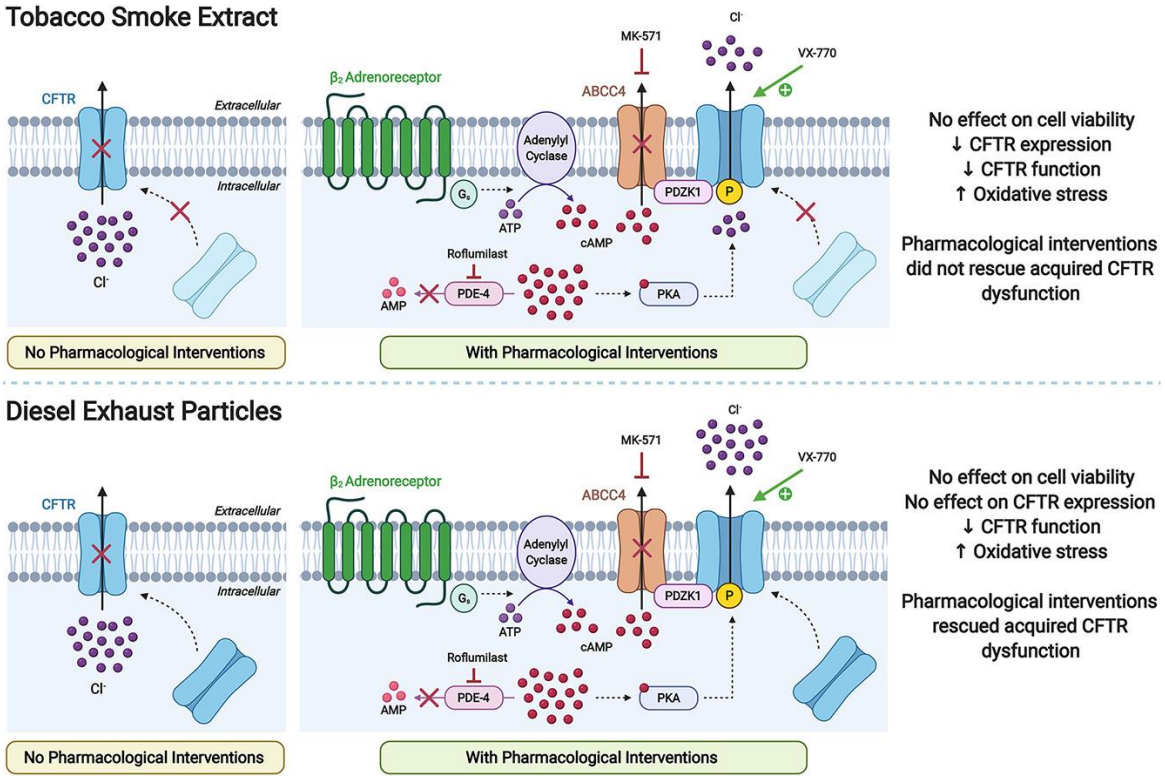
Keywords: Air pollution, Tobacco smoke, Airway epithelium, Cyclic AMP, ABCC4, ABCC7/CFTR

Abstract

The airway epithelium is exposed to a variety of air pollutants, which have been associated with the onset and worsening of respiratory diseases. These air pollutants can vary depending on their composition and associated chemicals, leading to different molecular interactions and biological effects. Mucociliary clearance is an important host defense mechanism against environmental air pollutants and this process is regulated by various ion transporters including cystic fibrosis transmembrane conductance regulator (CFTR). With evidence suggesting that environmental air pollutants can lead to acquired CFTR dysfunction, it may be possible to leverage therapeutic approaches used in cystic fibrosis (CF) management. The aim of our study was to test whether environmental air pollutants tobacco smoke extract, urban particulate matter, and diesel exhaust particles lead to acquired CFTR dysfunction and whether it could be rescued with pharmacological interventions. Human airway epithelial cells (Calu-3) were exposed to air pollutant extracts for 24 h, with and without pharmacological interventions, with readouts of CFTR expression and function. We demonstrate that both tobacco smoke extract and diesel exhaust particles led to acquired CFTR dysfunction and that rescue of acquired CFTR dysfunction is possible with pharmacological interventions in diesel exhaust particle models. Our study emphasizes that CFTR function is not only important in the context of CF but may also play a role in other respiratory diseases impacted by environmental air pollutants. In addition, the pharmacological interventions approved for CF management may be more broadly leveraged for chronic respiratory disease management.

Graphical Abstract



Introduction

The airway epithelium lining the lungs is continuously exposed to a variety of harmful substances that are present in the environment, including tobacco smoke, urban particulate matter, diesel exhaust particles, and pathogens[1]. The airway epithelium plays a crucial role in the defense against these harmful substances through mucociliary clearance and other host defense mechanisms. An important component of mucociliary clearance is the airway surface liquid (ASL), which is regulated by a variety of ion transporters including cystic fibrosis transmembrane conductance regulator (CFTR)[2]. CFTR is a cyclic AMP (cAMP)-regulated ion channel that conducts chloride and bicarbonate ions across the epithelial cell membrane, contributing to the regulation of ASL volume and composition[3–7]. In cystic fibrosis (CF), the inheritance of loss of function CFTR-variants lead to dysregulated ASL, and consequently, CF subjects experience pulmonary complications including excess inflammation, increased mucus viscosity, recurrent infections, and respiratory decline[8–11]. Recently, there has been evidence suggesting that CFTR dysfunction is also implicated in several other respiratory diseases, including chronic bronchitis and chronic obstructive pulmonary disease (COPD)[12].

Chronic bronchitis, a respiratory disease caused by long-term inflammation of the bronchi, is characterized by chronic coughing, sputum production, and impaired mucociliary clearance[13]. The greatest risk factor for the development of chronic bronchitis is tobacco smoke exposure, however, other air pollutants are also major risk factors[13,14]. While it has been widely accepted that long-term tobacco smoke exposure causes chronic bronchitis, the underlying mechanisms are not completely understood. A possible

explanation linking tobacco smoke exposures with chronic bronchitis is acquired CFTR dysfunction. There have been numerous studies demonstrating that tobacco smoke exposures can lead to acquired CFTR dysfunction through various mechanisms, including CFTR internalization and trafficking, and altered channel gating[15–17]. This acquired CFTR dysfunction ultimately results in airway surface dehydration and impaired mucociliary clearance, thus, further exploration regarding the effect of tobacco smoke on CFTR is needed[15,17–20].

Air pollution is a large environmental health risk that consists of several solid and liquid particles, and gasses, including particulate matter, volatile compounds, ozone, sulfur dioxide, nitrogen oxides, and carbon monoxide. Air pollution has been associated with the potential onset of several respiratory diseases, including chronic bronchitis, COPD, and asthma[21–24]. In addition, susceptible populations experience greater adverse effects in response to air pollutant exposures[25–27]. For example, in CF subjects, air pollution exposures have been associated with an increase in pulmonary exacerbations, increased susceptibility to respiratory infections, and a decrease in pulmonary function[28–31]. In addition to tobacco smoke exposures, recent studies have suggested that exposure to other air pollutants, such as ozone and reactive oxygen nitrogen species, can also impact CFTR activity, thus leading to an acquired CFTR dysfunction phenotype[32,33]. Furthermore, it has been demonstrated that exposure to diesel particulate matter can reduce the amount of CFTR binding to the membrane, downregulate CFTR mRNA and protein expression, increase mucus viscosity, and inhibit ASL secretion[34]. With the emerging links between air

pollution and CFTR becoming more apparent, additional research is necessary to better understand its impact on respiratory diseases.

Currently, there exists pharmacological interventions that target CFTR and are clinically approved for treating select CF populations. These are called CFTR modulators, which are small molecules that directly target CFTR defects by either increasing CFTR channel opening – CFTR potentiators, or by improving CFTR protein folding and trafficking to the apical membrane – CFTR correctors[35,36]. Presently, four CFTR modulators have been approved by the Federal Drug Administration, CFTR potentiator ivacaftor (VX-770), and CFTR correctors lumacaftor (VX-809), tezacaftor (VX-661), and elexacaftor (VX-445)[37–40]. While CFTR potentiators can be used alone, CFTR correctors are used in combination with a potentiator. The use of these CFTR modulators has been shown to be efficacious and beneficial for CF management, as demonstrated by several studies via improvements in FEV_1 and a reduction in pulmonary exacerbations[41–44]. In addition to CFTR modulators, investigations into other therapeutics that can influence CFTR activity, including drugs that modulate cAMP metabolism, have been explored. cAMP levels are regulated by various mechanisms, including adenylyl cyclases, cAMP-efflux transporters, and phosphodiesterases[45–49]. Due to CFTR being a cAMP-regulated ion channel that gets phosphorylated by protein kinase A (PKA), increasing CFTR open channel probability, several drugs that inhibit cAMP-efflux transporters or phosphodiesterases, leading to an increase in intracellular cAMP levels which activates PKA, have been investigated as a potential therapeutic for CF management[50–52]. Previously, we have demonstrated that pharmacological inhibition of ATP Binding Cassette Transporter

C4 (ABCC4), a cAMP-efflux transporter that is physically and functionally coupled to CFTR, is able to potentiate CFTR activity in combination with VX-770 beyond VX-770 alone in G551D-CFTR cells[45,46]. Additionally, we have also shown that cAMP modulation through pharmacological inhibition of ABCC4 and phosphodiesterase-4 (PDE-4), in combination with VX-770, is able to increase sensitivity and CFTR activity, when compared to VX-770 treatment alone[53]. These limited studies suggest that cAMP modulation and CFTR modulators can be effective at increasing CFTR function and could be leveraged as a potential strategy for the treatment of acquired CFTR dysfunction caused by environmental air pollutants, however, more research needs to be performed.

In this study, to investigate the effects of environmental air pollutants on CFTR expression and function, we exposed human airway epithelial cells to tobacco smoke extract (TSE), urban particulate matter (Urban PM), and diesel exhaust particles (DEP). We hypothesized that the environmental air pollutants would reduce CFTR expression and function, which could then be rescued with pharmacological interventions. Using a membrane potential plate-based assay, a cell viability assay, and an oxidative stress assay, our results demonstrate that 24 h treatments of tobacco smoke extract and diesel exhaust particles, but not urban particulate matter, led to reduced CFTR function and an increase in oxidative stress, without significantly affecting cell viability. Using western blotting, our results suggest that TSE and DEP may impact CFTR by different mechanisms. In addition, our results suggest that air pollutant-induced loss of CFTR function can be rescued via pharmacological intervention with CFTR potentiator VX-770. Collectively, our results suggest that exposures to environmental air pollutants, such as TSE and DEP, can lead to

acquired CFTR dysfunction. Our data indicates an importance of CFTR function beyond CF, including in healthy and those with chronic lung diseases living or working in areas of poor air quality, and a potential application for CFTR modulators to a broader patient population.

Materials and Methods

Reagents

All the following chemical reagents were dissolved in DMSO and were purchased from Cayman Chemical, apart from CFTR Inh-172 and Ivacaftor, which were purchased from Selleck Chemicals. L-Ascorbic Acid was dissolved in ddH_2O and was purchased from Sigma-Aldrich. For *in vitro* CFTR membrane potential assays, ABCC4 inhibitor MK-571 (1.8 μM) and PDE-4 inhibitor Roflumilast (RF – 1 μM) were used for cAMP modulation intervention, CFTR potentiator Ivacaftor (VX-770 – 1 μM) was used for VX-770 intervention, Forskolin (fsk – 10 μM) was used for cAMP elevation, and CFTR Inh-172 (Inh172 – 10 μM) was used to inhibit CFTR in order to abolish the CFTR activity signal. For *in vitro* CFTR membrane potential assays and oxidative stress assays, where the effectiveness of introducing an antioxidant was assessed, L-ascorbic acid (AA – 100 μM) was used.

Human airway epithelial cell culture

The human airway epithelial cell line Calu-3 (ATCC HTB-55), isolated from lung adenocarcinoma tissue, was cultured in Alpha-Minimum Essential Medium (α-MEM) (Corning) supplemented with 10% fetal bovine serum (VWR), 1% HEPES (Corning), and 1% penicillin-streptomycin (VWR) at 37°C. The medium was changed three times per week (every Monday, Wednesday, and Friday). Calu-3 cells were grown in submerged monolayers for either 14 days or 21 days, after reaching 100% confluency, prior to air pollutant treatment exposures and downstream *in vitro* experiments.

Tobacco smoke extract (TSE) treatment

TSE was produced by bubbling the smoke from one research cigarette for tobacco heath research (University of Kentucky Research Institute, Lot: 2R4F) through 4 mL of α-MEM culture media supplemented with 1% HEPES and 1% penicillin-streptomycin. The 100% tobacco smoked media was subsequently filtered using a 0.22 μm filter and then diluted for final concentrations of 10% and 20% TSE. Calu-3 cells were treated with either 10% or 20% TSE for 24 h at 37°C.

Urban particulate matter (Urban PM) and diesel exhaust particles (DEP) treatment

Urban PM and DEP mixtures were produced by combining standard reference material SRM 1648a (National Institute of Standards and Technology) or diesel exhaust particle material (Air Pollution Exposure Laboratory) respectively, with α-MEM supplemented culture media for a final concentration of 125 μg/mL. Following the addition of Urban PM and DEP to α-MEM supplemented culture media, mixtures were sonicated for 10 s, for a total of 10 rounds with 10 s of rest in between, to disperse particles that may have aggregated together. Urban PM has a mean particle diameter of 5.85 μm, as listed in its Certificate of Analysis, and DEP has a median size mobility of 0.109 μm as previously described[54]. The diesel exhaust particles used in this study were collected from the tailpipe of an EPA Tier 3-compliant, 6.0 kW Coliseum GY6000 generator located in the Air Pollution Exposure Laboratory (Vancouver, BC, Canada) as previously described[54]. Calu-3 cells were treated with either 125 μg/mL of Urban PM or DEP extract mixture for 24 h at 37°C.

Microscopy imaging

Calu-3 cells were seeded into 12-well plates at a density of 1.5 X 10^5 cells/well. After 21 days post-confluency, cells were treated with environmental air pollutants TSE, Urban PM, or DEP for 24 h. Calu-3 cells were viewed using the EVOS FL Cell Imaging System (Thermo Fisher Scientific) and were captured at the 10X and 20X objective pre- and post-treatment. After imaging, cells were used for downstream western blots.

Western blotting

Calu-3 cells were seeded into 12-well plates at a density of 1.5 X 10^5 cells/well prior to environmental air pollutant exposures for 24 h. Twenty micrograms of protein lysates were incubated with 1X Laemmli buffer and 0.1 M dithiothreitol for 15 min at 65°C. Samples were then loaded into a 4-20% Mini-PROTEAN TGX Stain-Free Protein Gel (Bio-Rad) and transferred to a Low-Fluorescence PVDF Transfer Membrane (Bio-Rad). Stain-free blot images were taken using the ChemiDoc Imaging System (Bio-Rad) prior to blocking. The membranes were then blocked with 5% Casein blocking powder in a mixture of 1X TBS and 0.05% Tween 20 (TBST) for 2 h at room temperature. The membranes were incubated overnight at 4°C with primary antibody (UNC-Chapel Hill, AB596), which was diluted in TBST with 3% Casein to a working concentration of 1:5000. The membranes were subsequently washed with TBST and then incubated with HRP-linked anti-mouse secondary antibody (1:3000, Cell Signalling Technology, 7076S) for 2 h at room temperature. After incubation, the membranes were washed with TBST and incubated with Clarity Western ECL Substrate (Bio-Rad). A chemiluminescence image was taken using

the ChemiDoc Imaging System (Bio-Rad). Full western blot images can be found in the supplementary information (**Supplementary Fig. 1**).

In vitro CFTR membrane potential assay

Calu-3 cells were seeded into 96-well plates at a density of 5 X 10^4 cells/mL for 21 days post-confluency prior to environmental air pollutant exposures for 24 h. Calu-3 cells were then washed with HBSS prior to the *in vitro* CFTR membrane potential assay as previously described[45,53]. The BLUE Membrane Potential Dye (Molecular Devices, #R8042) was dissolved in a chloride-free buffer (150 mM NMDG-gluconate, 3 mM potassium gluconate, 10 mM HEPES, pH 7.35, 300 mOsm), warmed at 37°C, prior to loading onto the Calu-3 cells. After the loading of the dye, CFTR activity measurements, using the SpectraMax i3 Multi-Mode Platform microplate reader (Molecular Devices), were taken for baseline (40 min), cAMP modulation interventions (30 min), VX-770 intervention (10 min), cAMP elevation (30 min), and CFTR inhibition (20 min). In assays assessing the effectiveness of introducing an antioxidant, L-ascorbic acid was added for the entire duration of the experiment. In the case where no interventions were introduced, DMSO was added as the vehicle control. After the completion of the assay, raw data was exported for subsequent statistical analysis.

Cell viability assay

Calu-3 cells were seeded into 96-well plates at a density of 7.5 X 10^4 cells/mL for 14 days post-confluency prior to environmental air pollutant exposures for 24 h. To assess

cell viability, a lactate dehydrogenase (LDH) cytotoxicity assay was performed to evaluate cellular cytotoxicity using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, 88953). 10 µL of 10X lysis buffer was added to the appropriate wells and 35 µL of the supernatant was collected and plated into a new 96-well plate. 50 µL of assay reaction buffer was added to each well and incubated for 30 min at room temperature to allow for colour development. After incubation, 50 µL of stop solution was added to each well. Absorbance was read using the SpectraMax i3 Multi-Mode Platform microplate reader. After the completion of the assay, raw data was exported for subsequent statistical analysis.

***In vitro* oxidative stress assay**

Calu-3 cells were seeded into 96-well black walled plates, for increased fluorescence sensitivity, at a density of 7.5 X 10^4 cells/mL for 14 days post-confluency. 100 µL of warmed HBSS with 10 µM H2DCFDA, a reactive oxygen species fluorescence sensor, were loaded onto the Calu-3 cells and incubated for 1 h at 37°C. Cells were then washed three times with HBSS prior to environmental air pollutant exposures. 0.5 mM hydrogen peroxide (H_2O_2) was used as a positive control to induce oxidative stress. To assess the effectiveness of introducing an antioxidant, L-ascorbic acid was added for the entire duration of the experiment for select conditions. H2DCFDA fluorescence was measured using the SpectraMax i3 Multi-Mode Platform microplate reader over a period of 24 h. Over the 24 h duration, measurements were taken every hour between the 0 to 6 h mark, the 22 h mark, and the 24 h mark. After the completion of the assay, raw data was exported for subsequent statistical analysis.

Statistical analysis

For western blotting, normalized volume values were obtained via total protein normalization. Total protein normalization was performed using Image Lab software (Bio-Rad) by comparing the band intensities from the chemiluminescence image to the respective total lane intensities of all protein in the stain-free blot image. SD was calculated using data from biological replicates (n=3) and an unpaired *t*-test was performed.

For the *in vitro* CFTR membrane potential assay, CFTR activity was determined by normalizing the relative fluorescence values to the stabilized baseline values over the DMSO vehicle control, represented by the dotted line. CFTR activity for all replicates and time points were averaged and plotted with a connecting line (n=3-4). An area under the curve (AUC) analysis, max change analysis, and max peak analysis was performed for each individual replicate and were subsequently averaged. SD was calculated using the data from biological replicates (n=3-4).

The AUC analysis, an aggregate measure of sensitivity and maximal response, was used to observe the total net response of the Calu-3 cells. Using the CFTR activity data collected, the AUC analysis was split into two separate time components, pre- and post-stimulation. The pre-stimulation component was defined as the AUC during the cAMP modulation and VX-770 interventions or DMSO controls. The post-stimulation component was defined as the AUC during the cAMP elevation step. In addition, the total AUC was calculated by summing the pre- and post-stimulation AUCs. The max change analysis was performed by subtracting the max CFTR activity value achieved during the cAMP elevation step by an averaged baseline CFTR activity value calculated in the step prior to the cAMP

elevation step. In the case for the VX-770 intervention, the averaged baseline CFTR activity value was calculated from the step immediately before the introduction of VX-770. The max peak analysis was performed by averaging the max CFTR activity value achieved during the cAMP elevation. Vehicle and air pollutant exposure controls (vehicle, 20% TSE, Urban PM – 125 µg/mL, and DEP - 125 µg/mL) were shared across pharmacological intervention experiments (CFTR potentiator – VX-770, cAMP modulation – RF + MK-571, and antioxidant - AA) during analysis. A one-way ANOVA with subsequent multiple comparisons was performed.

For the LDH assay, percent of cytotoxicity was determined by dividing the corrected LDH activity values over LDH Lysis Buffer, multiplied by 100. Corrected LDH activity values were determined by subtracting experimental absorbance values from background absorbance values. SD was calculated using data from biological replicates (n=4) and an unpaired *t*-test or one-way ANOVA with subsequent multiple comparisons was performed.

For *in vitro* oxidative stress assays, the fold change AUC was determined by averaging the technical replicates of the AUC of the treatment conditions over the AUC of the untreated conditions. SD was calculated using data from biological replicates (n=4) and an unpaired *t*-test or one-way ANOVA with subsequent multiple comparisons was performed. Statistical analysis was performed using GraphPad Prism 8.

Results

TSE reduces CFTR expression and function

Previous demonstrations have suggested that tobacco smoke leads to a reduction in CFTR expression and function[17–20,55–59]. To confirm these findings and to validate our model of study, human airway epithelial Calu-3 cells were grown in submerged monolayer conditions and were exposed to TSE for 24 h prior to downstream *in vitro* experiments. Microscopy images were taken pre- and post-exposures to observe the effect of TSE on cell monolayers. Post-TSE treatment wells had qualitatively more floating cells and debris present in comparison to the vehicle (**Fig. 1a**), without accompanying changes in cell viability (**Fig. 1h**). To assess the effect of TSE on CFTR expression, a western blot and total protein stain was performed (**Fig. 1b**). Cells that were treated with 20% TSE showed a significant reduction in CFTR expression (**Fig. 1c;** $*P \leq 0.05$), supporting a previous demonstration in the literature[19]. To assess the effect of TSE on CFTR function, an *in vitro* CFTR membrane potential assay was performed (**Fig. 1d**). Cells treated with 10% or 20% TSE showed a dose-dependent decline in CFTR activity and upon forskolin (fsk) stimulation, were only able to achieve CFTR activity levels close to unstimulated conditions, represented by the dotted line (**Fig. 1d**). An AUC analysis showed a significant decrease in CFTR activity in response to TSE in all measurements, indicating that TSE leads to dose-dependent reduction in CFTR function (**Fig. 1e**; $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$). Although the max change between the vehicle and TSE-exposed cells remained unchanged (**Fig. 1f**), a dose-dependent decrease in the max peak achieved was observed in response to TSE (**Fig. 1g**; $**P \leq 0.01$). Altogether, these demonstrations support

previous evidence in the literature suggesting TSE leads to a reduction in CFTR protein expression and consequently, a dose-dependent reduction in CFTR function, without significantly impacting cell viability.

Urban PM does not induce CFTR dysfunction

While several studies have investigated the role of tobacco smoke on CFTR expression and function, the role of other types of environmental air pollutants have not been pursued. To further investigate the effects of air pollutants on CFTR expression and function, the air pollutant Urban PM was tested. Calu-3 cells grown under submerged monolayer conditions were exposed to 125 μg/mL of Urban PM for 24 h prior to downstream *in vitro* experiments. Microscopy images were taken pre- and post-exposure to Urban PM (**Fig. 2a**). Cells treated with Urban PM experienced more cell lifting without impacting cell viability, as assessed by LDH secretion (**Fig. 2h**). To assess the effect of Urban PM on CFTR expression, a western blot and total protein stain was performed (**Fig. 2b**). In contrast to TSE, there was no reduction in CFTR expression in response to Urban PM (**Fig. 2c**). To assess the effect of Urban PM on CFTR function, an *in vitro* CFTR membrane potential assay was performed (**Fig. 2d**). Although a decline in CFTR activity was observed (**Fig. 2d**), the AUC, max change, and max peak analysis for Urban PM were not significant (**Fig. 2e-g**). These findings suggest that Urban PM does not impact CFTR expression and does not induce CFTR dysfunction.

DEP induce CFTR dysfunction

On account of the contrasting results observed between TSE and Urban PM, we next explored the effects of air pollutant DEP and its impact on CFTR. Calu-3 cells were grown under submerged monolayer conditions and were exposed to 125 μg/mL of DEP for 24 h prior to downstream *in vitro* experiments. Microscopy images were taken pre- and post-exposure to DEP (**Fig. 3a**). Similar to post-TSE and Urban PM exposures, post-DEP treatment displayed more cell lifting in comparison to the vehicle (**Fig. 3a**) with no impact on cell viability (**Fig. 3h**). To assess the effect of DEP on CFTR expression, a western blot and total protein stain was performed (**Fig. 3b**). Interestingly, in contrast to TSE and Urban PM, there was a modest trend suggesting an increase in CFTR expression in response to DEP, however it was not of significance (**Fig. 3c**). Although DEP did not reduce CFTR expression like TSE exposures, findings from the CFTR functional assay suggests that exposure to DEP leads to a decline in CFTR function (**Fig. 3d**). The AUC and max peak analysis for DEP showed significant decreases, suggesting the reduction in CFTR function caused by DEP is not attributed to CFTR protein expression (**Fig. 3e and g**; $*P \leq 0.05$, $**P \leq 0.01$). While the max change for DEP was not significant, a modest reduction was noted (**Fig. 3f**). Collectively, these findings suggest that DEP is causing CFTR dysfunction by alternative means, in comparison to TSE, and that its function could potentially be rescued with pharmacological interventions.

CFTR potentiator VX-770 rescues CFTR function in cells exposed to DEP

Due to our observations suggesting that both environmental air pollutants TSE and DEP lead to a reduction in CFTR function, we next investigated whether this reduction in

CFTR function could be rescued by pharmacological interventions. For our first pharmacological intervention, we tested the clinically approved CFTR potentiator ivacaftor (VX-770) and its ability to increase CFTR activity post-treatment of TSE (**Fig. 4a**) and DEP (**Fig. 4b**). The addition of VX-770 to 20% TSE-exposed cells was unable to rescue CFTR function (**Fig. 4a**). This observation was supported by the AUC, max change, and max peak analysis since the addition of VX-770 to 20% TSE-exposed cells remained unchanged (**Fig. 4c, e, and g**). In contrast, the addition of VX-770 to DEP-exposed cells appeared to rescue CFTR function to levels comparable to vehicle conditions (**Fig. 4b**). The AUC and max peak analysis for DEP, with the addition of VX-770, led to a significant increase in CFTR activity in comparison to no intervention (**Fig. 4d and h**; $*P \leq 0.05$, $**P \leq 0.01$). While the max change analysis showed an increasing trend with the addition of VX-770 to DEP-exposed cells, it was not significant (**Fig. 4f**). Altogether, these findings suggest that even though both air pollutants TSE and DEP lead to a reduction in CFTR function, rescue of CFTR function using CFTR potentiator VX-770 was only possible in DEP-exposed cells, thus warranting further investigations into the effects of air pollutants on CFTR and other pharmacological interventions that can promote CFTR activity.

Impact of cAMP modulation on TSE and DEP-exposed cells

Following our investigation of VX-770, our next pharmacological intervention was cAMP modulation by inhibiting ABCC4 and PDE-4 using MK-571 and roflumilast (RF) respectively. From our CFTR membrane potential assay, cAMP modulation with MK-571 and RF post-treatment of 20% TSE did not appear to increase CFTR activity and resembled

TSE-exposures without any interventions (**Fig. 5a**). When examining CFTR activity in response to cAMP modulation post-treatment of 20% TSE, the AUC, max change, and max peak analyses remained unchanged (**Fig. 5c, e, and g**). However, similar to VX-770 intervention but not of significance, the addition of MK-571 and RF post-treatment of DEP appeared to have subtle increases in CFTR activity compared to DEP treatments without interventions (**Fig. 5b**). The AUC, max change, and max peak analysis for cAMP modulation post-treatment of DEP showed increasing trends for CFTR activity, but were not of significance (**Fig. 5d, f, and h**). These demonstrations suggest that cAMP modulation alone is not sufficient in rescuing the loss of CFTR function caused by environmental air pollutants TSE and DEP.

TSE and DEP lead to an increase in oxidative stress

Although both environmental air pollutants led to a reduction in CFTR function, only TSE led to a reduction in CFTR expression. Furthermore, pharmacological intervention with CFTR modulator VX-770 was only able to rescue acquired CFTR dysfunction in DEP-exposed cells but not in TSE-exposed cells. Therefore, we next investigated whether oxidative stress was the primary cause of acquired CFTR dysfunction. From our oxidative stress assay, our results show that both TSE (**Fig. 6a**; ****$P \leq 0.0001$*) and DEP (**Fig. 6b;** ****$P \leq 0.0001$*) exposures led to a significant increase in oxidative stress, as measured by the fold change AUC of the 2′,7′-Dichlorodihydrofluorescein diacetate (H2DCFDA) reactive oxygen species fluorescence sensor. From the oxidative stress assay, we also assessed whether the addition of an antioxidant, ascorbic acid (AA),

could significantly reduce oxidative stress in TSE and DEP-exposed cells. The addition of AA significantly reduced oxidative stress in both TSE (**Fig. 6a**; *****P≤ 0.0001*) and DEP-exposed cells (**Fig. 6b**; *****P≤ 0.0001*).

Considering both TSE and DEP exposures led to a significant increase in oxidative stress, and that the introduction of an antioxidant can significantly reduce it, we next investigated the effect of AA on CFTR function to determine whether oxidative stress is leading to acquired CFTR dysfunction. Using our *in vitro* CFTR membrane potential assay, we demonstrate that the addition of AA did not lead to an increase in CFTR activity in 20% TSE-exposed cells (**Fig. 7a**). This observation was supported by the AUC, max change, and max peak analysis for 20% TSE-exposed cells where there were no significant changes in response to AA (**Fig. 7c, e, and g**). On the contrary, the addition of AA appeared to increase CFTR function in DEP-exposed cells (**Fig. 7b**). For the AUC and max peak analysis, a modest trend suggesting an increase in CFTR function, in response to AA, was observed but was not of significance (**Fig. 7d and h**). However, the max change analysis for DEP-exposed cells, with the addition of AA, led to a significant increase in CFTR activity (**Fig. 7f**; **P≤ 0.05*). In addition to TSE and DEP, we also tested the effect of Urban PM on oxidative stress (**Supplementary Fig. 2**). Like TSE and DEP, Urban PM exposures led to significant increases in oxidative stress and the addition of AA was able to significantly reduce oxidative stress in Urban PM-exposed cells (**Supplementary Fig. 2**; ****P≤ 0.001; ****P≤ 0.0001*). Taken together, these findings suggest that while oxidative stress may play a role in acquired CFTR dysfunction, it is not the only contributing mechanism that leads to acquired CFTR dysfunction.

Discussion

The airway epithelium acts as the first line of defense, via mucociliary clearance and other host defense mechanisms, against various environmental air pollutants. Mucociliary clearance is carefully regulated by various ion transporters, including CFTR, the protein affected in CF. With emerging evidence suggesting that select environmental air pollutants can lead to acquired CFTR dysfunction, there is interest in whether therapeutic approaches used in CF management could be leveraged for these instances. In the present study, we investigate the effect of TSE, Urban PM, and DEP on CFTR expression and function. We also assess whether pharmacological interventions can rescue acquired CFTR dysfunction caused by environmental air pollutants. Using Calu-3 cells, we demonstrate that both TSE and DEP, but not Urban PM, can lead to acquired CFTR dysfunction, either through a decrease in CFTR expression, an increase in oxidative stress, or a combination of the two. Pharmacological intervention with VX-770 rescued CFTR function in DEP-exposed cells. These findings emphasize that CFTR dysfunction is possible in non-CF individuals, including healthy individuals and those with other respiratory diseases. Additionally, the rescue of acquired CFTR dysfunction via pharmacological interventions post-air pollutant exposures suggests that therapeutic approaches for CF management can be leveraged, warranting further exploration.

TSE exposures to Calu-3 cells resulted in a dose-dependent decrease in CFTR function, as measured by the AUC and max peak analysis, and 20% TSE led to a significant decrease in CFTR expression. Interestingly, from the max change analysis, the magnitude of change between all the conditions were comparable in size, suggesting that there is a

capped amount of CFTR function possible that may be dependent on the amount of CFTR protein present. There were no significant changes in cell viability in response to 10% and 20% TSE exposures, thus the decreases in CFTR expression and function cannot be attributed to cell viability. Collectively, these findings are in alignment with previous evidence that demonstrate TSE exposures lead to acquired CFTR dysfunction in a dose-dependent manner, and that the decrease in CFTR function could be attributed, in part, to the reduction of CFTR protein[15,18,19,55,59].

While the effects of tobacco smoke on CFTR function have been well studied, little is known about the impact of other air pollutants regarding acquired CFTR dysfunction. Therefore, we decided to investigate the effects of Urban PM and DEP on CFTR expression and function.

Like TSE exposures, Urban PM exposures did not significantly affect cell viability. However, in contrast to TSE exposures, Urban PM did not decrease CFTR expression. While a decrease in CFTR function was observed, the AUC, max change, and max peak analysis were not significant. Together, these results suggest that Urban PM does not induce CFTR dysfunction.

On the other hand, DEP exposures induced CFTR dysfunction, as demonstrated by the significant reduction in AUC and max peak compared to the vehicle control. Like TSE and Urban PM, DEP exposures to Calu-3 cells did not impact cell viability. Interestingly, in contrast to TSE exposures, DEP exposures did not lead to a reduction in CFTR expression. Based on these results, exposure to DEP led to acquired CFTR dysfunction.

However, the reduction in CFTR function is not due to CFTR expression or cell viability, but via another mechanism.

Altogether, the different conclusions obtained from environmental air pollutants TSE, Urban PM, and DEP suggest that not all air pollutants lead to acquired CFTR dysfunction. The differences between these air pollutants and their findings could be due to a variety of factors, including differences in size and composition, variations in geographical location and season, and different manufacturing facilities[54,60–62]. In addition, it is important to recognize that the particles and associated chemicals of each of the air pollutants used in this study are distinct, thus they may have different molecular interactions and biological effects. As such, due to the variability between air pollutants, broad conclusions and interpretations must be made after careful considerations.

Since TSE and DEP led to acquired CFTR dysfunction, we therefore decided to investigate whether pharmacological interventions could be used to rescue CFTR function in these models. Previously, it has been demonstrated that pharmacological intervention with VX-770 is able to rescue acquired CFTR dysfunction caused by tobacco smoke[17,18]. However, to our knowledge, this has not been demonstrated in diesel exhaust particle models. In our study, while subtle trends suggesting an increase in CFTR activity, in response to VX-770, was present in TSE-exposed cells, it was not of significance and did not align with previous demonstrations[17,18]. However, in contrast to the tobacco smoke findings, pharmacological intervention with VX-770 rescued CFTR function in DEP-exposed cells via an increase in AUC and max peak. Our interpretation of this observation is that since DEP exposures only led to a reduction CFTR function, and not CFTR

expression, CFTR activity was able to be rescued since VX-770 directly targets CFTR to improve channel function. Conversely, TSE exposures resulted in both a reduction in CFTR expression and function, thus, it is possible that the pharmacological intervention with VX-770 was not as effective at rescuing CFTR function due to decreased amount of CFTR protein present.

Following pharmacological intervention with VX-770, we next investigated whether cAMP modulation, via ABCC4 and PDE-4 inhibition, can rescue acquired CFTR dysfunction. Similar to VX-770, there has been previous evidence supporting the use of cAMP modulation for increasing CFTR activity. In tobacco smoke exposures, inhibition of PDE-4 using roflumilast, a clinically approved PDE-4 inhibitor used in COPD management, has been shown to increase intracellular cAMP levels, improve CFTR function, and increase ASL[49,63–66]. Additionally, in diesel particulate matter-exposed cells, roflumilast was shown to increase intracellular cAMP levels, and increase CFTR mRNA and protein expression[34]. In our study, cAMP modulation did not rescue CFTR function in both TSE and DEP-exposed cells. However, trends suggesting an increase in CFTR activity was seen in DEP-exposed cells but were not significant. It is possible that cAMP modulation alone is not sufficient in rescuing acquired CFTR dysfunction caused by these environmental air pollutant models, thus, further studies exploring the combination of cAMP modulation with VX-770, which could have an additive effect, should be pursued.

In the interest of determining what mechanisms could explain the loss of CFTR function caused by TSE and DEP, we next investigated whether oxidative stress was the primary cause of acquired CFTR dysfunction. In our oxidative stress assay, both TSE and

DEP exposures led to a significant increase in oxidative stress. To assess whether the introduction of an antioxidant can reduce oxidative stress levels, AA was introduced. The introduction of AA led to a significant reduction in oxidative stress for both TSE and DEP-exposed cells. Following our oxidative stress experiments, we investigated whether the introduction of AA can rescue acquired CFTR dysfunction, since AA was able to reduce oxidative stress levels for both TSE and DEP exposures. The introduction of AA did not have any significant effect on CFTR function in TSE-exposed cells. However, the introduction of AA rescued CFTR function in DEP-exposed cells via an increase in the max change. While trends suggesting an increase in AUC and max peak was present in response to AA for DEP-exposed cells, it was not of significance. Collectively, these results suggest that acquired CFTR dysfunction can partially be attributed to oxidative stress, however, it is not the only mechanism involved in acquired CFTR dysfunction. Our findings are consistent with previous studies demonstrating oxidative stress suppresses CFTR expression[32,67]. In these studies, oxidative stress led to a decrease in both CFTR gene and protein expression, along with CFTR function[32,67]. Furthermore, other studies have demonstrated that oxidative stress interrupts ATP synthesis and mitochondrial function, which could be another possible explanation for how oxidative stress reduces CFTR function[68–70]. These explanations support why pharmacological intervention with VX-770 and AA was able to rescue CFTR function in DEP models. Further investigations on the role of oxidative stress in acquired CFTR dysfunction, in response to various environmental air pollutant models such as polycyclic aromatic hydrocarbons (PAHs), metals, and other combustion-associated chemicals, should be performed. It has been demonstrated that

PAHs and metals can induce xenobiotic, metal, and stress-dependent cellular responses[60]. As well, further investigations on the potential role of dietary antioxidant supplementation as an economical approach for managing oxidative effects of air pollutants should be investigated. It has been demonstrated that dietary antioxidants, such as vitamin C (ascorbic acid), can have protective effects against oxidative stress[71,72].

An integral component of this study is the utilization of the *in vitro* CFTR membrane potential assay for indirect measurements of CFTR activity, which involves the use of CFTR Inh-172, a selective and direct inhibitor of CFTR[73]. This assay has been demonstrated to recapitulate findings produced via Ussing chambers, a direct measurement of CFTR activity[45]. CFTR Inh-172 was first identified via high-throughput screening and was shown to inhibit CFTR without inhibiting calcium and volume-activated chloride channels, ATP-sensitive potassium channels, and other transporters at 5 μM concentration[73]. However, a subsequent specificity study demonstrated that CFTR Inh-172 inhibits volume-sensitive outwardly rectifying chloride and calcium-activated channels at concentrations greater than 5 μM and 10 μM respectively[74]. In this study, we administer 10 μM of CFTR Inh-172, thus it is possible that there may be minimal off-target effects on volume-activated chloride channels.

In the present study, we demonstrate that environmental air pollutants, TSE and DEP, both lead to acquired CFTR dysfunction. However, it appears that the mechanisms involved in acquired CFTR dysfunction for these different environmental air pollutant models may vary and could be due to reduced CFTR expression or increased oxidative stress. It is also possible that acquired CFTR dysfunction may be a synergism of the

mechanisms suggested. Future studies exploring the mechanisms in how these different models of environmental air pollutants achieve CFTR dysfunction should be performed. We also demonstrate that pharmacological intervention with VX-770 could rescue acquired CFTR dysfunction in diesel exhaust particle models, suggesting that therapeutic approaches for CF management can be leveraged and have broad applications for other respiratory diseases caused by environmental air pollutants. Further investigations exploring CFTR modulators, cAMP modulators, and combinations of them should be pursued.

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Figures

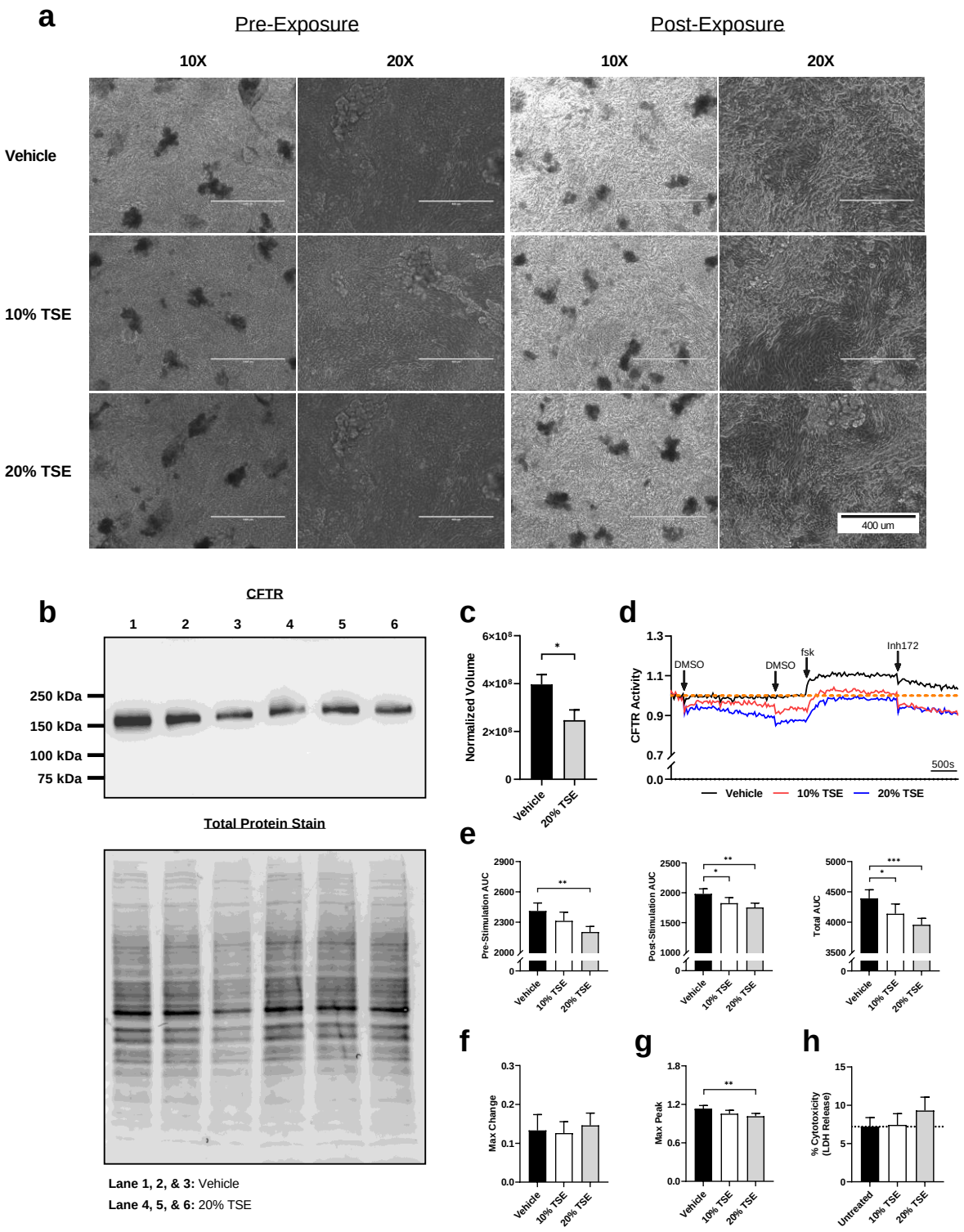


Figure 1: *Figure legend continued on the following page.*

Figure 1: Effect of TSE on CFTR expression and function in human airway epithelial cells. Human airway epithelial (Calu-3) cells were exposed to 10% and 20% TSE for 24 h treatment. (**a**) Microscopy images were taken at the 10X and 20X objective pre- and post-24 h treatment. (**b** and **c**) CFTR protein expression was characterized and measured with western blot and a total protein stain was performed as the loading control. Band intensities were normalized via total protein normalization with data presented as ± SD (n=3). Full-length blots are presented in Supplementary Fig. 1a. (**d**) CFTR function was measured using an *in vitro* membrane potential assay. The line graph shows the change in CFTR activity in response to different stimulations (DMSO, forskolin, and CFTR Inh-172). CFTR activity was normalized to stabilized baseline over vehicle control, represented by the dotted line (n=4). Analysis of the line graph was performed for (**e**) pre-stimulated, post-stimulated and total AUC, (**f**) max change, and (**g**) max peak with data presented as ± SD (n=4). (**h**) Cellular cytotoxicity was measured using a LDH assay on collected supernatants with data presented as ± SD (n=4). An unpaired *t*-test was used for the statistical analysis of CFTR protein expression, while a one-way ANOVA with subsequent multiple comparisons was used for the analysis of AUC, max change, max peak, and cellular cytotoxicity. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

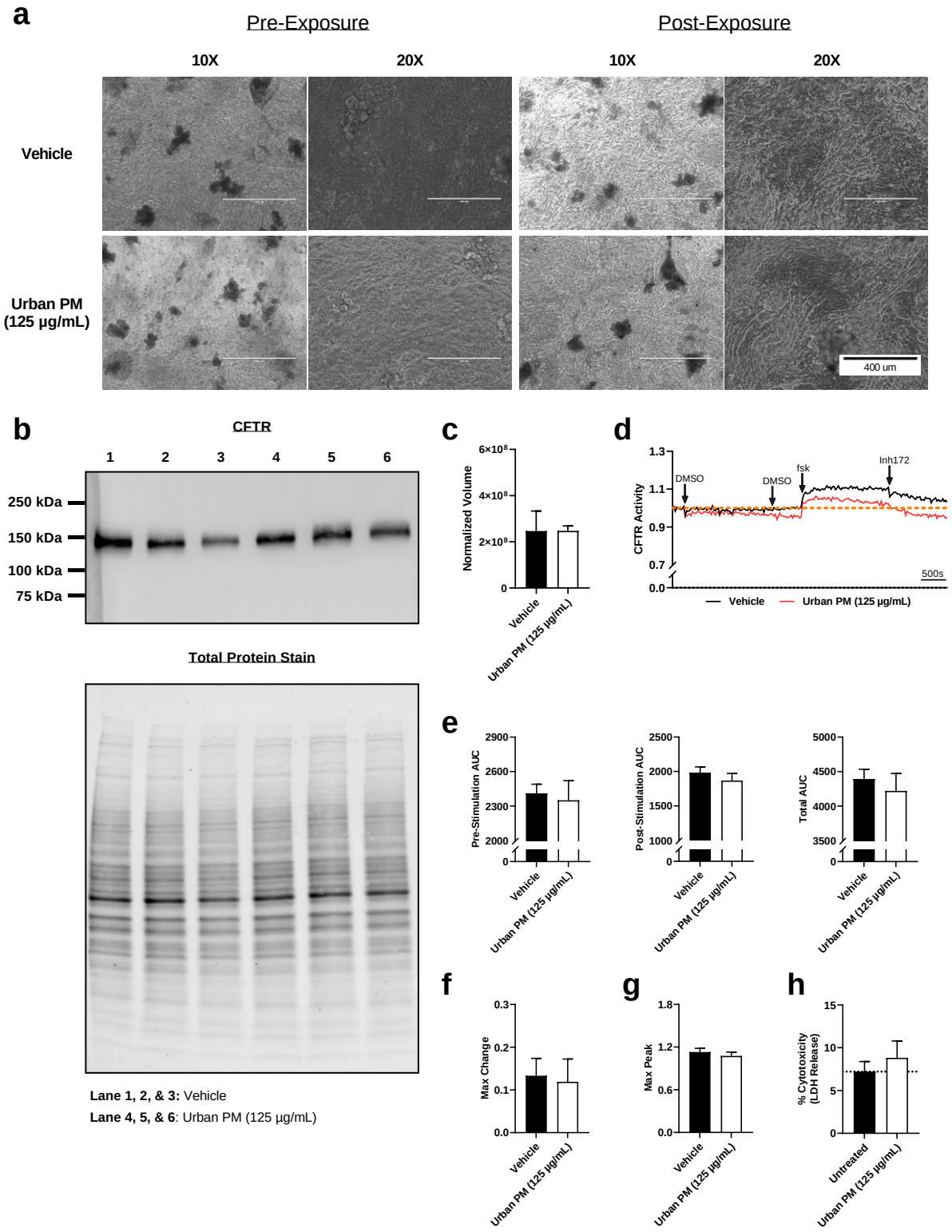


Figure 2: *Figure legend continued on the following page.*

Figure 2: Effect of Urban PM on CFTR expression and function in human airway epithelial cells. Human airway epithelial (Calu-3) cells were exposed to 125 µg/mL of Urban PM for 24 h treatment. (**a**) Microscopy images were taken at the 10X and 20X objective pre- and post-24 h treatment. (**b** and **c**) CFTR protein expression was characterized and measured with western blot and a total protein stain was performed as the loading control. Band intensities were normalized via total protein normalization with data presented as ± SD (n=3). Full-length blots are presented in Supplementary Fig. 1b. (**d**) CFTR function was measured using an *in vitro* membrane potential assay. The line graph shows the change in CFTR activity in response to different stimulations (DMSO, forskolin, and CFTR Inh-172). CFTR activity was normalized to stabilized baseline over vehicle control, represented by the dotted line (n=4). Analysis of the line graph was performed for (**e**) pre-stimulated, post-stimulated and total AUC, (**f**) max change, and (**g**) max peak with data presented as ± SD (n=4). (**h**) Cellular cytotoxicity was measured using a LDH assay on collected supernatants with data presented as ± SD (n=4). An unpaired *t*-test was used for the statistical analysis of CFTR protein expression and cellular cytotoxicity, while a one-way ANOVA with subsequent multiple comparisons was used for the analysis of AUC, max change, and max peak.

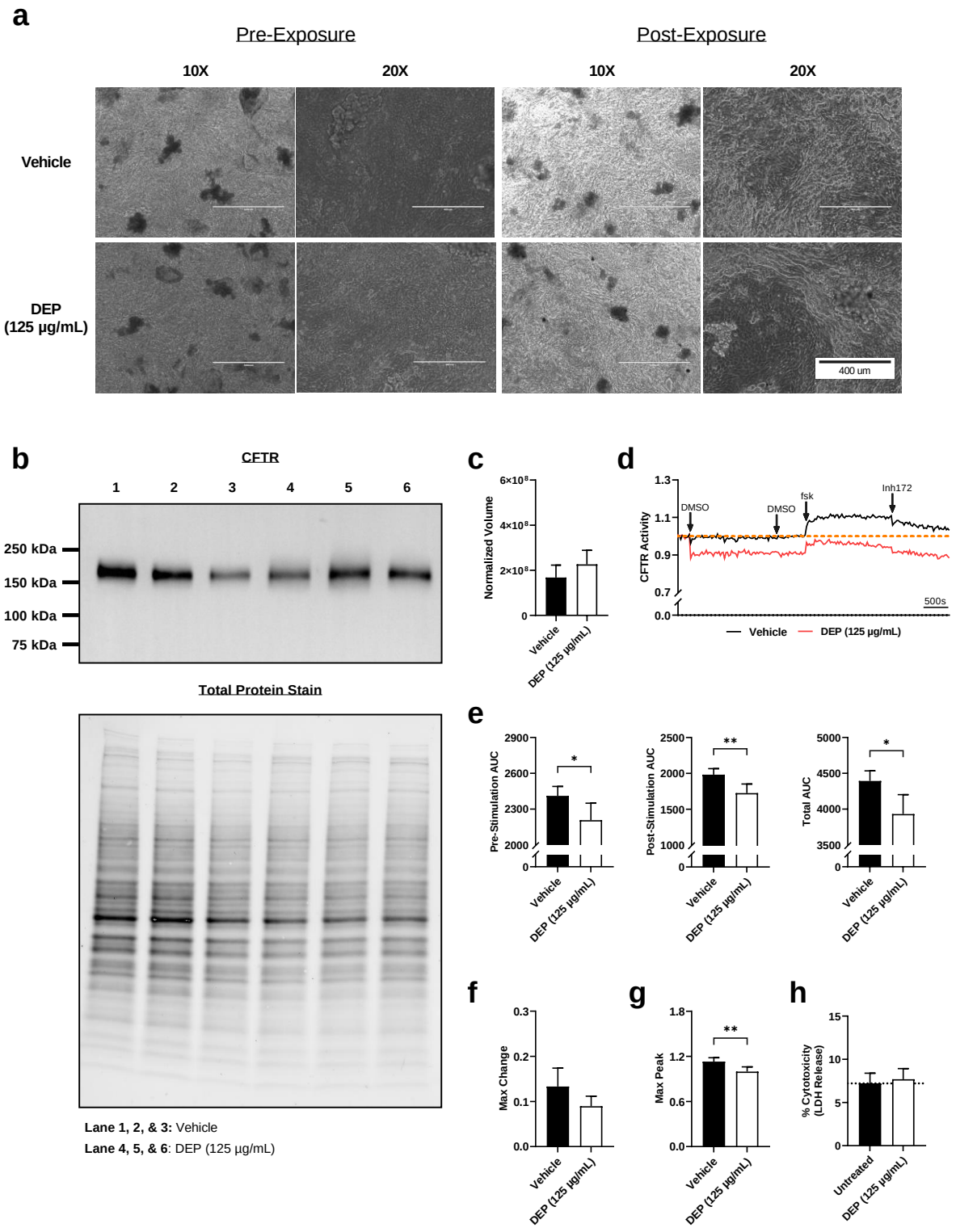


Figure 3: *Figure legend continued on the following page.*

Figure 3: Effect of DEP on CFTR expression and function in human airway epithelial cells. Human airway epithelial (Calu-3) cells were exposed to 125 µg/mL of DEP for 24 h treatment. (**a**) Microscopy images were taken at the 10X and 20X objective pre- and post-24 h treatment. (**b** and **c**) CFTR protein expression was characterized and measured with western blot and a total protein stain was performed as the loading control. Band intensities were normalized via total protein normalization with data presented as ± SD (n=3). Full-length blots are presented in Supplementary Fig. 1c. (**d**) CFTR function was measured using an *in vitro* membrane potential assay. The line graph shows the change in CFTR activity in response to different stimulations (DMSO, forskolin, and CFTR Inh-172). CFTR activity was normalized to stabilized baseline over vehicle control, represented by the dotted line (n=3-4). Analysis of the line graph was performed for (**e**) pre-stimulated, post-stimulated and total AUC, (**f**) max change, and (**g**) max peak with data presented as ± SD (n=3). (**h**) Cellular cytotoxicity was measured using a LDH assay on collected supernatants with data presented as ± SD (n=4). An unpaired *t*-test was used for the statistical analysis of CFTR protein expression and cellular cytotoxicity, while a one-way ANOVA with subsequent multiple comparisons was used for the analysis of AUC, max change, and max peak. *$P \leq 0.05$; **$P \leq 0.01$

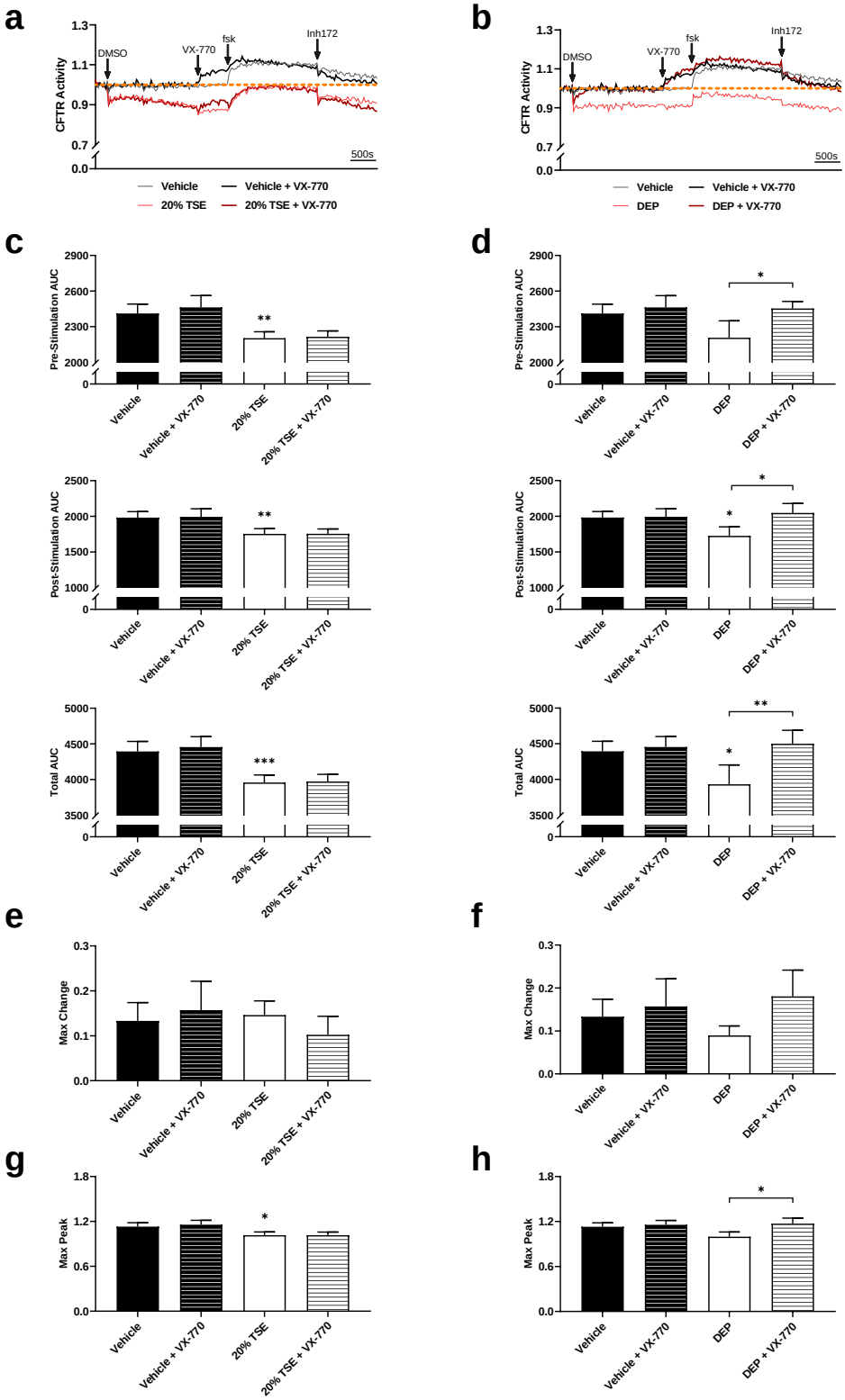


Figure 4: *Figure legend continued on the following page.*

Figure 4: Effect of CFTR modulator VX-770 on CFTR function in human airway epithelial cells after TSE or DEP exposures. Human airway epithelial (Calu-3) cells were exposed to either 20% TSE or 125 µg/mL of DEP for 24 h treatment. (**a** and **b**) CFTR function was measured using an *in vitro* membrane potential assay. The line graph shows the change in CFTR activity in response to different stimulations (DMSO, VX-770, forskolin, and CFTR Inh-172). CFTR activity was normalized to stabilized baseline over vehicle control, represented by the dotted line (n=3-4). Analysis of the line graph was performed for (**c** and **d**) pre-stimulated, post-stimulated and total AUC, (**e** and **f**) max change, and (**g** and **h**) max peak with data presented as ± SD (n=3-4). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

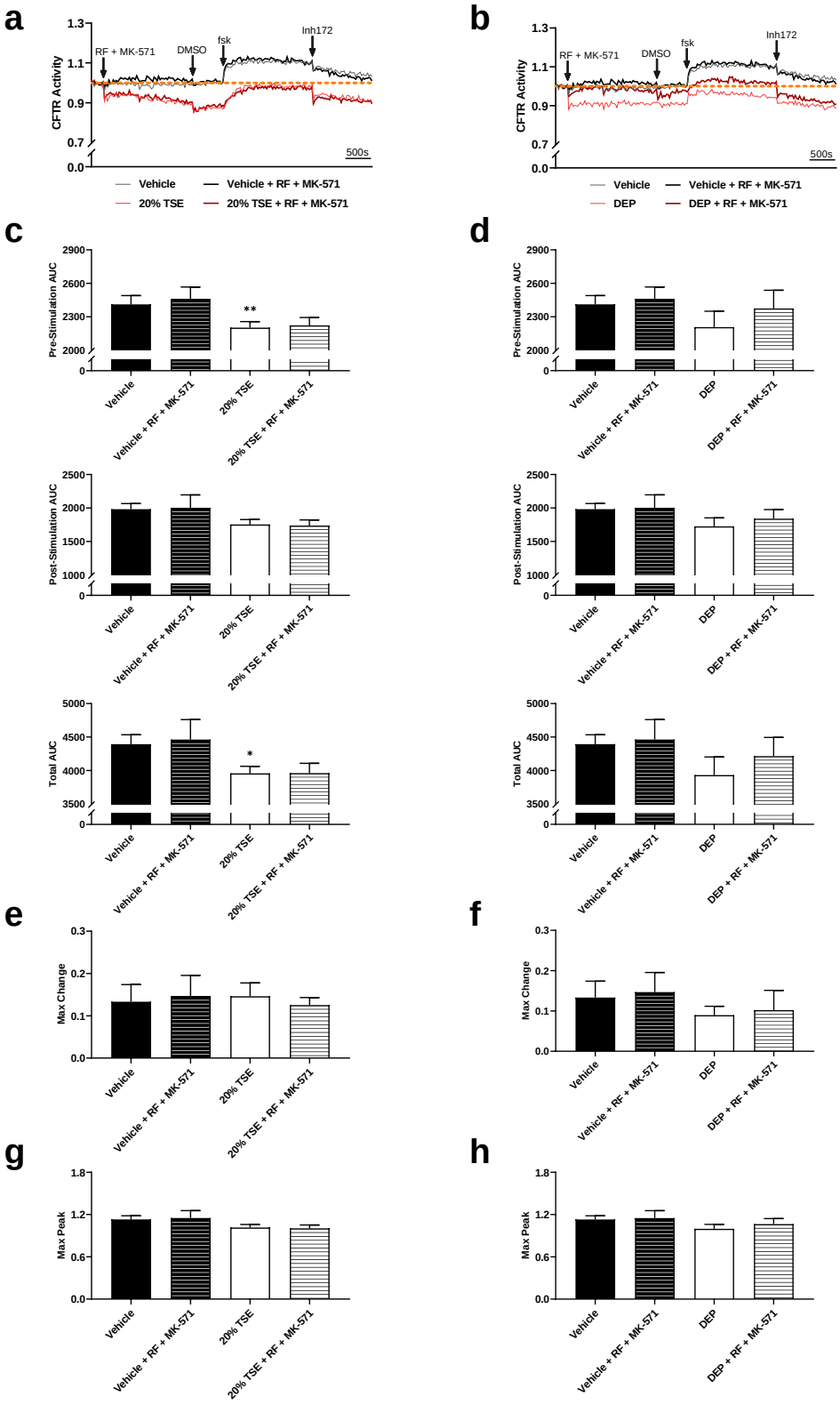


Figure 5: *Figure legend continued on the following page.*

Figure 5: Effect of cAMP modulation, through ABCC4 and PDE-4 inhibition, on CFTR function in human airway epithelial cells after TSE or DEP exposures. Human airway epithelial (Calu-3) cells were exposed to either 20% TSE or 125 µg/mL of DEP for 24 h treatment. (**a** and **b**) CFTR function was measured using an *in vitro* membrane potential assay. The line graph shows the change in CFTR activity in response to different stimulations (RF + MK-571, DMSO, forskolin, and CFTR Inh-172). CFTR activity was normalized to stabilized baseline over vehicle control, represented by the dotted line (n=3-4). Analysis of the line graph was performed for (**c** and **d**) pre-stimulated, post-stimulated and total AUC, (**e** and **f**) max change, and (**g** and **h**) max peak with data presented as ± SD (n=3-4). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. *$P \leq 0.05$; **$P \leq 0.01$

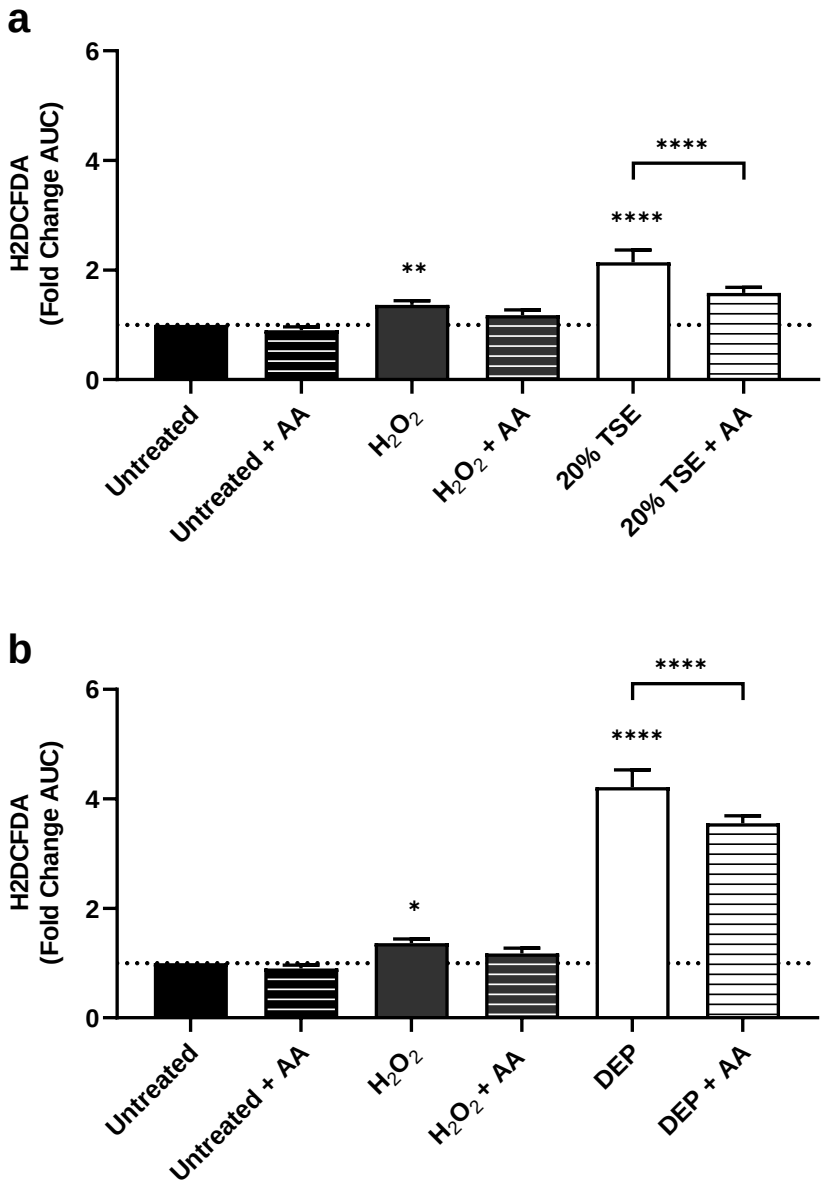


Figure 6: Effect of TSE and DEP on oxidative stress and evaluating the effectiveness of antioxidant ascorbic acid in human airway epithelial cells. Human airway epithelial (Calu-3) cells were exposed to either 20% TSE or 125 µg/mL of DEP for 24 h treatment with and without 100 µM ascorbic acid (AA) for the entire duration of the experiment. Oxidative stress was measured using the H2DCFDA reactive oxygen species fluorescence sensor in (**a**) TSE-exposed and (**b**) DEP-exposed cells. 0.5 mM hydrogen peroxide (H_2O_2) was used as a positive control to induce oxidative stress. All data is presented as ± SD (n=4). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. *$P \leq 0.05$; **$P \leq 0.01$; ****$P \leq 0.0001$

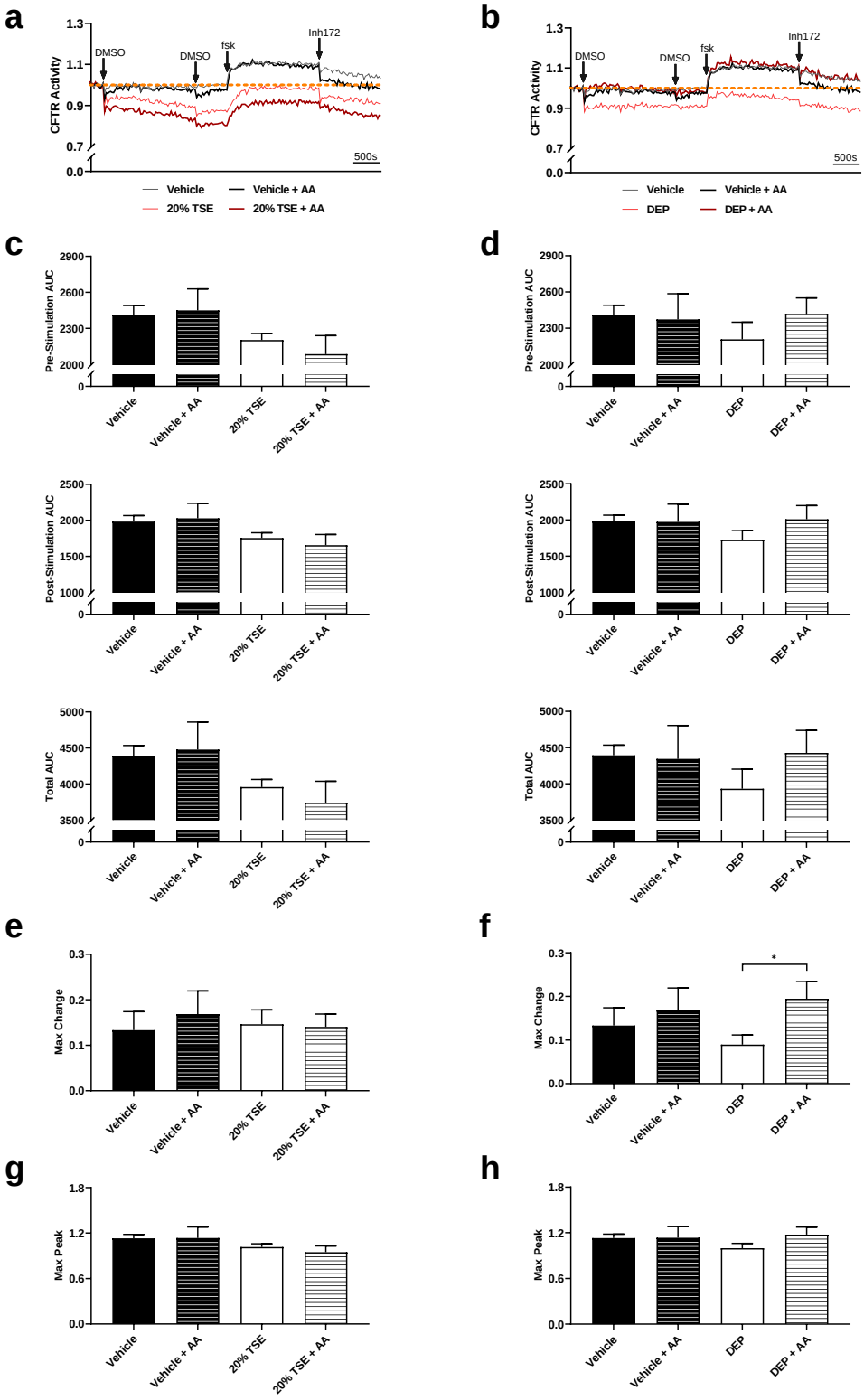
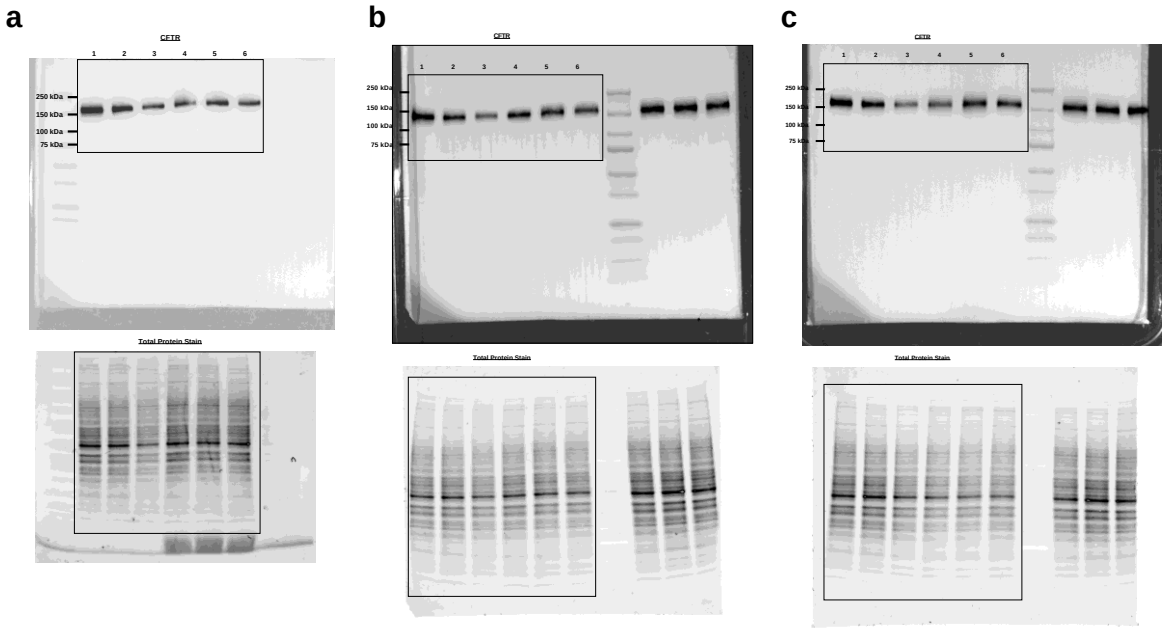


Figure 7: *Figure legend continued on the following page.*

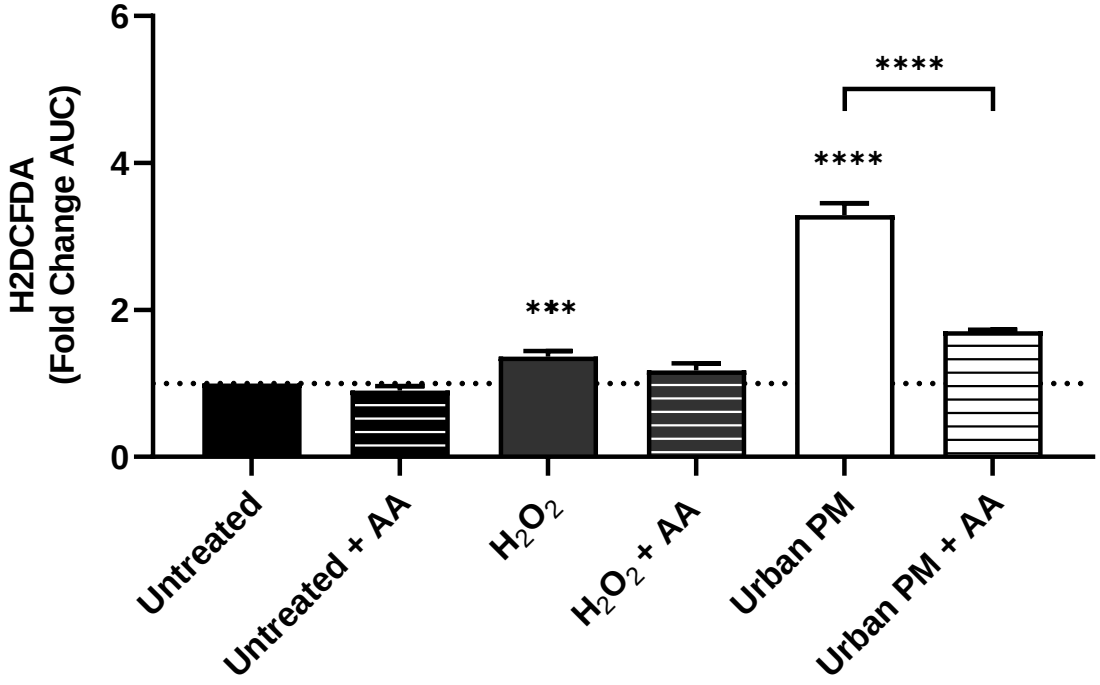
Figure 7: Effect of ascorbic acid on CFTR function in human airway epithelial cells after TSE or DEP exposures. Human airway epithelial (Calu-3) cells were exposed to either 20% TSE or 125 µg/mL of DEP for 24 h treatment with and without 100 µM ascorbic acid (AA) for the entire duration of the experiment. (**a** and **b**) CFTR function was measured using an *in vitro* membrane potential assay. The line graph shows the change in CFTR activity in response to different stimulations (DMSO, forskolin, and CFTR Inh-172). CFTR activity was normalized to stabilized baseline over vehicle control (n=3-4). Analysis of the line graph was performed for (**c** and **d**) pre-stimulated, post-stimulated and total AUC, (**e** and **f**) max change, and (**g** and **h**) max peak with data presented as ± SD (n=3-4). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. **P*≤ *0.05*

Supplementary Information

Supplementary Figures



Supplementary Figure 1: Full-length western blot images used in Fig. 1-3. CFTR and total protein stain blots post-24 h treatment with (**a**) TSE, (**b**) Urban PM, and (**c**) DEP.



Supplementary Figure 2: Effect of Urban PM on oxidative stress and evaluating the effectiveness of antioxidant ascorbic acid in human airway epithelial cells. Human airway epithelial (Calu-3) cells were exposed to 125 µg/mL of Urban PM for 24 h treatment with and without 100 µM ascorbic acid (AA) for the entire duration of the experiment. Oxidative stress was measured using the H2DCFDA reactive oxygen species fluorescence sensor in Urban PM-exposed cells. 0.5 mM hydrogen peroxide (H_2O_2) was used as a positive control to induce oxidative stress. All data is presented as ± SD (n=4). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. ****P≤ 0.001; ******P≤ 0.0001*

CHAPTER 4:

Effects of cAMP and CFTR modulation on airway surface liquid pH in human airway epithelial cells

Jenny P. Nguyen, Jeremy A. Hirota

The following study is to be submitted

Author Contributions:

J.P.N. conducted all experiments, performed data analyses, generated figures, conducted the literature review, contributed to the manuscript conception, and drafted the manuscript. J.A.H. (Principal Investigator and Corresponding Author) provided oversight for the entire study, including data collection, analyses, manuscript drafting, and finalization.

Study Overview:

This study investigates the impact of pharmacological interventions, specifically cAMP and CFTR modulators, on airway surface liquid pH in Calu-3 cells. Airway surface liquid regulation is crucial for host defense mechanisms, including mucociliary clearance and antimicrobial activity, and relies on the proper function of various ion transporters, such as CFTR. The aim of this study was to determine whether these interventions could effectively raise airway surface liquid pH, potentially serving as a therapeutic strategy for respiratory diseases characterized by airway surface liquid abnormalities, including CF and COPD. This study aligns with the thesis topic by exploring the role of cAMP and CFTR modulation in airway surface liquid pH regulation and contributes insights into the role of CFTR in maintaining airway surface liquid homeostasis. Moreover, our study proposes a potential therapeutic strategy for respiratory diseases with airway surface liquid abnormalities.

Effects of cAMP and CFTR modulation on airway surface liquid pH in human airway epithelial cells

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Abstract

The airway epithelium serves as the first line of defense against inhaled pathogens and particles present in the external environment by acting as a physical barrier and through various host defense mechanisms, including mucociliary clearance (MCC) and antimicrobial activity. The proper maintenance of these host defense mechanisms relies heavily on the regulation of airway surface liquid (ASL) volume, pH, and composition, a process that is tightly controlled by various ion transporters, including the cystic fibrosis transmembrane conductance regulator (CFTR) protein. With evidence suggesting dysfunctional CFTR-mediated bicarbonate secretion leads to airway acidification, resulting in impaired MCC and antimicrobial activity, there is an increased interest in therapies that can lead to improvements in ASL pH. The aim of our study was to determine whether pharmacological interventions, via cAMP and CFTR modulators, lead to an increase in ASL pH. Human airway epithelial (Calu-3) cells were exposed to various combinations of cAMP and CFTR modulating agents to assess their effectiveness at elevating ASL pH. Our results show that pharmacological interventions with cAMP elevating agents and CFTR modulator VX-770 led to significant increases in ASL pH, with combinations leading to greater increases compared to single drug interventions. Our study suggests that cAMP and CFTR modulation has potential as a therapeutic strategy for elevating ASL pH and may be beneficial for respiratory diseases with ASL abnormalities.

Introduction

The airway epithelium is the first line of defense against harmful pathogens and inhaled particles present in the external environment by acting as a physical barrier and by initiating various host defense mechanisms, including mucociliary clearance (MCC) and antimicrobial activity[1,2]. MCC is an innate host defense mechanism that protects the lungs by capturing pathogens and particulates in the mucus and expelling them out of the airways[1–3]. It is a highly coordinated process that is dependent on several different components, including proper ciliary function and airway surface liquid (ASL)[1,4]. The ASL, which is comprised of a periciliary layer and a mucus layer, contributes to proper airway hydration. In addition, the ASL also contains antimicrobial agents, thus contributing to antimicrobial host defenses[1–4]. The ASL composition, as well as the volume and pH, is highly regulated by various ion transporters, including the cystic fibrosis transmembrane conductance regulator (CFTR) protein[2–9]. Due to its critical role for normal airway function, dysregulation of ASL is associated with various diseases, including cystic fibrosis (CF)[3,4].

CF is a recessive genetic disease caused by the inheritance of two mutations in the CFTR gene[10]. CFTR is a phosphorylation-dependent ion channel that conducts chloride and bicarbonate ions across the epithelia, contributing to epithelial fluid transport in the lungs[11–14]. Due to CFTR dysfunction, individuals with CF experience several complications, including impaired MCC, reduced antimicrobial activity, abnormal inflammatory responses, persistent airway infections, and airway obstruction[6–9,15]. Collectively, these consequences can result in the loss of pulmonary function, the major

cause of morbidity and mortality in CF. The pathogenesis of CF lung disease has been suggested to be initiated by defective airway host defenses, thus predisposing CF individuals to persistent airway infections and increased inflammation[2,3,8,9].

Several studies have demonstrated that the loss of CFTR function, resulting in impaired bicarbonate secretion, leads to airway surface liquid acidification and consequently, impaired antimicrobial activity of cationic host defense peptides (CHDPs)[5,8,9,15–18]. CHDPs are an essential component of the innate immune system and are involved in modulating the adaptive immune response[2]. The two most well-characterized families of CHDPs in mammals are defensins and cathelicidins[2,19]. *In vitro* and *in vivo* studies using human bronchial epithelial cells and porcine models, an animal model that recapitulates hallmark features of CF lung disease, demonstrated that ASL pH is more acidic in CF models compared to healthy wild-type controls[7,8,15–17]. Furthermore, these studies also demonstrated that this acidification of ASL leads to reduced antimicrobial activity and that select CHDPs, such as human β-defensins and cathelicidin-related peptide LL-37, have pH-dependent and synergistic activity[9,15]. Additionally, normalization of pH was shown to improve bacterial killing[8,9,15,16]. In addition to CF, there has been increasing evidence supporting CFTR dysfunction and ASL abnormalities in other respiratory diseases, including chronic obstructive pulmonary disease (COPD)[4,20,21]. With emerging evidence demonstrating that CFTR dysfunction results in acidic ASL pH and reduced antimicrobial activity, which can lead to downstream complications, further research on potential therapeutics that can improve CFTR function, thus increasing chloride and bicarbonate secretion, should be pursued.

Currently, there exists pharmacological interventions for the treatment of CFTR dysfunction for select CFTR mutations called CFTR modulators, which are small molecules designed to correct and improve the function of CFTR[22–26]. Presently, there are four CFTR modulator treatments approved by the Federal Drug Administration, Kalydeco (ivacaftor; VX-770), Orkambi (lumacaftor/ivacaftor; VX-809/VX-770), Symdeko (tezacaftor/ivacaftor; VX-661/VX-770), and Trikafta (elexacaftor/tezacaftor/ivacaftor; VX-445/VX-661/VX-770)[27–30]. The use of CFTR modulators has been shown to be beneficial for CF management via improvement in lung function and reduction in pulmonary exacerbations[31–34]. In addition to existing CFTR modulators, there has been several investigations into other molecules that can impact CFTR function, including drugs that can modulate cAMP metabolism. Intracellular cAMP levels are regulated by various mechanisms, including cAMP-efflux transporters and phoshodiesterases[35–39]. Previously, we have demonstrated that pharmacological inhibition of cAMP-efflux transporter ABCC4, in combination with CFTR potentiator VX-770, increases CFTR function beyond VX-770 alone in G551D-CFTR subjects[35]. Additionally, we have also demonstrated that cAMP modulation, via inhibition of ABCC4 and PDE-4, in combination with VX-770, is able to increase sensitivity and CFTR activity in Calu-3 cells, when compared to VX-770 treatment alone[40]. Furthermore, we have also demonstrated that pharmacological intervention with VX-770 was able to rescue acquired CFTR dysfunction caused by diesel exhaust particles[41]. Altogether, these studies suggest that combining cAMP modulation with CFTR modulators can be used to improve CFTR function to potentially increase ASL pH and antimicrobial activity. However, more research needs to be performed.

In this study, we hypothesized that the potentiation of CFTR function in human airway epithelial cells, via cAMP modulation and CFTR modulators, will lead to an increase in ASL pH. To investigate the effect of cAMP and CFTR modulation on ASL pH, human airway epithelial cells were exposed to various pharmacological interventions. We demonstrate that pharmacological interventions with combinations of cAMP and CFTR modulating agents led to an increase in ASL pH, but these increases in pH cannot be solely attributed to CFTR. Our results indicate the involvement of additional ion transporters and mechanisms in maintaining airway surface liquid homeostasis, potentially compensating for impaired CFTR function. Collectively, our results provide supporting evidence of cAMP and CFTR modulation as a therapeutic strategy for elevating ASL pH and prompts future investigations into related downstream effects, particularly whether increases in ASL pH will lead to improvements in antimicrobial activity.

Materials and Methods

Reagents

All pharmacological intervention compounds were purchased from Cayman Chemical, excluding Ivacaftor, CFTR Inh-172, and GlyH-101, which were purchased from Selleck Chemicals. For cAMP modulation, cAMP elevating agent Forskolin (FSK) and Isoproterenol (ISO), ABCC4 inhibitor MK-571, and PDE-4 inhibitor Roflumilast (RF) were used. For CFTR modulation, CFTR potentiator Ivacaftor (VX-770), and CFTR inhibitors CFTR Inh-172 (CFTRinh-172) and GlyH-101 were used. All compounds were dissolved in DMSO.

Human airway epithelial cell culture

The human airway epithelial cell line Calu-3 (ATCC HTB-55), derived from lung adenocarcinoma tissue, was cultured in Alpha-Minimum Essential medium (α-MEM) (Corning) supplemented with 10% fetal bovine serum (VWR), 1% HEPES (Corning), and 1% penicillin-streptomycin at 37°C and 5% CO_2 for both submerged and air-liquid interface (ALI) culture conditions. The medium was changed three times per week (every Monday, Wednesday, and Friday). Calu-3 cells were grown until they reached 50% to 70% confluency and were subsequently passaged and plated onto 6.5 mm Transwell Inserts (Corning). Medium changes to both the apical (top) and basolateral (bottom) compartment were performed until 100% confluency was reached. Calu-3 cells were then grown at ALI for at least 21 days post-air lift prior to airway surface liquid pH experiments.

Transepithelial electrical resistance (TEER) measurements

Electrical resistance measurements were performed after washing the apical surface of the cell layer with 200 µL of warmed PBS (10 min) and feeding the basolateral compartment with medium using the Millicell ERS-2 Epithelial Volt-Ohm Meter (Millipore). TEER values were subsequently calculated and used as a method for quality control to confirm the integrity and permeability of the cell layer on the Transwell insert. For all experiments, the averaged TEER values for the Transwell inserts were typically ≥200 $\Omega*cm^2$.

Airway surface liquid pH measurements

Calu-3 cells were washed with warmed PBS prior to experimental conditions and TEER measurements were performed 1 hr prior to stimulation with pharmacological interventions. For ASL pH experiments, the apical compartment was loaded with 100 µL of HCO_3^- and K^+ -free saline Ringer's Solution (135 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.5 mM Na_2HPO_4; pH 6), while the basolateral compartment was replaced with HEPES-free α-MEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Pharmacological interventions were administered to the basolateral compartment for 3 h at 37°C, unless otherwise stated. After 3 h, plates were left at RT for 30 min prior to collection of the fluid in the apical compartment into microcentrifuge tubes. Tubes were spun down at 7500 rpm for 10 min to remove any cells and mucus, then subsequently transferred to a new set of tubes. After collection of the cell and mucus-free diluted ASL fluid, pH was measured using the commercially available Orion PerpHecT

ROSS Combination pH Micro Electrode (Thermo Scientific). Prior to all pH measurements, the pH microelectrode was calibrated using the appropriate buffers and was rinsed with ddH_2O between measurements. Following collection of the diluted ASL, if cells were intended for subsequent experiments, the basolateral compartment was replaced with complete α-MEM and transferred into the incubator at 37°C and 5% CO_2 for future use.

Statistical analysis

For all ASL pH experiments, a single pH measurement was taken for each Transwell insert and measured pH values were averaged across all biological replicates (n=3-7). SD was calculated using data from all biological replicates (n=3-7) and a one-way ANOVA with subsequent multiple comparisons was performed. Statistical analysis was performed using GraphPad Prism 10.

Results

cAMP elevating agents increase ASL pH

In our previous study, we demonstrated the ability of cAMP elevating agents FSK and ISO to induce CFTR activity in human airway epithelial Calu-3 cells[40]. Furthermore, we also demonstrated the capacity of FSK to elicit a rise in ASL pH[42]. To validate whether enhanced CFTR activity corresponds to increases in ASL pH, Calu-3 cells grown under ALI conditions were exposed to FSK or ISO for 3 h in the basolateral compartment (**Fig. 1**). Cells treated with FSK showed a dose-dependent increase in ASL pH (**Fig. 1a**) and comparisons between treatment groups confirmed these increases to be significant (**Fig. 1b**; ****$P \leq 0.0001$*). In contrast, ISO did not show a dose-dependent increase in ASL pH (**Fig. 1c**), but the changes in ASL pH were still significant (**Fig. 1d**; ****$P \leq 0.0001$*). These findings align with previous studies demonstrating that the introduction of cAMP elevating agents result in a rise in ASL pH[7,17,42]. Altogether, these findings indicate that cAMP elevating agents FSK and ISO not only induce CFTR activity but also increase ASL pH.

pH is unaltered in the absence of cells

To confirm whether the observed changes in ASL pH are due to cellular responses, rather than the direct addition of the drugs, we tested various pharmacological interventions in the absence of Calu-3 cells (**Fig. 2**). No significant differences in pH were observed in response to FSK, ISO, CFTR potentiator VX-770, PDE-4 inhibitor RF, ABCC4 inhibitor MK-571, or combinations of these drugs. This suggests that the previously observed

increases in ASL pH are likely attributed to cellular responses to the pharmacological interventions rather than the drug compounds themselves.

Impact of pharmacological interventions on ASL pH

Having established that the observed changes in pH are linked to cellular responses, we next investigated the effect of various pharmacological interventions on ASL pH (**Fig. 3).** Notably, the administration of VX-770, FSK, and ISO, individually and in combination, resulted in significant increases in ASL pH (**Fig. 3**; ****$P \leq 0.0001$*). This aligns with previous studies demonstrating that CFTR modulators can influence ASL properties[22,43,44]. In contrast, the use of RF and MK-571 alone did not lead to increases in ASL pH (**Fig. 3**). However, when RF and MK-571 were used in combination with a cAMP elevating agent, this led to a rise in ASL pH (**Fig. 3**; ****$P \leq 0.0001$*). Furthermore, drug combinations led to greater increases in ASL pH compared to their individual use (**Fig. 3**; *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$*). These findings suggest that combinational therapies are better at elevating ASL pH than single therapies, emphasizing the potential of combinatorial approaches.

CFTR inhibition does not reduce ASL pH

Following our observations that pharmacological interventions modulating cAMP and CFTR, which have been shown to induce CFTR activity, can elevate ASL pH, we next explored whether inhibiting CFTR would result in a decrease in ASL pH.

Calu-3 cells were exposed to CFTR inhibitors CFTRinh-172 and GlyH-101 in the basolateral compartment for 3 h at various concentrations (**Fig. 4**). Basolateral administration of CFTRinh-172 did not lead to a reduction in ASL pH (**Fig. 4a and b**). Likewise, inhibition of CFTR using GlyH-101 also failed to reduce ASL pH (**Fig. 4c and d**). Since basolateral administration of CFTR inhibitors did not reduce ASL pH, we exposed Calu-3 cells to CFTR inhibitors in the apical compartment (**Fig. 5**), considering that CFTR is localized to the apical membrane[45,46]. Similarly, apical administration of CFTRinh-172 (**Fig. 5 and b**) and GlyH-101 (**Fig. 5c and d**) did not lead to a reduction ASL pH.

Considering that neither basolateral nor apical administration of CFTR inhibitors reduced ASL pH, we next investigated whether pre-treatment with CFTR inhibitors would prevent the observed increases in ASL pH resulting from pharmacological interventions (**Fig. 6**). While pre-treating Calu-3 cells with CFTR inhibitors for 30 min to the apical compartment resulted in reduced magnitude of elevated ASL pH in response to pharmacological interventions FSK, ISO, and VX-770, it was not of significance (**Fig. 6**). Neither CFTRinh-172 (**Fig. 6a**) nor GlyH-101 (**Fig. 6b**) prevented increases in ASL pH. Collectively, these findings suggest that while pharmacological interventions modulating cAMP or CFTR can elevate ASL pH, increases in ASL pH cannot be solely attributed to CFTR. This implies the involvement of alternative ion channels or transporters which may compensate for CFTR.

Discussion

The airway epithelium plays a crucial role in protecting us against inhaled pathogens and particulates, relying on effective host defense mechanisms such as MCC and antimicrobial activity. These host defense mechanisms require proper regulation of ASL composition and properties. CFTR, the protein implicated in CF, plays a key role in ASL regulation, with dysfunction in CFTR being associated with airway acidification and impaired host defenses. In the present study, we investigate the effect of pharmacological interventions, specifically cAMP and CFTR modulators, on ASL pH. Using Calu-3 cells, we demonstrate that cAMP elevating agents FSK and ISO, along with CFTR potentiator VX-770, lead to significant increases in ASL pH. These results are consistent with our previous study which showed the ability of these compounds to induce CFTR activity in Calu-3 cells[40]. Additionally, cAMP elevating agents, when used in combination with VX-770, resulted in greater increases in ASL pH, suggesting a potential synergistic effect. These findings prompt further exploration into whether pharmacological interventions that enhance CFTR activity and elevate ASL pH, can translate into improvements in host defense mechanisms.

The addition of FSK to Calu-3 cells resulted in a dose-dependent increase in ASL pH. In contrast, while ISO also led to significant increases in ASL pH, a dose-dependent increase was not observed, likely due to saturation of the response at the lowest concentration used. Importantly, we confirmed that alterations in pH do not occur in the absence of cells. These findings are consistent with our previous demonstration that cAMP elevating agents can potentiate CFTR activity and elevate ASL pH, supporting the notion

that increases in CFTR activity may translate to elevations in ASL pH[40,42]. Our results align with other studies demonstrating a rise in ASL pH in response to cAMP elevation but contradict previous studies that indicated no significant impact of cAMP elevation on ASL pH[7,15,17,47].

To further explore this, we next investigated the impact of pharmacological interventions, targeting cAMP and CFTR, on ASL pH. VX-770, FSK, and ISO individually resulted in significant increases in ASL pH. In contrast, individual administration of PDE-4 inhibitor RF or ABCC4 inhibitor MK-571 did not elevate ASL pH. These findings align with our previous studies that assessed their ability to potentiate CFTR[40,41]. However, when VX-770, RF, or MK-571 was administered in combination with either cAMP elevating agent FSK or ISO, we observed significant increases in ASL pH. Moreover, these increases were more substantial than individual interventions, suggesting synergistic effects with drug combinations. Our findings are supported by previous demonstrations of these compounds influencing ASL properties[7,17,48]. Altogether, these results highlight the potential of these interventions as a therapeutic strategy for elevating ASL pH.

Since pharmacological interventions using cAMP and CFTR modulators elevated ASL pH, given that dysfunctional CFTR has previously been linked to airway acidification, we next investigated whether CFTR inhibition would result in a reduction in ASL pH[8,16,17]. Neither basolateral nor apical administration of CFTR inhibitors, CFTRinh-172 and GlyH-101, led to a decrease in ASL pH. Furthermore, pre-treatment with CFTR inhibitors did not prevent the increases in ASL pH induced by cAMP and CFTR modulators. This suggests that while CFTR activation contributes to elevated ASL pH, other ion channels and

transporters are likely involved, compensating for impaired CFTR function to maintain pH levels[3]. Notably, VX-770 elevated ASL pH even after CFTR inhibition with GlyH-101. VX-770, recognized as a selective potentiator of CFTR, binds directly to CFTR and enhances its gating[49,50]. CFTRinh-172 inhibits CFTR by stabilizing the closed channel, impacting gating without directly occluding the pore, while GlyH-101 inhibits CFTR by pore occlusion[51–53]. Both CFTRinh-172 and GlyH-101 are known to be potent inhibitors, however, they may have off-target effects on other channels at the concentrations used in our study, perhaps partially explaining the observed rise in ASL pH[54].

Ion channels and transporters involved in ASL regulation include ATP12A – an apical proton pump, pendrin (SLC26A4) – an apical chloride/bicarbonate exchanger, TMEM16A – a calcium-activated chloride channel, and ENaC – an epithelial sodium channel[55–59]. While there has been strong evidence demonstrating the importance of pendrin activity in regulating ASL pH, it has been suggested that most bicarbonate secretion in Calu-3 cells is through CFTR[60]. Another important factor to take into consideration when studying ASL pH regulation is inflammation, which has been shown to play a key role in ion transport by modifying various mechanisms involved[18,61–63]. Studies have shown that the presence of inflammatory cytokines, such as TNF-α and IL-17 – two cytokines elevated in CF individuals – are able to increase ASL pH[18,63]. This pH elevation is further enhanced with the administration of CFTR modulators[18,63]. Altogether, the regulation of ASL pH involves a complex interplay of various ion channels and is further influenced by inflammation. Thus, future studies on ASL regulation will need to consider all these variables.

In this study, we demonstrate the potential of combinatorial therapies targeting cAMP and CFTR for improving ASL pH. Our findings suggest the effectiveness of targeting multiple pathways, as drug combinations led to greater increases in ASL pH compared to individual interventions. However, it is important to note that the elevations in ASL pH observed cannot be solely attributed to CFTR, thus further investigation into other ion channels and transporters involved in ASL regulation, along with the influence of inflammatory cytokines, should be pursued to better understand the underlying mechanisms involved. Moreover, future studies exploring the effect of these pharmacological interventions on host defense mechanisms, such as antimicrobial activity and MCC, should be performed. Altogether, this study supports further investigation into the development of therapeutic strategies aimed at elevating ASL pH. This therapeutic strategy has the potential to enhance antimicrobial activity and MCC, benefiting respiratory diseases characterized by ASL abnormalities, including CF and COPD.

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Figures

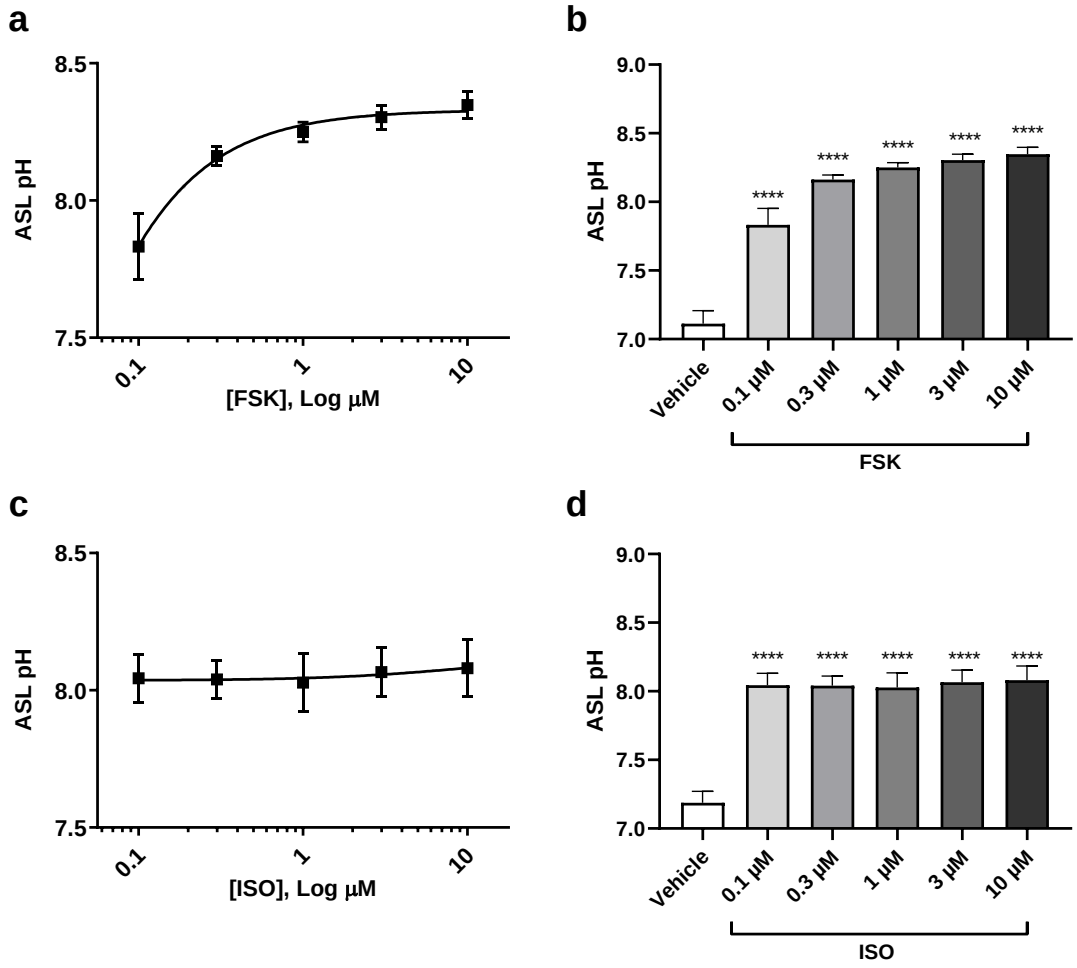

Figure 1: Concentration-response analysis of cAMP elevating agents on airway surface liquid pH. Human airway epithelial (Calu-3) cells were exposed to cAMP elevating agent (**a** and **b**) forskolin and (**c** and **d**) isoproterenol in the basolateral (bottom) compartment for 3 h at various concentrations. (**a** and **c**) A dose-response curve for measured ASL pH is depicted and (**b** and **d**) comparisons between treatment groups were performed. Data presented as means ± SD (n=5). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. **** *P≤0.0001*

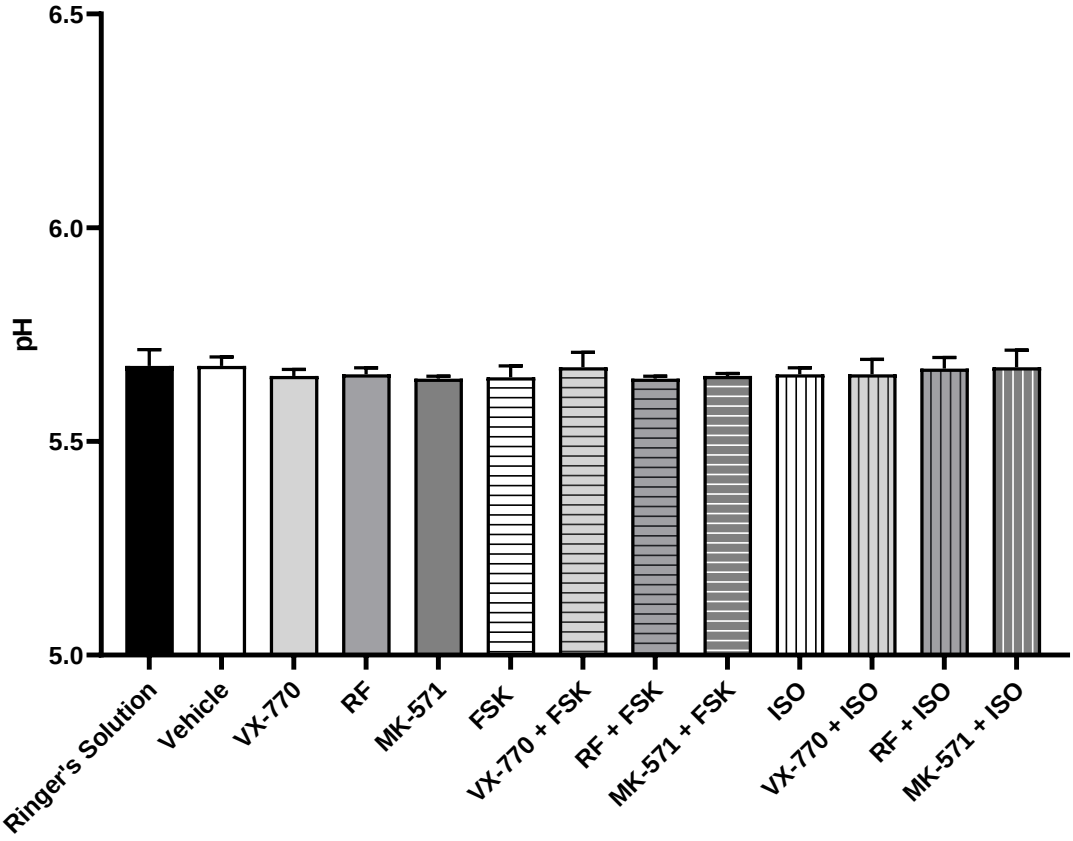

Figure 2: Control experiment in Ringer's solution to validate drug-induced pH changes in the absence of cells. Various combinations of cAMP elevating agents forskolin (0.1 μM) and isoproterenol (0.01 μM), CFTR potentiator VX-770 (1 μM), PDE-4 inhibitor roflumilast (1 μM), and ABCC4 inhibitor MK-571 (10 μM) were administered to the basolateral (bottom) compartment for 3 h to evaluate the change in pH in the absence of human airway epithelial (Calu-3) cells. Data presented as means ± SD (n=3). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis.

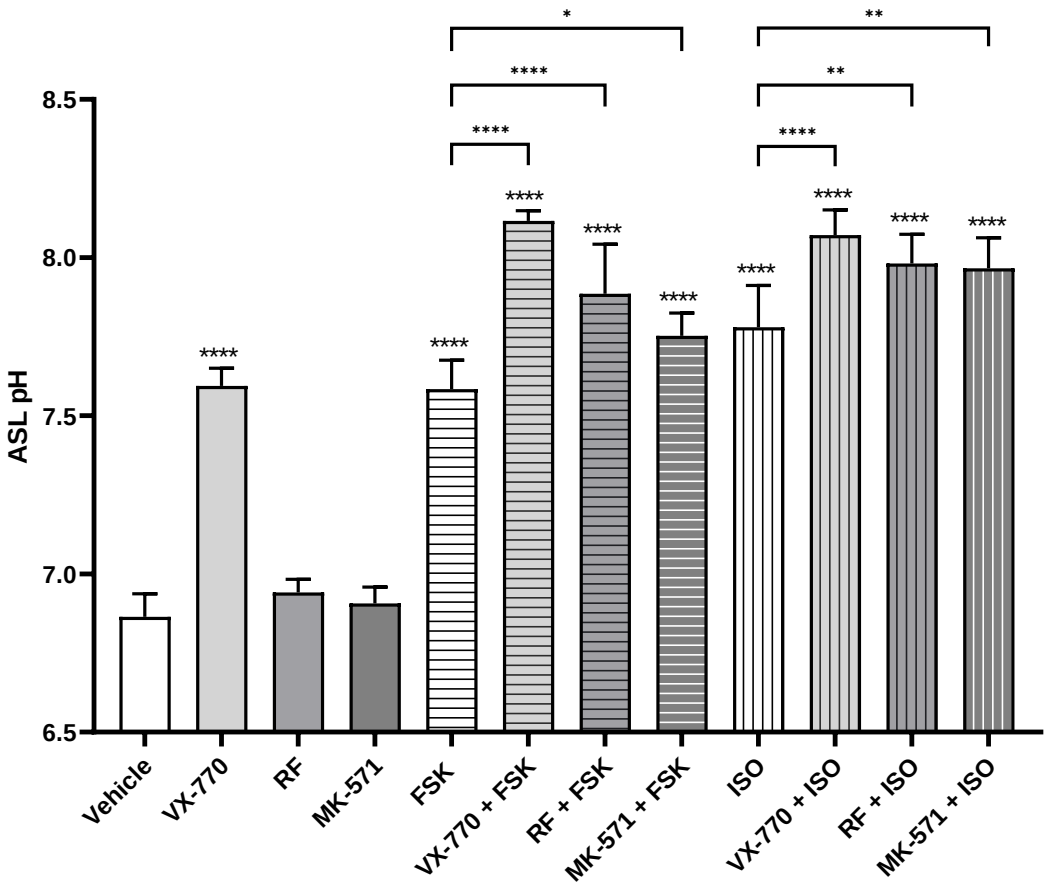

Figure 3: Effect of various pharmacological interventions on airway surface liquid pH. Human airway epithelial (Calu-3) cells were exposed to various combinations of cAMP elevating agents forskolin (0.1 μM) and isoproterenol (0.01 μM), CFTR potentiator VX-770 (1 μM), PDE-4 inhibitor roflumilast (1 μM), and ABCC4 inhibitor MK-571 (10 μM) to the basolateral (bottom) compartment for 3 h. Measured ASL pH is depicted and comparisons between treatment groups were performed. Data presented as means ± SD (n=7). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. *$P \leq 0.05$; **$P \leq 0.01$; **** $P \leq 0.0001$

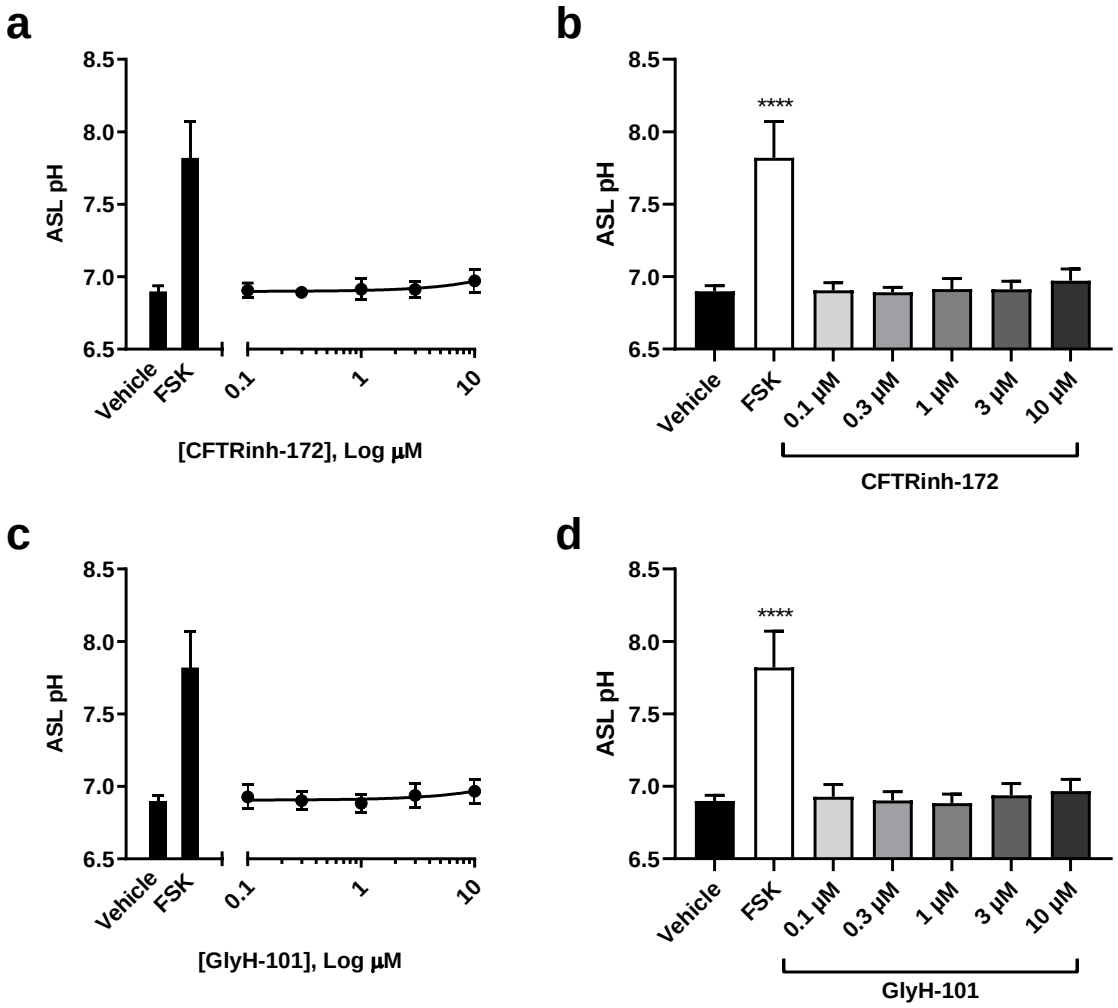

Figure 4: Concentration-response of CFTR inhibitors on airway surface liquid pH with basolateral compartment administration. Human airway epithelial (Calu-3) cells were exposed to CFTR inhibitors (**a** and **b**) CFTRinh-172 and (**c** and **d**) GlyH-101 in the basolateral (bottom) compartment for 3 h at various concentrations. cAMP elevating agent forskolin (0.1 µM) was used as a positive control. (**a** and **c**) A dose-response curve for measured ASL pH is depicted and (**b** and **d**) comparisons between treatment groups were performed. Data presented as means ± SD (n=5). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. **** *P≤0.0001*

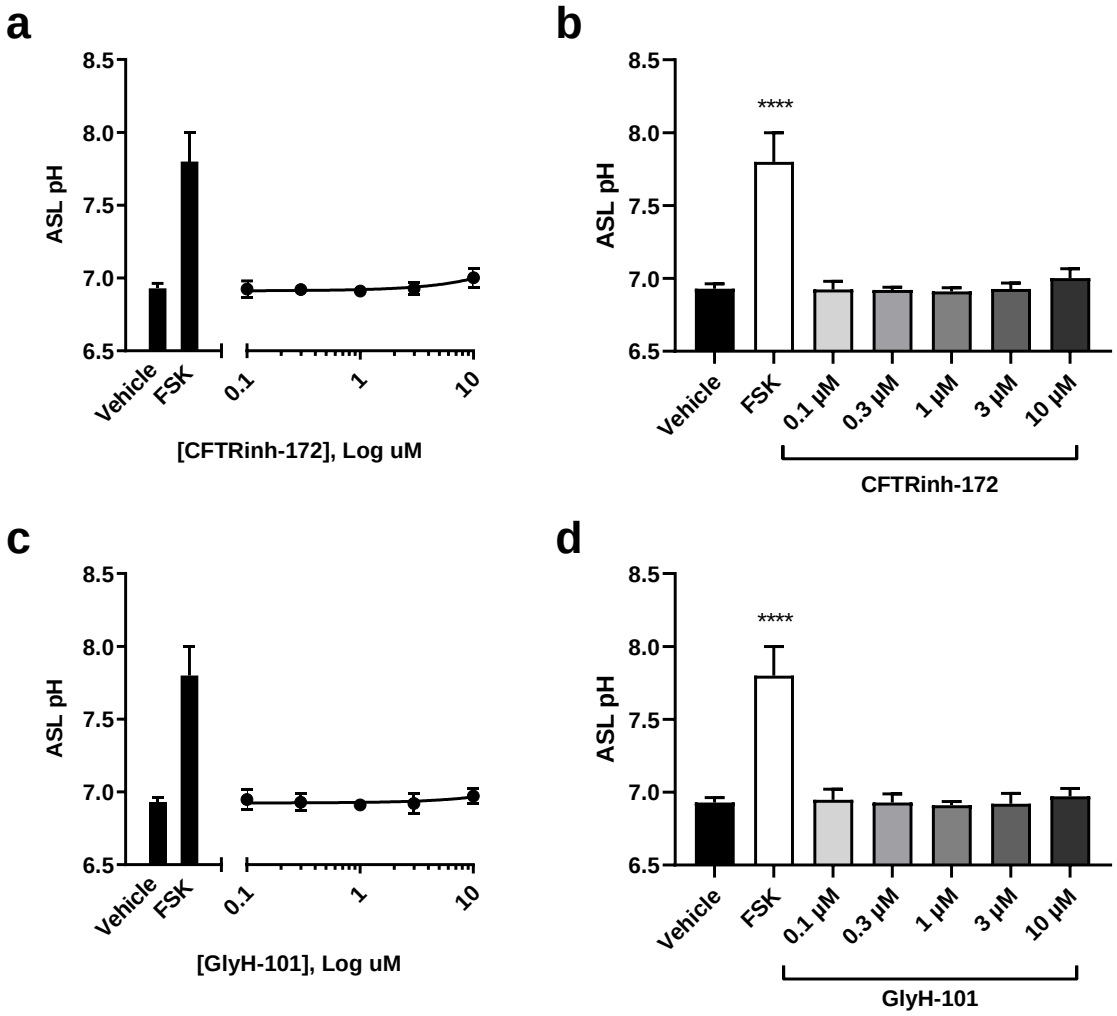

Figure 5: Concentration-response of CFTR inhibitors on airway surface liquid pH with apical compartment administration. Human airway epithelial (Calu-3) cells were exposed to CFTR inhibitors (**a** and **b**) CFTRinh-172 and (**c** and **d**) GlyH-101 in the apical (top) compartment for 3 h at various concentrations. cAMP elevating agent forskolin (0.1 µM) was used as a positive control. (**a** and **c**) A dose-response curve for measured ASL pH is depicted and (**b** and **d**) comparisons between treatment groups were performed. Data presented as means ± SD (n=5). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. **** *P≤0.0001*

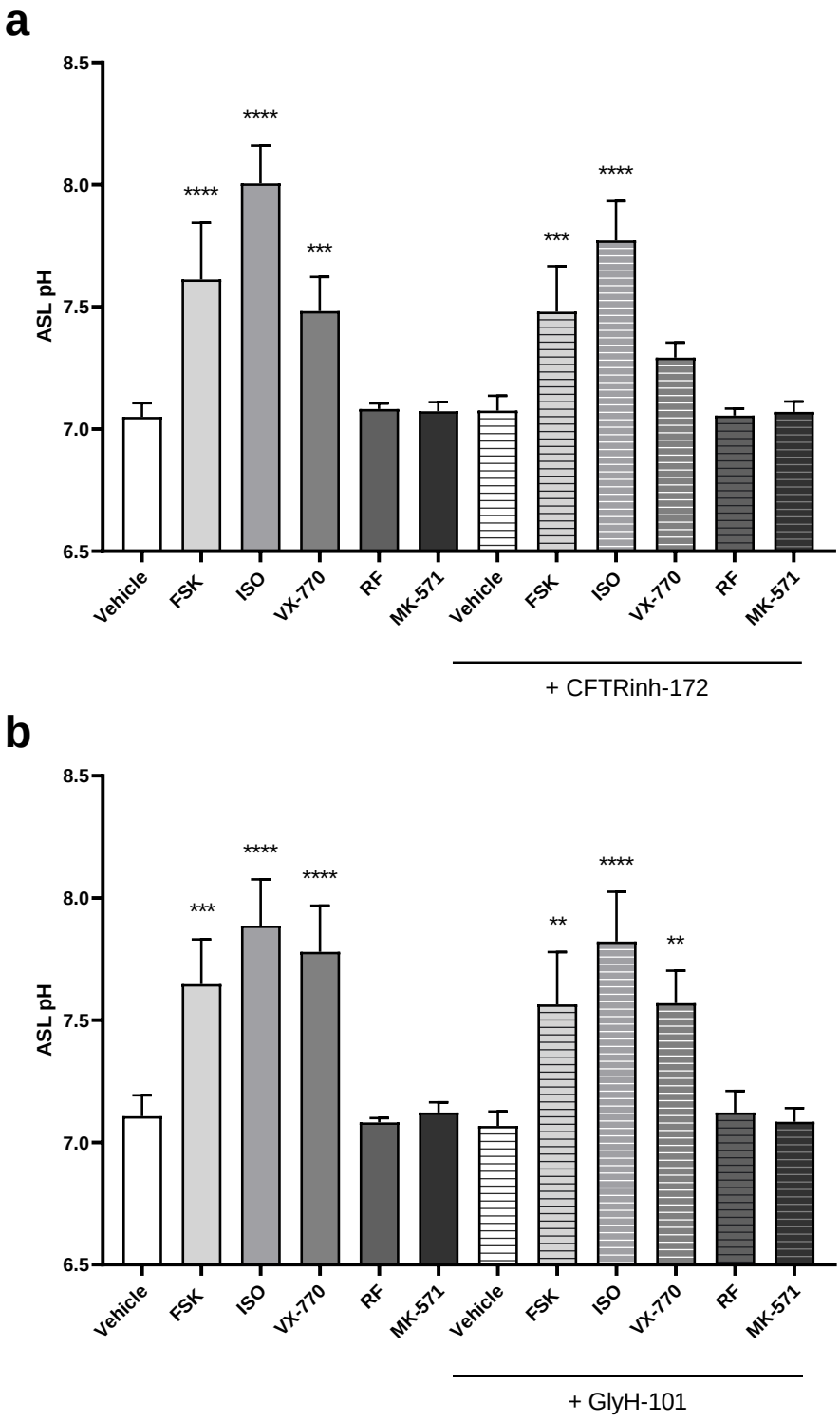

Figure 6: *Figure legend continued on the following page.*

Figure 6: Effect of various pharmacological interventions post-CFTR inhibition on airway surface liquid pH. Human airway epithelial (Calu-3) cells were pre-treated with CFTR inhibitors (**a**) CFTRinh-172 (10 μM) and (**b**) GlyH-101 (10 μM) to the apical (top) compartment for 30 min prior to various combinations of cAMP elevating agents forskolin (0.1 μM) and isoproterenol (0.01 μM), CFTR potentiator VX-770 (1 μM), PDE-4 inhibitor roflumilast (1 μM), and ABCC4 inhibitor MK-571 (10 μM) to the basolateral (bottom) compartment for 3 h. Measured ASL pH is depicted and comparisons between treatment groups were performed. Data presented as means ± SD (n=4). A one-way ANOVA with subsequent multiple comparisons was used for statistical analysis. ***P≤ 0.01*; ****P≤0.001*; **** *P≤0.0001*

CHAPTER 5:

General Discussion

Section Overview:

In this section, the key findings of the three primary research studies are consolidated and discussed, emphasizing their contributions to the field. This section is intended to supplement the earlier discussions outlined in the preceding chapters and will cover the significance of the conducted research within the broader literature. Furthermore, this section will describe how Chapters 2 to 4 are conceptually intertwined, explore potential implications, acknowledge limitations, and pave the way for future research directions.

Introduction

Cystic fibrosis is the most prevalent autosomal recessive genetic disease affecting Canadian newborns, with an incidence of 1 in 3,850 live births[1]. CF is caused by mutations within the CFTR gene, a phosphorylation-dependent ion channel responsible for conducting chloride and bicarbonate ions across the epithelia, contributing to ASL regulation[2–7]. Currently, over 2000 different CFTR variants have been identified, with F508del being the most common CFTR variant, accounting for approximately 85% to 90% of cases[8–11]. Inheritance of these disease-causing variants leads to dysfunctional CFTR, leading to several complications such as impaired MCC, mucus obstruction, persistent airway infections, and modulated inflammatory responses[10,12–17]. Collectively, these complications often result in poor lung function and pulmonary disease, the major cause of morbidity and mortality in CF individuals[10,11].

Presently, there is no cure for CF. However, the development of small molecule therapeutics, known as CFTR modulators, has transformed the management of CF, significantly improving the quality of life for many individuals with CF[10,18–25]. These CFTR modulators, which directly target the CFTR protein, have demonstrated remarkable efficacy in enhancing lung function and alleviating respiratory symptoms in responsive CF individuals[10,18–25]. Furthermore, the development of combination therapies, utilizing both CFTR potentiators and correctors, has expanded treatment options to include a larger patient population[10,11,18–21,23].

Despite these advancements, some patients continue to remain unresponsive to current therapies, particularly those with CFTR variants resulting in premature stop codons,

leading to the absence of functional CFTR protein[25]. Additionally, while CFTR modulators have been demonstrated to improve lung function, the progression of disease persists, partly due to persistent airway infections and inflammation leading to lung damage[26]. Moreover, the financial burden of these therapies, with annual costs exceeding $300,000 poses significant challenges for patients without adequate health coverage[27]. As of September 2022, Trikafta (elexacaftor/tezacaftor/ivacaftor; VX-445/VX-661/VX-770) is covered by all provinces and territories in Canada for eligible individuals aged six and older[28]. Additionally, as of December 2023, this coverage has been extended to include Canadians aged two to five with at least one F508del-variant in Alberta, British Columbia, Ontario, and Nunavut[29]. This extension not only promises to significantly enhance the health outcomes of many affected CF individuals but also ensures greater accessibility to the life-changing treatment.

While CFTR modulator therapies have revolutionized CF treatment, the pressing need for additional and complementary therapies remains. An alternative strategy involves the use of cAMP modulators as an add-on combinatorial therapeutic, targeting cAMP levels instead of CFTR directly. Intracellular cAMP levels, regulated by various mechanisms, including ACs, cAMP-efflux transporter ABCC4, and PDEs, could be elevated to activate PKA, phosphorylating CFTR and thereby increasing its function[30–36]. Therefore, gaining a comprehensive understanding of the role and interplay between cAMP and CFTR in lung health and disease is crucial for the development of novel therapeutics for lung disease management.

Central Focus and Theme of Thesis

This Ph.D. thesis is centered around the exploration of cAMP and CFTR modulation within the context of lung health and disease. The central hypothesis proposes that the combination of cAMP modulating agents with CFTR modulators will serve as an effective therapeutic strategy for CF and other respiratory diseases with similar pathological features. This dissertation is comprised of three studies, each contributing to this overarching theme.

The collective body of work, detailed across **Chapters 2 to 4**, investigated the impact of this therapeutic approach on CFTR activity, on acquired CFTR dysfunction induced by environmental insults, and on ASL pH. **Chapter 2 and 3** directly investigated the effects of cAMP and CFTR modulation on CFTR activity in human airway epithelial cells, providing insights for the management of CF and other related respiratory diseases. **Chapter 4** focused on downstream consequences typically associated with CFTR dysfunction, specifically examining the impact of cAMP and CFTR modulation on ASL pH.

The following discussion aims to complement the existing discussion sections found in **Chapters 2 to 4**, providing a more comprehensive understanding of the potential therapeutic benefits that arise from combining cAMP and CFTR modulators.

Enhancing CFTR Modulator Therapies with cAMP Modulation

The discovery of CFTR modulators has facilitated treatment to a larger subset of the CF patient population, however, some patients continue to remain unresponsive to therapy. Additionally, specific subsets of the CF patient population are ineligible for CFTR

modulator therapies. This emphasizes the need for alternative or complementary combinatorial therapies, using precision medicine approaches, to address the gaps in both treatment responsiveness and eligibility.

In **Chapter 2**, we explore an alternative strategy for enhancing CFTR function, in which we target intracellular levels of cAMP, in addition to CFTR, due to its key role in regulating CFTR ion channel activity within the CFTR-containing macromolecular signaling complex[31,36,37]. Our initial hypothesis was that cAMP modulation, in the presence of CFTR modulator VX-770, would potentiate CFTR activity. The rationale behind this is due to the involvement of ABCC4 and PDE-4 in cAMP signaling within the CFTR-containing macromolecular signaling complex, thus inhibition of these proteins would lead to a rise in intracellular cAMP levels within the microenvironment, thereby regulating CFTR function and activity[37,38]. To evaluate cAMP modulation as a therapeutic strategy, we used pharmacological inhibiters to attenuate extracellular transport and breakdown of cAMP, in the absence and presence of VX-770, in the human airway epithelial Calu-3 cells. Our findings demonstrated that modulation of cAMP levels resulted in increased sensitivity to VX-770 and increased CFTR activity.

Our study in **Chapter 2** builds upon prior work conducted in the Hirota lab, in collaboration with other research groups. Previously, we described the presence and function of ABCC4 in human airway epithelial cells as a cAMP-efflux transporter[39]. Furthermore, we have also demonstrated that inhibiting ABCC4 reduced extracellular cAMP transport, enhancing PKA activity, leading to CFTR potentiation in patient-derived tissues from CF patients[30,39]. Aside from our contributions, Schnur et al. have previously

demonstrated that ABCC4 inhibition via MK-571 or acute tobacco smoke condensate exposure resulted in CFTR activation through phosphorylation by activated PKA in airway epithelial cells[40].

In our previous investigation with Ahmadi et al., we demonstrated that VX-770 enhancement by MK-571 varied depending on the CFTR variant[30]. This variability in effectiveness suggests that cAMP modulation as an add-on therapeutic may be limited to certain subsets of the CF population. However, in our current study, we did not observe a comparable level of VX-770 enhancement by MK-571. Although, using fold-change analyses, we did observe an increase in sensitivity to VX-770, without changes in the maximal response, for both MK-571 and roflumilast. A possible explanation is that in Calu-3 cells, wild-type CFTR may already be maximally active, thus making it difficult to further potentiate this activity with cAMP modulation.

Interestingly, a recent study exploring the triple combination therapy Trikafta for rescuing F508del-CFTR variant function demonstrated that pre-incubation with MK-571 led to a significant increase in basal short-circuit currents, indicative of chloride secretion by CFTR[41]. Notably, this effect was significantly reduced in the absence of VX-770[41]. Moreover, short-circuit currents generated by MK-571 were inhibited by CFTR inhibitors in the absence of additional stimulation[41]. The authors propose that increases in basal and forskolin-stimulated short-circuit currents by MK-571 is due to local elevations of cAMP within the microenvironment, providing additional support for the pursuit of cAMP modulators[41].

Prior to our study, a few studies had already demonstrated the potential of PDE-4 inhibitors, particularly roflumilast, in potentiating CFTR activity[34,35,42–44]. These studies ultimately supported the plausibility of cAMP modulators serving as a complementary therapeutic strategy to existing CFTR modulators. Roflumilast, a selective PDE-4 inhibitor, is currently employed in the treatment of COPD, a respiratory disease that shares many phenotypic characteristics with CF[45–47]. In the study conducted by Lambert et al., the authors demonstrated that roflumilast is capable of increasing CFTR activity alone in HBECs[35]. Additionally, they observed an additive effect when combining roflumilast and VX-770 in HBE monolayers exposed to whole tobacco smoke[35]. In contrast to their findings, our study did not reveal significant increases in CFTR activity with roflumilast alone or in combination with VX-770, consistent with our MK-571 experiments. However, within their study, Lambert et al. observed that roflumilast failed to enhance short-circuit currents induced by maximal forskolin addition, with no additive effects in Calu-3 cells[35]. Our study aligns with this observation, reinforcing our explanation that cAMP modulation is unable to further potentiate CFTR since it is already maximally active in Calu-3 cells. In addition to their cAMP modulating effects for CFTR activation, PDE inhibitors could also be beneficial for the management of CF due to their anti-inflammatory and bronchodilator effects[48]. This is particularly important given that CF disease progression persists despite the use of CFTR modulator therapy, which could lead to lung damage.

The effect of cAMP modulation with VX-770 in Calu-3 cells was not as impactful as observed in previous studies. This suggests that cell-specific factors may influence the efficacy of cAMP modulation, emphasizing the importance of selecting an appropriate cell

model for research. Despite this, our findings support the potential application of cAMP modulators with existing CFTR modulators for optimal CF disease management, given their synergistic effects. However, further investigation is warranted to better understand the potential clinical implications of this therapeutic approach. Several studies have already suggested that monotherapies are insufficient for addressing CFTR defects, indicating that further investigations into combination therapies will be key for optimal CF management[20,23,24,49–51].

CFTR Modulation for Environmental-Induced Dysfunction

The airway epithelium is continuously exposed to various environmental air pollutants, which have been associated with the onset and worsening of respiratory diseases[52–62]. In addition, there have been emerging links between air pollution and acquired CFTR dysfunction, primarily attributed due to tobacco smoke exposure, a major risk factor for the development and progression of COPD, the third leading cause of death worldwide[63–65]. Building upon our previous study in **Chapter 2,** which demonstrated that modulating cAMP levels increased sensitivity to VX-770 for CFTR activity compared to monotherapy with VX-770, this prompted our next investigation which aimed at exploring the potential application and leveraging of therapeutic approaches used in CF management for addressing acquired CFTR dysfunction.

In **Chapter 3**, to better understand the consequences and impact of various environmental air pollutants on CFTR, we exposed Calu-3 cells to an aqueous extract of tobacco smoke, urban particulate matter, and diesel exhaust particles. We hypothesized that

exposures to environmental air pollutants would result in acquired CFTR dysfunction, which could potentially be rescued through pharmacological interventions. In our studies, we reveal that exposure to tobacco smoke extract and diesel exhaust particles induces acquired CFTR dysfunction due to increased oxidative stress or reduced CFTR expression. Furthermore, to our knowledge, we provide the first evidence that acquired CFTR dysfunction can be rescued through the administration of CFTR modulator VX-770 and pre-treatment with ascorbic acid in DEP-exposed models. Our findings reveal potential therapeutic approaches for addressing the adverse effects of environmental air pollutants on CFTR functionality.

Prior to our investigation, several studies have identified tobacco smoke exposure as the primary cause of acquired CFTR dysfunction, demonstrating that exposure to tobacco smoke leads to decreased CFTR expression and function via CFTR internalization, trafficking, and altered gating.[35,42,43,66–71]. Moreover, studies have also suggested the use of cAMP and CFTR modulators as a potential therapeutic strategy for addressing acquired CFTR dysfunction in COPD patients[35,43,70]. Notably, studies conducted by the Rowe group have demonstrated a dose-dependent reduction in CFTR function following tobacco smoke exposure and have shown that treatment with either roflumilast or VX-770 can partially restore CFTR activity in smoke exposure models, leading to improvements in downstream consequences associated with CFTR dysfunction such as ASL volume, secretion, and mucociliary transport[35,43,70]. Importantly, they demonstrate that rescue of CFTR function with roflumilast is additive when combined with CFTR modulator VX-770[35]. In our study, we similarly observed a dose-dependent reduction in CFTR expression and function to

tobacco smoke exposure, however, in contrast to previous findings, we did not observe a rescue of CFTR activity in response to cAMP or CFTR modulators. This discrepancy may be due to the lower levels of CFTR protein present and specific characteristics of Calu-3 cells used for our study.

In addition to tobacco smoke exposure, although not as extensively studied, there has been emerging evidence of various air pollutants leading to acquired CFTR dysfunction, including ozone, reactive oxygen nitrogen species, heavy metals, and particulate matter, through oxidative stress[71–75]. In our study, we reveal that exposure to DEP leads to acquired CFTR dysfunction through a decrease in CFTR function, without affecting CFTR protein expression. Importantly, we are the first to demonstrate that VX-770 can rescue CFTR function in DEP models. We propose that rescue of CFTR function was possible in cells exposed to DEP, as opposed to tobacco smoke extract, due to the unaffected presence of CFTR protein. While no rescue with cAMP modulators was observed, subtle trends of improvement in CFTR activity were noted following exposure to DEP.

Altogether, our findings strongly support the notion that CFTR function is not only important in the context of CF but has potential implications for healthy individuals and those with respiratory diseases who are exposed to poor air quality. Additionally, different air pollutants have varied effects on CFTR, thus it is important to consider the specific characteristics of each pollutant. Furthermore, existing therapeutics used for CF management could have broad applications for other respiratory diseases with acquired

CFTR dysfunction, such as COPD, and for mitigating the adverse effects of air pollution, however, further investigation is necessary to fully understand its potential application.

cAMP and CFTR Modulation on ASL pH

In **Chapter 2**, we demonstrated the potential of leveraging cAMP modulation as an add-on therapeutic approach to existing CFTR modulators for enhancing CFTR function in the context of CF. In **Chapter 3**, we highlight that exposure to environmental insults can induce acquired CFTR dysfunction, and that therapies used for CF could have broader applications and be leveraged for other respiratory diseases such as COPD. Overall, our studies suggest that combining cAMP and CFTR modulation could be used to improve CFTR function and holds promise for addressing respiratory diseases. We next wanted to investigate whether this therapeutic approach leads to improvement in downstream consequences commonly associated with CFTR dysfunction.

In **Chapter 4**, our aim was to explore the potential of cAMP and CFTR modulation as a therapeutic strategy for elevating ASL pH, potentially leading to downstream improvements in consequences associated with CFTR dysfunction, such as MCC and antimicrobial activity by CHDPs. To achieve this, Calu-3 cells were treated with various combinations of cAMP and CFTR modulators. We hypothesized that this therapeutic strategy would result in elevated ASL pH. Our findings revealed significant increases in ASL pH, with combination therapies outperforming monotherapies. Although we did not investigate whether elevated ASL pH translated into improvements in MCC and antimicrobial activity, highlighting a potential future study, our findings indicate the

potential of cAMP and CFTR modulation as a therapeutic strategy for addressing ASL abnormalities. This therapeutic strategy could benefit several respiratory diseases impacted by ASL abnormalities, including CF and COPD. However, further investigations are required to determine the effectiveness of this approach at improving MCC and antimicrobial activity.

Prior to our investigations, several studies have highlighted the potential of both cAMP and CFTR modulators for improving ASL properties, such as pH, volume, and viscosity[16,35,42,70,76,77]. Moreover, past investigations into the rescue of CFTR function have shown promising outcomes, demonstrating significant improvements in MCC and antimicrobial activity by CHDPs[13,78,79]. However, conflicting results have been reported, with some studies observing no improvements, thus warranting further investigations[80]. The application of cAMP modulators, such as forskolin, IBMX, roflumilast, and adenosine, has been demonstrated to improve ASL properties, including pH, viscosity, and volume, along with ciliary beat frequency[16,42,81,82]. Similarly, VX-770 has also been shown to increase ASL volume, ciliary beat frequency, and mucociliary transport, particularly in tobacco smoke models[70,77]. Recently, Ludovico et al. demonstrated the efficacy of Trikafta in restoring CFTR function, revealing improvements in ASL pH and viscosity in HBECs from CF subjects[76]. Additionally, greater improvements in ASL viscosity and pH were observed in combination therapies compared to monotherapies[76]. Moreover, insights from single cell RNA sequencing analyses conducted by Loske et al., on nasal epithelial and immune cells from CF children, revealed pharmacological improvement of CFTR function via Trikafta restored airway homeostasis and improved host defenses[83]. Exploring the

potential synergy between cAMP and CFTR modulators to address downstream consequences linked with CFTR dysfunction offers an intriguing avenue for further investigation. This therapeutic strategy could potentially lead to greater improvements.

In summary, our study focusing on the combined effects of cAMP and CFTR modulators on ASL pH, along with supporting evidence from other research groups, provides valuable insights into this potential therapeutic strategy for addressing ASL abnormalities and enhancing host defense mechanisms. This therapeutic strategy offers potential benefits for individuals impacted by these downstream consequences, including individuals with CF and COPD. Our study revealed significant increases in ASL pH in response to this therapeutic strategy, laying the groundwork for future investigations on mucociliary clearance and antimicrobial activity. While past studies have demonstrated the individual potential of cAMP and CFTR modulators, our therapeutic strategy emphasizes the synergy between these two approaches.

Concluding Remarks

In conclusion, the three studies presented within this Ph.D. thesis dissertation collectively contribute to the understanding of cAMP and CFTR modulation in the context of lung health and disease, highlighting the potential of this therapeutic strategy for improving the treatment of CF and other respiratory diseases. In **Chapter 2**, we demonstrate that cAMP modulation increases sensitivity to VX-770, revealing its potential application in select CF subjects, particularly those expressing CFTR at the apical membrane. In **Chapter 3**, we next explored acquired CFTR dysfunction induced by

environmental insult exposure. We show that VX-770 was successful in rescuing acquired CFTR dysfunction in DEP models. Finally, in **Chapter 4**, we assessed the effectiveness of this therapeutic strategy for elevating ASL pH, as a potential target for improving downstream consequences associated with CFTR dysfunction, demonstrating that this therapeutic approach led to significant increases in ASL pH.

It is important to acknowledge the limitations of our studies. All studies in **Chapters 2 to 4** were performed in Calu-3 cells expressing wild-type CFTR, with **Chapters 2 and 3** performed in submerged cultures. Future investigations should take into consideration cell-specific factors and utilize more relevant cell culture models, such as primary cells grown under ALI. Additionally, expanding the experiments to cover a wide range of CFTR variants would help identify the subset of the CF population that could benefit from this therapeutic strategy. Despite these limitations, these studies revealed the potential of this therapeutic strategy for respiratory diseases and will serve useful for future investigations.

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