Role of CFTR and MRP1 in determining intra- and extra-cellular glutathione in Calu-3 cells

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Abstract

Cystic Fibrosis (CF), the most common fatal genetic disorder, is due to mutations of the cystic fibrosis transmembrane regulator protein (CFTR). Loss of functional CFTR leads to mucus thickening with the consequences of chronic pulmonary infection and inflammation, the most common fatal complication of this disorder. This thesis aimed to examine one aspect of the pathophysiology of CF, the potential role of glutathione (GSH) and oxidant stress. CFTR is permeable to GSH, at least *in vitro*, where evidence of the ability of CFTR to transport GSH is strong. Furthermore, extracellular GSH is diminished in CF airway lining fluid and it has been hypothesized that deficiency of CFTR leads to higher intracellular GSH coupled with lower extracellular GSH. This would be consistent with the observation of low extracellular GSH in CF patients and increased resistance to apoptosis of CF cell lines, where falling intracellular GSH concentrations ([GSH]) are required to initiate apoptosis. In addition to CFTR, other channels transport GSH, including Multi-drug Resistance Protein 1 (MRP1) which has structural and functional homology to CFTR. Higher expression of MRP1 has been associated with fewer symptoms in CF patients, suggesting that MRP1 may contribute to CF pathophysiology. We therefore set out to study GSH at the cellular level, with or without functional CFTR, in an airway epithelial cell model. Given the importance of oxidant stress in CF, we tested whether cells were more susceptible to stress in the absence of CFTR function. We hypothesized that lack of CFTR function would increase intracellular and decrease extracellular [GSH]. We also hypothesized that MRP1 function would influence intracellular and extracellular [GSH].

Blockade of CFTR function in Calu-3 cells failed to alter either intra- or extracellular [GSH], independent of oxidant stress conditions, suggesting the channel was not a determinant of [GSH]. However, inhibition of other anion channels did increase intracellular [GSH], suggesting an alternate mechanism for regulating cellular [GSH]. In contrast to CFTR, inhibition of MRP1 increased intracellular [GSH], suggesting a role for this channel in regulating intracellular [GSH]. Paradoxically however, extracellular [GSH] was higher after administration of the MRP1 antagonist MK-571. In addition, we also observed higher CFTR activity with use of MK-571. These findings suggest that CFTR could be indirectly responsible for the changes in [GSH] through interaction with MRP1. CFTR blockade in the presence of MK-571 administration returned extracellular [GSH] to normal, which reinforces a role for CFTR in the extracellular [GSH] increases. Administration of either MK-571 or Montelukast increased both wild-type and ΔF508 CFTR expression and function in the BHK cell model. Montelukast may have beneficial effects in restoring CFTR expression and function, which if confirmed in other models and *in vivo*, may pave the way for future therapeutic treatments for CF patients.

Résumé

La fibrose kystique (FK), la maladie génétique mortelle la plus répandue au Canada, est causée par des mutations dans la protéine CFTR (pour « Cystic fibrosis transmembrane conductance regulator »). La perte de fonction de CFTR mène à l'épaississement des muqueuses des voies respiratoires et l'avènement d'infections pulmonaires chroniques avec réponse inflammatoire, la cause primaire de mortalité chez les personnes affligées de la maladie. Cette thèse va examiner une partie de la pathophysiologie de la FK, celle du rôle du glutathion (GSH) et du stress oxydatif. Le CFTR est perméable au GSH et les données expérimentales in vitro suggèrent fortement que le CFTR puisse transporter le GSH. De plus, le GSH extracellulaire est diminué dans le liquide de surface recouvrant les voies respiratoires des patients avec la FK et de cela découle l'hypothèse que la déficience en CFTR mène à une augmentation du GSH intracellulaire, avec une diminution concomitante du GSH extracellulaire. Cette hypothèse s'alignerait bien avec le bas taux de GSH extracellulaire observé chez les patients affectés par la FK et la résistance accrue contre l'apoptose vue dans les lignées cellulaires de la FK. Cette résistance découle du fait que la cellule a besoin d'une diminution de concentration de GSH ([GSH]) intracellulaire pour entamer le processus d'apoptose. À part du CFTR, il existe d'autres canaux membranaires qui transportent le GSH, tels que le *Multi-drug Resistance Protein 1* (MRP1). Ce canal a une forte homologie structurelle et fonctionnelle avec le CFTR et son expression se trouve liée avec moins de symptômes cliniques chez les patients atteints de FK. Ces observations suggèrent que le MRP1 pourrait jouer un rôle important dans l'évolution de la physiopathologie de la maladie. Notre but est d'étudier l'état du GSH du point de vue de

la cellule, avec ou sans stress oxydatif, dans une lignée de cellules épithéliales des voies respiratoires. Considérant l'importance du stress oxydatif dans la FK, il a fallu déterminer si les cellules étaient plus susceptibles au stress en absence du fonctionnement du CFTR. On a mis devant l'hypothèse que le manque de fonctionnement du CFTR amène à l'augmentation de [GSH] interne avec diminution de [GSH] externe. De plus, nous croyions que le fonctionnement du MRP1 aurait des effets sur le [GSH] intracellulaire ou le [GSH] extracellulaire.

En utilisant un bloqueur de canal visant le CFTR, nous n'avons pas observé de changement dans le [GSH] intracellulaire ou extracellulaire, chez la lignée cellulaire Calu-3, avec ou sans stress oxydatif. Ces observations suggéraient que le CFTR n'était pas impliqué dans la régulation du [GSH]. Cependant, nous avons observé une augmentation du [GSH] intracellulaire avec les traitements utilisant les bloqueurs de canaux anioniques, ce qui amenait la possibilité qu'un autre mécanisme soit impliqué dans la régulation du [GSH]. Contrairement à l'inhibition de la fonction du CFTR, le blocage de MRP1 causait une augmentation du [GSH] intracellulaire, apportant au canal un rôle possible dans la régulation du GSH. De façon paradoxale, le [GSH] extracellulaire augmentait avec l'utilisation de MK-571, bloqueur de MRP1. De plus, nous avons constaté une augmentation de l'activité de CFTR avec l'utilisation de MK-571. Ces observations prises ensemble suggéraient que CFTR pouvait être impliqué de façon indirecte dans les changements de [GSH], à travers une interaction avec MRP1. En bloquant CFTR et MRP1 ensemble nous avons constaté que le [GSH] extracellulaire était revenu au niveau de base, ce qui venait appuyer un rôle pour le CFTR dans l'augmentation du [GSH] extracellulaire. Par après, nous avons constaté qu'un traitement

de MK-571 ou de Montelukast, utilisant le modèle cellulaire BHK, causait une augmentation de l'expression et de la fonction du CFTR normal ainsi que du CFTR ΔF508. Dans ce contexte, le Montelukast pourrait alors avoir des effets bénéfiques sur le rétablissement de l'expression et de fonction de CFTR ce qui, après confirmation dans d'autres modèles cellulaires ainsi qu'*in vivo*, pourra ouvrir la porte à de nouvelles thérapies pour les personnes atteintes de la FK.

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List of diagrams, tables and figures

Publications during thesis training

1. K. Govindaraju, **C. Pavlovic**, W. S. Powell, F. Morin and D. H. Eidelman. Myeloperoxidase as an oxidant: Effects on intracellular glutathione. Manuscript in preparation.
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3. K. Govindaraju, **C. Pavlovic** and D. H. Eidelman. Interaction between NO Production and Oxidative Stress in A549 Cells. Am J. Respir. Crit. Care Med. 169: A238, 2004.
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Abbreviations

Abbreviation	Meaning
4-HNE	4-hydroxy-2-nonenal
8-bromo-cAMP	8-bromoadenosine 3′,5′-cyclic monophosphate
ABC	ATP-binding cassette
ABCC	ATP-binding cassette channel
ADP	Adenosine diphosphate
aG_{M1}	Surface glycolipid asialo ganglioside M1
ALF	Airway lining fluid
AP-1	Activator protein 1
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BHK	Baby hamster kidney
BSO	L-buthionine SR-sulfoximine
cAMP	Cyclic adenosine monophosphate
CAVD	Congenital absence of the vas deferens
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator protein
CHAPS	3-[(3-cholamidopropyl)-dimethyl-ammonio]-l-propanesulfonate
cpt-cAMP	8-(4-chlorophenylthio)-adenosine-3',5'-monophosphate
cys	Cysteine
DCFDA	2',7'-dichlorofluorescein diacetate
DIDS	4,4'-diisothiocyano-2,2'-stilbene disulfonic acid
DMSO	Dimethyl sulfoxide
DNP	Dinitrophenyl
DNPH	2,4-dinitrophenylhydrazine
DPC	Diphenylamine 2, 2'-dicarboxylic acid
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)
ENaC	Epithelial sodium channel
EtOH	Ethanol
FBS	Fetal bovine serum
GCL	Gamma-glutamylcysteine ligase
GCLC	GCL catalytic subunit
GCLM	GCL modulating subunit
GCS	Gamma-glutamylcysteine synthetase
GGT	Gamma-glutamyl transferase
glu	Glutamate

Abbreviation	Meaning
gly	Glycine
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthase
GS-4HNE	Glutathione S-conjugate of 4HNE
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
$HO^{\cdot}$	Hydroxyl racial
HOCl	Hypochlorous acid
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
HTS	High throughput screening
IL	Interleukin
inh-172	Abbreviation of CFTRinh-172, the CFTR channel blocker
LDH	Lactate dehydrogenase
LT	Leukotriene
MCP	Monocyte chemoattractant protein
MDA	Malondialdehyde
MDR	Multi-drug resistant
mRNA	Messenger RNA
MRP	Multidrug resistance protein
MSD	Membrane spanning domain
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Muc5AC	Mucin 5AC
NAC	N-acetylcysteine
NBD	Nucleotide binding domain
NF-κB	Nuclear factor-kappa B
$NO^{\cdot}$	Nitric oxide
Nrf2	Nuclear erythroid 2 p45-related factor 2
$O_2^{-\cdot}$	Superoxide anion
OD	Optical density
$ONOO^-$	Peroxynitrite
PBS	Phosphate buffered saline
PEEK	Polyetheretherketone
PK	Pyurvate kinase

Abbreviation	Meaning
PKA	Protein kinase A
RISC	RNA-induced silencing complex
ROH	Alcohol
ROOH	Hydroperoxide
ROS	Reactive oxygen species
Rp-8-bromo-cAMP	8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer
RP-HPLC	Reverse phase HPLC
SEM	Standard error
siRNA	short interference RNA
TBARS	Thiobarbituric acid reactive substances
TBST	Tris buffered saline with Triton
TCA	Trichloroacetic acid
TMD	Transmembrane domain
TNF	Tumor necrosis factor

Chapter 1: Introduction

1.1 Overview

Cystic Fibrosis (CF) is the most prevalent fatal genetic disorder, affecting approximately one in 2500 births in Caucasian populations [1]. CF is caused by mutations of the cystic fibrosis transmembrane regulator protein (CFTR), which translates into the symptoms of the disease: mucus thickening and plug formation, deficient mucus clearance leading to inefficient clearance of infection, and increased state of inflammation [2]. This thesis aims to examine one aspect of the pathophysiology role of CFTR, the potential role of glutathione and oxidant stress.

Mutations of CFTR may lead to disease through a variety of mechanisms. One aspect of CFTR deficiency is the lack of normal chloride transport. This in turn has been linked to mucus thickening although the exact mechanism by which this occurs is still debated [3]. This mucus thickening compromises mucociliary clearance and contributes to airway obstruction [4]. Although differences in glycosylation and sulfation between normal and CF mucins have been found [5], it has been difficult to link these differences to defective CFTR expression since CFTR expression is absent in airway mucus-producing cells [6]. A direct link between mucin modification and CFTR function has been elusive, but it has been shown that the changes in mucus are linked to the pro-inflammatory and chronic infectious state of the CF lung [7]. Another potential role for CFTR deficiency relates to its function as a receptor for internalization of infectious agents, such as the common CF pathogen *Pseudomonas aeruginosa*. Within this context,

the lack of CFTR expression has been suggested as one of the reasons for compromised infection clearance [8]. A third aspect of CFTR which has come under study is its ability to act as a transporter of the antioxidant glutathione [9-11]. Each of these mechanisms will be discussed further in sections 1.3.8, 1.3.9, 1.5.4 and 1.6.

The immune response in CF is marked by chronic infiltration of neutrophils [12, 13], due to the inability to properly clear infectious agents which have implanted themselves in the thick mucus of the lung [4]. With this response comes the release of oxidants whose role is to destroy the invading pathogens [14, 15]. It is understood that glutathione (GSH), the main antioxidant in mammalian cells, is present in decreased amounts in CF airway, when measured in bronchial alveolar fluid, compared to normal lungs [16]. Within this context, oxidant stress plays a major role not only in the destruction of pathogens but also in tissue damage. It is interesting to note that in contrast to CF, under normal conditions infection can lead to increased glutathione levels in the airways [17]. Yet GSH in the airways can also be increased in CF: Dauletbaev *et al.* found that GSH was increased in CF sputum, representative of airway surface secretions, which is in contrast with alveolar measurements [18]. Within this context, understanding how CFTR mediates this response, and its implications in CF, is important in understanding the disease and how a defect in one protein can subsequently lead to numerous problems which lead to the demise of the patient.

1.2 Cystic Fibrosis

CF (also known as mucoviscoidosis, or mucoviscidosis) is an autosomal recessive disease affecting the exocrine glands. It is characterized by pancreatic insufficiency, intestinal obstructions, impaired nutrient absorption, male sterility, and chronic episodes of pneumonia and bronchitis. Although most gastrointestinal complications can be managed through diet and enzyme replacement therapy, the treatment of pulmonary complications is more challenging and it is respiratory failure which is the primary cause of mortality in CF [19]. CF was first known as a disease of the pancreas, where fibrosis was observed pathologically [20]. The first comprehensive description of CF, correlating the pancreatic disease with the lung and gut disease, was done by Dorothy Hansine Andersen in 1938 [20]. In the early 1950s, abnormalities were described in sweat of CF patients [21]. In that study, Paul di Sant' Agnese found a difference in electrolytes which would become the basis of the sweat test, used in the diagnosis of CF. The gene responsible for CF, the product of which became known as CFTR, was discovered in 1989 by the group led by Lap-Chee Tsui at the Hospital for Sick Children in Toronto [22, 23]. The gene was mapped to the q31.2 locus of chromosome 7.

1.3 CFTR and the ATP-binding cassette channel (ABC) family

1.3.1 CFTR

CFTR is a member of the C sub-group of the ATP-binding cassette channel (ABC) family of proteins [9]. Two nucleotide-binding domains are found in the cytoplasmic parts of the protein and ATP binding and hydrolysis is required to provide

the driving force to pump substrates such as chloride or glutathione [24, 25]. One characteristic of this group is the presence of 2 transmembrane domains (TMD), also known as membrane spanning domains (MSD), each containing six transmembrane α-helices [26-29]. MRP1, MRP2, MRP3, MRP6, and MRP7, which all belong to the Multidrug Resistance Protein family (MRP), also contain an additional third transmembrane domain which comprises five transmembrane α-helices at the N-terminus region of the protein [26, 28, 30]. It has been suggested that this extra domain confers a specialized function to these proteins and it has been shown that leukotriene C4 (LTC4) transport by MRP1 requires that domain [30].

1.3.2 Multi-drug resistant proteins (MRPs)

The multidrug resistant protein (MRP) family, also known as the multidrug resistance-associated family (to avoid confusion with Multi-Drug Resistant proteins, MDRs), is part of the greater group of ABC-binding cassette channels, or ABCC [31], of which CFTR is also a member (known as ABCC7). These membrane proteins were first discovered in drug-resistant cancer cells and found responsible for the export of chemotherapy drugs [32]. Further reports have shown these channels to be implicated in the protection of cells from oxidant stress, serving as exporters of the antioxidant glutathione (GSH), its oxidized form (GSSG) and GSH adducts [9, 33-36]. MRPs are expressed throughout the human body, with different expression patterns depending on the protein in question. MRP1 is found in most cells with relatively high levels in the lung, testes and kidney, and is present in several blood organ barriers [31, 37-39]. In contrast to the ubiquitous presence of MRP1 in the body, MRP4 is mainly found in the gut, liver and kidneys [37, 40]. This difference in expression amongst the proteins is also

reflected in their different functions: MRP1 transports GSH while MRP4 transports cAMP [41]. The function of MRPs is regulated differently from CFTR, due to the lack of the R-domain [25]. Furthermore, some MRPs have varying numbers of nucleotide-binding domains, not just two as found in CFTR [9, 31, 32]. We will further examine MRPs later in Sections 1.3.12 and 1.5.4.2 of the Introduction.

1.3.3 CFTR localization

Originally described as a disease of the pancreas but known best for its pulmonary complications, CF affects multiple systems as a function of its expression in different cell types and organ systems in the human body. It is found in endothelial cells of the umbilical vein, red blood cells, sweat gland, colon, liver, proximal tubules (and cortex and medulla) of kidney, the brain (hypothalamus), and lymphocytes and cardiac myocytes [42-45]. One clinical manifestation of defective CFTR is the high-salt content found in sweat, which is 3 to 5 times higher than normal, which serves as the basis for a diagnostic test for the disease [46]. Another clinical manifestation is the obstruction of the gut due to formation of mucus plugs [46]. In relation to digestive tract problems, mucus thickening can also lead to the occlusion of ducts in the pancreas. This can prevent pancreatic enzymes from reaching the lumen of the intestine, leading to premature activation of digestive enzymes within the pancreas and causing pancreatitis. Furthermore the retained digestive enzymes can cause diabetes mellitus through the destruction of insulin-producing beta cells [46]. Defective CFTR also leads to obstructive azoospermia caused by thickened mucus having blocked the vas deferens during development [47-49], resulting in sterility in 97% to 99% of males with CF. Furthermore, a mutated CFTR gene

on at least one allele is found in 80% of cases of congenital absence of the vas deferens (CAVD), which also leads to azoospermia [50].

1.3.4 CFTR structure

CFTR is a 168-kD integral membrane protein located on the apical cell membrane of secretory cells [25]. CFTR contains 2 transmembrane domains (TMD) and nucleotide-binding domains (NBD), as well as a regulatory domain (R domain). In the next few sections we will examine in more detail the roles of these structures.

1.3.4.1 The Transmembrane Domains (TMDs)

There are two transmembrane domains (TMDs), also known as membrane spanning domains (MSD), found in CFTR, each comprised of six transmembrane α-helices [26-29]. They are involved in the formation of the pore, which is selective to chloride ions, and in the trafficking of CFTR to the cellular membrane [25]. The selectivity of CFTR is determined by a combination of binding sites and secondary structures found within the pore formed by the TMDs [25].

1.3.4.2 The Nucleotide Binding Domains (NBDs)

CFTR has 2 nucleotide-binding domains, each connected to a TMD. They serve as regulators of channel gating, thus determining the opening or closing of the CFTR channel [51, 52]. This is accomplished through ATP hydrolysis at the NBD sites [25, 52]. Two models were initially suggested to explain how the sites are implicated in regulation: differential NBD regulation and symmetrical NBD regulation [25, 53, 54]. The first model is based on the hypothesis by Carson, in which ATP hydrolysis at NBD1 controls channel opening and ATP hydrolysis at NBD2 regulates channel closing. Within this

context, ATP binds to the NBDs during the closed state of the channel, followed by hydrolysis at NBD1, which opens the channel. Channel closure occurs with hydrolysis at NBD2 and the resulting ADP then departing the site. The cycle repeats itself with the arrival of ATP to both sites. The symmetrical hypothesis is based on the idea that both NBDs have the same functional role, so hydrolysis of ATP at one NBD causes the release of ADP from the other. With the binding of ATP to the vacant NBD, the channel changes state, resulting in the transition either from an open to a closed state or vice versa.

Recently a third model to explain channel gating has been suggested, where the NBDs form a sandwich when ATP is bound [55, 56]. This model was inspired from observations that the structure of CFTR NBD1 was different from the structure of other typical NBD domains due to added regulatory segments and was subsequently found to be incapable of hydrolyzing ATP on its own, therefore requiring NBD2 for hydrolysis to be performed [57].

1.3.4.3 The R domain

The R domain acts as the link between the two MSD-NBD motifs and is a regulator of CFTR channel activity [25]. The activity of CFTR is enabled through phosphorylation of multiple sites within the R domain, though action by protein kinase A, PKA [25, 58-61]. Both the R domain and the NBDs act on CFTR channel activity yet have different roles: ATP hydrolysis on the NBDs is essential for the opening (or closing) of CFTR, while the R domain is needed to stabilize the open channel and allow it to function [25, 62]. The regulatory role of the R domain was demonstrated using R domain-deleted CFTRs, which showed absent channel function even with intact NBDs [63, 64].

1.3.5 CFTR mutations

Mutations in CFTR cause CF either through defective protein folding, premature deletion or functional mutants [65, 66]. Welsh and Smith have proposed a classification for the more than 1000 mutations identified so far [1, 67]. The common thread among all these types of mutations is defective chloride transport.

Type 1 mutations cause no synthesis of the CFTR protein, such as mutation G542X, due to premature deletion or stop codon insertions. In a previous classification, these mutants were classified separately from those with reduced, but measurable synthesis such as mutation G576A. However, both types of mutation lead to either reduced or absent synthesis of protein and are now classified as type 1 [1].

Type 2 mutations are due to a problem in processing, causing protein misfolding and subsequent retention and degradation. The most common CF mutation, ΔF508, is of this type. The ΔF508 mutation is a deletion of the three bases encoding a phenylalanine residue at position 508 found in the first NBD. This deletion results in CFTR protein misfolding and consequently its mislocalisation [68]. This specific type of mutation is temperature sensitive: the processing and expression of ΔF508 CFTR is increased at lower temperatures in either non-epithelial or epithelial cell lines [69, 70]. The mechanism by which proper ΔF508 protein folding and subsequent cell membrane expression is obtained, when the temperature is dropped to 25-27 °C, has yet to be determined. Since it has been observed that the structure of NBD1 of both wild-type ΔF508 CFTR is nearly identical when properly folded [56], an explanation was proposed in which the mutation induces a kinetic trap, where folding efficiency of the defective

CFTR protein is diminished and a drop in temperature would permit the proper conformation to be attained [56, 71]..

Type 3 mutations are functionally defective, such as G551D, in which case the protein does fold correctly and migrates to the cell membrane but is unable to function due to a mutation in the NBDs. These mutations affect the binding of ATP or the coupling of ATP binding which prevents the conformational changes needed for channel activation [1].

Type 4 mutations are of altered conductance, such as R117H [72]. These mutations produce a correctly trafficked CFTR protein which has a functional channel yet generate a reduced Cl^- current. The majority of the mutations are located in the TMDs [1].

1.3.6 CFTR function

CFTR is responsible for transporting chloride as well as organic anions, through coupling with ATP hydrolysis [25]. The hydrolysis occurs at the level of the NBDs (see 1.3.4.2), which causes conformational changes in the protein, resulting in the opening of the CFTR channel with phosphorylation of the R domain permitting channel function (see 1.3.4.3) Specificity of CFTR for chloride and organic anions is determined by sites found the in the TMDs, which line the pore of CFTR (see 1.3.4.1).

In addition to its involvement in the pathogenesis of CF, CFTR activity may also play an important pathophysiological role in other diseases. In particular, cholera is a disease marked by severe dehydration due to the excessive excretion of watery diarrhea [46]. This is due to the production of cholera toxin by the bacteria responsible for the

disease, *Vibrio cholerae*, which contains binding and enzymatically active subunits that activate the adenylate cyclase system in intestinal mucosa cells [73]. Over activation of adenylyl cyclase leads to increase in levels of intracellular cAMP, causing PKA activation and subsequent phosphorylation of the R domain, resulting in increased CFTR function. Since CFTR-related chloride transport into the intestinal lumen is continuously activated by intracellular cAMP, an osmotic environment is formed which draws water into the intestinal lumen leading to the characteristic large volumes of watery diarrhea. In this case, it may prove beneficial to have a defect in CFTR, so as to minimize the loss of water. It has been hypothesized that an improved resistance to the cholera toxin may be the cause behind the high prevalence of CFTR heterozygotes [46, 74]. Against this hypothesis is that CFTR is predominately found in populations of European descent who live in regions in which cholera is not frequent. Nevertheless, selection for this gene by diarrheal diseases due to other organisms, such as *Escherichia coli*, may have occurred in the past [75].

1.3.7 Regulatory role of CFTR

Although the genetic cause of CF has been determined, the steps by which malfunctioning CFTR leads to the clinical manifestations of the disease in the lung are still not well understood. One theory suggests that the lack of chloride transport via CFTR leads to accumulation of more viscous mucus in the lungs, thus allowing bacteria to escape the body's immune system [76]. Another theory puts forward an increase in sodium uptake leading to increased water reabsorption, thus dehydrating and thickening the mucus [4, 77]. Ultimately, all the proposed theories take into account the thickening of secretions. This in turn precipitates the blockade of small airways and permits the

establishment of chronic infections. Repeated infections and immune responses lead to tissue damage and airway remodeling, culminating in respiratory failure and death [78].

The expression and activity of CFTR can have important physiological effects in the lung. For example, lack of CFTR has been linked to the increased function of the sodium membrane channel ENaC [79-81]: the lack of CFTR causes increased Na^+ reabsorption into the cell by ENaC, leading to dehydration of the air surface liquid. This would be due to CFTR regulating ENaC [65, 82] and is observed in studies with mutant forms of CFTR, such as ΔF508 and G551D, where no inhibition of ENaC-mediated sodium transport occurred [83, 84]. Observations made using mutant mice that overexpress ENaC demonstrated increased Na^+ absorption followed by CF-like symptoms, including thickening of the mucus, poor bacterial clearance and neutrophilic inflammation [77]. These findings are consistent with the notion that at least part of the pathogenesis of CFTR relates to excessive reabsorption of sodium.

1.3.8 CFTR in host defense

CFTR has been shown to be a receptor protein required for internalization of bacteria, suggesting the possibility that deficiencies in CFTR in the airways may explain why infections, such as *P. aeruginosa*, are notoriously resistant to eradication in CF. It has been observed that *P. aeruginosa* was able to adhere more to CF epithelial cells from patients [8, 85, 86]. Further investigation has found that CF cells expressed more receptors for *P. aeruginosa* adhesins, and this receptor, surface glycolipid asialo ganglioside M1 (aG_{M1}), is expressed in higher levels due to CFTR dysfunction [8]. The higher expression of aG_{M1} would cause *P. aeruginosa* to remain in the airways longer

and, in conjunction with defective airway clearance, promote the implantation of chronic infections.

1.3.9 CF in the lung

Within the respiratory system, CF is characterized by abnormally increased neutrophilic infiltration and a hyper-inflammatory state [12, 87-89]. Oxidants, such as superoxide [14], are released by the immune response to counter pathogens and these same oxidants can also damage lung tissue. Due to the chronic nature of infections in cystic fibrosis, it has been suggested that this cycle of repetitive oxidant challenge contributes to irreversible lung damage with development of severe bronchiectasis [90]. Bronchiectasis is typically progressive and will eventually lead to respiratory failure or other fatal complications such as massive hemoptysis [78]. It has been hypothesised that deficiency of airway glutathione (GSH), the most abundant antioxidant in mammalian cells, may play an important role in cellular defence against repeated oxidative injury caused by the immune system, which occurs in response to chronic bacterial infection [90]. In support of this hypothesis, GSH levels in the airway lung fluid (ALF) are lower than normal in cystic fibrosis [91]. The report that CFTR can serve as a GSH transporter [11], raises the possibility that CFTR deficiency in CF lung epithelium contributes to the exacerbation of the disease by promoting a diminished antioxidant response and thereby facilitating repetitive tissue damage.

Mucus buildup and subsequent airway clogging are other characteristics of CF in the lung and in the early stages of the disease it is common to observe incessant coughing, increased phlegm production, and a decrease in the ability to exercise. Pulmonary infection appears to begin early in life in CF patients, as demonstrated by the presence of

microorganisms and inflammatory markers in bronchoalveolar lavage (BAL) fluid collected from infants and young children diagnosed with CF [92-95]. Initial infections in CF are caused primarily by *Staphylococcus aureus*, while later infections are caused by *Hemophilus influenzae* and *P. aeruginosa* [96]. These infections occur as a consequence of impeded airway clearance and can turn into pneumonia when bacteria grow uncontrolled in the thick mucus. The immune response to these infections is predominantly characterized by neutrophilic infiltration and inflammation. It is still debated whether the hyper-inflammatory state is inherent to CF [95, 97] or whether the presence of neutrophilia reflects the immune response to infection [92, 98]. Irrespective of the origin of inflammation, increases in different pro-inflammatory markers are found in CF lung and other systems such as the gut including tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, and IL-8 [99]. IL-8 is of particular interest in CF since it is a neutrophil chemoattractant [100], which could help to explain the predominantly neutrophil immune response to infection. Moreover, as a consequence of the destruction and remodeling of the lung associated with the disease, individuals with CF tend to develop other lung conditions. An example of this is allergic bronchopulmonary aspergillosis, in which breathing problems are exacerbated by the response to *Aspergillus fumigatus*.

Within the respiratory tract, CF symptoms in the airways are not just limited to the lung, but can affect the upper respiratory tract, particularly the paranasal sinuses. Thickening of the mucus in that location is also prominent and can lead to blockade of sinus passages followed by infection. This can cause nasal drainage, fever, facial pain, and headaches. In addition, CF individuals can develop nasal polyps caused by

inflammation from repeated sinus infections. These polyps can obstruct the nasal passages and add to the breathing difficulties already experienced by individuals with CF [101, 102].

1.3.10 Study of CFTR function

1.3.10.1 Measurement of channel function

Different methods exist in the measurements of channel function, of which patch clamping and efflux assays are the most commonly used [103-107]. Iodide efflux is a useful way to measure chloride transport [105-109], taking advantage of measuring the exit of iodide as a surrogate for chloride. While radio-labeled iodide [107] was originally used, this has been replaced in favor of using an iodide probe for detection of non-radioactive iodide [105, 108-110]; the probe detects the conductance of current in the solution. This technique is particularly useful in measuring macroscopic ion fluxes such as those seen in intact cell cultures.

Although iodide efflux will detect any form of chloride channel function, careful selection of the model system allows the assay to be used for the study of CFTR function [105, 108, 109]. In particular cells such as the immortalized cell line Calu-3, which express very high levels of CFTR, allows for the study of CFTR channel function since in such a system the entire iodide efflux can be ascribed to that channel. One limitation of this technique is the measurement of total channel function from a group of cells, not on an individual per cell basis.

1.3.10.2 Use of anion channel blockers

Anion channel blockers have been widely used in the study of the function of CFTR. Diphenylamine 2, 2'-dicarboxylic acid (DPC) is a commonly used anion channel inhibitor which can also block CFTR [109-111]. DPC binds to specific residues lining the CFTR channel; once bound to these sites, the channel is occluded [112]. Due to its non-selective blockade, it cannot be used to specifically study CFTR function in a cell model. As an alternative, early studies made use of the pharmacological compound glybenclamide (also known as glibenclamide) to block CFTR anion transport. First used in the treatment of diabetes mellitus through binding on the sulfonylurea receptor of pancreatic beta cells [113], causing inhibition of associated K+ channels, glybenclamide was also found to inhibit CFTR function by physically blocking the open channel [114].

DIDS (4,4'-diisothiocyano-2,2'- stilbene disulfonic acid), a disulfonic stilbene, is another anion channel blocker which can act as a blocker of CFTR-mediated anion transport [115] or as a non-CFTR inhibitor [107, 111, 116]. The inhibition is caused by the insertion of the blocker into the open pore on the cytoplasmic side of activated CFTR, which is large enough to allow entry of the blocker [117]. It has been suggested, from data obtained through site-directed mutations, that site R347 contributes to the binding site for DIDS [117]. In using DIDS as a non-CFTR inhibitor one must be careful of the blocker entering the cell, which could lead to CFTR channel inhibition [118]. This is due to the size of DIDS which does not permit entry into the channel from the extracellular side; therefore extracellular administration of the blocker fails to cause CFTR blockade [119].

The search for more specific CFTR inhibitors has been ongoing, not only for the study of CF but also for the study of diarrhea resulting from cholera [46]. Recently, the laboratory of Dr. Alan Verkman used a high throughput screening approach to identify a novel, CFTR selective inhibitor of anion transport, which they named CFTRinh-172 [120]. This thiazolidinone channel inhibitor has been used extensively since its discovery and has been shown to be effective at a range of concentrations, from 1 μM to 100 μM, and on different cell types [110, 120, 121]. Furthermore use of CFTRinh-172 has been able to induce increased IL-8 secretion in human primary airway epithelial cells [121], suggesting a role for CFTR function in the inflammatory process. Since these results suggest that use of the blocker can mimic CF-like conditions, it has become a useful tool in the study of CF on CFTR-expressing cell models, such as Calu-3 [115]. In addition, CFTRinh-172 has not been shown to interfere with the function of other anion channels [120].

1.3.10.3 CFTR knockdown

Although pharmacological inhibitors provide an easy means of blocking channel function, these compounds can have undesirable side effects and are frequently not sufficiently selective for the targeted channel. An alternative is to use short interfering RNA (siRNA) to inhibit the production of the targeted protein [122-126]. Short interference RNAs are 20 to 25 nucleotides long double stranded RNA molecules involved in the RNA interference pathway [122, 123]. These siRNAs are recognized by the RNA-induced silencing complex (RISC), known as DICER1 in humans, which then degrades the complementary mRNA [127]. One disadvantage with the administration of siRNA is its transient effect, especially in rapidly dividing cells [128, 129]. A way around

this problem is the insertion the siRNA into the cell using a vector such as a plasmid, allowing it to be expressed constitutively. In this case, a loop is introduced between the two strands, forming a single transcript, which is processed into functional siRNA [127]. CFTR has been successfully knockdown in different cells types, including Calu-3, either by transient transfection or by constitutive expression [130-133].

1.3.10.4 Correction of defective CFTR expression or function

In the quest to correct the underlying chloride transport defect found in CFTR, two approaches have been taken: correction of CFTR expression (and by definition, function) or increasing and activating alternate chloride channel function. Various compounds have been found to potentially correct CFTR expression and function, such as gentamicin [134], genistein [135] and curcumin [136]. Gentamicin has proven to be beneficial in correcting stop-codon mutations and restoring CFTR function *in vivo* [134, 137], although administration to CF individuals has not shown any significant clinical benefit [138]. Genistein has been shown to activate CFTR and even ΔF508, which has residual functional activity [135, 139], so long as some protein is present in the membrane, since it had no effect in promoting expression of either protein [140]. Curcumin had been initially shown to help increase ΔF508 expression [136], but subsequent work has put these observations in question [141].

Another approach to correcting the CF defect is gene therapy. Initially, attempts were made at inserting foreign genes, using an adenovirus as a vector for transmission, in rat primary cells [142]. This proved successful and was followed by *in vivo* work using viral vectors to insert human CFTR in both mouse and rat lung epithelium [143, 144]. The results of these experiments were long term expression of the human CFTR gene in

rat lung, up to 6 weeks post-treatment [143]. With promising outcomes in an animal model, the gene therapy work was extended to human subjects [145-148]. Unfortunately, the results showed inefficient gene transfer into intact epithelium and an inflammatory response to high doses of the viral vector [145, 146]. In addition, CFTR gene expression was shown to be transient, indicating the need for repeated adenoviral treatments, which would be expected to lead to loss of efficacy due to antibody production against the vector [147]. Some clinical data suggests persistent expression is possible in human sinuses using a viral vector, although further study would be required to determine if this would be possible in lung epithelium [148]. Liposome-entrapped plasmid DNA, an alternate gene delivery mechanism, has also been used for gene insertion in humans, with CFTR gene expression in the nasal epithelium of CF patients, sustained through repeated treatments [149, 150]. It is important to note that although CFTR gene expression was detected in nasal cells, it did not translate directly into expressed CFTR or even detectable chloride transport function [150].

An indirect method, circumventing CFTR altogether, has been used to correct the underlying channel defect in CF. Gao and colleagues have shown that the insertion of an artificial chloride channel in the membrane of CF mouse airways restored extracellular GSH levels [151]. This finding suggested a link between chloride transport and one of the known characteristics of the CF airway, decreased concentrations of GSH in the airway lining liquid. Use of this technique has not progressed beyond the lab however, due to the lack of efficient gene delivery in humans (see previous paragraph), yet it may be possible to utilize a pharmacological approach instead to boost alternate chloride channel function. Current trials in CF patients using denufosol, a chloride channel stimulator, have shown

promising results in improving lung function [152, 153]. Besides restoration of the underlying defect in CF, it would be interesting to determine during these trials if airway GSH levels are also restored, as would be predicted by the animal-based work of Gao *et al*. [151].

1.3.11 Other ABC channel proteins of interest: MRP1

Multi-drug Resistance Protein 1, MRP1, the first member of the MRP family is the MRP with the most sequence homology to CFTR and is expressed in the lung [9, 37, 154]. Furthermore, it has been shown to transport a multitude of GSH forms: GSH, GSSG, and GS-conjugates such as GS-4-hydroxy-2-nonenal (GS-4HNE) [33, 34, 36, 155, 156]. The function of MPR1 is ATP-dependent but not regulated by an R-domain, since it is non-existent in MRPs [39]. MRP1 function can be inhibited pharmacologically using cyclosporine A or MK-571 [33, 34, 36, 155, 157, 158]. Cyclosporine A is used more as a wide spectrum MRP inhibitor [33, 34], while MK-571 is more specific with reported effects on MRP1 and MPR4 [158, 159].

In CF, the presence of MRP1 correlates with lower respiratory tract symptom severity, as reflected in the Shwachman score [160]. Considering the similarity of MRP1 to CFTR, at least with reference to GSH transport function, it has been suggested that MRP1 could play a role in inflammatory [157] and anti-oxidant responses [160]. An example of the role in anti-oxidant response is the export of GS-4HNE, which is produced by GST and exported by MRP1 (see *4-HNE, section 1.4.2.2*), due to the affinity of GS-conjugates for the channel [155]. This export is important since accumulation of conjugates formed by GST, such as GS-4HNE, can lead to its inhibition [161], thus

MRP1 plays an important role in protecting against oxidant stress damage and in the proper functioning of the detoxification process.

1.4 Oxidant stress

1.4.1 Overview

Oxidative stress can be defined as an imbalance between the production of reactive oxygen species (ROS) and the ability of biological systems to mitigate their effects by detoxifying reactive intermediates or repairing oxidative damage; this imbalance favors the generation of ROS [162, 163], a group of molecules that include molecular oxygen and its derivatives. ROS are produced in all aerobic cells and can be found in the environment. Most biological processes require a reducing environment within cells and this environment is maintained by enzymes that provide protection against reactive oxygen species, which in turn require metabolic energy to function [163]. An imbalance can cause toxic effects via production of ROS, such as free radicals, which can damage different cellular components through oxidation of biological molecules, such as DNA, proteins, and lipids [164]. Therefore the balance which exists between reduction and oxidation and GSH plays an important role in the ability of the cells to respond to redox changes. Besides causing damage to cellular components, ROS can also be involved in cell signaling by oxidatively modifying proteins in a variety of signaling cascades, which is known as redox signaling [165].

1.4.2 Types of oxidant stress

1.4.2.1 Reactive oxygen species

Molecules containing unpaired electrons are known as free radicals. In biological systems these include superoxide anion ($O_2^{-\cdot}$), hydroxyl racial ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$), and lipid radicals. Other biologically important ROS, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl), are not free radicals per se, but have oxidizing effects which contribute to oxidant stress nevertheless.

Biologically active ROS may have their origin within the cell or in its environment. One of the main sources of endogenous stress are the mitochondria [166], which generate ROS as byproducts of oxidative metabolism. Other exogenous sources of oxidative stress can come from the body itself, such as the free radicals or hydrogen peroxide, which are released during an immunological response [167]. In the case of cells which are exposed to the outside world, such as airway epithelial cells, other potential sources include pollutants such as sulfur dioxide or ozone [168, 169].

Excessive oxidative stress is thought to contribute to the pathogenesis of human diseases such as Alzheimer's and Parkinson's [170-173]. In these conditions characterized by neuronal apoptosis, 4-HNE, a lipid peroxidation product produced by oxidation of cellular lipids, may be the mediator of cell death [173]. In support of this hypothesis, high levels of 4-HNE are found in the brain of both diseases [170, 172, 173]. In case of Alzheimer's disease, the substrate of cellular lipid oxidation is the amyloid beta-peptides [171, 172].

Reactive oxygen species also play a role in tissue damage caused by ischemia-reperfusion, as may occur after organ transplantation [174] where hypoxic conditions in the harvested organ are followed by a rapid influx of oxygen-rich blood when the organ is connected to the circulatory system of the recipient. This sudden increase in oxygenation is thought to overwhelm the cellular antioxidant systems, like GSH, through the formation of ROS and subsequent cellular damage, such as lipid peroxidation as seen with the formation of 4-HNE [175].

1.4.2.2 4-hydroxy-2-nonenal (4-HNE)

4-HNE is a highly reactive and diffusible end-product of lipid peroxidation which results from the action of oxidants, such as superoxide or hydrogen peroxide on lipids in the cellular membrane, causing the formation of aldehydes [175]. Specifically, peroxidation of ω-6-polyunsaturated fatty acids (e.g., linoleic acid and arachidonic acid) promotes the formation of lipid hydroperoxides. These lipid hydroperoxides are further degraded, leading to the formation of a variety of biologically active aldehydes, such as 4-HNE. These products are longer lasting than the free radical that create them and are highly diffusible between and within the cells [175]. There is growing evidence that aldehydes formed during the lipid peroxidation process are involved in most pathophysiological effects related to oxidative stress in tissues and cells. Among the different aldehydes produced, 4-HNE is found at concentrations of 10 μM to 5 mM in cells, and is considered to be principally responsible for the cytopathological effects observed *in vivo* [175]. 4-HNE is known to be a potent agent of modification of cellular DNA, RNA, proteins or other lipids and studies have shown it to act as a neutrophil chemoattractant *in vitro* and *in vivo* [175, 176].

The process by which 4-HNE is neutralized and exported out of the cell involves adduction to GSH followed by export via different channels (see 1.5.2 and 1.5.4.2). Briefly, the formation of the GSH adduct, GS-4HNE, is accomplished by glutathione-S-transferase, GST (see Diagram 1). Accumulation of 4-HNE itself can lead to GST inhibition, which makes its export a crucial step in protecting the cell against oxidant damage. The export of GS-4HNE is accomplished by the multi-drug resistance family of proteins, one of them being MRP1.

It has been reported that 4-HNE can activate GSH synthesis *via* induction of the glutamyl-cysteine ligase (GCL) gene and activate the synthesis of a variety of pro-inflammatory genes, such as IL-8, monocyte chemoattractant protein (MCP-) 1, and mucin 5AC (Muc5AC). This suggests that 4-HNE may play a role in gene transcription by acting on signaling molecules [177], a role further reinforced by reports of 4-HNE present and maintained in cells at sub-lethal concentrations [178].

4-HNE may be physiologically relevant in CF since it could help promote neutrophilic infiltration and it may be involved in increases of IL-8 via AP-1 [176, 179-181]. Furthermore 4-HNE involvement in mucin regulation could contribute to the thickening of the mucus observed in the airways as well as in the digestive tract of CF patients [182]. Although it has been reported that mucin gene expression is decreased in CF nasal epithelia [76], gene expression and protein production do increase with neutrophil elastase in lung epithelial cells [183] and it has been suggested that mucus hypersecretion could be associated with neutrophilic inflammation.

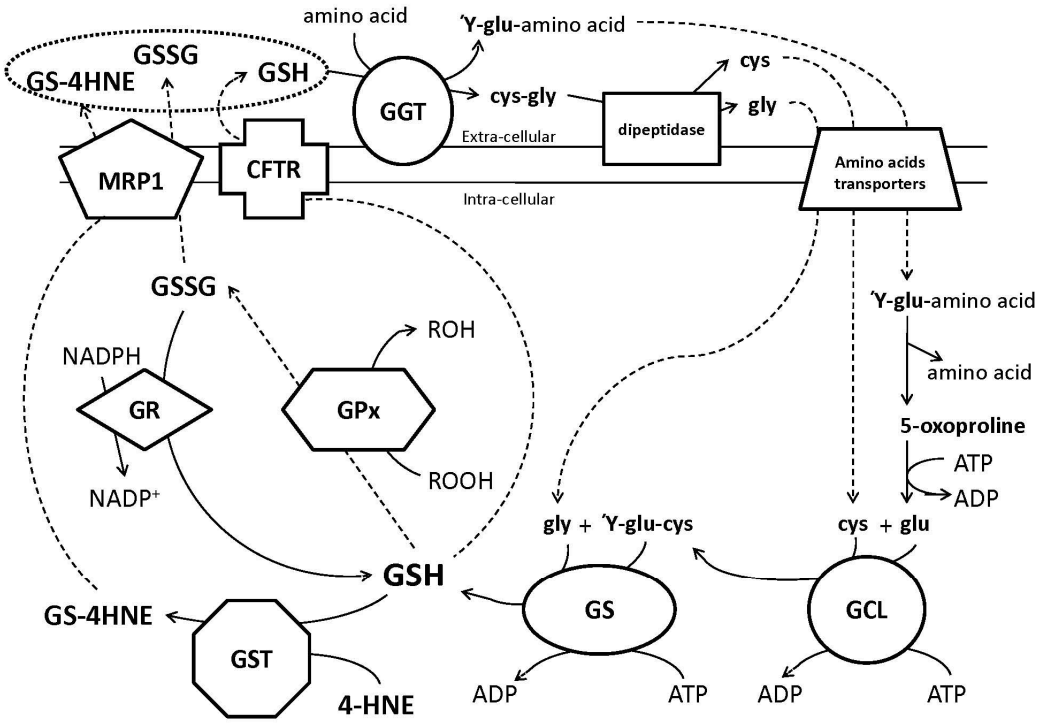


Diagram 1. The metabolism of GSH. Transport pathways are shown as dashed lines while metabolic pathways are represented by solid lines. For detailed explanation of the different pathways and enzymes implicated, see Section 1.5. (Diagram was adapted from Dickinson and Forman [165])

1.4.3 Measurement of oxidative stress in cells

Oxidative stress can be detected and measured within the cell in a variety of ways including the measurement of intracellular GSH concentrations. One approach is to use decreases in intracellular GSH levels as an indicator of oxidative stress [184]. It is also

possible to track changes in the ratio between reduced and oxidized GSH, which can be indicative of a change in the redox state of the cell [185]. Another approach is to use fluorescent markers, such as 2',7'-dichlorofluorescein diacetate (DCFDA), to determine the presence of different types of oxidants, such as hydrogen peroxide [186]; an increase in fluorescence, caused by the marker reacting with the oxidant, would indicate an increase in the oxidant measured.

End products of oxidation are also a means to determine the occurrence of oxidant stress. Cellular protein or cellular lipid modifications can be used as markers of oxidant stress. Protein carbonyl formation is used as an indicator of oxidant stress and is assayed using derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), leading to the formation of a stable dinitrophenyl (DNP) hydrazone product that can be easily detected using different techniques, such as a spectrophotometric assay [187]. Detection of malondialdehyde (MDA), a product of the lipid peroxidation, can be accomplished using thiobarbituric acid (also called the TBARS assay) which leads to the formation of a red substrate that can be easily detected using different methods, such as HLPC [187-190]. Finally, 4-HNE can form protein adducts whose epitopes can be detected using a western blot system [191-195].

1.4.4 Antioxidant mechanisms in the cell

Sulphur containing compounds such as thioredoxin and GSH [165, 196] can be easily oxidized and rapidly regenerated, which allows them to be involved in various biochemical reactions [162, 197, 198]. They are thus well suited for their role as intracellular antioxidants.

Given that GSH is the most abundant antioxidant in mammalian cells, it is important in the regulation of various cellular processes, such as transcriptional activation, and for cellular viability due to constant generation of ROS [196]. It is possible to define the cellular redox state as the ratio between the reduced and oxidized forms of GSH. [199]. Specifically, this would be the ratio between the oxidized form of GSH (GSSG) and the reduced form. A shift in the ratio can be associated with the activation of transcription factors such as NF-κB and AP-1 [200] as well as initiation of apoptosis [201].

1.5 Glutathione

GSH is a non-protein tri-peptide and is the most abundant antioxidant in mammalian cells, known as a nucleophilic scavenger and an enzyme-catalyzed antioxidant. Composed of precursor amino acids cysteine, glycine and glutamate, it is principally synthesised *de novo* in the liver [196]. GSH plays several important roles in the cell, including xenobiotic and eicosanoid metabolism, antioxidant defense, and regulation of gene expression and of the cell cycle [165].

1.5.1 GSH synthesis

De novo synthesis of GSH is dependent on two enzymes: glutamyl-cysteine ligase (GCL) and glutathione synthase (GS) [165]. As seen in Diagram 1, glutamate and cysteine are covalently linked by GCL, considered the rate limiting enzyme in GSH synthesis. The compound which is formed, gamma-glutamylcysteine, is then processed by GS, which adds glycine to form gamma-glutamyl-cysteinyl-glycine, GSH.

GCL, also known as gamma-glutamylcysteine synthetase (GCS), is a heterodimer composed of a heavy subunit, called the *GCL Catalytic* subunit (GCLC), and a light subunit, called the *GCL Modulatory* subunit (GCLM) [165]. GCLC is a 73 kDa protein that provides all of the catalytic activity of the heterodimer and is the target of GSH feedback inhibition. In contrast, the 31 kDa protein GCLM has no catalytic activity by itself yet exhibits regulatory function on GCLC. GCL activity can be regulated by factors such as oxidation, phosphorylation, and S-nitrosylation. It has been hypothesized that the modulation of GCL activity by GCLM is mediated through formation of disulfide bonds between the subunits. As there are oxidants in the cellular environment increase in concentration, the double bonds between GCLC and GCLM increase, thereby increasing the activity of GCL [202, 203]. As the GSH concentration rises, GSH forms its own disulfide bonds decreasing the number of bonds within the GCL heterodimer, thereby leading to a drop in GCL activity. Thus GCL is partly regulated through a GSH-mediated negative feedback loop. GCL can also be inhibited through the use of pharmacological inhibitors, such as L-buthionine-(S,R)-sulfoximine (BSO), causing an inhibition of GSH production [204]. Overall GCL activity is however, primarily transcrptionally regulated via the transcription factors Nrf2 and AP-1 [205-207]. For example, 4-HNE can increase transcription of GCL [207, 208], leading to an increase in GSH synthesis [209].

GS is a 118 kDa homodimer and less is known about how its activity is regulated. Even though GCL is considered the main determinant of GSH synthesis, there are reports that GS may have a more important role at least under some conditions. For example, reduced skeletal muscle GSH levels, such as those found in response to surgical trauma, have been found to relate to a decrease in GS activity with no significant change in GCL

activity [210]. In addition, studies in the rat found that GSH synthesis was further enhanced with increased GS and GCL expression than with GCL expression increase alone [211]. Finally Lu *et al*. focused on understanding the regulation of human GS and found that GS gene expression was regulated by Nrf2, similar to GCL [212]. Based on these findings, GS may play an equally important role in GSH synthesis.

1.5.2 Neutralization of oxidants

Two enzymes play a role in glutathione-mediated detoxification of oxidants: glutathione peroxidase (GPx) and glutathione-S-transferases (GSTs). GSH is a substrate of the glutathione peroxidase (GPx) enzyme, used to neutralize hydrogen peroxide [165]. The oxidant is reduced to water by GPx and GSSG, glutathione disulfide, is formed by the use of two GSH molecules. It is important to note that GSH does not react nonenzymatically to H_2O_2 [165].

Other products of oxidation, such as lipid peroxides, are mainly detoxified by enzyme-assisted adduct formation of GSH with GST. In this process, GSH is conjugated to electrophilic compounds. This detoxification process is important due to the effects lipid peroxidation products have on the cell. One such product, 4-HNE, can have cell signalling effects at low doses, but is lethal at high doses to cell function [178, 213, 214]. GST will form the conjugation product GS-4HNE, to neutralize the effects of the lipid peroxide. However, GS-4HNE can still threaten cell survival through inhibition of the GST enzyme, compromising a part of the cellular antioxidant mechanism [214]. Therefore export of adducts such as GS-4HNE, primarily handled by MRP1 (see 1.5.4.2), is critical.

1.5.3 GSH recycling

Two enzymes are needed for the rebuilding of the GSH pool, either by reducing GSSG to GSH or cleaving GSH-derived adducts into their constitutive parts for their reuptake and subsequent use in synthesis. Intracellularly, glutathione reductase (GR) is the enzyme which reduces GSSG, through cleavage of the double disulfide bond, back to GSH [165]. Extracellularly, γ-glutamyl transferase (GGT) cleaves the γ-glutamyl moiety to initiate the breakdown of extracellular GSH, GSSG or GSH adducts [165, 215, 216]. Those resulting products, which are the substrate components of GSH, are then transferred back into the cells to be reused for GSH synthesis. Thus GGT plays an important role in the cells antioxidant systems since it protects from GSH depletion. The function of GGT can be inhibited irreversibly by the glutamine analog acivicin, leading lead to an increase in extracellular GSH as shown in immortalized human tracheal epithelial cells [91].

1.5.4 Transporters of GSH

1.5.4.1 CFTR

Clinical and experimental observations indicate that CFTR has an important role in the extracellular transport of GSH [10, 11, 91, 217]. In one clinical study, investigators found significantly lower concentrations of GSH in the airway lining fluid (ALF) from patients with CF compared with non-CF control subjects [16]. ALF is a biochemically complex thin layer of fluid that covers the entire airway surface of the lung and is recognized as a first line of defense against inhaled chemicals and pathogens [87, 218]. *In vitro* studies have demonstrated that functional CFTR was linked to apical GSH secretion

[10, 11, 91] and *in vivo* studies with CFTR knockout (KO) mice have shown ~50% lower ALF GSH concentration, strongly suggesting an important role of CFTR in ALF GSH regulation [217]. Furthermore, an adaptive GSH response to *Pseudomonas* lung infection has been suggested as well [17], where CFTR was linked to the increase in ALF GSH seen during infection in mice.

1.5.4.2 MRP1

CFTR is not the sole transporter of GSH. GS-4HNE and other conjugated or non-conjugated forms of GSH are transported out of the cell using a variety of channels from the Multi-Drug Resistant family of proteins, MDRs, or the Multidrug Resistance-associated family of proteins, MRP [9]. Within the MRP family, MRP1 is known to transport several forms of GSH [33, 34, 36, 155, 156] and, as noted above, is the most similar in sequence homology to CFTR [9, 154]. In principle, given its organisational and functional similarity to CFTR, MRP1 could compensate for the lack of CFTR-dependent GSH transport.

1.6 Role of CFTR and GSH in CF

Evidence of an imbalanced redox system has been found in CF patients [219], suggesting that part of the pathogenesis of CF pulmonary disease involves oxidant stress damage. In support of this notion, increased activity of erythrocyte GR as well as increased amounts of the lipid peroxidation product MDA and protein carbonyl concentrations have been found in the plasma of CF patients, all of which are indicators

of oxidative injury. Moreover, analysis of proteins obtained from the BAL fluid of CF patients has shown evidence of oxidative stress modification [220].

The change in extracellular GSH is dependent on the type of oxidant challenge: stress which is chronic in nature, such as in smokers, causes an increase in extracellular GSH, while an acute challenge, such as an infection, causes a decrease [221-225]. In addition, the type of disease is also a factor in determining the concentrations of GSH. Diseases associated with active inflammation, including CF, typically have lower extracellular values [226]. This is in contrast with asthma where, despite chronic inflammation, extracellular GSH concentrations are increased [16, 227].

In addition to its role as an antioxidant, GSH can have an effect on signalling. In particular, a fall in the intracellular GSH concentration is associated with initiation of the caspase-3 system, leading to apoptosis [228, 229]. This mechanism has been invoked as a possible explanation of the observation that CF epithelial cells are relatively resistant to apoptosis compared to normal cells. It has been proposed that the higher intracellular concentrations of GSH that have been found in CF act to prevent the onset of caspase-mediated apoptosis [31, 32].

In view of the potential importance of oxidative tissue damage, a number of strategies have been proposed to increase resistance to oxidative damage. In particular, several methods of increasing intracellular GSH levels have been proposed. Aerosolized GSH was found to increase fluid-lining GSH and diminish inflammatory cell-derived oxidants in CF lungs [230, 231]. A recent study suggested that oral administration of N-acetylcysteine (NAC), a precursor molecule to GSH, has beneficial effects for modulating

inflammation in CF, although relatively high doses were used [232]. Dietary GSH supplementation has also been attempted with a reasonable degree of success in augmenting antioxidant defences. Whey protein has been more successful by increasing the GSH levels in clinically stable CF patients [233]. These approaches may be beneficial in maintaining optimal levels of GSH and counteracting the damaging effects of oxidative stress in CF lung. However, long term studies would be required to determine if diminishing the extracellular oxidant load translate into decreased tissue scarring and remodelling. Although augmenting lung GSH shows promise, it is important to be aware in the central defect in CF, deficient chloride transport, is still not corrected by this type of treatment.

Besides its antioxidant properties, GSH may decrease the mucus viscosity by disrupting disulfide bond formation in mucins. It has been previously shown that viscosity of mucus is augmented by an increase of mucin disulfide bonds [234]. NAC, a mucolytic agent and also a precursor in the formation of GSH, has been used to reduce mucus viscosity [235-237]. There are contradictory reports regarding its efficacy. One review suggesting no beneficial effects in aerosolized form for CF patients [238] while the same review pointed to beneficial effects through oral administration, pertaining to mucus viscosity. In addition to work with NAC mentioned above within the context of ameliorating the inflammatory state in CF patients, a study of aerosolized GSH was conducted and suggested that this approach could modify mucus viscosity. The sputum of treated CF patients was less viscous than that of the placebo group [239]. In addition, some studies have found a link between GSH and mucin secretion. GSH depletion lead to an increase in mucin secretion in tracheal epithelial cells [240] and GSH deficiency

coupled with NF-kB expression caused an increase in secretion from airway epithelial cells in the presence of *P. aeruginosa* [241]. Hence it seems that GSH does not just affect mucus directly, but may be a factor modulating its production.

1.7 Relationship between MRPs and Cystic Fibrosis

Hurbain *et al.* were the first to suggest that MRPs may play a role in modulating disease severity in CF [160]. Their study focused on MRP1 and MRP5, which were found to be expressed in nasal epithelium. Higher levels of MRP1 expression were correlated with less severe symptoms. This raises the possibility that MRP1 is acting as an alternative chloride channel to CFTR, providing another mechanism for reabsorption of chloride (with sodium) across the epithelial surface.

Besides being a potential alternate chloride channel, it has been suggested that MRP1 could play a role in inflammatory [157] and anti-oxidant responses [160]. An example of the role in anti-oxidant response is the export of GS-4HNE, which is produced by GST and exported by MRP1 (see *4-HNE*) [155]. This export is important since accumulation of conjugates formed by GST, such as GS-4HNE, can lead to GST inhibition and compromise antioxidant defense [161]. Within the context of CF, MRP1 could play an important role in protecting against oxidant stress damage resulting from chronic immune responses. In addition, it has been shown that MRP1 function is linked to transport of leukotrienes (LTs) [242, 243], which are themselves mediators of inflammation and agents of neutrophilic recruitment, such as LTB4 and LTC4 [244-247]. To further underscore the importance of LTs in CF, elevated levels of these mediators

having been measured in the sputum and urine of CF children [248]. Hence MRP1 could prove important in the regulation of inflammation, which is an issue in CF.

A major issue concerning MRP1 is its localization to the basolateral membrane of epithelial cells rather than the apical side as CFTR in most tissues (with certain exceptions, such as placenta [249]). Expression on the basolateral membrane would lead to its substrates being transported into the interstitial space [31, 39]. Furthermore, the localization of MRP1 would preclude it from having any direct interaction with CFTR. Despite this, as noted above there are hints that MRP1 may be a physiologically important alternative channel that has the potential to act as a modifier gene in CF.

1.8 Summary

1.8.1 Rationale

It has been proposed that alteration of pulmonary host defense can be influenced through CFTR, by its role in mediating the response of respiratory epithelial cells to oxidative stress. Specifically, lack of CFTR may affect GSH, which can play a role in the pathogenesis of CF due to its role as an antioxidant, a mucolytic, and a regulator of inflammation, immune response, and cell viability *via* its redox status in the human body. Numerous reports on the status of GSH in the CF airway have been published, yet not much is known about the intracellular domain of epithelial cells, especially under oxidant stress conditions. Within this context, we hypothesized that CFTR function plays a role in determining intracellular GSH under oxidative stress.

We also conjectured that other channels could be involved in GSH regulation, due to the involvement of oxidant stress. This was investigated in light of possible CFTR down-regulation under oxidant stress conditions [108]. Alternate means of GSH transport could play an important role and have repercussions in CF. We hypothesized that one such alternative channel could be MRP1, a GSH transporter linked to CFTR function [159].

1.8.2 Hypothesis

Firstly, it is hypothesized that CFTR can affect intracellular GSH in airway epithelial cells, with blockade of CFTR leading to an increase in intracellular concentrations of GSH. Secondly, it is hypothesized that intracellular and extracellular GSH concentrations can be changed with blockade of MRP1 function.

1.8.3 Objectives

1.8.3.1 General objectives

The general objective of this thesis was to examine how CFTR function affects intra- and extra-cellular GSH, under normal or oxidant stress conditions. An investigation of the mechanism behind the changes in intra- and extra-cellular GSH was conducted in a relevant cellular airway model. In addition, the role of MRP1 in determining GSH amounts was investigated, under normal or oxidant stress conditions.

1.8.3.2 Specific objectives

1. To determine how CFTR affects intra- and extra-cellular GSH, under oxidant stress conditions, in the CFTR expressing airway cell model, Calu-3. This was

accomplished using pharmacological blockers to affect anionic channel function or using CFTR knockdown cells, which inherently lack CFTR function, and then comparing GSH values obtained to untreated cells. The oxidant 4-HNE was used to create the oxidative stress conditions needed.

2. To determine if another GSH transporter, MRP1, played a role in determining intra- and extra-cellular GSH from Calu-3 cells. This was done by comparing GSH amounts obtained using pharmacological inhibition of the channel, using MK-571, to that from untreated cells.

3. To determine the mechanism behind the changes in CFTR function observed with use of MK-571, the blocker of MRP1 function. This was done using a BHK cell line expressing either wild-type or ΔF508 CFTR, by first examining expression of the proteins and then studying their chloride channel function, after administration of MK-571.

Chapter 2: Materials and Methods

2.1 Summary of Experimental Procedures

The study in this thesis was divided into 3 parts, in accordance with the number of objectives previously laid out in the Introduction. We chose the Calu-3 serous cell line for our studies of the relationship between CFTR and GSH, and our studies of MRP1, under oxidant stress conditions. Serous cells in lung submucosal glands express high levels of CFTR [250] and are important in the secretion of anti-microbial compounds [251]. Considering the defect in CF, these cells would be most affected by the disease and thus are a relevant model for study. Calu-3 cells are a human airway cell line derived from human lung cancers [252]. More specifically, the cells are human lung carcinoma serous cells [106, 253, 254] and therefore serve as a potentially useful tool to investigate CFTR function [104, 255-257]. In particular, in an air-liquid culture system, the cells will produce chloride-secreting apical CFTR channels and develop tight junctions. Furthermore, CFTR-deficient Calu-3 cells were available to determine if CFTR was required for any changes observed. We used the BHK cell model to study of CFTR expression and function in the final part of this thesis, which allowed us to examine the effects of MRP1 blockade/MK-571 treatment on wild-type and ΔF508 CFTR expression and function, which was important considering ΔF508 is the most common mutation found in CF patients.

In the first part, the role of CFTR in determining intra- and extra-cellular GSH amounts, under oxidative stress, was investigated using Calu-3 cells. We used a

pharmacological approach to inhibit CFTR channel function and examine the outcome in intra- and extra-cellular GSH concentrations. Any changes or lack thereof in GSH amounts with CFTR blockade were confirmed using a Calu-3 CFTR knockdown model. Iodide efflux was used to determine if CFTR function was inhibited with administration of blockers specific to that task. Cell viability was also studied to determine that the blockers and oxidant stress used were not causing unwanted cell death.

The second part dealt with the role of MRP1 in influencing GSH concentrations under oxidative stress, with the same Calu-3 model. A pharmacological approach was taken to block MRP1, using MK-571 to determine if blockade of the channel caused changes in intra- and extra-cellular GSH concentrations. The role of CFTR in these changes was also investigated through concomitant administration of both CFTR and MRP1 blockers, considering the overlap in function and structural homology these proteins have. If any changes were observed, the role for CFTR would be confirmed with the use of CFTR knockdown cells. Since pharmacological inhibitors can have unforeseen side-effects, some of which could affect GSH amounts and be unrelated to channel blockade, GSH synthesis was studied by measuring GCL activity. To further understand the changes in GSH amounts and knowing MK-571 is also a leukotriene receptor antagonist, we studied the possible involvement of leukotrienes in determining the amounts of GSH. In addition, since MK-571 is also an inhibitor of cAMP transport, the role of cAMP was studied to determine if any increases in extra-cellular GSH amounts were the results of cAMP blockade which could result in an increase of CFTR activity.

The final part of this study investigated the mechanism of action behind the changes in GSH seen in the second part. The MRP1 channel blocker MK-571 was

administered to a CFTR-expressing BHK cell model. We measured cell surface and whole cell CFTR protein expression, as well as CFTR channel function. This model was used since a ΔF508-CFTR expressing variant was available, allowing us to study changes in function and expression in not just the normal protein but that of ΔF508-CFTR, the most common mutation found in CF. Cell surface expression of CFTR was determined using a fluorescence-based high throughput screen assay, while whole cell expression was examined using western blots and antibodies targeted against the protein. CFTR function, either for normal or ΔF508 protein, was measured using iodide efflux. The experiments from all three parts of the study provided enough data to allow us to answer the hypotheses put forward in this thesis and to draw a conclusion.

2.2 Description of methods

2.2.1 Epithelial Cell Culture

For the first and second parts of this thesis, Calu-3 immortalized cell lines (*ATCC, Rockville, MD*), either wild-type or with CFTR knockdown, obtained from the lab of Dr. John Hanrahan, were cultured in either 6-well (at 0.3 X 10^6 cells/well) or 24-well (at 0.075 X 10^6 cells/well) plates in phenol red-free MEM medium *(Invitrogen, Burlington, Ontario, Canada)* containing 10% FBS (*Wisent, Montreal, Quebec, Canada)*. Medium also contained 2 mM L-glutamine (Wisent), 1 mM sodium pyruvate (*Wisent)*, 1x non-essential amino acids *(Wisent*), 100 mg/ml penicillin, 100 U/ml streptomycin (*Wisent)* and 0.1 mg/ml normocin (*Amaxa, Cologne, Germany*). Cells were maintained in a humidified incubator at 37^{o}C with 95% (vol/vol) air and 5% (vol/vol) CO_2. The cells

were replenished with fresh media every 2 to 3 days. At 90% confluence (approx. 5 to 6 days of growth) the cells were serum deprived for 24 h in MEM medium containing 0.5% FBS before various experiments were carried out (2.2.2 through 2.2.11).

2.2.2 Cell viability

Cell viability was assessed by MTT assay as described previously [18]. Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (*Sigma-Aldrich, St. Louis, MO*) was dissolved in phosphate buffered saline (PBS) at 5 mg/ml concentration and filtered to sterilize and remove any insoluble residue present in the solution. After 24 h serum deprivation and with different treatments where appropriate (vehicles, blockers, stress), stock MTT solution (10 ml per 100 ml medium) was added to all wells in a plate and were incubated at 37°C for 2 h. Acid-isopropanol (100 ml of 0.04 N HCI in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After 5 min at room temperature (to ensure that all crystals were dissolved), the plates were read using a test wavelength of 550 nm and a reference wavelength of 690 nm. Plates were read within 1 h of adding the isopropanol. The viability was expressed as a percentage of the viability of control cells exposed to 0 μM of the oxidant, vehicle or blocker in question. The concentration that caused more than a 20% decrease in cell viability was considered to be cytotoxic.

2.2.3 Channel blockade in Calu-3 cells

In the first part of the study, Calu-3 cells were exposed to different channel blockers during 24 h serum deprivation and oxidant stress challenge: 250 μM diphenylamine 2, 2'-dicarboxylic acid (DPC), 500 μM 4,4'-diisothiocyano-2,2'-stilbene

disulfonic acid (DIDS), 2 μM CFTR-inh-172 (*Sigma-Aldrich, St. Louis, MO*). In the second part of the study, MK-571 at 30 μM (*Calbiochem, San Diego, CA*) was used to inhibit MRP1 channel function. In both parts of the thesis, 30 μM 4-hydroxy-2-nonenal (4-HNE; *Cayman Chemical, Cedarlane Laboratories, Hornby, Ontario, Canada)* was used to generate oxidative stress and was administered after 24 h serum deprivation, in newly changed serum deprived medium. At 6 h after challenge, the extracellular media were collected and the cells were lysed and used for various analyses (2.2.3 through 2.2.11).

2.2.4 Iodide efflux assay on Calu-3 cells

CFTR channel functional activity was measured using the iodide efflux method as described elsewhere [105]. Briefly, cells were incubated with iodide loading buffer (136 mM NaI, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose and 20 mM Hepes, pH 7.4) for 45 min at room temperature. Extracellular NaI was removed by rapidly rinsing the cells three times with iodide-free efflux buffer, which was identical to the loading buffer except $NaNO_3$ replacing NaI. Samples were collected by completely replacing the efflux buffer volume (1 ml) with fresh solution at 1 min intervals. The first two samples were averaged to establish the baseline efflux rate, then 8-(4-chlorophenylthio)-adenosine-3',5'-monophosphate (cpt-cAMP), a membrane-permeable cAMP analogue, was added and samples were collected every minute for 6 min in the continued presence of cpt-cAMP. The iodide concentration of each aliquot was determined using an iodide-sensitive electrode (*Orion Research Inc., Boston, MA, USA*). The amount of iodide released during the 1 min interval is expressed in nanomoles (nM). If channel blockers were used for experiments, they were included in all buffers used for the assay.

2.2.5 Measurement of GSH using RP-HPLC

In the first part of the study, intra- and extra-cellular GSH was measured by reverse phase high pressure liquid chromatography (RP-HPLC) using a post-column derivatization procedure as previously described [258]. Briefly, following incubation of Calu-3 cells with 4-HNE and/or channel blockers, the medium was removed and cells were lysed in 500 µl PBS containing 200 mM phosphoric acid and 12 mM 3-[(3-cholamidopropyl)-dimethyl-ammonio]-l-propanesulfonate (CHAPS) at 0 °C . 50 µl aliquots were loaded on to RP-HPLC column and eluted using a gradient (flow rate, 1 ml/min) prepared from solvents A (0.05% trifluoroacetic acid in water) and solvent B (0.05% trifluoroacetic acid in acetonitrile) as follows: 0 min, 0% B; 10 min, 15% B. The stationary phase was a 15 cm column of Ultracarb ODS (5 µm particle size; *Phenomenex, Torrance, CA*). The column eluate was mixed with o-phthalaldehyde (370 µM) in 0.2M Na_3PO_4, pH 12 (flow rate, 1 ml/min), and then passed through a loop of polyetheretherketone (PEEK) tubing (6 m x 0.5 mm, i.d.; volume, 1.2 ml) in a water bath at 70 °C. Under these conditions both GSH and GSSG were converted to an isoindole adduct, which was measured using a fluorescence detector (λex, 336 nm; λem, 420 nm). The concentrations of GSH were determined from a standard curve using the authentic compounds as external standards.

2.2.6 GSH spectrophotometric assay

For the second part of the study, Calu-3 cells were collected for measurement of intra- and extra-cellular GSH using a spectrophotometric assay, according to a previously published protocol [259], with minor modifications. Briefly, cells were scraped off culture wells in a small amount of PBS, re-suspended thoroughly by pipetting and then

transferred into a microtube containing ice-cold trichloroacetic acid (TCA; 2.5% final). After vigorous vortexing, cells were incubated for 30 min on ice with intermittent vortexing to complete cell lysis and deproteinization. Proteins were pelleted by high-speed centrifugation (microcentrifuge, 12,000 g, 10 min, 4°C). Acid-soluble supernatants and protein pellets were collected and then stored at -80°C for subsequent analyses of GSH and protein. For extracellular GSH, culture medium was collected and then centrifuged to pellet any floating cells (microcentrifuge, 12,000 g, 10 min, 4°C). Supernatants were collected and mixed with TCA (1% final) and then centrifuged again to pellet any remaining protein (microcentrifuge, 12,000 g, 10 min, 4°C). Supernatants were collected and then stored at -80°C for subsequent GSH analyses.

The GSH assay was conducted using an automated enzymatic kinetic assay as described previously [260], using reagent concentrations and reaction conditions from the literature [18]. Briefly, samples were prepared using TCA (5%) and the concentration of GSH was determined by the glutathione reductase recycling method of Tietze [261] adapted for the Cobas Mira spectrophotometer (*Roche Diagnostics*) [262]. The Cobas Mira loads into cuvettes 210 µl NADPH (0.3 mmol/l), 30 µl DTNB (6.0 mmol/l), and 95 µl of sample or standard. After a 4-min incubation at 37°C, 15 µl glutathione reductase (1.0 U/100 µl) was added, and the reaction is monitored every 24 s for 12 min. Under these conditions, the method is linear for GSH concentrations between 0.5 and 5 µmol/l. The instrument constructs a calibration curve by assaying known GSH standards, and from this the GSH concentration of the unknown is determined. The intra-assay coefficient of variation for this assay was 2%. Values were normalized for volume (1 ml, for extracellular GSH) or protein (mg of protein, for intracellular GSH).

2.2.7 Glutamate-cysteine ligase (GCL) activity assay

GCL activity, an indicator of *de novo* synthesis of GSH, in epithelial cell lysate was measured using a published protocol [263], with some modifications. In summary, Calu-3 cells were grown in 6-well plates until they reached confluence and then were treated with 4-HNE and/or a channel blocker. Cells were then washed twice with PBS and lysed with 500 µl of lysis buffer (20 µl Triton X-100 in 20 ml Tris buffer, pH 8.0). The cells were then scraped and the whole cell lysates were spun down at 10,000g for 10 min. Supernatant was collected and 100 µl was added to 880 µl reaction buffer (100 mL of 0.1 M Tris buffer, pH 8, containing 0.14g KCl, 0.034g NaATP, 0.018g glutamate, 0.013g aminobutyrate, 0.051g $MgCl_2$, 0.009g EDTA), containing freshly prepared 10 µl lactate dehydrogenase (LDH) solution (1.7 mg/ml LDH in Tris buffer pH 8.0) and 10 µl pyurvate kinase (PK) solution (1.7 mg/ml PK in Tris buffer pH 8.0). Then the absorbance of the mixture was measured at 338 nm for every min from time 0 to 5 min using a spectrophotometer. Then GCL activity was determined using the delta OD per min of the measurements as in the equation below:

GCL activity (IU) = [Rate (ΔOD/min, sample) – Rate (ΔOD/min, sample Blank) x total volume] / [6.22 x sample volume].

The activities were normalized per mg of protein.

2.2.8 GGT enzyme inhibition

The GGT enzyme inhibitor acivicin (*Sigma-Aldrich, St. Louis, MO*) was administered during the 6 h time period after 24 h serum deprivation (for methodology see 2.2.2), at final concentrations of 100 µM. At 6 h post-challenge, the extracellular

media were collected and the cells were lysed and used for GSH analyses, using the method in 2.2.6.

2.2.9 Leukotriene synthesis inhibition

Leukotriene synthesis inhibitors MK-886 (*Calbiochem, San Diego, CA*) and zileuton (1-(1-benzothiophen-2-ylethyl)-1-hydroxy-urea; *Critical Therapeutics, Lexington, MA*) were administered as the channel blockers (for methodology see 2.2.2), at final concentrations of 200 nM and 30 μM respectively. At 6 h post-challenge, the extracellular media were collected and the cells were lysed and used for GSH analyses, using the method in 2.2.6.

2.2.10 8-bromo-cAMP and Rp-8-bromo-cAMP administration

Treatment of Calu-3 cells with either 8-bromo-cAMP (8-bromoadenosine 3′,5′-cyclic monophosphate) or Rp-8-bromo-cAMP (8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; *Sigma-Aldrich, St. Louis, MO*), was preformed after 24 h serum deprivation, at a final concentration of 250 μM. At 6 h post-challenge, the extracellular media were collected and the cells were lysed and used for GSH analyses, using the method in 2.2.6.

2.2.11 MRP1 and MRP4 Western blot

Calu-3 cell lysates were prepared by adding 250 μl RIPA lysis buffer (*Santa Cruz, Santa Cruz, CA*) per well of a 6-well culture plate. Then the cell lysates (20 μg protein/sample) were loaded on a 4%-12% gradient acrylamide gel (*Bio-Rad, Mississauga, Ontario*), and run for 2 h at 150 V. Proteins were then transferred onto a nitrocellulose membrane for 30 min at 100 V. Non-specific binding sites were blocked

with 2% BSA in Tris-Buffered Saline with Tween-20 (TBST) for 45 min. The membrane was then rinsed with TBST and incubated overnight at 4^{o}C with MRPr1 primary antibody (MAB4124, *Chemicon Chemicals, Temecula, CA)* for MRP1 detection or with M4I-10 primary antibody (ab15602, *Cedarlane., Hornby, Ontario, Canada)* for MRP4 detection, with light shaking. The membrane was washed three times with TBST for 5 min and then incubated with the second HRP anti-rat antibody (*BD Biosciences, Mississauga, Ontario*) for 1 h. The membrane was washed three times with TBST for 5 min and incubated with Immun-Star (*Bio-Rad, Mississauga, Ontario*) for 5 min. An image of the membrane was then made using a FluroChem imaging device (*Alpha Innotech Corp., San Leandro, CA*). Anti-GAPDH primary antibody (*Ambion, Austin, TX*) was also used to determine if gel was loaded correctly.

2.2.12 BHK Cell Culture

These experiments were conducted in the laboratory of Dr. D. Thomas. We used Baby Hamster Kidney cells (BHK) already stably transfected with constructs expressing either wild-type or ΔF508 CFTR bearing three tandem haemagglutinin-epitope tags (3HA) and linker sequences in the fourth extracellular loop after amino acid 901 [264, 265]. Cells were cultured at 37^{o}C and 5% CO_2/95% air. The culture medium contains DMEM (F12) (*Wisent, Montreal, Quebec, Canada*), 5% fetal bovine serum (*Wisent*), 20 U/ml Penicillin/Streptomycin (*Wisent*) and 500μM Methotrexate (*Mayne Pharma, St-Laurent, Quebec, Canada*). Methotrexate was used as a selection agent to ensure expression of either either wild-type or ΔF508 CFTR in the transfected BHK cells. These cells were used in methods 2.2.13 through 2.2.15.

2.2.13 HTS Protocol for detection of CFTR cell-surface expression in BHK cells

High throughput screening (HTS) was performed using BHK cells which stably express either wild-type or ΔF508 CFTR [264]. Briefly, cells were seeded in 96-well plates at 15,000 cells per well and incubated for 24 h at 37^{o}C. Each well was then treated with different concentrations of MK-571 or Montelukast for 1h to 24 h. The remaining wells were used for control conditions. Cells were fixed in paraformaldehyde, washed with PBS, and incubated with PBS containing 5% fetal bovine serum (FBS) for 1 h at 4^{o}C. This was replaced with primary antibody solution containing 1% FBS and mouse monoclonal anti-HA antibody (1:150 dilution, *Sigma*) in PBS. The plates were sealed and left at 4^{o}C overnight. After washing with PBS, cells were incubated for 1 h with secondary antibody solution containing 1% FBS and anti-mouse IgG conjugated with FITC (1:100 dilution, *Sigma*) in PBS. Cells were again washed in PBS and analyzed in a plate reader (Analyst^{TM} HT96.384, *Biosystems*) (488nm excitation, 510nm emission). A change of 11.5 % in cell surface expression was considered significant ($p < 0.05$).

2.2.14 Western blots of whole-cell CFTR from BHK cells

Cell lysates were quantified for protein by Bradford assay (*BioRad, Mississauga, Ontario, Canada*) and separated by SDS-PAGE (6% polyacrylamide gels) and analyzed by Western blotting. Western blots were blocked using 5% skimmed milk in PBS and were probed overnight at 4^{o}C with a primary anti-CFTR monoclonal mouse antibody at a dilution of 1:1000 (*Chemicon*). The blots were washed four times in PBS before the addition of the secondary HRP-conjugated anti-mouse antibody, at a dilution of 1:5000 (*Amersham*) for 1h at room temperature. The blots were washed five times in PBS and

probed for chemiluminescence (*Pierce*). All samples were run with equal protein loading as determined using the Bradford assay (*Biorad*). Densitometry of the immunoblots was performed using the ImageJ program (*http://rsbweb.nih.gov/ij/*).

2.2.15 Iodide efflux assay on BHK cells

Iodide efflux was assayed as described previously. Experiments were performed with a robotic liquid handling system (BioRobot 8000, *Qiagen, USA*) using Qiagen 4.1 software. Cells were cultured in 24-well plates until they reached confluence in order to perform parallel experiments and comparison analysis. After the test compound treatment period, the medium of each well was replaced with iodide loading buffer (in mM: 136 NaI, 3 KNO_3, 2 $Ca(NO_3)_2$, 11 glucose and 20 Hepes pH 7.4) for 1 hour at 37^oC to permit the I^- to reach equilibrium. At the beginning of each experiment, the loading buffer was removed by aspiration and cells were washed eight times with efflux buffer (same as loading buffer except that NaI was replaced with 136 mM $NaNO_3$) to remove extracellular I^- in each well. The loss of intracellular I^- was determined by removing the medium with efflux buffer every 1 min for up to 11 min. The first four aliquots were recovered at one-minute intervals into an empty 24-well plate and used to establish a stable baseline in efflux buffer alone. Then, a stimulation buffer (efflux buffer containing 50 μM genistein + 10 μM forskolin) was added and samples were also collected every minute in its continued presence. The iodide concentration of each aliquot was determined using an iodide-sensitive electrode (*Orion Research Inc., Boston, MA, USA*) and converted to iodide content (i.e. the amount of iodide released during the 1 min interval). Curves were constructed by plotting concentration versus time. Data are presented as means ± SEM.

2.2.16 Statistics

Statistical Analyses were performed using the chart graphing and statistical analysis software GraphPad Prism 5 (GraphPad Software Inc., *San Diego, California*, *USA*). For all experimental outcomes in this thesis, the Student's t-test (two tailed) analysis was performed. The Student's t-test was chosen to determine if the outcome of a given treatment was different to its respective vehicle control or another group. In the case of a combination of 2 different compounds of the same class, the analysis was performed by comparison to either compound used alone. The Student's t-test analysis shows if a difference was present between 2 groups where one single condition was changed. Statistical significance was determined for P values <0.05. Statistical analysis was reviewed by an in-house consultant, Dr. Heberto Ghezzo.

Chapter 3: Results

3.1 Results from Part 1: Study of CFTR

3.1.1 Cell viability

Cytotoxicity studies were carried out to assess the effect of channel blocker treatments on cell viability. Overall, the use of the different anion channel blockers had no significant effect on cell viability (all values are % of control, n=4): DPC: 91.16%; DIDS: 106.94%; CFTR-inh-172: 88.17%. The treatment with HNE (94% of control) did not have a major impact on cell viability. Vehicles used for the experiments also had no effect on Calu-3 cells: Ethanol (EtOH) and DMSO gave cell viabilities of 99.74% and 92.37%, respectively.

3.1.2 CFTR channel activity

Iodide efflux measurements were used to determine CFTR channel activity and iodide values are expressed as the difference between baseline and activated cells in the presence of different blockers. The CFTR channel was functionally active under baseline conditions (21.0 μM ± 1.5 iodide/min at 2 min) as well as with DIDS treatment (maximum efflux of 11.2 μM ± 1.1 iodide/min at 2 min). CFTR activity was almost completely abrogated with DPC (maximum efflux of 1.4 μM ± 0.5 iodide/min at 2 mins) or CFTR-inh-172 (maximum efflux of 3.6 μM ± 2.1 iodide/min at 2 mins) (Figure 3.1). CFTR Knockdown Calu-3 cells showed no significant efflux (-1.6 ± 2.1 iodide/min at 2 mins).

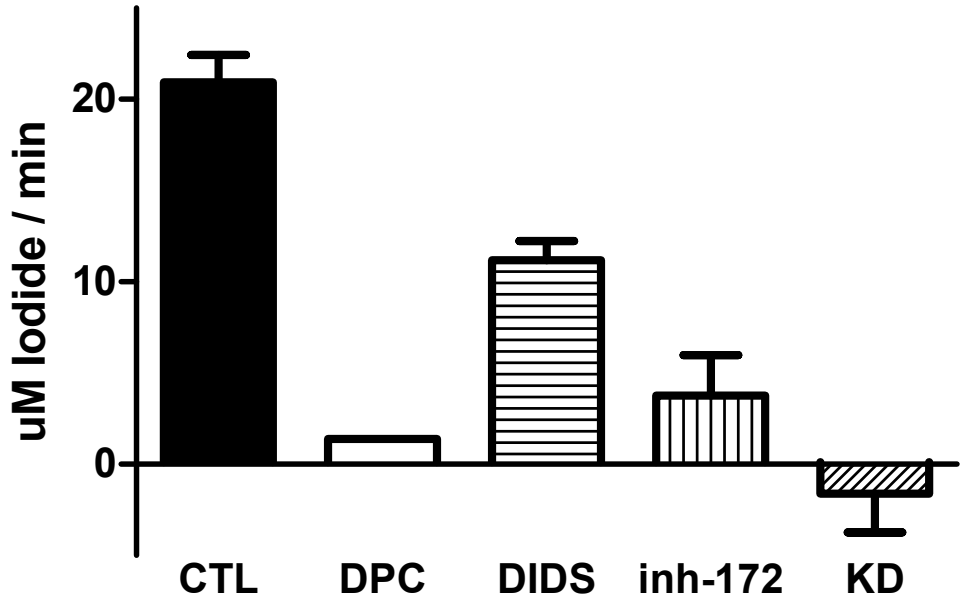


Figure 3.1. Inhibition of anion channel activity using anion channel blockers in Calu-3 cells. Iodide efflux assay on Calu-3 cells after treatment with different anion channel inhibitors: DPC, a general anion channel function inhibitor, DIDS, a non-CFTR channel blocker, and CFTRinh-172 (Inh-172), a CFTR-specific channel blocker. The peak activity of CFTR knockdown (KD) Calu-3 cells is also shown. Cells were treated with blockers for 24 h before analysis by iodide efflux (n = 3 for each blocker). Data are presented as an average of cAMP-stimulated peak iodide efflux (at 2 mins).

3.1.3 Blocker and 4-HNE treatment effects on intracellular GSH amounts

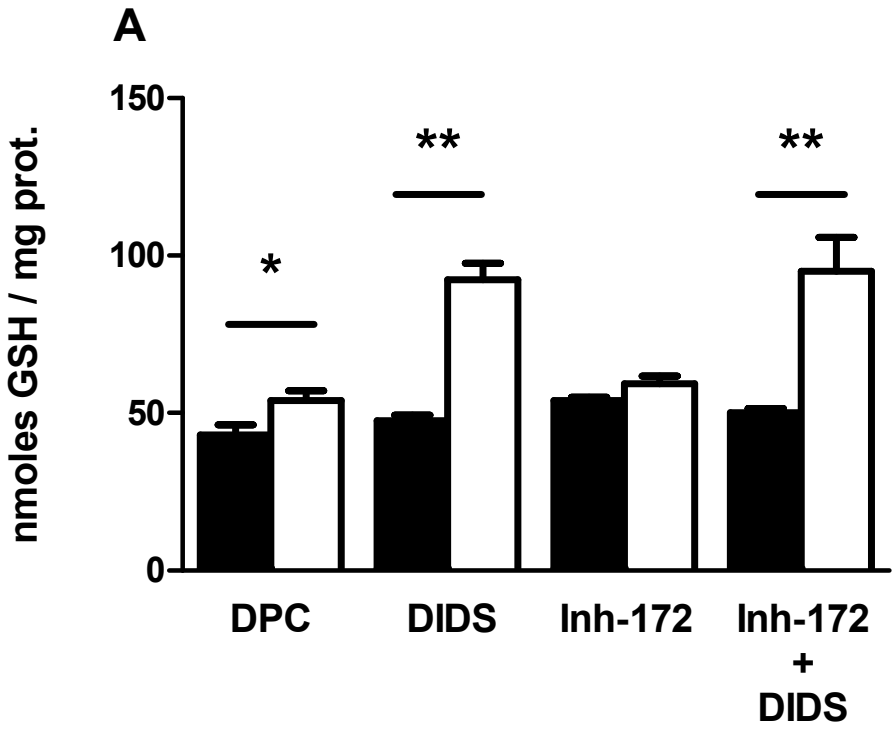
Administration of anion channel blockers caused an increase of intracellular GSH: DPC (53.96 ± 3.14 nM/mg protein, vs 43.04 ± 3.10 (control), $p<0.03$, n=6), DIDS (92.27 ± 3.46 vs 47.59 ± 2.01 (control), $p<0.002$, n=10), and when combining DIDS and inh-172 together (95.10 ± 10.76 vs 50.08 ± 1.40 (control) $p<0.002$, n=6). Yet we did not observe any changes with intracellular [GSH] using CFTR channel blocker CFTR-inh-172 alone (59.37 ± 2.36 vs 53.96 ± 1.08 (control) $p<0.06$, n=6) (figure 3.2A).

A similar pattern emerged with addition of 4-HNE. Cells exposed to 4-HNE and either DPC or CFTR-inh-172 showed no significant increases in [GSH] levels (DPC: 19.55 ± 2.90 vs 15.16 ± 1.93(control) $p<0.24$; n=6; CFTR-inh-172: 33.79 ± 1.89 vs 24.91 ± 4.34 (control) $p<0.08$, n=6). Administration of either DIDS (50.90 ± 3.84 vs 20.55 ± 0.85, (control) $p<0.002$, n=8) or both DIDS and CFTR-inh-172 together (61.87 ± 10.14 vs 20.54 ± 3.73 (control) $p<0.002$, n=6) resulted in increased intracellular [GSH] (figure 3.2B).

3.1.4 Blocker and 4-HNE treatment effects on extracellular GSH amounts

None of the anion channel blockers had any effect on extracellular [GSH] (figure 3.3A) compared to controls Results were similar in the presence of oxidant stress induced by the administration of 4-HNE (figure 3.3B).

Figure 3.2. Changes in intracellular GSH after administration of anion channel blocker to Calu-3 cells. Black bars represent vehicle controls while white bars represent treatment with anion channel blockers: DPC, general anion channel function inhibitor (n=6), DIDS, non-CFTR channel blocker (n=10), CFTR-inh-172 (Inh-172; n=6), the CFTR channel inhibitor, and combined DIDS + CFTR inh-172 treatment (n=6). Panel A shows intracellular [GSH] from Calu-3 cells after addition of blockers. Panel B shows intracellular [GSH] after blocker and 4-HNE administration. Data are presented as mean intracellular [GSH] normalized by cellular protein concentration. Blocker treatments are compared with vehicle control cells: *, $p < 0.05$; **, $p < 0.01$.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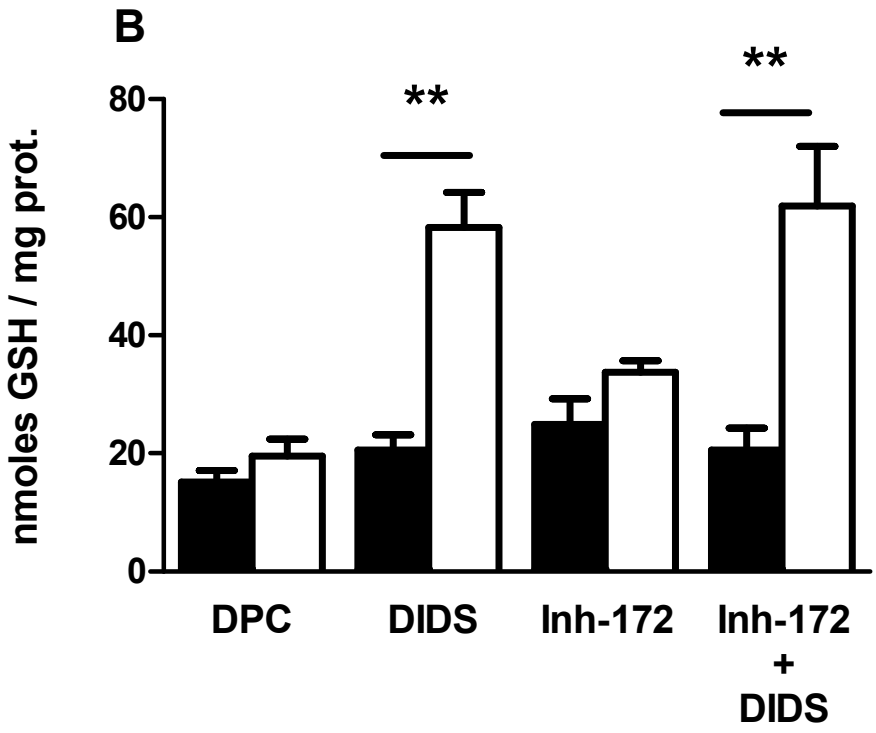
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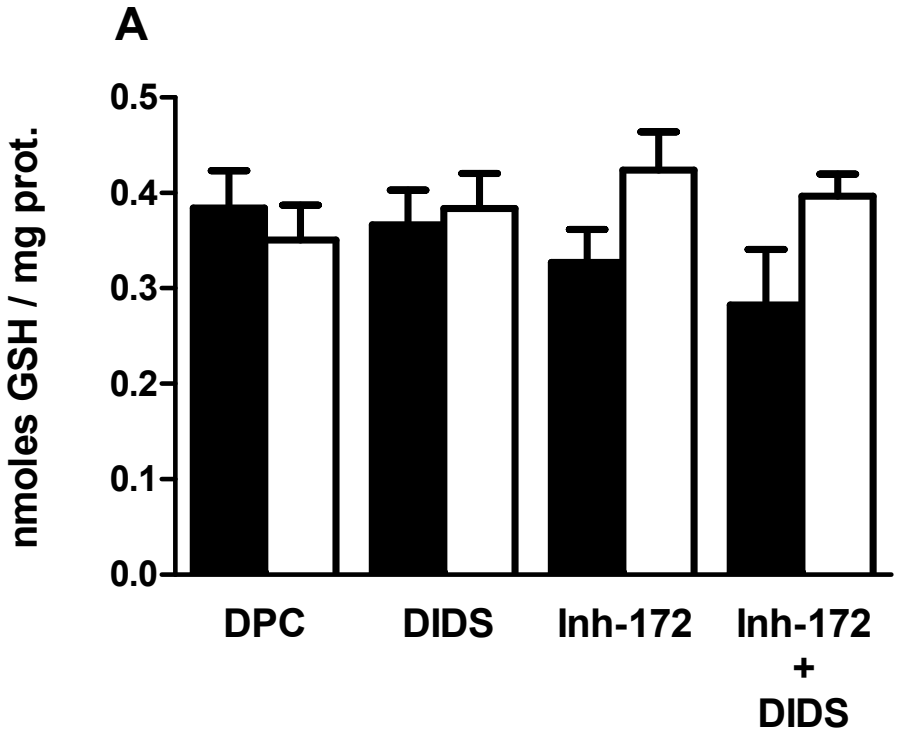
DIDS

Figure 3.3. Changes in extracellular GSH after addition of anion channel blockers in Calu-3 cells. Black bars represent vehicle controls while white bars represent treatment with anion channel blockers: DPC, general anion channel function inhibitor (n=6), DIDS, non-CFTR channel blocker (n=8), CFTR-inh-172 (Inh-172), CFTR-specific blocker (n=6), and combined DIDS + CFTR inh-172 (Inh-172) treatment (n=6). Panel A shows extracellular [GSH] from Calu-3 cells after addition of blockers. Panel B shows extracellular [GSH] after blocker and 4-HNE administration. Data are presented as mean extracellular [GSH] normalized by cellular protein concentration. Blocker treatments are compared with vehicle control cells. No significant changes were observed with any blocker treatment.


A
nmoles GSH / mg prot.
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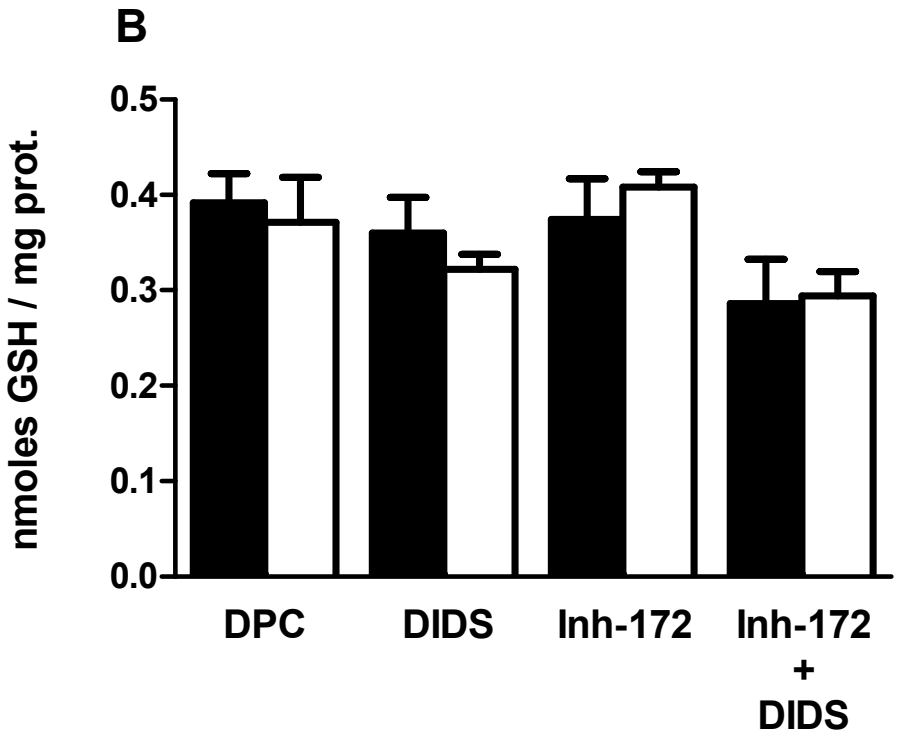
Inh-172
Inh-172 + DIDS


B
nmoles GSH / mg prot.
0.5
0.4
0.3
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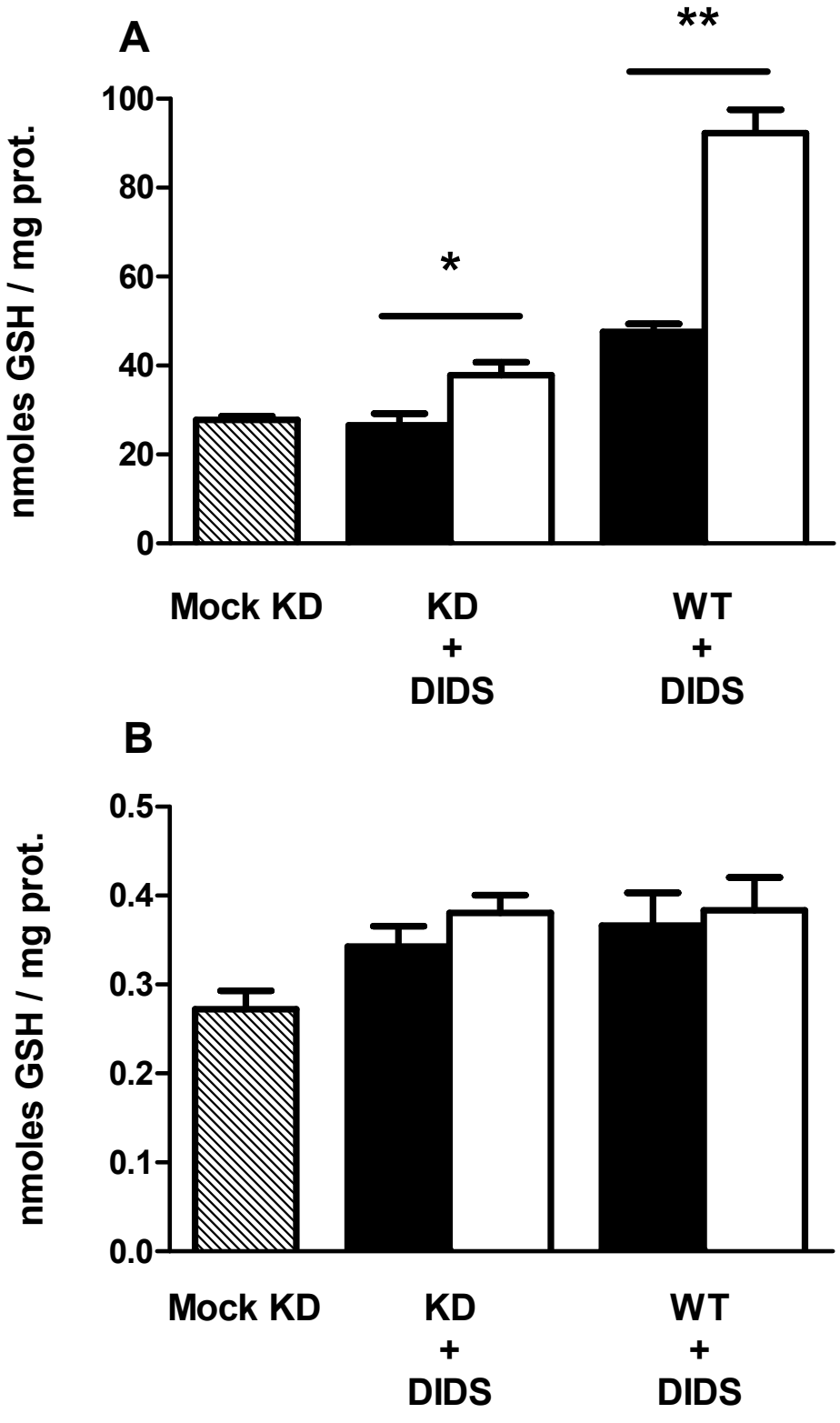
DPC
DIDS
Inh-172
Inh-172 + DIDS

3.1.5 Blocker treatment effects on intracellular and extracellular GSH in CFTR KD cells

The addition of the non-CFTR anion channel blocker DIDS to CFTR KD Calu-3 cells resulted in higher intracellular [GSH] (Figure 3.4A), similar to results obtained with administration of DIDS on unmodified Calu-3 cells (WT + DIDS, from Figure 3.2A). No changes were seen in extracellular [GSH], similar to results obtained with administration of DIDS on unmodified Calu-3 cells (WT + DIDS, from Figure 3.2B).

Figure 3.4. Increase of intracellular GSH in CFTR knockdown Calu-3 cells with DIDS-mediated anion channel blockade. Administration of DIDS to KD CFTR Calu-3 cells (KD, n=6) led to an increase in intracellular [GSH] (panel A), similar to that obtained with unmodified Calu-3 cells (WT, n=10). No significant changes in extracellular [GSH] were observed (panel B). The baseline [GSH] of KD cells was measured in cells expressing a non-reactive scrambled siRNA (Mock KD, hatched column, n=4). Black bars are vehicle control and white bars are treatment with DIDS. In panel A, data are presented as mean intracellular [GSH] normalized by cellular protein concentration. In panel B, data are presented as mean extracellular [GSH] normalized by cellular protein concentration. Inhibitor treated cells are compared with vehicle control cells: *, $p < 0.05$; **, $p < 0.01$.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WT + DIDS
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0.1
0.0
nmoles GSH / mg prot.
Mock KD
KD + DIDS
WT + DIDS

3.2 Results from Part 2: Role of MRP1

3.2.1 Cell viability

MK-571 treatment had no detectable cytotoxic effect on Calu-3 cells and the viability was found to be 108.31%, compared to control condition (100%). Vehicles used for the experiments also had no detectable effect on Calu-3 cells: EtOH and DMSO gave cell viabilities of 99.74% and 92.37%, respectively. The treatment with 4-HNE (94% of control) did not have a major impact on cell viability. No changes were observed with the other anion channel blockers as well: DPC: 91.16% (n=4); DIDS: 106.94% (n=4); inh-172: 88.17% (n=4). Changes over 20% would have been considered significant.

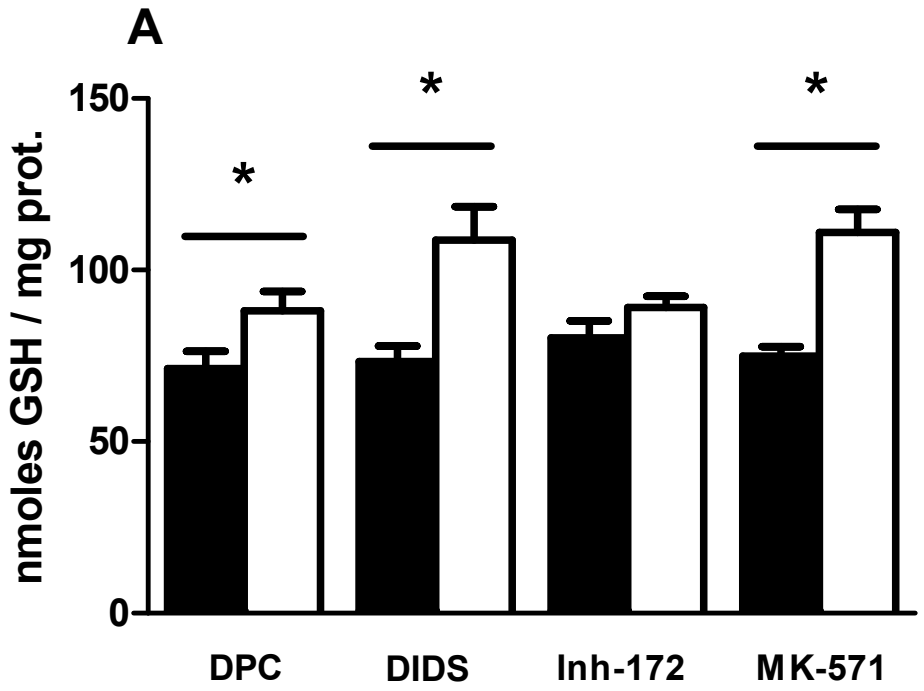
3.2.2 Effect of channel blockers on intracellular GSH levels

Administration of the anion channel blockers DPC and DIDS increased intracellular [GSH] compared to control (DPC: 88.11 ± 5.68 nM GSH/mg protein, $p<0.05$, n=7; DIDS: 108.70 ± 9.72 nM GSH/mg protein, $p<0.01$, n=5). No significant change was observed with CFTR channel blocker inh-172 (89.01 ± 3.30 nM GSH/mg protein, $p<0.18$, n=5; control: 80.16 ± 5.08 nM GSH/mg protein, n=5). Treatment with the MRP1 channel blocker MK-571 was associated with a substantial increase of intracellular [GSH] (110.50 ± 6.79 nM GSH/mg protein $p<0.001$, n=10) compared to control (Figure 3.5A).

The administration of 4-HNE (figure 3.5B) did not change this pattern (DIDS: 118.70 ± 5.90 nM GSH/mg protein, $p<0.05$, n=4; inh-172: 96.72 ± 5.66 nM GSH/mg protein, $p<0.24$, n=4; MK-571: 106.82 ± 6.48 nM GSH/mg protein, $p<0.04$, n=4), except

for DPC, which failed to change intracellular [GSH] (82.27 ± 8.06 nM GSH/mg protein, $p<0.66$, n=5; control: 77.65 ± 6.25 nM GSH/mg protein, n=5).

Figure 3.5. Increase in intracellular GSH after addition of certain anion channel blockers. Calu-3 cells were pre-treated with different blockers for 24 h, during 0.5% FBS serum deprivation, and subsequently for 6 h after administration of the lipid peroxidation product 4-HNE. Black bars represent vehicle controls while white bars represent treatment with anion channel blockers. Panel A shows the effect of blocker administration alone: 250 μM general anion channel function inhibitor DPC (n=6), 500 μM non-CFTR channel blocker DIDS (n=10), 2 μM CFTR-inh-172 (n=6), and 30 μM MK-571 treatment (n=6). Panel B shows [GSH] after administration of the oxidant 4-HNE. Data are presented as mean intracellular [GSH], normalized by cellular protein concentration. Blocker treatments are compared with vehicle control cells. *, $p < 0.05$.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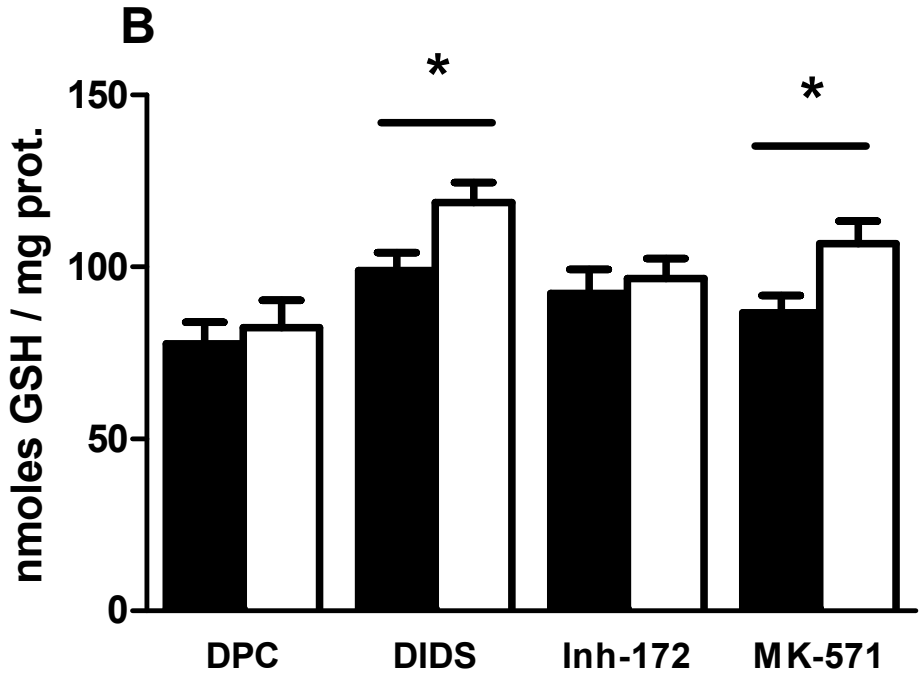
DPC
DIDS
Inh-172
MK-571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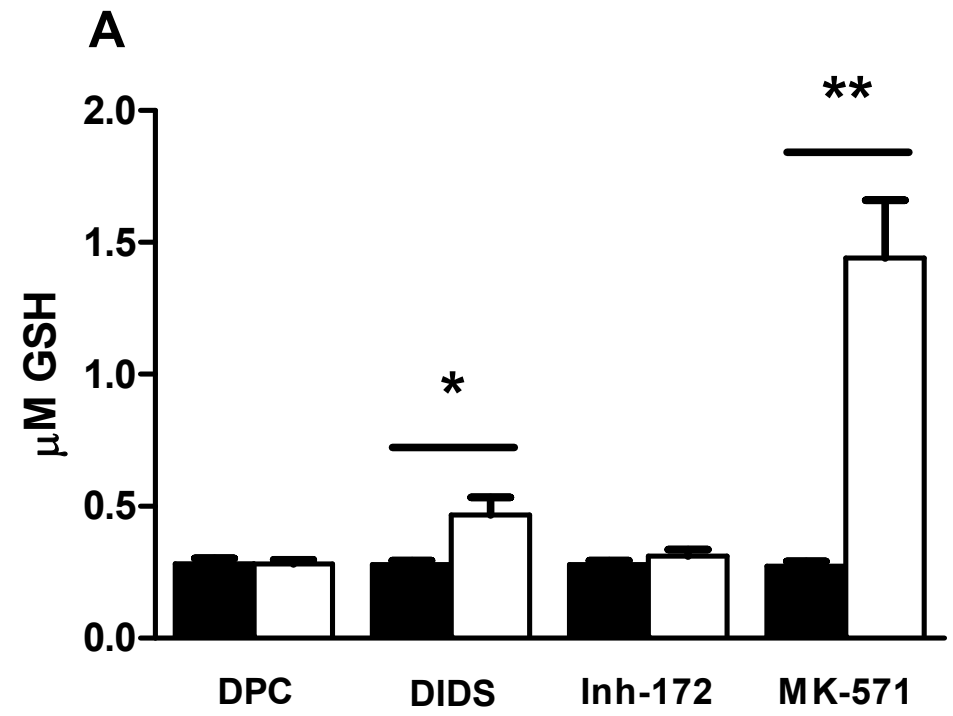
DPC
DIDS
Inh-172
MK-571

3.2.3 Effect of channel blockers on extracellular GSH levels

Administration of the anion channel blockers DPC and DIDS (figure 3.6A) caused no significant change in extracellular [GSH] (0.28 ± 0.02 μM GSH, $p<0.93$, n=4) compared to control cells (0.28 ± 0.02 μM GSH, n=4). Similarly, the CFTR specific channel blocker inh-172 failed to modify extracellular [GSH] (0.31 ± 0.03 μM GSH, $p<0.29$, n=4; control: 0.28 ± 0.02 μM GSH, n=6). In contrast, administration of the MRP1 blocker MK-571 led to a marked increase in extracellular [GSH] (1.44 ± 0.22 μM GSH, $p<0.001$, n=9; control: 0.27 ± 0.02 μM GSH, n=5). A more subdued increase in [GSH], compared to MK-571 treatment, was observed with use of non-CFTR anion channel inhibitor DIDS (0.46 ± 0.07 μM GSH, $p<0.02$, n=6; control: 0.28 ± 0.02 μM GSH, n=6).

Administration of 4-HNE caused no change in extracellular GSH (figure 3.6B) in cells treated with the general anion channel blocker DPC (0.32 ± 0.02 μM GSH, $p<0.07$, n=3) and the CFTR blocker inh-172 (0.37 ± 0.04 μM GSH, $p<0.15$, n=3) compared to control (control: 0.27 ± 0.02 μM GSH, n=5). In contrast, extracellular [GSH] increased in cells treated with non-CFTR anion channel inhibitor DIDS (0.71 ± 0.09 μM GSH, $p<0.01$, n=4) and MRP1 inhibitor MK-571 (1.42 ± 0.16 μM GSH, $p<0.001$, n=5).

Figure 3.6. Increase in extracellular GSH after addition of certain anion channel blockers. Calu-3 cells were pre-treated with different blockers for 24 h, during serum deprivation (0.5% FBS), and subsequently for 6 h after administration of the lipid peroxidation product 4-HNE. Panel A shows the effect of blocker administration alone: 250 μM general anion channel function inhibitor DPC (n=6), 500 μM non-CFTR channel blocker DIDS (n=10), 2 μM CFTR-inh-172 (n=6), and 30 μM MK-571 treatment (n=6).). Panel B shows [GSH] after administration of the oxidant 4-HNE. Data are presented as mean extracellular [GSH]. Blocker treatments are compared with vehicle control cells. *, $p < 0.05$; **, $p < 0.01$.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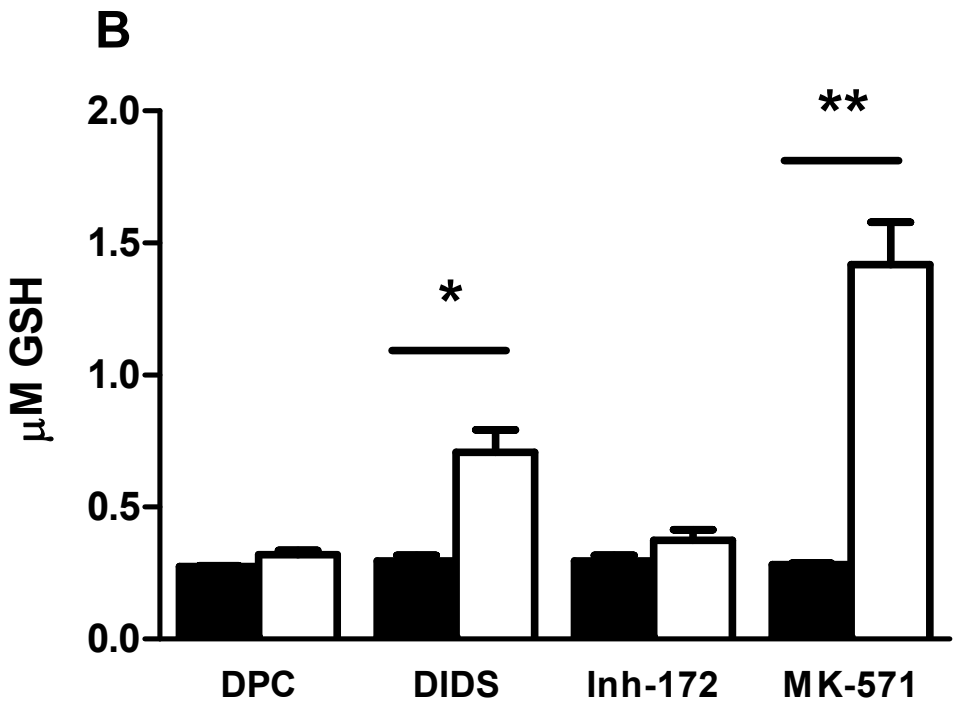
MK-571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Inh-172
MK-571

3.2.4 GCL activity assay

During 24h serum deprivation, MK-571-treated and non-treated control cells showed increased GCL activity at 18h, followed by a decrease at 24h which was more pronounced with MK-571 treatment (Table 3.1).

Table 3.1. GCL enzyme activity over a 24 hour time period. GCL activity values during 24hr blocker pre-treatment of Calu-3 cells (n=3-5). Values are normalized to cellular protein concentrations.

		Time (hours)					
		0.5	1	3	6	18	24
Activity (U/mg protein ± SEM)	**Vehicle Control**	0.077 ± 0.001	0.104 ± 0.013	0.089 ± 0.012	0.073 ± 0.009	0.198 ± 0.012 $p < 0.05$ *	0.106 ± 0.22
	MK-571	0.072 ± 0.001	0.082 ± 0.001	0.078 ± 0.012	0.077 ± 0.012	0.162 ± 0.014 $p < 0.05$ *	0.046 ± 0.007 $p < 0.05$ +

+: vs 24h vehicle control.

*: vs 6hr identical treatment

3.2.5 Effect of GGT function blockade on GSH

GGT is responsible for the breakdown outside the cell of GSH in its various forms (reduced, oxidized and adducts). We used to acivicin to inhibit GGT in order to determine if the enzyme had any effect on either intra- or extra-cellular [GSH] in cells treated with anion channel blockers, including MK-571.

Acivicin administration did not alter intracellular [GSH] (figure 3.7A) (69.68 ± 4.20 nM GSH/mg protein, p=0.81; control: 71.31 ± 5.03 nM GSH/mg protein; n=7). In contrast, extracellular [GSH] was higher than control cells (0.65 ± 0.09 μM GSH, $p<0.01$; control: 0.28 ± 0.02 μM GSH; n=7) (figure 3.7B). There was no difference in the effects of acivicin in the presence of anion channel blockers (figure 3.8) compared to experiments without acivicin treatment (figures 3.5A and 3.6A).

Administration of acivicin caused no change in intracellular [GSH] (figure 3.8A) in cells treated with the CFTR blocker inh-172 (89.87 ± 8.322 nM GSH/mg protein, p= 0.48, n=5) compared to vehicle control (82.35 ± 5.58 nM GSH/mg protein, n=5). Intracellular [GSH] increased in cells treated with the general anion channel blocker DPC (95.65 ± 8.03 nM GSH/mg protein, $p<0.01$; control: 69.68 ± 4.20 nM GSH/mg protein; n=7), the non-CFTR anion channel inhibitor DIDS (108.10 ± 12.70 nM GSH/mg protein, $p<0.05$; control: 74.46 ± 5.16 nM GSH/mg protein; n=5) and MRP1 inhibitor MK-571 (106.40 ± 8.30 nM GSH/mg protein, $p<0.01$; control: 73.90 ± 3.33 nM GSH/mg protein; n=9).

The addition of acivicin also did not influence extracellular GSH (figure 3.8B) in cells treated with the general anion channel blocker DPC (0.83 ± 0.18 μM GSH, p=0.32;

control: 0.50 ± 0.04 μM GSH; n=4) and the CFTR blocker inh-172 (1.26 ± 0.14 μM GSH, p=0.17; control: 0.96 ± 0.13 μM GSH; n=4) compared to their respective controls. Extracellular [GSH] increased in cells treated with the non-CFTR anion channel inhibitor DIDS (1.34 ± 0.23 μM GSH, $p<0.05$; control: 0.77 ± 0.12 μM GSH; n=6) and the MRP1 inhibitor MK-571 (5.00 ± 0.76 μM GSH, $p<0.001$; control: 0.67 ± 0.07 μM GSH; n=9).

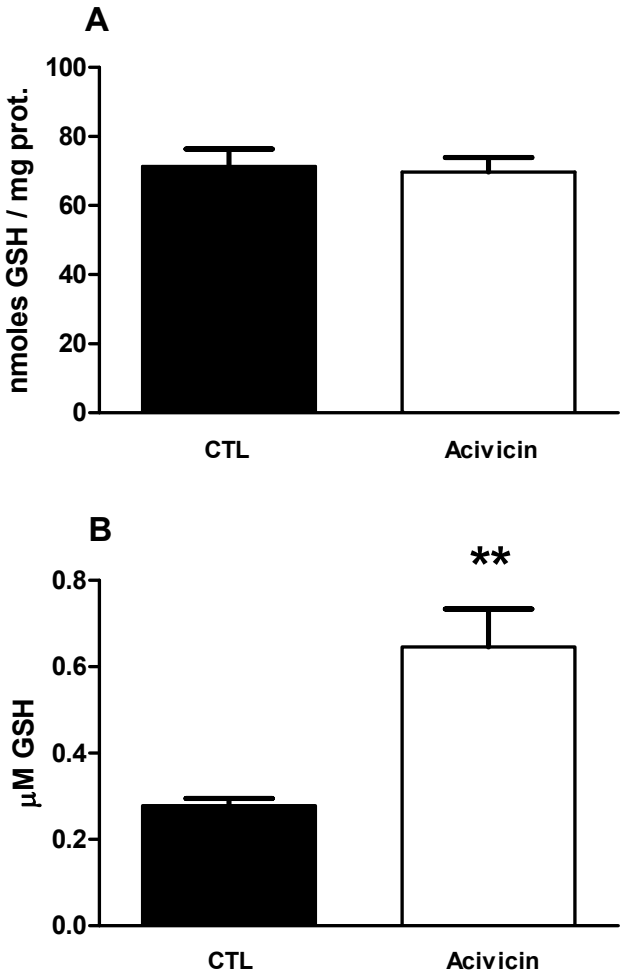
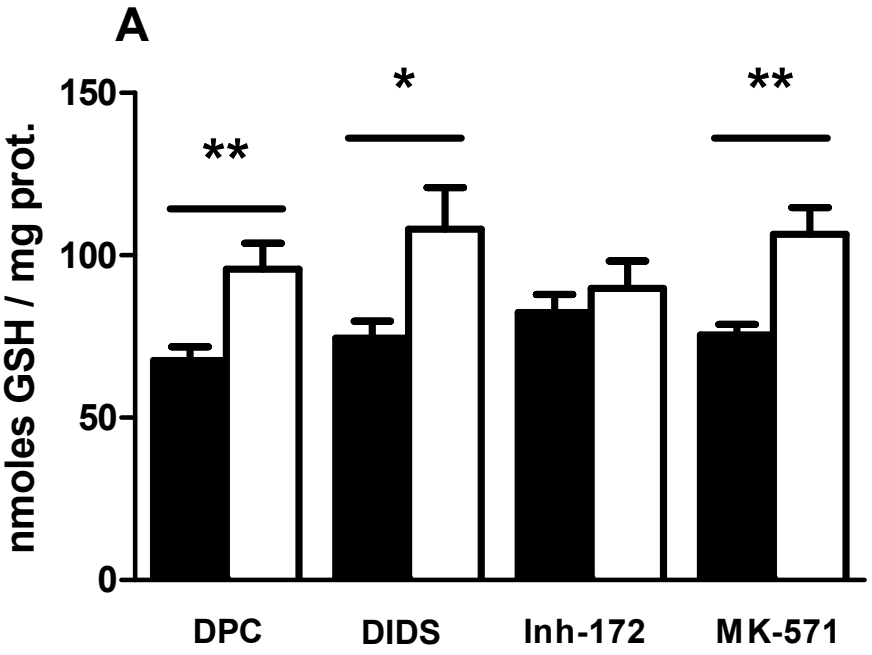


Figure 3.7. Acivicin treatment increases extracellular [GSH] without changing intracellular [GSH]. Calu-3 cells were serum deprived (0.5% FBS) for 24 h and subsequently cultured for 6 h with or without the GGT enzyme inhibitor acivicin (100 mM). In panel A, intracellular [GSH] was unchanged after inhibitor administration (n=7). In panel B extracellular [GSH] increased after administration of acivicin (n=7). Data are presented as mean [GSH]. Acivicin treatment compared with vehicle control cells. **, $p < 0.01$.

Figure 3.8. No change in intra- and extra-cellular GSH with anion channel blockers after GGT blockade. Calu-3 cells were pre-treated with different blockers for 24 h under serum deprived conditions (0.5% FBS). Cells were then cultured 6h in the presence of acivicin (100 mM) and an anion channel blocker. Result are shown for the general anion channel function inhibitor DPC (250 μM, n=4), the non-CFTR channel blocker DIDS (500 μM ,n=5), the CFTR-inh-172 (2 μM, n=4), and MK-571 (30 μM, n=8). Despite the administration of acivicin, results are similar to those in figures 3.5A and 3.6A. Data are presented as mean [GSH]. Blocker treatments are compared with vehicle control cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.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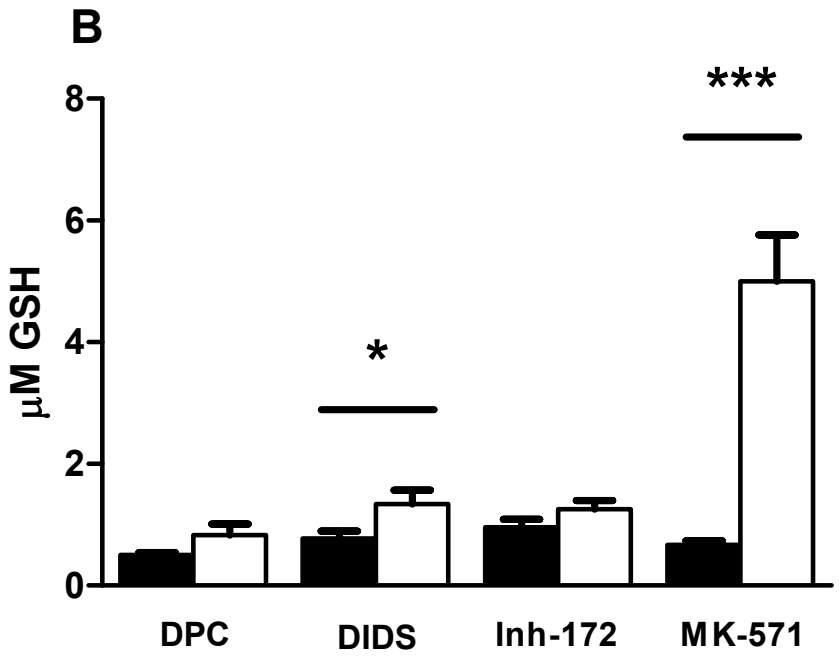
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3.2.6 Effect of MK-571 on CFTR channel activity

Iodide efflux measurements were used to determine CFTR channel activity and iodide values are expressed as the difference between control conditions and activator-administered cells in the presence of different blockers (figure 3.9). As expected in Calu-3 cells, CFTR was functionally active under baseline conditions (maximum efflux of 31.56 μM at 2 min). Increased activity was also observed in the presence of MRP1 channel blocker MK-571 (iodide efflux peak of 40.27 μM at 3 min). Significantly increased efflux was observed with MK-571 treatment after peak efflux (points at 4, 5, and 6 min, $p<0.05$ vs. control).

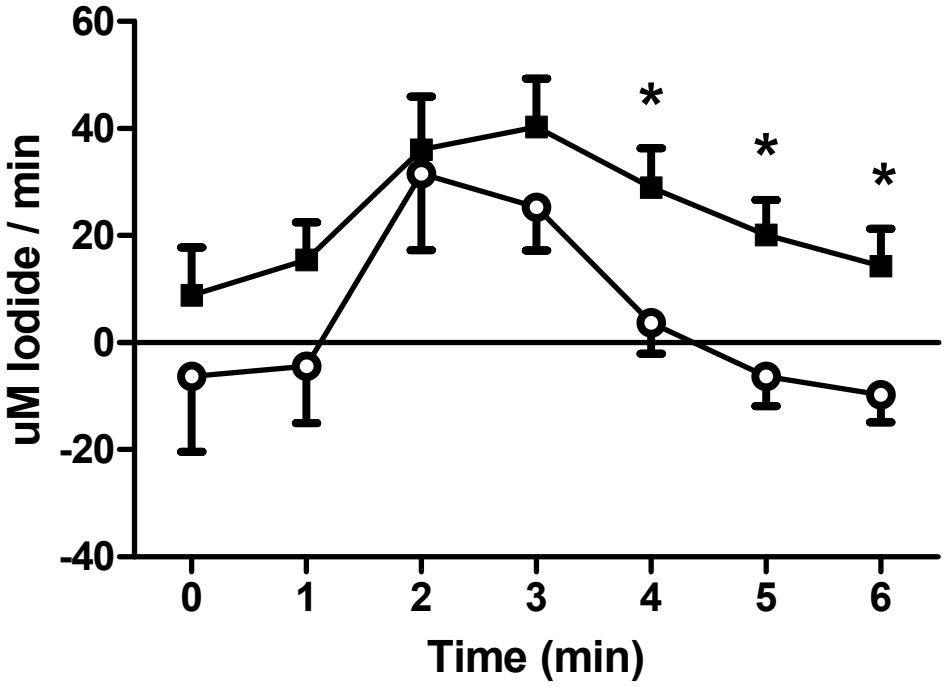


Figure 3.9. Anion channel activity present with MK-571 treatment. Iodide efflux assay on Calu-3 cells: vehicle control cells (white circles) and treatment with 30 μM MRP1 channel blocker MK-571 (black squares). Cell were pre-treated with either vehicle control or blocker for 24 h before analysis by iodide efflux (n = 4). Data are presented as the average of cAMP-stimulated iodide efflux of collected samples every minute starting from t=0 (baseline). Stimulation started at t=0 and maintained for the duration of the assay. MK-571 treatment is compared with vehicle control cells for each time point. *, $p < 0.05$.

3.2.7 Effect of CFTR blockade on GSH in MK-571-treated cells

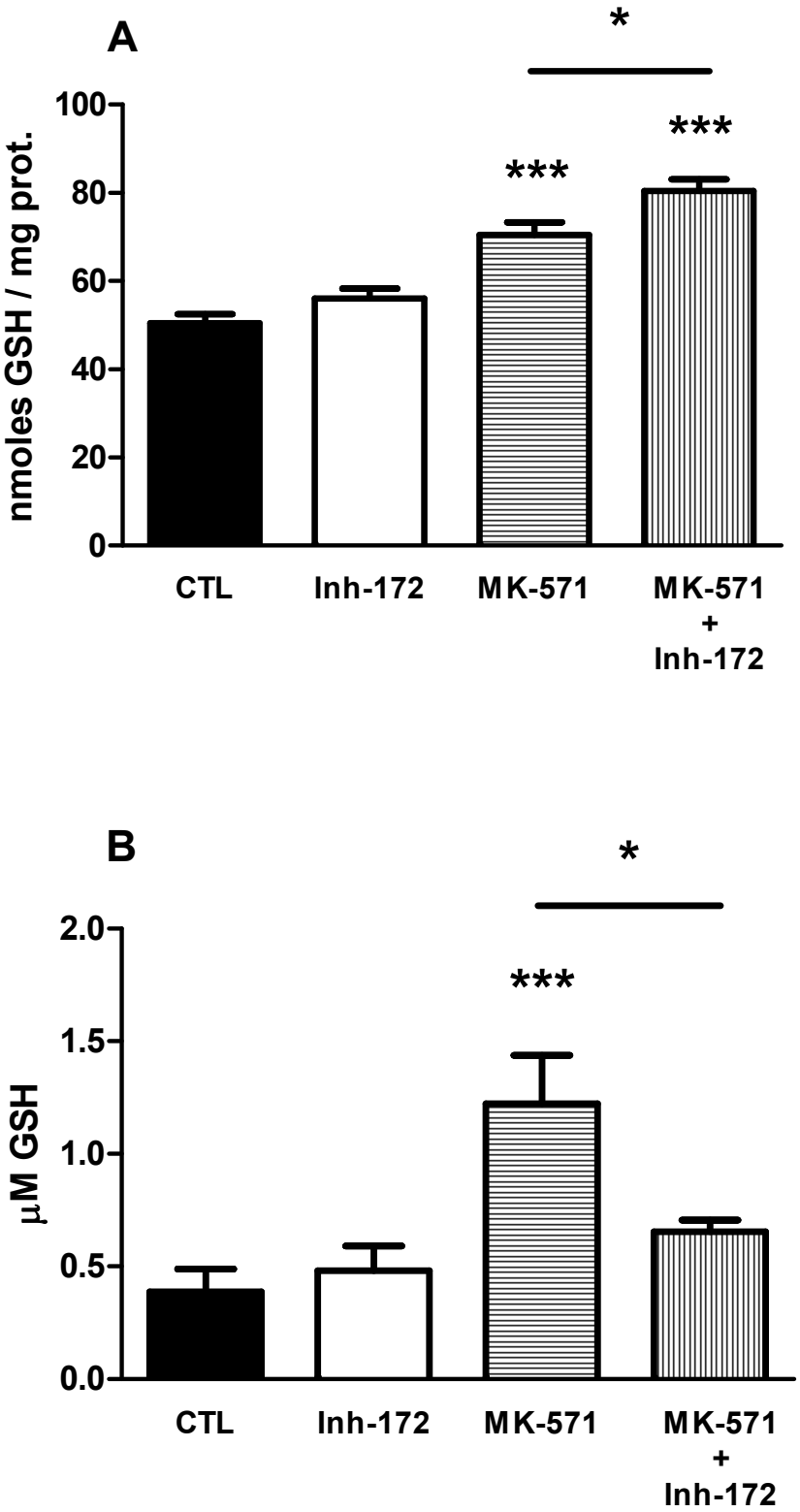
We investigated whether CFTR blockade would alter intracellular [GSH] in MK-571 treated cells (figure 3.10A). Administration of both inh-172 and MK-571 resulted in increased extracellular [GSH] (80.48 ± 2.54 nM GSH/mg protein, $p<0.001$, n=8), as did MK-571 treatment alone (70.46 ± 2.86 nM GSH/mg protein, $p<0.001$, n=6), compared to vehicle control (50.48 ± 2.04 nM GSH/mg protein, n=4). In addition, combined blocker treatment obtained higher [GSH] than MK-571 treatment alone (80.48 ± 2.54 vs. 70.46 ± 2.86, $p<0.05$).

We then studied whether CFTR blockade altered extracellular [GSH] in MK-571 treated cells (figure 3.10B). The administration of both MK-571 and inh-172 resulted in a decrease of extracellular [GSH] (0.65 ± 0.05 μM GSH n=8) compared with MK-571 treatment alone (1.22 ± 0.22 μM GSH, $p<0.035$, n=10). Although the result was higher than vehicle control (0.39 ± 0.10 μM GSH, $p<0.001$, n=7), it was the same when compared to [GSH] obtained from CFTR blockade alone (0.48 ± 0.11 μM GSH, $p<0.16$, n=7).

Since inhibition of GGT can affect [GSH], mainly through the increase of extracellular [GSH] (figure 3.7), we examined whether this would alter the outcomes observed with combined inh-172 and MK-571 treatment (figure 3.10). In figure 3.11A, Administration of both inh-172 and MK-571 with acivicin resulted in an increase of extracellular [GSH] (80.66 ± 6.30 nM GSH/mg protein, n=2), compared to vehicle control (65.17 ± 4.77 nM GSH/mg protein, n=2), and MK-571 treatment alone did provide an increase as well (106.40 ± 8.30 nM GSH/mg protein, $p<0.001$; control: 73.90 ± 3.33 nM GSH/mg protein; n=8).

We then studied whether the addition of acivicin altered extracellular [GSH] in MK-571 treated and CFTR blocked cells (figure 3.11B). The administration of acivicin on cells treated with both MK-571 and inh-172 resulted in a decrease of extracellular [GSH] (1.145 ± 0.1650 μM GSH, n=2) compared with MK-571 treatment alone (5.00 ± 0.76 μM GSH, n=8), which is similar to what was seen without acivicin treatment (Figure 3.10B).

Figure 3.10. Changes in GSH with combined MK-571 and inh-172 treatment. Calu-3 cells were pre-treated with different blockers for 24 h, during serum deprivation (0.5% FBS). Cells were then cultured for 6h in the presence of an anion channel blocker. Results are shown for CFTR-inh-172 (2 μM, n=4-7), MRP1 channel blocker MK-571 (30 μM, n=5-10) and combined MK-571+inh-172 treatment (n=8). No significant change found between extracellular [GSH] between inh-172 treatment and combined MK-571+inh-172 treatment. Data are presented as either mean intracellular GSH amounts (Panel A), normalized by cellular protein concentration, or mean extracellular [GSH] (Panel B), normalized to volume. Blocker treatments are compared with vehicle control cells (n=4), unless otherwise stated. *, $p < 0.05$. ***, $p < 0.001$. Black bars represent vehicle control and black bars represent blocker treatment.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CTL
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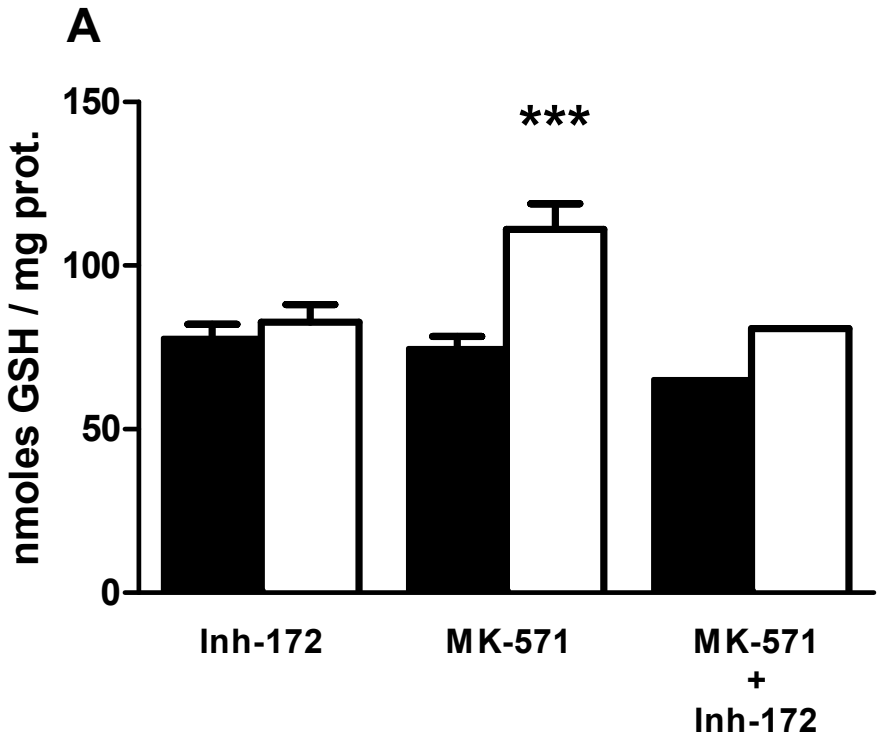
MK-571
MK-571
+
Inh-172

Figure 3.11. GGT blockade and changes in GSH obtained with combined MK-571 and inh-172 treatment. Calu-3 cells were pre-treated with different blockers for 24 h, during serum deprivation (0.5% FBS). Cells were then cultured for 6h in the presence of acivicin (100 mM) and an anion channel blocker. Results are shown for CFTR-inh-172 (2 μM, n=4), the MRP1 channel blocker MK-571 (30 μM, n=8) and a combination of MK-571 and inh-172 (n=2). Despite the administration of acivicin, results are similar to those in figure 3.10. Data are presented as either mean intracellular [GSH] (Panel A), normalized by cellular protein concentration, or mean extracellular [GSH] (Panel B), normalized to volume. ***, $p<0.001$. Black bars represent vehicle control and black bars represent blocker treatment. No statistics conducted with combined MK-571+inh-172 treatment due to low experiment number ($n < 3$).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Inh-172
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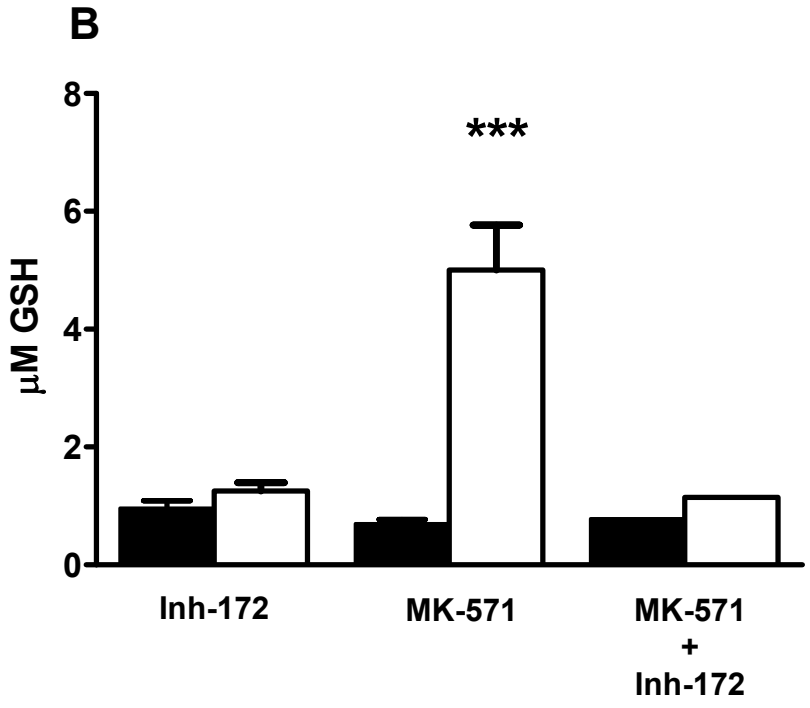
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3.2.8 Effect of MK-571 treatment on GSH levels in CFTR-knockdown cells

Treatment of CFTR-knockdown Calu-3 cells with MK-571 caused no significant change in intracellular [GSH] (21.11 ± 0.66 nM GSH/mg protein, $p<0.21$, n=8) compared to control cells (19.70 ± 0.65, n=4), unlike the higher intracellular [GSH] observed with treatment of wild-type Calu-3 cells (figure 3.12A).

When examining extracellular [GSH], we again observed no change with MK-571 treatment (0.45 ± 0.04 μM GSH, $p<0.72$, n=5), compared to control cells (0.47 ± 0.03 μM GSH, n=7) (figure 3.12B). This was unlike the increases in [GSH] observed with MK-571 treatment of wild-type Calu-3 cells.

3.2.9 MRP1 and MRP4 expression in Calu-3 cells

MRP1 protein was detectable on Western blots of intracellular proteins from Calu-3 cells (figure 3.13) and MRP4 expression was not detectable. A549 cells were used as positive control for both MRPs, since they are known to express both proteins [266].

3.2.10 Role of leukotrienes on intracellular and extracellular GSH levels

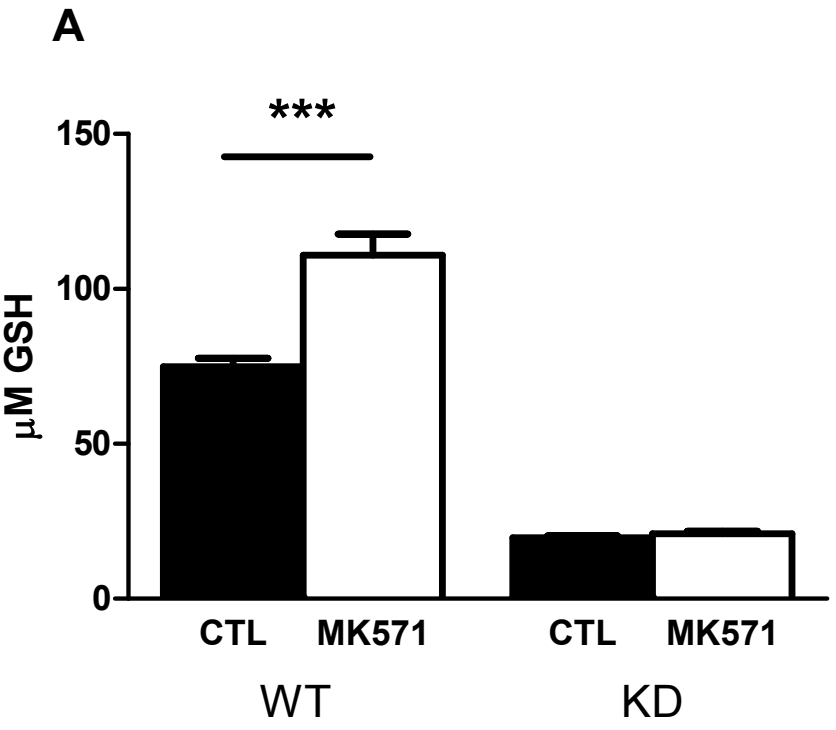
In figure 3.14A, Montelukast administration led to increased intracellular [GSH] (65.94 ± 1.06 nM GSH/mg protein, $p<0.001$, n=4) similar to results obtained with MK-571 (70.46 ± 2.86, $p<0.001$, n=6), both compared to control cells (51.28 ± 1.16, n=8).

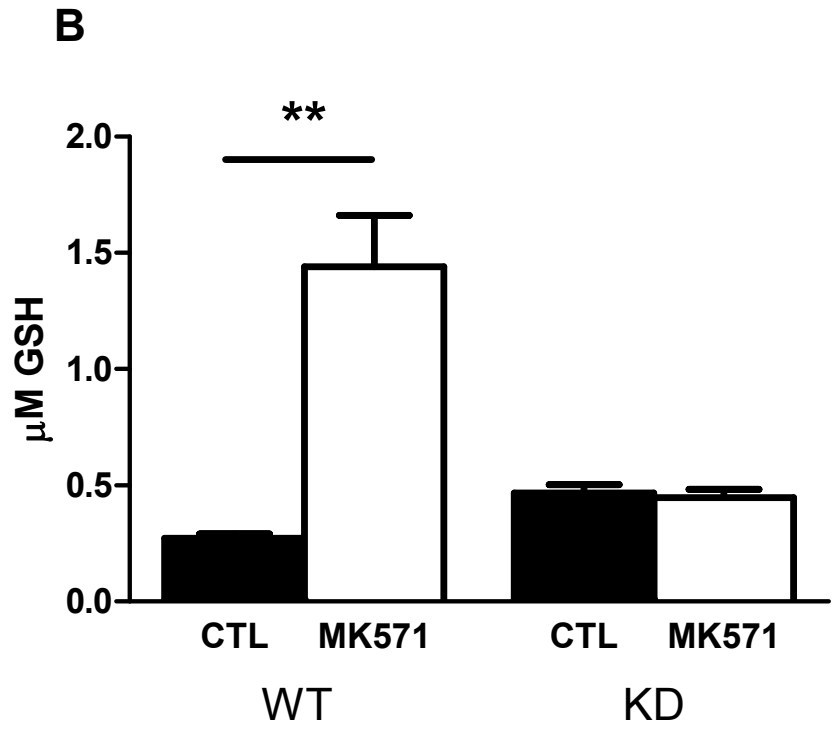
We then administered leukotriene (LT) synthesis inhibitors MK-886 and Zileuton and found no change to intracellular [GSH] (MK-886: 74.34 ± 1.40 nM GSH/mg protein, $p<0.14$ n=3; Zileuton: 71.60 ± 1.82 nM GSH/mg, $p<0.29$, n=4) compared to vehicle control (67.34 ± 3.22 nM GSH/mg, n=4). This contrasts with MK-571 treatment which

still caused higher [GSH] (103.78 ± 7.86 nM GSH/mg, $p<0.005$, n=4), even in combination with the LT synthesis inhibitors (MK-571+MK-886: 111.24 ± 4.88 nM GSH/mg protein, $p<0.0015$, n=4; MK-571+zileuton: 100.40 ± 5.66 nM GSH/mg protein, $p<0.003$, n=4), compared to control (figure 3.14B).

Extracellular [GSH] also did not change with the administration of the LT synthesis inhibitors (MK-886: 0.38 ± 0.01 μM GSH, $p<0.83$ n=4; Zileuton: 0.38 ± 0.01, $p<0.65$, n=4), compared to control (0.38 ± 0.01 μM GSH, n=4). The increases in [GSH] following MK-571 administration (0.97 ± 0.16 μM GSH, $p<0.011$, n=4) persisted with the addition of the LT synthesis inhibitors (MK-571+MK-886: 0.81 ± 0.10 μM GSH, $p<0.01$, n=4; MK-571+zileuton: 0.95 ± 0.21 μM GSH, $p<0.034$, n=4) compared to control (figure 3.14C).

Figure 3.12. MK-571 does not change GSH in CFTR-knockdown cells. CFTR-KD Calu-3 cells were pre-treated with different blockers for 24 h, under serum deprivation conditions. Following medium change, cells were cultured for an additional 6 h in the presence of channel blockers. Black bars represent vehicle controls while white bars represent treatment with 30 μM MK-571 treatment. KD indicates CFTR-knockdown cells while WT indicates unmodified Calu-3 cells. Panel A: intracellular GSH amounts (n=8) after MK-571 administration. Panel B: extracellular [GSH] (n=5) after MK-571 administration. Data are presented as either mean intracellular GSH amounts (Panel A), normalized by cellular protein concentration, or mean extracellular [GSH] (Panel B). MK-571 treatment is compared with vehicle control: **, $p < 0.01$; ***, $p < 0.001$. No significant changes between treatment and control in KD cells were found.


A
***
150
100
50
0
μM GSH
CTL
MK571
CTL
MK571
WT
KD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**
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1.5
1.0
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0.0
μM GSH
CTL
MK571
CTL
MK571
WT
KD

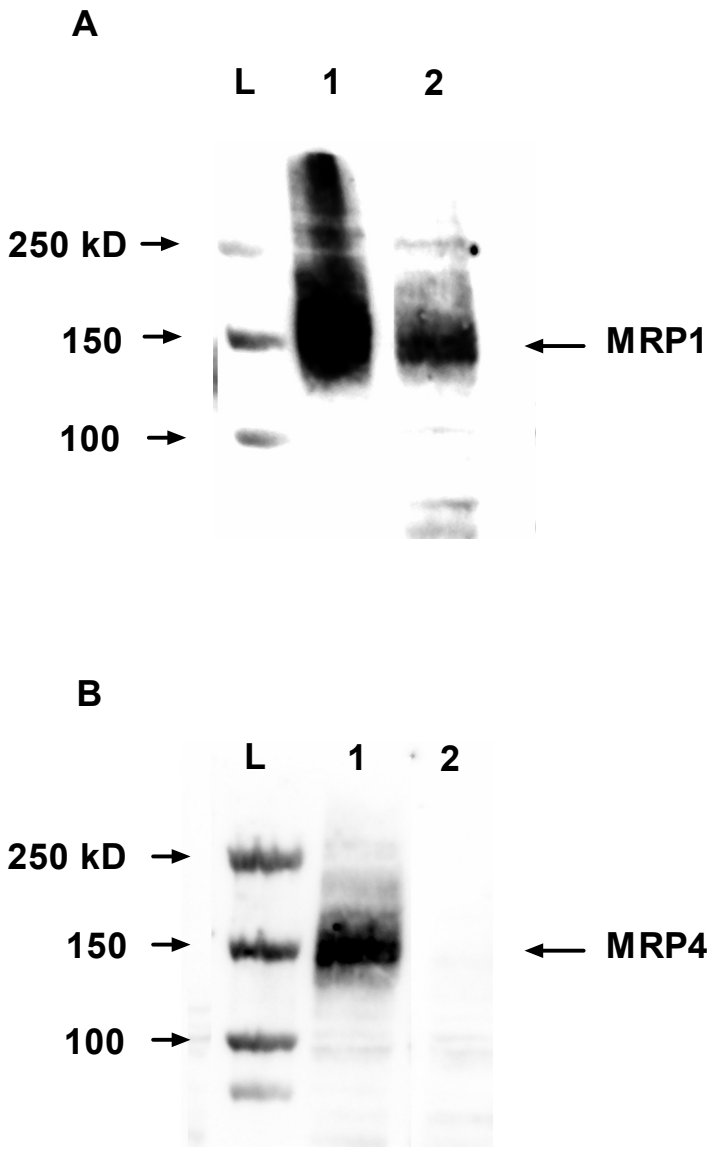
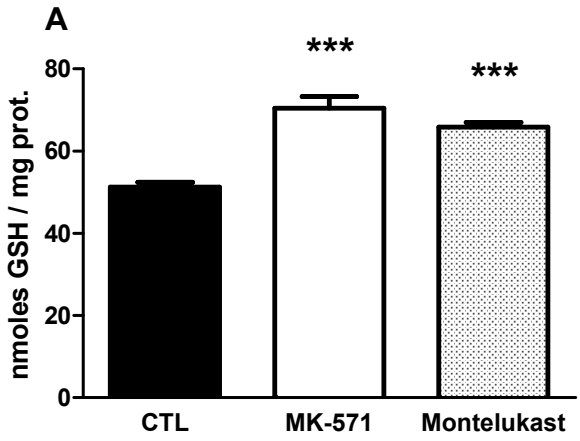


Figure 3.13. Expression of MRP1 but not MRP4 in Calu-3 cells. Whole-cell expression for MRP proteins was determined in Calu-3 cells. Lane L is for the protein ladder, Lane 1 is for A549 cell lysate, used as a positive control for MRPs, and Lane 2 is for the Calu-3 cell lysate. In panel A we find, as expected, expression of MRP1 in Calu-3 cells (lane 2). In panel B we find no evidence of whole-cell expression of the MRP4 protein (lane 2) in Calu-3 cells.

Figure 3.14. Leukotriene (LT) inhibition does not modify GSH levels. Calu-3 cells were treated with compounds then incubated for 24 h under serum deprivation conditions. Following medium change, cellus were cultured for for 6 h in the presence of LT inhibitors or controls. Panel A) Intracellular GSH. The black bar represents vehicle control (n=8), the white bar represents MK-571 treatment (30 μM, n=4) and the gray bar represents Montelukast treatment (15 μM, n=4). Panel B) Intracellular GSH (n=4 unless otherwise stated). vehicle control, 30 μM MK-571, 200 nM MK-886 (n=3), 30 μM Zileuton, combined MK-571+MK886 treatment and combined MK-571+Zieluton treatment. Panel C) Extracellular [GSH] after respective treatments (n=4), same order as for intracellular GSH. Data are presented as either mean intracellular GSH amounts, normalized by cellular protein concentration, or mean extracellular [GSH]. Treatments are compared with vehicle control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.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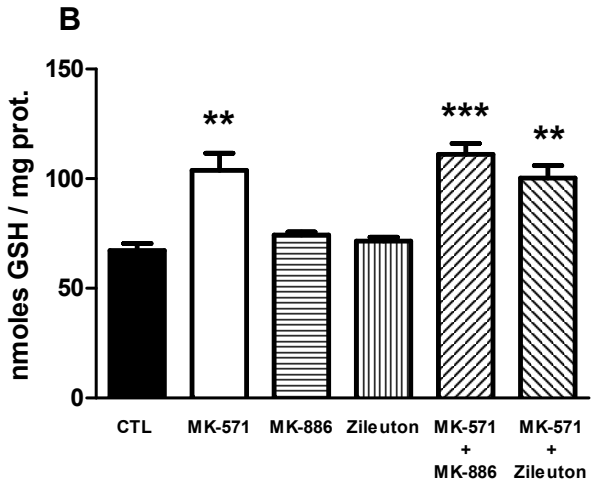
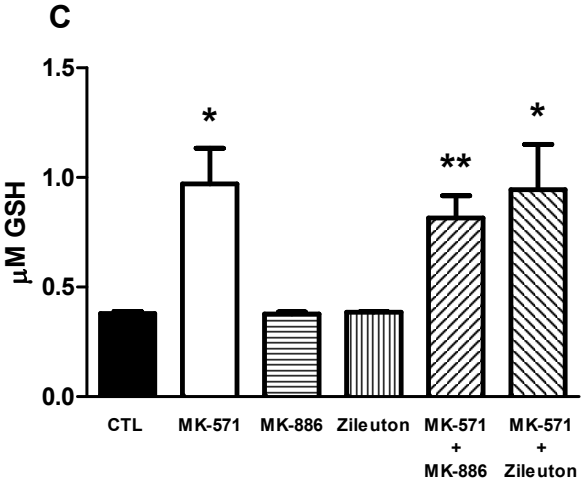
nmoles GSH / mg prot.
80
60
40
20
0
***
***
CTL
MK-571
Montelukast


B
nmoles GSH / mg prot.
150
100
50
0
**
***
**
CTL
MK-571
MK-886
Zileuton
MK-571 + MK-886
MK-571 + Zileuton

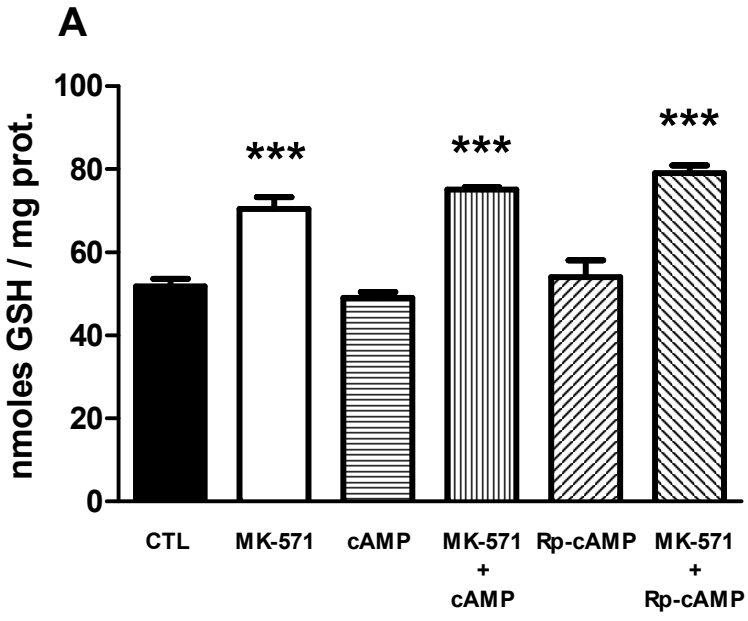

C
µM GSH
1.5
1.0
0.5
0.0
*
**
*
CTL
MK-571
MK-886
Zileuton
MK-571 + MK-886
MK-571 + Zileuton

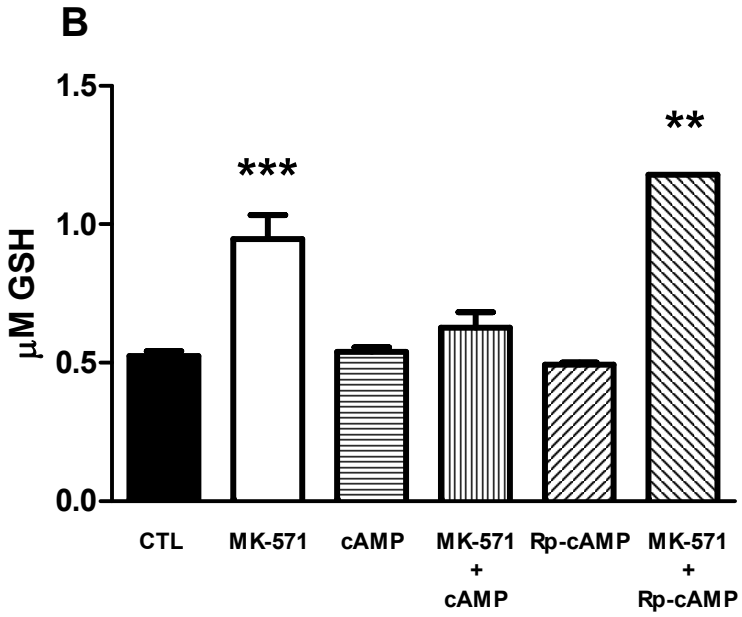
3.2.11 Role of cAMP in mediating changes in GSH

In figure 3.15A, we examined the effect of 8-bromo-cAMP treatment, which can activate CFTR function via PKA activation, alone or in conjunction with MK-571. As MK-571 is an inhibitor of MRP- based cAMP transport it may lead to a localized increase in CFTR function [159]. The addition of 8-bromo-cAMP yielded similar intracellular [GSH] to that seen with vehicle control alone (control: 51.83 ± 1.781 nM GSH/mg protein, n=6; cAMP: 49.03 ± 1.425 nM GSH/mg protein, $p<0.35$, n=3) in the presence of MK-571 (MK-571: 70.46 ± 2.851 nM GSH/mg protein, $p<0.001$, n=6; cAMP+MK-571: 75.14 ± 0.5401 nM GSH/mg protein, $p<0.001$. n=3). When we used Rp-8-bromo-cAMP, a specific inhibitor of cAMP-dependent protein kinase (PKA) activation, we again found no change in [GSH], compared to control (Rp-cAMP: 54.08 ± 3.973 nM GSH/mg protein, $p<0.56$, n=3), or in the presence of MK-571 (Rp-cAMP+MK-571: 79.01 ± 1.964 nM GSH/mg protein, $p<0.001$, n=3).

With regard to extracellular [GSH] (figure 3.15B), we found that administration of 8-bromo-cAMP or Rp-8-bromo-cAMP [GSH] was unchanged (cAMP: 0.54 ± 0.02 μM GSH, $p<0.60$, n=3; Rp-cAMP: 0.49 ± 0.01 μM GSH, $p<0.26$, n=3) compared to control (0.53 ± 0.02 μM GSH, n=6). In contrast, administration of MK-571 continued to increase GSH (0.95 ± 0.09 μM GSH, $p<0.0008$, n=6) except when combined with 8-bromo-cAMP (0.63 ± 0.06 μM GSH, $p<0.22$, n=3; compared to cAMP alone).

Figure 3.15. Effect of administration 8-bromo-cAMP and its antagonist on [GSH]. Calu-3 cells were treated with for 24 h, during 0.5% FBS serum deprivation. Following a change in medium, cells were then cultured for 6 h in the presence of a variety of agents, as follows. Panel A) Intracellular [GSH] after respective treatments: vehicle control (n=6), 30 μM MK-571 (n=6), 250 μM 8-bromo-cAMP (cAMP in graph) (n=3), combined MK-571+8-bromo-cAMP treatment (n=3), 250 μM Rp-8-bromo-cAMP (Rp-cAMP in graph) (n=3), and combined MK-571+Rp-8-bromo-cAMP treatment. Panel B) Extracellular GSH after respective treatments, same order as for intracellular GSH. Data are presented as either mean intracellular GSH amounts, normalized by cellular protein concentration, or mean extracellular [GSH]. Treatments are compared with vehicle control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.


A
nmoles GSH / mg prot.
100
80
60
40
20
0
***
***
***
CTL
MK-571
cAMP
MK-571 + cAMP
Rp-cAMP
MK-571 + Rp-cAMP


B
µM GSH
1.5
1.0
0.5
0.0
***
**
CTL
MK-571
cAMP
MK-571 + cAMP
Rp-cAMP
MK-571 + Rp-cAMP

3.3 Results from Part 3: Effect of MK-571 treatment on CFTR

3.3.1 WT and ΔF508 CFTR cell surface expression after MK-571 and Montelukast treatment

Treatment with MK-571 or Montelukast led to an increase in WT CFTR expression (figures 3.16) at 1h after administration, with results varying from approx. 10% to approx. 30%. At 24h after treatment, we see expression still increased at lower concentration and no change or drop in expression at higher concentrations. The most effective concentrations for increased expression were 30 μM for Montelukast at 1h, 100 nM for MK-571 at 1h, 1 pM for both compounds at 24h. The most pronounced drop in cell surface expression (5.75%) was found with 30 μM MK-571 treatment after 24h.

Treatment with either MK-571 or Montelukast resulted in an increase in cell surface expression of WT and ΔF508 CFTR in BHK cells, which persisted 24h after administration of the compounds. MK-571 treatment in three of four experimental conditions, 100 nM significantly increased cell surface expression. Similarly, montelukast at 1 pM was the most effective concentration.

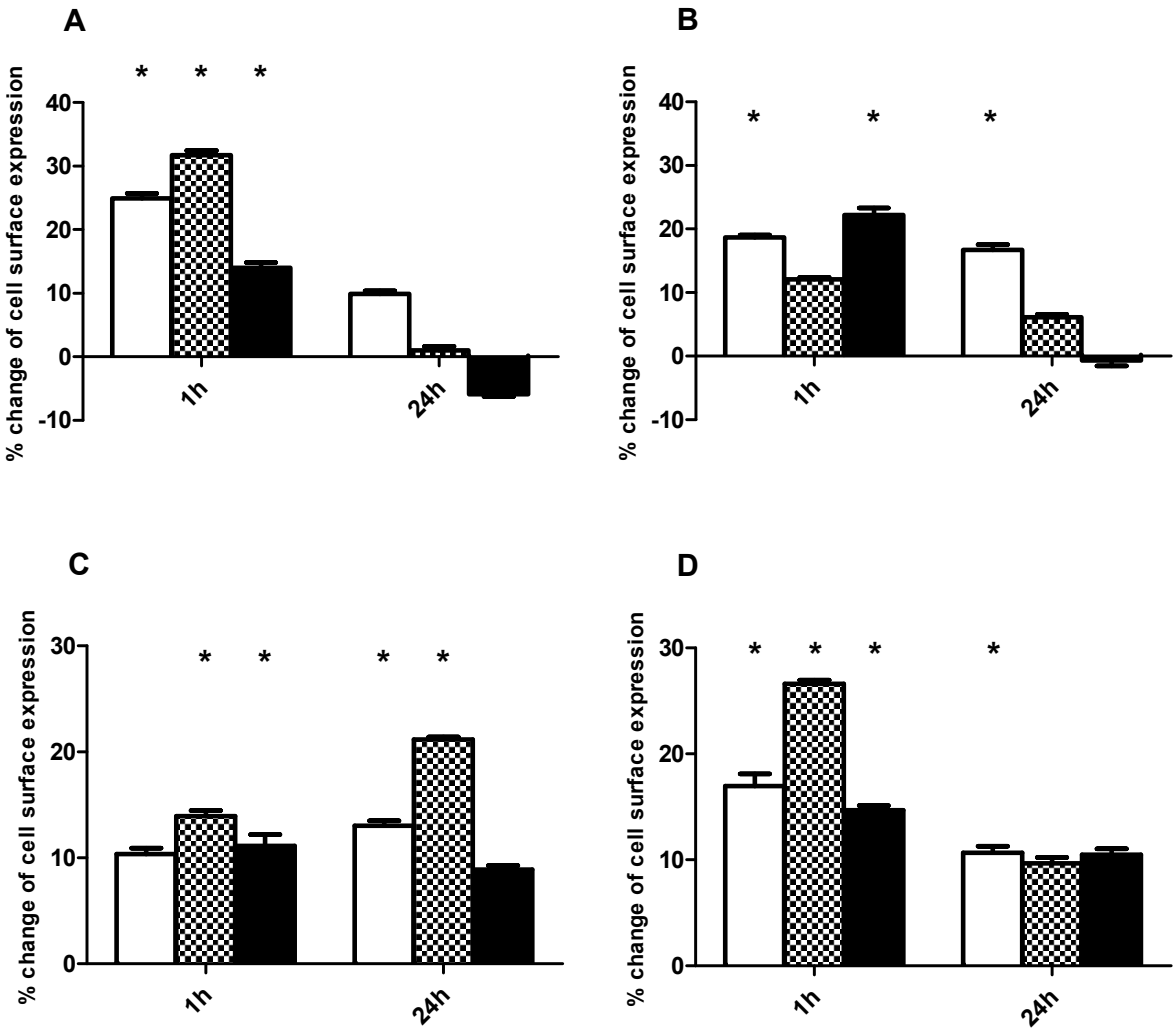


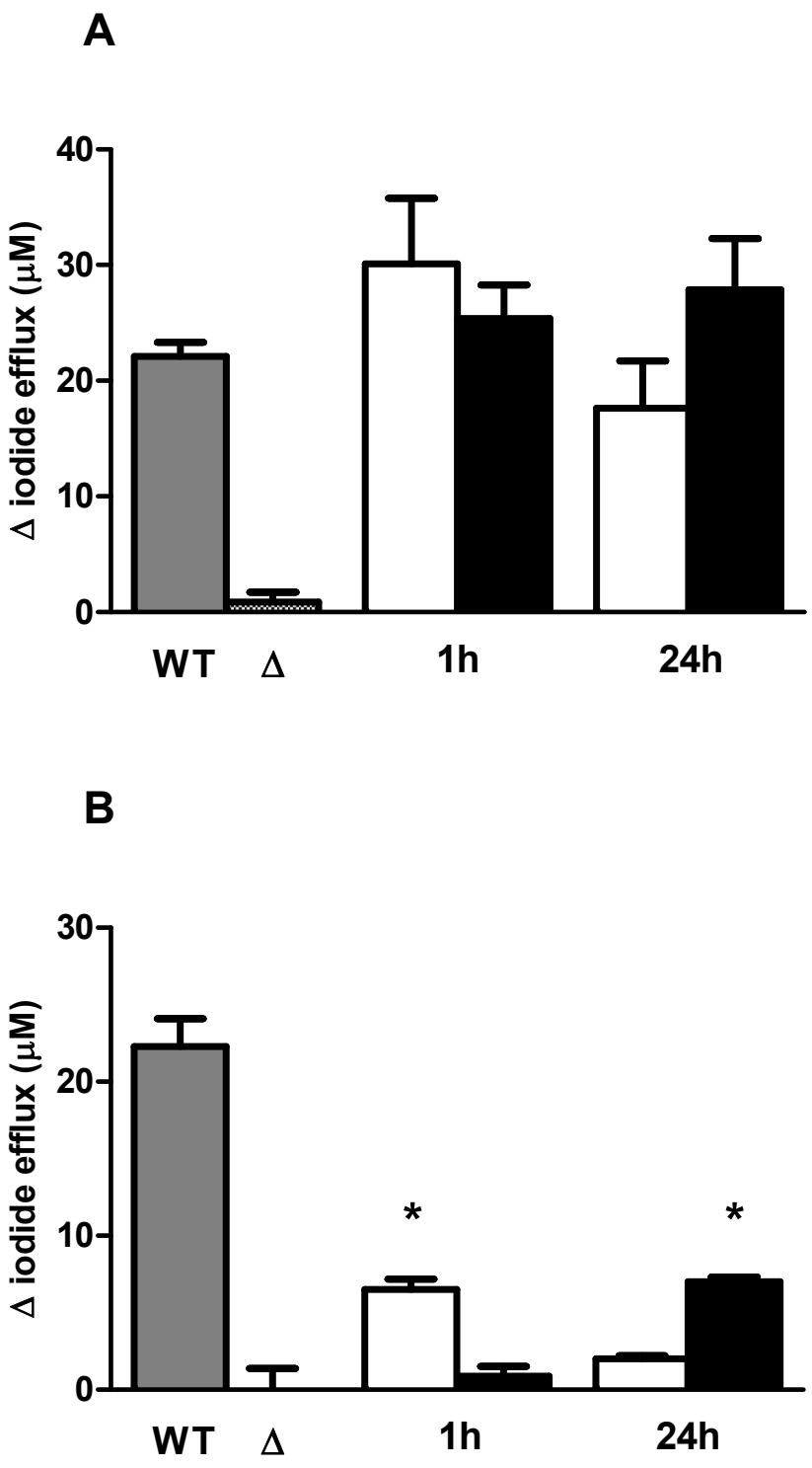
Figure 3.16. Changes in wild-type and ΔF508 CFTR cell surface expression with administration of MK-571 and Montelukast. Expression of either wild type CFTR (panels A and B) or ΔF508 CFTR (panels C and D) on the cell surface, shown as a percent change in expression compared to non-treated controls, examined at 1 h or 24 h post-administration of different concentrations of MK-571 (panels A and C) and Montelukast (panels B and D): White bars represent 1 pM, chequered bars represent 100 nM, and black bars represent 30 μM (n=10). Any change over 11.5 % in cell surface expression is considered significant (*, $p < 0.05$).

3.3.2 Chloride channel function in WT- and ΔF508 CFTR cells after MK-571 and Montelukast treatment

In WT cells, CFTR channel function was the same or increased with either MK-571 or Montelukast, 1h after administration (figure 3.17A). At 24h post-administration, we observed a dose-response effect in which an increase in compound concentration caused a decrease in efflux, with activity at lower concentrations equal or higher to control efflux.

Efflux was detected in ΔF508 CFTR-expressing BHK cells (figure 3.17B) with MK-571 or Montelukast treatment and persisted up to 24h, while control cells showed none. At 1h, we observed a dose-response effect with either MK-571 or Montelukast treatment such that higher efflux occurred with increasing concentrations. This dose-response effect reverses itself at 24h, where the lowest concentrations of compounds are associated with the highest efflux.

Figure 3.17. Changes in anion channel activity from wild-type and ΔF508 CFTR-expressing BHK cells after administration of MK-571 or Montelukast. Iodide efflux assay on BHK cells treated either with 100 nM MK-571 (white bars) or with 1 pM Montelukast (black bars). Panel A represents efflux measurements from wild-type CFTR-expressing cells. Panel B represents efflux measurements from ΔF508 CFTR-expressing cells. Effluxes from non-treated wild-type cells (WT) and ΔF508 CFTR-expressing cells (Δ) are shown along with positive and negative controls respectively. Cells were treated with blockers and then 24h later analysis by iodide efflux was done (n = 4). Data are presented as an average of cAMP-stimulated peak iodide efflux. In panel A, treatments are compared to WT control; in panel B, treatments are compared to Δ control. *, $p < 0.05$.


A
40
30
20
10
0
Δ iodide efflux (μM)
WT
Δ
1h
24h
B
30
20
10
0
Δ iodide efflux (μM)
*
*
WT
Δ
1h
24h

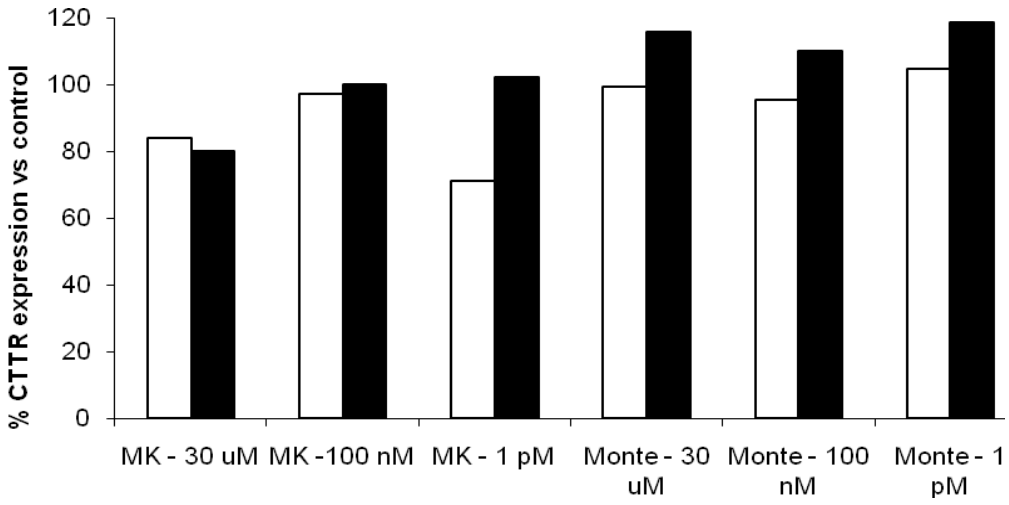
3.3.3 Whole cell CFTR expression after MK-571 and Montelukast treatment

For WT CFTR expression, treatments with either compound caused no change in expression with the exception of treatment with 30 μM MK-571, which indicated a drop of 20% in expression, at either 1h or 24h, and a 30% drop at 1h with 1 pM of MK-571 (figure 3.18A). Expression was increased by 10% for all concentrations (30 μM, 100 nM, and 1 pM) of Montelukast used, at 24h.

Whole cell expression of ΔF508 CFTR did not change with Montelukast treatment (figure 3.18B). The same was found with MK-571 treatment at 1h. Contrary to this, MK-571 showed a dose-response effect with ΔF508 expression, higher expression with lower concentration, at 24h post-treatment.

Figure 3.18. Preliminary study of whole-cell expression of CFTR in BHK cells after administration of either MK-571 or Montelukast. White bars represent data from 1 h after initial treatment of either MK-571 or Montelukast, black bars represent data collected at 24 after administation of the compounds. Panel A shows expression data from wild-type CFTR-expressing cells. Panel B shows expression from ΔF508 CFTR-expressing cells. Expression is shown as a percent of expression versus vehicle control cells. Values obtained from normalized densitometry data, obtained using Fluorchem 8000 equipment and software. n=2 for each time-point, no statistics done due to lack of sufficient number of experiments ($n < 3$).

A


% CTTR expression vs control
120
100
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60
40
20
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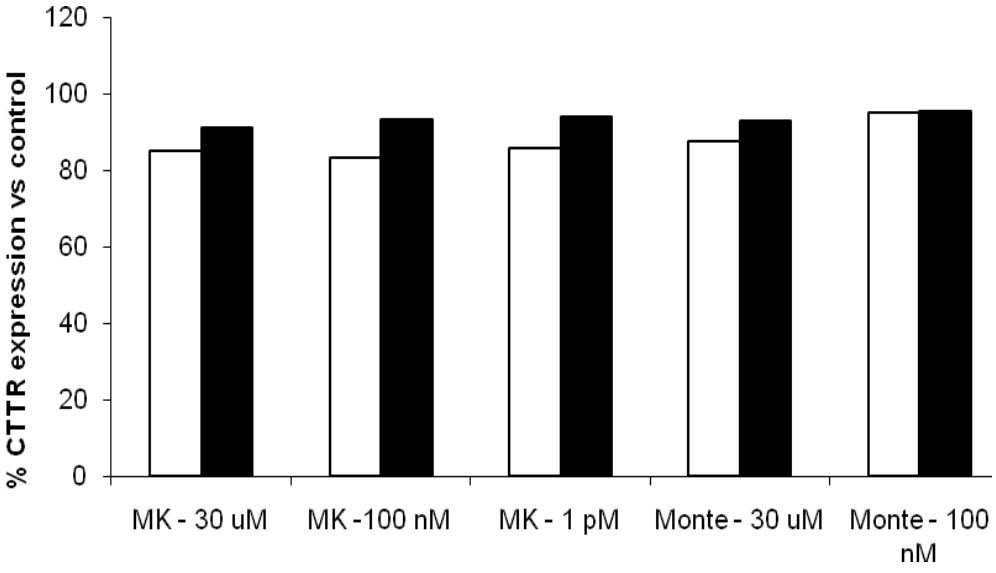
MK - 30 uM
MK -100 nM
MK - 1 pM
Monte - 30 uM
Monte - 100 nM
Monte - 1 pM

B


% CTTR expression vs control
120
100
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0
MK - 30 uM
MK -100 nM
MK - 1 pM
Monte - 30 uM
Monte - 100 nM

Chapter 4: Discussion

4.1 Introductory Remarks

The goal of this thesis was two-fold: to understand the role of CFTR in determining [GSH] using a relevant cellular model, in this case Calu-3, and to understand as well what role MRP1, a structural and functional homologue of CFTR, had in determining [GSH]. Much has been published in regard to GSH being a substrate of CFTR [11] and *in vitro* experiments, such as those by Bear *et al.,* showing the ability of the channel to transport GSH [10], with the addition of *in vivo* work showing a link between CF and diminished extracellular GSH [91]. It has been suggested that there is a direct link between CFTR dysfunction and higher intracellular GSH coupled with lower extracellular [GSH]. Hudson proposed the hypothesis, based on the review of literature and the results of others, by which the lack of functional CFTR leads to decreases in extracellular GSH and to maintenance of intracellular GSH [267]. This was proposed to explain why extracellular GSH was low in CF patients or airway epithelia [91, 217] and why CF cell lines were observed to be more resistant to apoptosis [268, 269], since decreased intracellular GSH is required for the apoptotic system to be engaged [198, 201, 228, 229, 270-272]. We set out to examine experimentally how levels of the antioxidant are changed, at the cellular level, with or without functional CFTR. Since oxidant stress can play a major role in CF pathogenesis [219], we wanted to study if the cells were more susceptible to stress with lack of CFTR function which would lead, within the context of the protein being the main GSH transport pathway, to diminished extracellular GSH.

The results for the first part of this thesis indicated that CFTR is not a determinant of GSH in Calu-3 cells, whether under oxidant stress conditions or not. However, inhibition of other anion channels did increase intracellular [GSH], suggesting an alternate mechanism for regulating cellular [GSH]. We therefore examined, in the second part of this thesis, the possible role of MRP1 in determining GSH handling in epithelial cells and the results showed that MRP1 does play a role in intracellular GSH handling. In the case of extracellular [GSH], the changes seen after administration of the MRP1 antagonist MK-571 were dependent on CFTR. How MK-571 affected CFTR activity was the focus of the third and final part of the study. The results suggested that both MK-571 and Montelukast can increase either wild-type or ΔF508 CFTR expression and function, although the differences between function and expression suggest a more complex relationship.

4.2 Discussion of the results

We first set out to understand if CFTR was determining intra- and extra-cellular GSH concentrations in Calu-3 cells, under oxidant stress conditions. CFTR was functional in Calu-3 epithelial cells under our experimental conditions (figure 3.1), an expected result based on previously published work [108, 273]. Oxidant stress can modify cellular proteins, altering their structures and/or functions [274-276], and can diminish CFTR expression [108]. We confirmed that in our model the CFTR channel was functional using both the iodide efflux assay and a pharmacological approach. CFTR inh-172, the CFTR channel blocker, and DPC, the general anion channel blocker known to

inhibit CFTR, diminished efflux to less than 10% of control efflux activity. It is noteworthy that DIDS, which does not inhibit CFTR-mediated anion transport unless it enters the cell, also diminished efflux activity, dropping to over 50% of control activity. This is likely the result of CFTR blockade, which is known to occur when DIDS enters the cell and acts on the cytoplasmic side of the channel. Although there was partial blockade of CFTR function with DIDS administration, the channel activity measured was well above the 20% to 30% reported to permit adequate functional activity [140, 277]. Within this context we considered the activity obtained as functional CFTR and DIDS as a non-CFTR inhibitor for the course of our studies.

The administration of anion channel blockers caused higher intracellular GSH concentrations (figure 3.2A), in line with a relationship between anionic channel function and GSH transport [151]. The exception to this increase was with the CFTR blocker inh-172; this was surprising considering previous work had demonstrated CFTR as a GSH transporter [10, 11, 278]. Furthermore, it has been hypothesised that lack of CFTR function would lead to increased intracellular GSH concentrations [267]. Our results suggest that CFTR function is not itself a major determinant of GSH. To extend these observations, we used a combination of DIDS, the non-CFTR anion channel blocker, with CFTR-inh-172 and we obtained similar results to DIDS administration alone, which we interpreted as further evidence that CFTR does not on its own determine intracellular GSH. Use of 4-HNE as a source of oxidant stress did not cause any deviation from the pattern seen previously (figure 3.2B), indicating that changes in intracellular GSH are independent of oxidant stress, or at least of 4-HNE treatment. These novel observations seem to contradict the hypothesis that CFTR is a major determinant of GSH [10, 11, 91],

irrespective of oxidant stress. Of course these observations were made in one cell model *in vitro* and the lack of CFTR involvement in GSH determination cannot be generalized till these observations are confirmed is other cell models as well as primary cells. The same can be said for the lack of effect of oxidant stress, although a relevant stress was used; increased lipid peroxidation products have been reported to be increased in CF airways [279].

Administration of the anion channel blockers had no effect on extracellular [GSH] (figure 3.3A), even in combination with oxidant stress (Figure 3B), and no change was found with extracellular GSH concentrations after administration of CFTRinh-172, the CFTR blocker. This was unexpected since a drop of extracellular [GSH] due to CFTR blockade was predicted, based on the reported association between low GSH, detected in the epithelial lining fluid of human subjects and CF mice, and lack of CFTR function [91]. This finding suggests the possibility of another channel, which compensates for the lack of CFTR activity.

It is also possible that early changes in extracellular [GSH] are being missed by measuring at a single time-point. A time course examining [GSH] changes from the onset of addition of fresh medium would allow us to determine if there are differences in the rate of GSH replenishment in the medium, with or without CFTR channel blockade, which would further explain if CFTR plays a role in determining extracellular [GSH] amounts. Furthermore, this approach would help in the study of GGT as well, since changes in GSH amounts could be then compared to enzyme activity to determine if a correlation existed. Under our current experimental conditions and within the limitations

of our experimental model, our observations suggest that CFTR is not a major determinant of intra- or extra-cellular [GSH].

To ensure that the changes in [GSH] were not due to CFTR involvement, we utilized another experimental system, CFTR-knockdown Calu-3 cells. This model provided us with Calu-3 cells lacking functional CFTR, allowing us to confirm that CFTR function was not a major determinant of observed [GSH] changes, using the same experimental conditions. In addition, the cell model mimicked the lack of CFTR function seen in CF. We observed that use of the non-CFTR anion channel blocker DIDS, on the CFTR knock-down cells had the a similar effect of increasing intracellular [GSH] as seen with wild-type cells (figure 3.4). This confirmed that changes in [GSH] were CFTR-independent. A concern is that that under our experimental conditions, the baseline intracellular [GSH] found in the transformed Calu-3 cells (either with mock or CFTR siRNA) was lower than that of wild-type cells (figures 3.4 and 3.12). It is important to note that transformed cells, such as the CFTR KD Calu-3 cells, may not behave in the same way as wild-type cells. Although the lower intracellular [GSH] in these cells did not have an effect on the outcome of our studies, it is important to be aware of this difference, which could account for the discrepancy in baseline intracellular [GSH].

At first glance, these results suggest that CFTR does not play a major role in GSH transport contradicting the idea of CFTR as the major GSH transporter. Given the importance of GSH as an antioxidant, we set out to study the role of CFTR under oxidant stress conditions but found that even under control conditions its functional presence in Calu-3 cells had no major impact on [GSH]. One potential explanation is CFTR can be made to favour either GSH transport or chloride transport [10]. Bear and colleagues

demonstrated that use of an non-hydrolyzable analogue of ATP, called AMP-PNP, caused CFTR to shift from chloride transport to GSH transport [10]. This effect was linked to the binding of nucleotides within the NBDs. where hydrolysis of ATP was not a requirement for the transport of GSH by CFTR, due to use of the non-hydrolyzable analogue of ATP. An explanation of the mechanism was put forward where binding of nucleotides within the NBDs led to possible conformational changes favouring GSH trafficking, instead of chloride, through the CFTR pore. Although this work gave invaluable insight into the working of CFTR, it was performed on inside-out membrane vesicles, not a whole cell model, a limitation of the model which can exclude variables such as possible protein-protein interactions. Exploring this possibility of differential nucleotide binding was beyond the scope of our experiments and further study would be required to determine if this were the case, using AML-PNP and then determining the effect on [GSH].

It is important to note that the non-CFTR involvement in [GSH] changes we report are thus far only confined to one *in vitro* model, the Calu-3 cell line. Other airway epithelial cell types would need to be subjected to the same experimental conditions to be able to determine if these observations can be generalized. Example of other models would be Normal Human Bronchial Epithelial cells (NHBE) [280, 281] and CF Bronchial Epithelial cells (CFBE) [265, 282, 283].

There is also a growing body of evidence suggesting that the presence of CFTR in the cell membrane can influence other channels and interact with them. This has been most clearly shown with ENaC [79-81], where the lack of CFTR expression brings an increase in function of the sodium channel. It is important to note that Calu-3 cells, one of the models used in this thesis, do not express ENaC [132]. This limitation of the Calu-3

cell model does not have any bearing in this thesis given that no reports have been found to date linking ENaC function and GSH transport. Therefore it is still conceivable that other channels could have an impact on GSH regulation. There is also evidence that CFTR-independent chloride function can facilitate GSH transport. This is observed in the work of Gao *et al*., which demonstrated that insertion of an artificial chloride channel in airway cells of CF mice was able to rectify GSH transport and restore airway GSH to non-diseased levels [151]. Since the changes in GSH we observed are occurring without CFTR involvement, this would indicate that another mechanism was playing a major role in GSH regulation. Elevated intracellular [GSH] observed with administration of general anion channel blockers (DPC, DIDS) suggests a role for other anionic channel(s), with or without oxidant challenge. A more pronounced [GSH] change is observed with DIDS, a blocker of chloride transport, which could give an indication that a channel-based mechanism may be responsible. To further reinforce the role of a channel-based mechanism, a DIDS-sensitive non-CFTR chloride channel in airway epithelia has been reported, located on the basolateral side [284]. Within this context and with the observations that GSH transport has been linked to non-CFTR chloride transport, this would suggest a role for a channel-based mechanism; blockade of this channel would then result in an increase in intracellular [GSH]. The literature is replete with examples of other channels involved in GSH regulation, such as the MRP channel family or the organic anionic transporter proteins (OATPs) [9]. One particular study demonstrated that MRP1, not CFTR, was the determinant of GSH in rat brain astrocytes [36]. The authors did not counter the observations that CFTR can transport GSH, but did suggest that CFTR could be involved in regulating GSH without actually transporting it. Within this context,

we sought to determine which other anionic channels could account for the GSH differences we've observed thus far.

MRP1 was chosen as the target for further study since it is functionally and structurally related to CFTR, although there is no evidence to date that MRP1 is DIDS-sensitive. MRP1 is generally located on the basolateral membrane, just as the DIDS-sensitive non-CFTR chloride channel (see above). If MRP1 played a role in determining GSH amounts, blockade of the channel would lead to increased intra-cellular GSH and a possible decrease in extra-cellular [GSH], results we initially expected to see with CFTR blockade. As shown in figure 3.5A, administration of anion channel blockers, with the exception of inh-172 administration, caused an increase of intracellular GSH, consistent with our previous observations. Use of MK-571, a known blocker of MRP1 [1, 2], also led to increases in [GSH], suggesting that MRP1 may be involved in GSH handling. Of note, administration of 4-HNE yielded results similar to those without 4-HNE (figure 3.5B), suggesting that the effects of anion channel blockers on GSH are independent of oxidant stress.

There are previous reports to the effect that MK-571 inhibition of MRP1 could cause increased intracellular [GSH] and decreased extracellular [GSH] [33, 36, 270, 285], presumably by preventing the transport of GSH out of the cell. The concentrations used in those studies (15 to 30 μM) were in the same range as we used (30 μM), therefore we would expect a similar outcome and as expected we do detect an increase in intracellular [GSH].

Administration of 4-HNE failed to alter the pattern of extracellular [GSH] (figure 3.6), consistent with the notion that the changes in GSH concentration following channel blockers is independent of oxidative stress. Under these conditions, MK-571-treated cells again showed a marked increase in extracellular [GSH]. This was surprising since a decrease in extracellular [GSH] was expected with MK-571 treatment (see previous paragraph). A possible explanation could be that MK-571 had a direct effect independent of its ability to inhibit MRP1, since it has been reported to stimulate GSH release [33, 34, 36]. However, this effect is typically seen at concentrations of 1 μM and less [33, 34, 36], much lower than that used in the present study. Another potential explanation for increased extracellular [GSH] after MK-571 administration, is release of GSH due to cell death. It has been proposed that in apoptosis, MK-571 can increase extracellular GSH while decreasing intracellular GSH [228]. However, we found no evidence of cell death by MTT assay. Furthermore, we did not observe depletion of intracellular GSH, in contrast to the report by Franco and Cidlowski [228]. In addition, work by Hammond *et al* has shown that blockade of MRPs with MK-571 would inhibit GSH transport as well as the appearance of apoptosis [270], which is the opposite of the findings of Franco and Cidlowski.

Increased synthesis could also account for the increase in the extracellular concentration of GSH following MK-571 administration but we found no evidence that MK-571 administration altered GCL activity (table 3.1). The decrease in synthesis in Calu-3 cells seen between 18h and 24h of serum deprivation can be ascribed to feedback inhibition of GSH synthesis [286], a well described characteristic of the GSH cycle [165] Within the cell, an increase of [GSH] is in response to a decrease of its normal

concentrations, which vary between cell types, primarily caused by a challenge such as an oxidant stress. As intracellular [GSH] is increased and restored, the GCL enzyme is concomitantly downregulated by GSH itself, thus the presence of feedback inhibition by GSH on its synthesising enzyme. Since administration of MK-571 did not increase GCL activity compared to vehicle control cells, enhanced synthesis is not the explanation for higher [GSH], intra- or extra-cellular, following MK-571 treatment.

It is also possible that the increase in extracellular [GSH] reflects the action of GGT, the main enzyme responsible for GSH recycling. Although GGT activity was not studied directly, inhibition of the enzyme's activity was performed to determine what changes would occur with extracellular [GSH]. Decreases in GGT activity would be expected to result in an increase of extracellular [GSH], due to accumulation of GSH [215, 216]. This would reduce reuptake into the cell. MK-571, through a yet unknown side-effect, might be able to diminish or abrogate GGT activity leading to the increases in extracellular [GSH] that we observed (figure 3.6). When acivicin was added, extracellular [GSH] increased by a factor of almost 3, without a change in intracellular [GSH] (figure 3.7). The same pattern was seen in the presence of anion channel blockers (figure 3.8). . Therefore, there is no reason to believe that GGT blockade can account for increased extracellular [GSH] seen with MK-571. If GGT is being inhibited, no decrease in extracellular [GSH] should be observed with combined MK-571 and inh-172 versus MK-571 blockade alone (figures 3.10B and 3.11B). CFTR mediated transport of GSH is a more likely explanation although further elucidation of this would require a more formal time course study. The absence of a role for GGT in our data is consistent with the observation that GGT activity is increases in BAL from CF patients [165, 279].

It is important to note that GSH can be dependent on factors other than oxidative stress. Specifically Cantin *et al.* have shown that depletion of albumin led to depletion of cellular and tissue GSH levels [287]. However, albumin was required for the proper maintenance of GSH. The serum deprivation conditions used during our experiments (0.5% FBS), with the goal of synchronizing the cell cycle, could affect [GSH] outcomes and cellular viability. Our finding of increased [GSH] is not consistent with the notion that our data is explained by insufficient albumin in the culture media.

To determine what other mechanism might account for the increase in GSH, we tested whether administration of MK-571 affected CFTR channel function. We found that administration of MK-571 led to higher post-peak efflux readings from Calu-3 cells (figure 3.9), which indicates that an open channel is being maintained for a longer period of time. Having an open channel would provide a path for GSH to be exported out of the cell, which in turn would increase extracellular [GSH] the longer the channel remained open. Therefore the results suggest a possible role for CFTR in determining extracellular [GSH]. In this regard, Li et al, studying rat intestinal epithelia, found that the open state of CFTR to be increased with use of MK-571 [159], which would translate into longer channel function in efflux studies. To further investigate this possibility, we blocked CFTR using inh-172 in MK-571-treated cells and observed that extracellular [GSH] returned to near control levels but with a further increase in intracellular [GSH], compared to MK-571 treatment alone (figure 3.10). Since blockade of CFTR effectively abrogated the changes in [GSH] induced by MK-571, we used CFTR-knockdown cells to confirm a role for CFTR. We found no effect of MK-571 administration on intracellular [GSH] and extracellular [GSH] in knockdown cells (figure 3.12), consistent with the

notion that MK-571 requires functional CFTR to exert its effects on GSH. Since MRP1 is expressed on the basolateral membrane in numerous cells types [31, 39], questions have been raised as to its relevance as a GSH transporter in the airways [160]. Contrary to the apically located CFTR, the location of MRP1 would suggest that it cannot mediate changes directly in airway GSH. Therefore, at first glance the channel would be a questionable target to ameliorate GSH deficiency observed in the airways of CF patients. Yet it is important to note that Hurbain *et al.* showed a correlation between MRP1 expression and the severity of CF symptoms [160], indicating a possible role for the channel *in vivo*. In the present study, we used submerged non-polarized cultures for our studies, causing GSH exchanges to occur within a single extracellular compartment; hence channel localization was not a factor. Further studies would need to examine whether the same GSH changes occur in a polarized Calu-3 cell model. A polarized cell model would assist in determining where the extracellular [GSH] changes are occurring, on the apical side or basolateral side, and therefore help in determining which channels are involved. Thus if the changes in extracellular [GSH] are indeed due to CFTR, the changes would only occur on the apical side. In addition, there would be no possibility of direct physical interaction between the 2 channels, CFTR and MRP1, unlike what is suggested between MRP4 and CFTR [159], considering the usual basolateral localisation of MRP1 [31, 39, 160]

The mechanism by which MK-571, or MRP1 blockade, modifies the role of CFTR in GSH regulation remains unclear and no studies have investigated this possibility. One possibility is that inhibition of MRP1 induces a compensatory increase in CFTR expression since when the expression of one is increased, the other is decreased

[26, 288]. This could have important implications in GSH regulation considering they both transport GSH [9]. Another potential explanation is that MK-571 itself, unrelated to MRP1 inhibition, may modify CFTR activity through a yet unreported side effect, which is commonly seen when using pharmacological inhibitors. For example, glybenclamide, originally developed as a K^+ ATP channel inhibitor was later found to block CFTR [114, 289-291]. Our current experimental model cannot differentiate between the two possibilities. Further studies would need to examine the use of MK-571 on a MRP1-knockdown model, which would clarify if this was a MRP1-dependent mechanism or a novel pharmacological effect of MK-571 on CFTR. We can conclude thus far that MK-571 treatment alters intracellular [GSH], possibly by its effects on MRP1.

Another possibility is that MRP1 influences CFTR indirectly. Li *et al.* have reported that blockade of MRP4 can lead to cAMP accumulation and subsequent increased CFTR activation [159]. As noted above, in the presence of MK-571, CFTR activity as measured by iodide efflux increased progressively after initial stimulation, (figure 3.9). As MK-571 can also block MRP4, this mechanism might explain our observations. However, Calu-3 cells do not express MRP4 (figure 3.13), making a cAMP-based explanation unlikely. Moreover, this hypothesis would require MRP1 to be the main vehicle for cAMP transport, which is not possible since MRP1 is not a cAMP transporter [9]. In the absence of an obvious cAMP transporter in our cell model, a cAMP-based mechanism is less likely. Furthermore our initial studies on the role of cAMP in mediating GSH, either with administration of 8-bromo-cAMP or its functional antagonist Rp-8-bromo-cAMP [292], gave no indication of involvement (figure 3.15). The only exception was combined administration of cAMP and MK-571 where

extracellular [GSH] returned to near control levels. A limitation of this observation is that the number of replicates was low. As the difference with control is close to significance ($p<0.059$, $n=3$), it is possible that with more experiments a significant effect of these compounds might still be found. It would be of interest to conduct experiments over shorter time frames to make proper conclusions on the relevance of cAMP in modulating the effects on extracellular [GSH] through CFTR, with patch clamping to confirm if CFTR function is indeed being affected. The rationale behind the shorter time frame is based on the work by Li *et al.* [159], where the study of cAMP on CFTR function were done at 30 minutes (or less). This does not still exclude CFTR as an explanation for the changes observed with extracellular [GSH], since iodide efflux experiments have shown that CFTR chloride channel can be active under our experimental conditions (figures 3.1 and 3.9). Furthermore, cells do have cAMP inherently, which is needed for CFTR channel activation, and which is exploited by diseases like cholera as a means to induce over activity of the channel [46].

We investigated the potential role of leukotrienes (LTs) in modulating [GSH] as MK-571 is an LTD4 receptor antagonist in addition to its action on MRP1 [293, 294]. LTs have also been shown to be increased in CF. Moreover, Montelukast, a known LTD4-receptor antagonist [295-297], has been shown to lower IL-8 levels and improve lung function in CF patients [298]. To study the possible role of LTs in our study, we treated cells with Montelukast, which led to increased intracellular [GSH], as had been seen with administration of MK-571 (figure 3.14A). However, using LT synthesis inhibitors we found no evidence of changes in intracellular [GSH], nor were the actions of MK-571 on extracellular [GSH] affected (figure 3.14B and 3.14C). Taken together

these observations are not consistent with a role of LTs in the intra- and extra-cellular [GSH] changes observed with MK-571 treatment. Additional experiments might be of interest, particularly studies at different time points, which would help to determine if there was a change in [GSH] which may have been missed. Ideally, direct administration of LTs should be tried. Finally, examination of extracellular [GSH] upon administration of Montelukast would be needed to determine if LT receptor inhibition could be the cause of the [GSH] changes seen with MK-571 administration. Nevertheless, our results so far do not support a major role for LTs in mediating [GSH] in this cell model.

Although our preliminary data suggests no role for LTs in the changes of [GSH] observed under our experimental conditions, further investigation would be warranted due to their possible roles in the pathogenesis of CF. The neutrophil chemoattractant and activator IL-8 is an important mediator of inflammation in CF [89]. Of interest, IL-8 has known interactions with different LTs, such as LTB4 and LTC4; IL-8 can either enhance production of LTs [247, 299] or be induced by LTs [300-302]. MK-571 and Montelukast are known LTD4 receptor antagonists [246] and LTD4 is synthesised from LTC4 [303], thus providing a possible link back to IL-8.. Furthermore, LTB4 is a known neutrophil chemoattractant [245, 304, 305], which may also play a role in neutrophilic infiltration during response to infection in CF [244]. It is important to note that synthesis of some of these mediators of inflammation depends on GSH. For example, LTC4 is synthesised by the conjugation of LTA4 with GSH [306]. Within this context, it could be hypothesised that higher intracellular [GSH] promotes the synthesis of LTs, which could then facilitate in the creation or maintenance of a pro-inflammatory state. Hence relationships between MRP1 (see section 1.7), LTs and GSH do exist, which would have important

consequences in CF. This is especially important considering that, in addition to being a GSH transporter, MPR1 also transports LTs, which means any change in its expression or function could potentially either ameliorate or aggravate the pro-inflammatory state in CF.

In the final part of the study, we wanted to understand how MK-571, a precursor of Montelukast that is a MRP1 channel blocker and a leukotriene D4 (LTD4) receptor antagonist, could increase CFTR function. We continued our studies in a BHK cell model, which afforded us the opportunity to study both wild-type and ΔF508 CFTR, providing us with observations relevant to the defect found in the majority of CF cases. In figure 3.16, we observed that cell surface expression of wild-type and ΔF508 CFTR increases with administration of both compounds. For MK-571 treatment the most consistent significant increase in expression was with 100 nM over a 24h period, with either wild type or ΔF508 CFTR. With Montelukast treatment the 1 pM concentration provided a consistent significant increase in either wild type or ΔF508 CFTR cell surface expression. Since both compounds could cause an increase in expression this would suggest that this class of compounds may have some beneficial effects for CFTR expression. To our knowledge, this is the first time that this phenomenon has been reported. Furthermore, it has been suggested before that ΔF508 CFTR is functional if steps are taken to promote its migration to the cell surface, hence this would be a very interesting observation if an already available and tested drug can promote an increase of ΔF508 CFTR expression at the cell surface. In figure 3.17, we do not observe a correlation between CFTR function and changes in expression with wild-type CFTR (figure 3.16). An explanation for this would be that an increase of 5% to 10% of cell

surface CFTR did not translate into any significant functional changes, in a cell model without defective chloride channel function. If the channel function is already at its peak in the wild-type CFTR expressing cells, a change in expression of 5% to 10% would not necessarily be detectable functionally. In contrast, in cells expressing ΔF508 CFTR, a model where there is no significant channel function is present at baseline, we observed complete restoration of chloride channel function. Both MK-571 and Montelukast restored chloride channel function, which is present after 24 h from administration. Although we used a CFTR-expressing BHK cell model and detected evidence of CFTR mediated chloride channel function [265], further confirmation of the role of CFTR could be obtained by testing the effect of CFTRinh-172 on the functional increases associated with administration of MK-571 and Montelukast.

Finally, we examined if the whole cell expression of CFTR (figure 3.18) would correlate to the changes found on the cell surface, giving us an indication whether total amount of protein was changing or only surface expression. Our preliminary results did not confirm a correlation between whole-cell CFTR expression, either with wild-type CFTR or ΔF508 CFTR, and cell surface changes: the lack of change seen in whole cell versus the differences in membrane expression. This would suggest that we may be dealing with either a change in the trafficking of CFTR or in the turn-over of the protein in the membrane and not in an increase in protein production. More work would be needed to confirm the lack of whole cell changes in CFTR amounts.

It is interesting to note that there is a difference between the two compounds with regard to the time course of their action on chloride channel function. Where MK-571 treatment is most potent at 1h, with function at 30% of control (dropping to 10% of

control function at 24h), Montelukast is most potent at 24h, with function at 30% of WT control as well (increased from 10% of WT function at 1h). These differences do not correlate with the changes seen in cell surface expression; the general increase in expression is not matched by equal changes in function. This would suggest that both MK-571 and Montelukast may have different, independent, effects on CFTR function, even if both increase CFTR expression. Irrespective of the differences in efflux, both compounds caused a restoration of chloride efflux in cell lines which did not have any significantly detectable levels of efflux prior to treatment. Furthermore, the restoration attained a maximum 30% - 35% of WT efflux, with a minimum of 10%, at optimal concentrations. Based on published data, this degree of function (10% to 30%) is sufficient to compensate for channel function defects in CF and restore normal epithelial cell function [140, 277]. Thus these results show that, at least in vitro, it is possible to restore CFTR function to levels needed for normal cell function. Confirming these observations in a relevant airway epithelial cell model, such as NHBE or CFBE, would point to a general effect and not a cell model- specific aberration.

Since these results were obtained using BHK cells, functional changes in CFTR could be caused by the action of cAMP. At first glance this would seem to contract the preliminary data in this thesis which indicates no change with cAMP use (figure 3.15), yet this data is from Calu-3 cells, which do not express MRP4 (figure 3.13). This is important to note, since the work done by Li *et al.* suggested that MK-571 could block cAMP transport by MPR4, causing localized accumulation of cAMP and increased CFTR function [159]. Besides the difference in cell model, there is the issue of when the results were collected: the changes in CFTR function observed by Li *et al.* were at 30 minutes

(or less), which is a time frame much shorter than the 6 hours used for cAMP experiments on Calu-3 cells, yet close to the 1 h time point used for measuring the chloride channel function in the BHK cells. Therefore, it is possible that MK-571 treatment caused an increase in intracellular cAMP within the BHK cell model, at the 1h time point, thus promoting increased ΔF508 CFTR function, which is cAMP-dependent [109, 159, 307]. In support of this hypothesis, kidney cell models have been reported to export cAMP and to express MRP4 [308, 309]. In the case of Montelukast however, we find the early functional increase was not significant, yet over time it became so, suggestive of a gradual accumulation. In support of this possibility, Rovati *et al*. had shown in an asthma model that cAMP accumulation in lung tissue occurred with Montelukast treatment [310]. Within this context, the increase in CFTR activity could be due to cAMP accumulation. The potential role of cAMP in the functional changes of CFTR would need to be investigated more thoroughly, yet cAMP alone cannot explain the changes in expression seen with either wild-type or ΔF508 CFTR. Therefore we could be dealing with two separate mechanisms of action: increasing CFTR function, possibly mediated by cAMP, and increase CFTR expression through yet to be determined mechanisms.

4.3 Implications of the results

First, the results in this thesis demonstrated that, at least in Calu-3 cells, GSH can be regulated through channels other than CFTR. This is an important finding since changing the intra- and extra-cellular [GSH] need not be solely dependent on CFTR. This raises the possibility that other channels could play a role in this phenomenon and points

the way to additional experiments. Within this context and with the results obtained in this study, MRP1 may act to regulate intra-cellular [GSH], similar to what Hudson predicted for CFTR [267]. However, instead of CFTR, MRP1 would act as the major pathway for GSH transport.

Second, this thesis demonstrated that CFTR can play an indirect role in GSH, possibly through interaction with MRP1. Such an interaction is not unprecedented. Li et al. demonstrated in rat intestinal epithelium that blockade of MPR4 using MK-571, caused an increase in CFTR function [159]. The findings in this thesis suggest for the first time that similar treatment is affecting CFTR function in cells derived from airways, although with another member of the MRP family present. Given that the changes in GSH were correlated to the presence of functional CFTR, the question remains what mechanism is affecting CFTR function. This can be due to MRP1 blockade causing a compensatory increase in CFTR expression, which would provide a pathway for increased GSH transport and an increase in extracellular [GSH]. This could also be due to a novel pharmacological effect on CFTR, as seen with other compounds like sildenafil, which increase CFTR trafficking to the cell surface [311]. This increase in CFTR expression to the cell membrane could also provide a pathway for increased GSH transport out of the cell and increased extracellular [GSH].

Further examination of the mechanism behind the changes in CFTR function, with MK-571 administration, revealed its capacity to increase CFTR expression, either with wild-type or ΔF508 CFTR. Subsequently we found that MK-571 and Montelukast, a member of the same family of pharmacological compounds, could also change CFTR function, yet another novel observation. Further work would be required to understand the

mechanisms behind the changes in CFTR expression and function caused by MK-571 and Montelukast treatment, but first these results would need to be reproduced in a clinically relevant model. This could be done first in a CF immortalized cell line, such as CFBE, or CF primary cells and later extended *in vivo*, to a CF mouse model.

4.4 Therapeutic implications

These results suggest that new treatments could be used in the regulation of GSH, which could target channels other than CFTR, such as MRP1. Examples of non-CFTR GSH regulation are found in rat brain cells where changes in [GSH] are mediated through MRP1 [34]. Our findings suggest that this could also be possible in the airways. Further investigation would be required to determine if our results can be reproduced in other models. If it were the case, this would open the door to changing extracellular [GSH] via non-CFTR pathways, and to assist the cell in defending against oxidant stress, stemming from the repetitive immune response to chronic infection found in CF. Another outcome would be a decrease in intracellular [GSH], which could promote the normal function of the apoptotic pathway in CF cells. This is important since defective bacterial clearance has been linked to defective apoptosis in CF cells, which may contribute to the pathogenesis of the disease [269].

In this thesis we also find CFTR to be a target for therapeutic treatment, using a compound originally intended to affect MRP1 function. Administration of both MK-571 and Montelukast led to an increase in both wild-type and ΔF508 CFTR expression followed by increased function, observations which have never been reported before. This

would suggest that this class of compounds may have some beneficial clinical effects. It would be very important finding if Montelukast, an already available and tested drug, could promote an increase of ΔF508 CFTR expression at the cell surface, followed by functional activity. Interestingly enough, Montelukast has been used on CF patients in a clinical study where the drug was shown to have a beneficial effect by lowering IL-8 levels and improving lung function [298]. Of course the focus of that study was to improve the inflammatory state in CF patients by blockade of LT receptors, while this thesis provides a novel and unreported effect of Montelukast on both CFTR expression and function. In effect, increasing ΔF508 CFTR expression in CF patients could bring about an increase in ALF [GSH] promoting protection against oxidative stress. In addition if the restoration of expression, and subsequent function, of defective CFTR was sufficient, it could bring about proper hydration of the mucus and even ameliorated mucociliary clearance of infectious agents. It would be important to confirm the effects of the drug on wild-type and ΔF508 CFTR first in vitro with an airway epithelial cell model, then *in vivo* with studies in CF mouse models and later CF patients, since this would open the door to a new therapeutic treatment for CF patients.

4.5 Future Directions

Although this thesis has shown that CFTR is not the sole determinant of [GSH] in Calu-3 cells, that MRP1 can play a role in determining intracellular [GSH], and that CFTR can play a role in determining extracellular [GSH] in conjunction with MK-571 treatment, further work is needed to complete or confirm observations seen in this thesis.

1. To examine the use of MK-571 on a MRP1-knockdown model. This would determine if changes seen with [GSH] in Calu-3 cells were related to a MRP1-dependent mechanism or a novel pharmacological effect of MK-571.

2. Completing the cAMP studies to determine whether activation of CFTR function in BHK and Calu-3 cells could be responsible for changes in GSH. This would be accomplished by examining the effect of cAMP treatment at a shorter time point (30 mins to 1 h) on intra- and extra-cellular [GSH], with patch clamping to confirm changes in CFTR function.

3. Completing the LT studies to determine the cause of the changes in CFTR function in Calu-3 cells. This would be accomplished by examining the effect of LTs and their respective receptor and synthesis inhibitors over time, on intra- and extra-cellular [GSH], with patch clamping to confirm changes in CFTR function.

4. Repeat BHK experiments in an airway lung cell model, such as normal human bronchial epithelial cells (NHBE) or cystic fibrosis bronchial epithelial cells (CFBE), to determine whether the observations and conclusions can be generalized and not limited to this particular model.

5. Examine if Montelukast promotes an increase in wild-type and ΔF508 CFTR expression in a CF cell line, followed by study in a CF mouse animal model and then in humans, if the results from the previous steps were positive.

4.6 Summary

This thesis demonstrated that CFTR, under our experimental conditions, was not the major determinant of intra- or extra-cellular [GSH]. Furthermore, this thesis showed that MRP1 does play a role in determining intra-cellular [GSH] amounts and that MK-571, and related molecule Montelukast, can affect expression and function of either wild-type or ΔF508 CFTR.

Initially we set out to determine what role CFTR had in determining intra- and extra-cellular GSH under oxidative stress. The first part of the study showed that CFTR was not a determinant of [GSH] in Calu-3 cells, whether under oxidant stress conditions or not. Although this result cannot be generalized, it did point to an alternate mechanism in GSH regulation. The second part of the study further investigated the implication of another channel in GSG regulation and found that MRP1 did play a role in GSH handling. The increased intracellular [GSH] observed with MRP1 channel blockade suggested a role for this channel in regulating intracellular [GSH]. Paradoxically however, extracellular [GSH] was higher after administration of the MRP1 antagonist MK-571. Furthermore, we also observed higher CFTR activity with use of MK-571. The last two findings suggest that CFTR could be indirectly responsible for the changes in [GSH] through interaction with MRP1. The results from the last part of our study, which dealt with the mechanism causing increases in extra-cellular [GSH] through CFTR, suggested that both MK-571 and Montelukast can increase expression and function of either wild-type or ΔF508 CFTR, although the differences between function and expression suggest a more complex relationship. Understanding how both MK-571 and Montelukast alter CFTR expression, whether it be wild-type or ΔF508, would advance our understanding of

the mechanisms involved and pave the way for future CF treatments. One possible future treatment for CF patients may be Montelukast, which may have beneficial effects in restoring CFTR expression and function, if confirmed in other models and *in vivo*.

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