Identification of Chemical Correctors for the Trafficking Defect of Mutant SLC4A11

by

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Abstract

Congenital Hereditary Endothelial Dystrophy, Harboyan Syndrome and Fuchs' Endothelial Corneal Dystrophy are three forms of heterogeneous, genetic corneal blindness. Mutations in the membrane protein SLC4A11 have been found to cause cases of these diseases. Most mutations in SLC4A11 cause the protein to misfold and become retained in the endoplasmic reticulum. The loss of function of SLC4A11 is thought to give rise to the symptoms of these diseases. Currently, the only permanent therapy for these diseases is corneal transplant. A pharmacological therapy is needed to better treat these diseases caused by SLC4A11. Screening techniques for the assessment of small molecule correctors is the first step toward a pharmacological therapy. A high throughput screening assay was developed to detect SLC4A11 at the plasma membrane. This technique could also be applied to other plasma membrane proteins. A small scale screen revealed that three non-steroidal anti-inflammatory drugs rescue the trafficking defect of some mutations of SLC4A11. Glafenine was effective in rescuing the trafficking defect in all three mutations screened. Functional analysis revealed that these mutants, when rescued to the plasma membrane, retained functional activity. These data suggest that glafenine may be a viable therapeutic for some cases of endothelial corneal dystrophies.

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List of Abbreviations

4-PBA; 4-phenylbutyric acid

AE; anion exchanger protein

AQP1; Aquaporin 1

ASA; acetylsalicylic acid

BCA; bicinchoninic acid

BiP; binding immunoglobulin protein

Carb; carbamazepine

cDNA; complementary deoxyribonucleic acid

CFTR; cystic fibrosis transmembrane conductance regulator

CHED; Congenital Hereditary Endothelial Dystrophy

COL8A2; collagen alpha-2(VIII) chain

COX; cyclooxygenase enzyme

DIDS; 4,4'-diisothiocyanatostilbene-2,2'-disulfonate

DMEM; Dulbecco's Modified Eagle Media

DMSO; dimethyl sulfoxide

DNA; deoxyribonucleic acid

EC_{50}; half maximal effective concentration

EDTA; ethylenediaminetetraacetic acid

ER; endoplasmic reticulum

ERAD; endoplasmic reticulum associated degradation

FECD; Fuchs' Endothelial Corneal Dystrophy

GAPDH; glyceraldehyde 3-phosphate dehydrogenase

GFP; green fluorescent protein

HA; hemagglutinin

HEK293; Human Embryonic Kidney 293 cells

HEPES; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP; horseradish peroxidase

HS; Harboyan Syndrome

KM60; 7-Chloro-4-[4-[4-chlorophenyl)sulfonyl]-1-piperazinyl]quinolone

LOXHD1; lipoxygenase homology domain 1

MG-132; N-(benzyloxycarbonyl)leucinylleucinylleucinal

MIP; major intrinsic protein

NSAID; non-steroidal anti-inflammatory drug

PCR; polymerase chain reaction

PPCD; posterior polymorphous corneal dystrophy

PSG; penicillin-streptomycin-glutamine

SAHA; N-hydroxy-N'-phenyl-octanediamide

SDS; sodium dodecyl sulfate

SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM; standard error of mean

SLC; solute carrier

SLC4A11; solute carrier family 4 member 11

T; total protein

TCF4; immunoglobulin transcription factor 2

TMAO; trimethylamine N-oxide

U; unbound protein

UGGT; (UDP)-glucose:glycoprotein glucosyltransferase

UPR; unfolded protein response

WT; wild type

Chapter 1: General Introduction

1.1 Thesis Overview

The objective of this thesis is to develop and validate a high throughput assay to screen potential correcting compounds for the trafficking defect in mutant SLC4A11. A novel high throughput assay to identify HA tagged SLC4A11 at the cell membrane was developed. A small screen of compounds shown to be effective in other membrane protein diseases was performed using this assay. A correcting molecule was identified and functional studies of corrected SLC4A11 mutants were also performed.

The exact function of SLC4A11 is still a matter of contention. SLC4A11 has been implicated in endothelial corneal dystrophies. This introduction discusses endothelial corneal dystrophies and current therapies for their treatment. The next section of this introduction discusses the function of SLC4A11 and its role in endothelial corneal dystrophies. The final section of this review includes a brief review of other diseases resultant of mistargeting of membrane proteins and chemical methods with which they have been rescued.

1.2 The Cornea and Corneal Dystrophies

The human cornea is an anterior structure of the eye covering the iris, pupil and anterior chamber. The cornea is responsible for focusing light onto the retina and is therefore important in visual acuity. The cornea is separated into five layers (Fig. 1.1). From anterior to posterior, these are the epithelial layer, Bowman's layer, corneal stroma, Descemet's membrane and endothelial monolayer. The endothelial monolayer is a single layer of polygonal cells (1). In general, human corneal endothelial cell density deceases with age.

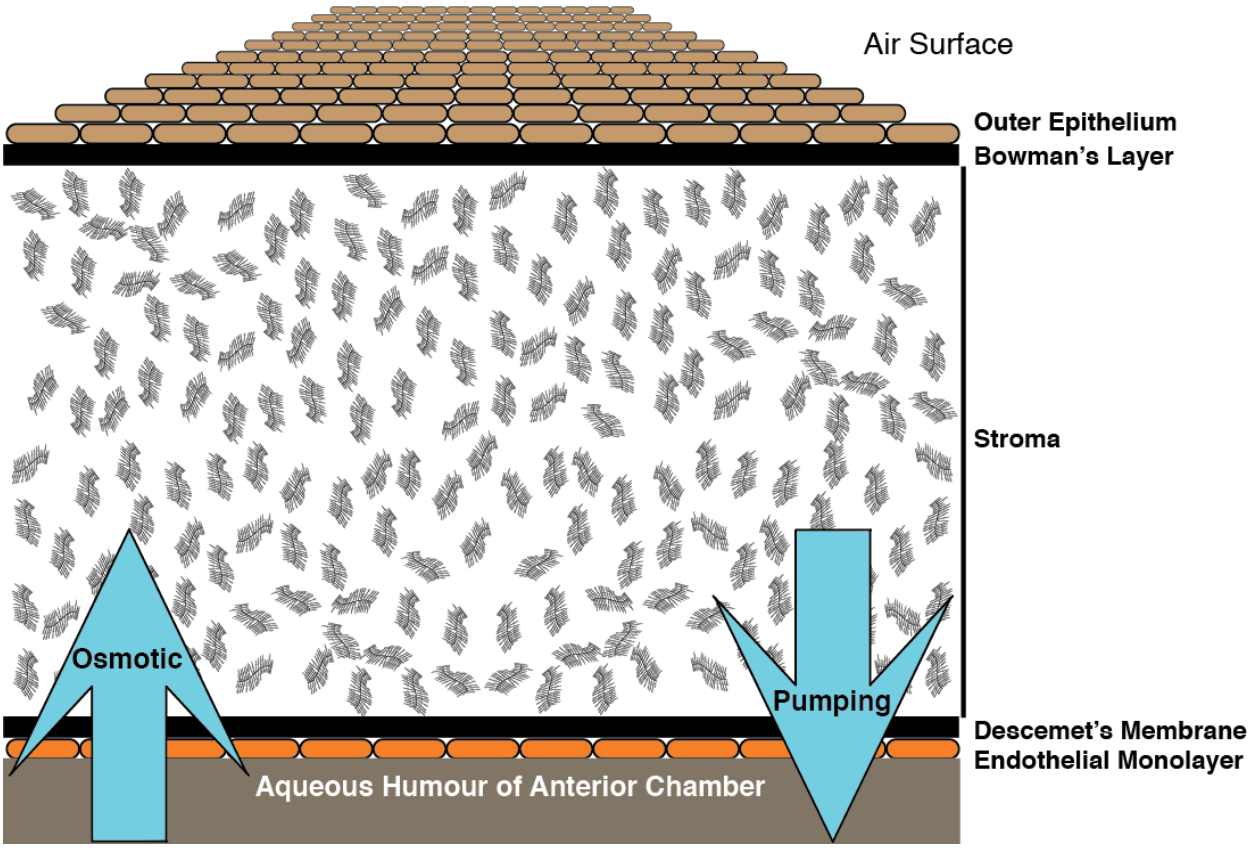

Figure 1.1 Schematic representation of the human cornea (based on Pepose and Ubels, 1992). The anterior face of the cornea faces up. The human cornea consists of five layers. From anterior to posterior they are; the outer epithelium, Bowman's layer, stroma, Descemet's membrane and the endothelial monolayer. The stroma consists of a high concentration of collagen and proteoglycans, which produces an osmotic gradient between the aqueous humor and stroma. Endothelial cells are responsible for the movement of water back from the stroma to the aqueous humor.

Newborn babies can have cell densities greater than 5500 cells/mm^2 whereas adults typically range from 2500 to 3000 cells/mm^2. The endothelial layer forms a leaky barrier between the aqueous humor and the corneal stroma (1), which allows the osmotic movement of water from the aqueous humor into an area of high solute concentration of the stroma (2, 3).

Corneal dystrophies refer to a large group of bilateral, genetic and non-inflammatory diseases that affect the cornea. Corneal dystrophies can be divided into three main groups based on the layers of the cornea they primarily affect. These are dystrophies of the epithelial and Bowman's layers (anterior), dystrophies of the corneal stroma (stromal) and dystrophies of the Descemet's and endothelial layers (posterior). Most corneal dystrophies result in corneal opacities, which affect visual acuity and in some dystrophies can eventually lead to blindness (2). This thesis will deal with three posterior corneal dystrophies of the endothelial layer; Congenital Hereditary Endothelial Dystrophy Type 2 (CHED2), Harboyan Syndrome (HS) and Fuchs' Endothelial Dystrophy (FECD). These diseases share similar symptoms in which those suffering from these diseases develop corneal edema of the stroma and eventually cloudiness of the cornea resulting in vision loss.

1.2.1 Congenital Hereditary Endothelial Dystrophy Type 2

Congenital hereditary Endothelial Dystrophy (CHED; MIM 217700) (4) is characterized by a ground glass, blue-white opaque appearance of both corneas and markedly thickened corneas from birth or infancy (2). There are two types of CHED classified as type 1 (CHED1) and type 2 (CHED2). CHED1 manifests in the first two years of life, but notably does not present with nystagmus. Nystagmus is a condition represented by involuntary movement of the eyes

(5). CHED1 is inherited in an autosomal dominant manner (6). CHED2 individuals are born with ground glass corneas and present with nystagmus (2). CHED2 is inherited in an autosomal recessive manner. In both types, the corneal stroma is swollen due to extensive stromal edema with some enlargement of the collagen fibrils in the stroma. This results in a scattering of light and the characteristic ground glass appearance (2). The clinical classification of CHED1 has been recently changed based on evidence that mutations causing this disease are similar to those of another endothelial dystrophy, posterior polymorphous corneal dystrophy (PPCD) (7). As such, CHED1 has been eliminated as a distinct clinical classification and CHED2 can now be referred to as simply, CHED (7).

1.2.2 Harboyan Syndrome

Harboyan Syndrome (HS; MIM 217400) (8) is a disease affecting both the eyes and ears. Patients present with the same symptoms as CHED2, accompanied with progressive hearing loss (6). Typically, the first symptoms of hearing loss are reported in teenage years. HS is inherited in an autosomal recessive manner (6). The physiological cause of hearing loss in HS is unknown. A current theory is that the absence of SLC4A11 disrupts the transport of potassium to the stria vascularis leading to decreased potassium secretion by intermediate cells (9). Intermediate cells are essential for establishing the endocochlear potential and without the proper potential, hair cells in the cochlea are unable to amplify auditory signals (9).

1.2.3 Fuchs' Endothelial Corneal Dystrophy

Fuchs' Endothelial Corneal Dystrophy (FECD; MIM 136800) (10) shares the same "ground glass" appearance of the cornea with CHED, but is distinctive in that the disease is

asymptomatic in early life (2). Symptoms do not start to appear until the fourth or fifth decades of life. One of the defining characteristics of FECD is a thickened Descemet's membrane accompanied with posterior excrescences (guttae) (2, 11) along with corneal edema (12). These guttae are more confluent and centrally located than those expected from normal aging (2, 12). FECD is also characterized by a marked decrease in endothelial cell density, greater than expected from normal aging (2, 11). Degeneration of the other layers of the cornea is common in FECD, but these are seen as secondary to the degeneration of the endothelial layer (13). FECD is inherited in an autosomal dominant manner and has a 4% occurrence in North Americans (2). The rate of occurrence has been reported to be as high as 9% in certain populations around the world (11). Interestingly, females are affected by FECD at a higher rate compared to males (14, 15). FECD has also been associated with hearing disability (16), higher incidence of age related macular degeneration (17), structural changes of the cornea (kerataconus) (18, 19) and cardiovascular disease (20).

1.2.4 Treatment for Endothelial Dystrophies

Vision loss severely affects the quality of life of those who suffer from these diseases. Currently, the only lasting treatment is corneal transplant. Currently, FECD accounts for 10-25% of all corneal transplants in North America (2). Tissue availability and compatibility are major problems associated with treatment method. Other treatments revolve around removing excess water from the corneal stroma. These include hypersaline eye drops and using a hair dryer to dry out the surface of the cornea. These are only temporary solutions that address the symptoms of the disease and not the root cause. Development of a chemical therapy for these

diseases could be important for not only those who suffer from endothelial dystrophies but could also free up valuable corneal tissues for transplant in other diseases.

1.3 SLC4 Family of Proteins

The SLC4 family of the solute carrier (SLC) group of proteins (Fig. 1.2) includes ten unique human genes (SLC4A1-5 and SLC4A7-11) which encode integral membrane proteins (21). Of these, eight genes encode proteins that fall into two major functional groups. The first group is the chloride bicarbonate (Cl^-/HCO_3^-) exchangers including SLC4A1-3. These proteins are also known as anion exchanger proteins (AE). The second group is the sodium coupled bicarbonate (Na^+/HCO_3^-) transporters, also known as NBCs, including SLC4A4, 5, 7, 8 and 10. This group is further divided into electrogenic (SLC4A4-5) and electroneutral (SLC4A7-8 and 10). SLC4A8 is unique in that while it is Na^+-driven, it is a Cl^-/HCO_3^- exchanger (22). SLC4A9 has been reported to have Cl^-/HCO_3^- exchange activity (23-25). In the kidney, SLC4A9 has demonstrated Na^+-coupled HCO_3^- exchange activity (26). Phylogenetically, SLC4A9 clusters with the Na^+-coupled bicarbonate transporters (27). The CO_2/HCO_3^- equilibrium is an important buffering system in the human body and so SLC4 family members are implicated in a number of diseases. The function of the remaining protein, SLC4A11, has not been conclusively established (21).

The SLC4 family shares a high degree of sequence similarity although SLC4A11 only shares approximately 14-20% identity with the other members (21). All members of the SLC4 family are characterized by a long N-terminal hydrophilic domain and a short C-terminal hydrophilic domain, both of which are intracellular (21). Several members of the SLC4 family are inhibited by disulfonic stilbene derivatives (DIDS) (28). SLC4 family members are widely spread

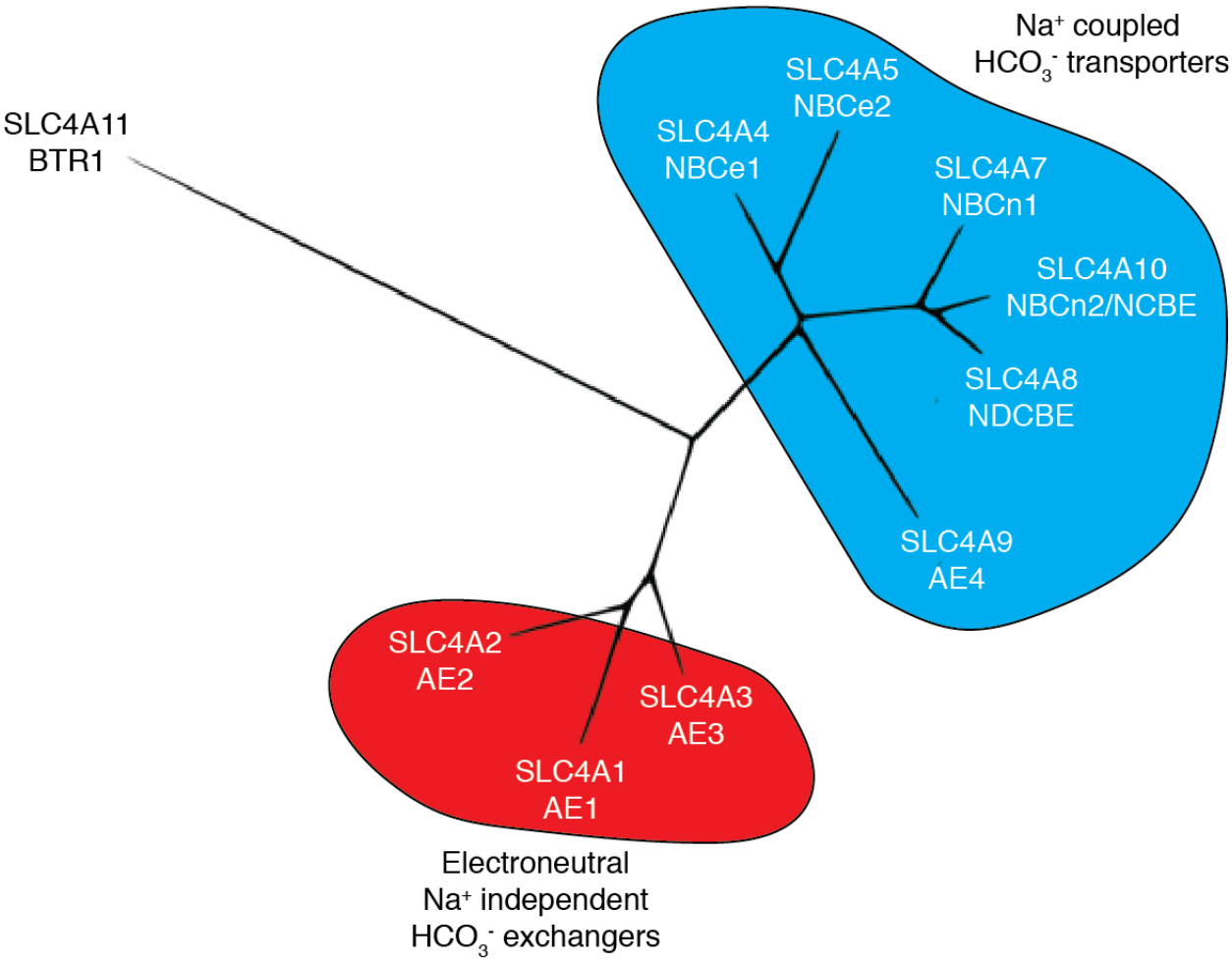

Figure 1.2 Phylogenetic dendrogram of the SLC4 family of bicarbonate transporters. The SLC4 family contains two main groups; electroneutral Na^+ independent HCO_3^- exchangers (red), and Na^+ coupled HCO_3^- transporters (blue). SLC4A11 is represented outside of these groups as its function has not been conclusively established. This dendrogram was created using Clustal W2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

throughout a variety of human tissues. They are also implicated in a wide variety of dysfunctions and diseases which includes cataracts (29), autism (30), epilepsy (31), heart failure (32), kidney failure (27) and even breast cancer (33). As such, therapeutics targeting SLC4 family proteins have received a lot of attention (34-38).

1.4 Membrane Protein Biosynthesis and Endoplasmic Reticulum Retention

Membrane proteins are synthesized and folded at the endoplasmic reticulum (ER). Folding can also be aided by chaperones in the ER (39). The folded protein is then subsequently transported to the Golgi apparatus and eventually its final destination to perform its function. When a protein is misfolded it is recognized by (UDP)-glucose:glycoprotein glucosyl transferase (UGGT) or immunoglobulin binding protein (BiP) (39, 40). In the case of UGGT, the N-glycosylated protein is given more time to fold by entering the calnexin/calreticulin cycle (40). If the protein does not fold properly after several cycles in the calnexin/calreticulin cycle, it is marked for degradation and sent to the proteasome in a process known as endoplasmic reticulum associated degradation (ERAD). In the case of mutant proteins, where presumably all of the mutant protein synthesized is misfolded, the calnexin/calreticulin and ERAD mechanisms can be overwhelmed with misfolded proteins, which can lead to unfolded protein response (UPR) (40). UPR increases expression of folding chaperones and decreases protein translation (40). If these measures are insufficient to restore the ER to a normal state, the cell can eventually undergo apoptosis (40). Apoptosis involves the activation of caspases 3, 6 and 7, which form proteases that cleave various proteins, leading to cell death (41).

1.5 SLC4A11

1.5.1 Structure and Function

SLC4A11 is an integral membrane protein comprised of 891 amino acids. It has 14 transmembrane domains, a long N-terminal hydrophilic domain and a short C-terminal hydrophilic domain common to SLC4 family members (42) (Fig. 1.3) Limited proteolysis analysis support this proposed topology model (42). Studies on the expression of SLC4A11 show that on immunoblots, two bands are detected at approximately 110 kDa and 90 kDa (42, 43). The upper band is likely a complex glycosylated form of the protein whereas the lower band is likely core glycosylated. The complex glycosylated form is present at the plasma membrane and the core glycosylated form is ER associated (44, 45). SLC4A11 localizes on the basolateral surface of the endothelial cells of the human cornea (46).

SLC4A11 was originally cloned on the basis of homology to the SLC4 family and was given the name BTR1 (bicarbonate transporter 1) (47). The function of SLC4A11 is a matter of contention. SLC4A11 is a member of the SLC4 family of bicarbonate transporters but does not demonstrate bicarbonate transport ability (48). Plant SLC4A11 orthologs are known borate transporters (47). Human SLC4A11 was originally reported to be a Na^+ coupled borate transporter but in the absence of borate transported Na^+ and OH^- and was subsequently renamed NaBC1 (48). However, other groups have not been able to replicate this finding and instead SLC4A11 is reported to function as a Na^+/OH^- cotransporter as well as a NH_4^+ permeation pathway (49, 50). Another group also identified SLC4A11 as an electrogenic $H^+(OH^-)$

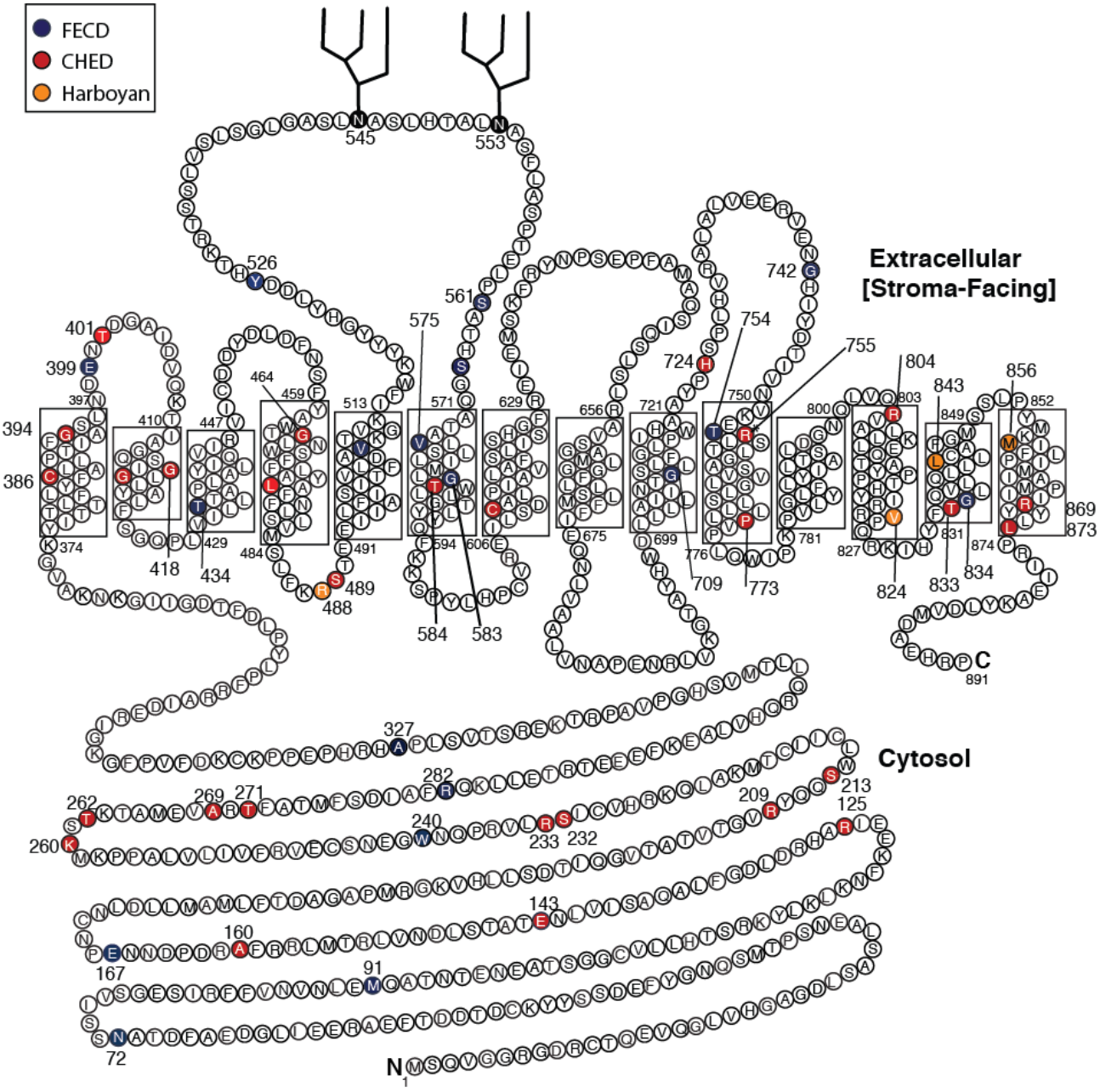

Figure 1.3 Topology model of SLC4A11. This model was originally published in (42). Residues implicated in FECD, CHED and HS are labelled in blue, red and orange respectively. Currently, there are 57 point mutations associated with these three diseases. Residues in black denote N-linked glycosylation sites. Boxes denote transmembrane domains. Mutations: (6, 44, 45, 51-65).

permeation pathway (66). Work in our lab has shown that SLC4A11 when expressed in *Xenopus laevis* oocytes and HEK293 cells facilitates water movement (46, 67). Whether this movement of water is resultant of movement of ions or by directly transporting water remains unclear. If SLC4A11 is directly moving water, this would make it the first non-major intrinsic protein water transporter to be identified (46). Aquaporin 1 (AQP1) was found to be localized to the apical surface of endothelial cells (46). SLC4A11 is proposed to work in conjunction with AQP1 to move water from the stroma to the aqueous humor (Fig. 1.4) (46).

1.5.2 Expression

According to the UniGene expression profile from the National Centre for Biotechnology Information, SLC4A11 is expressed in a variety of tissues, including testes, uterus, trachea, skin, brain, lung, mouth, eye, ovary, blood, kidney, embryonic tissue, intestine, prostate and pancreas. In the kidney, SLC4A11 is expressed in the thin descending loop of Henle (9). Analysis of kidney function of knock out *slc4a11* mice showed that their urine had a reduced osmolarity and a larger volume, suggesting that SLC4A11 in the kidney is important for water reabsorption (9).

Currently, three spliceforms of SLC4A11 have been identified. These three forms differ only in the N-terminal sequence encoded by the first exon. The last 861 amino acids in each

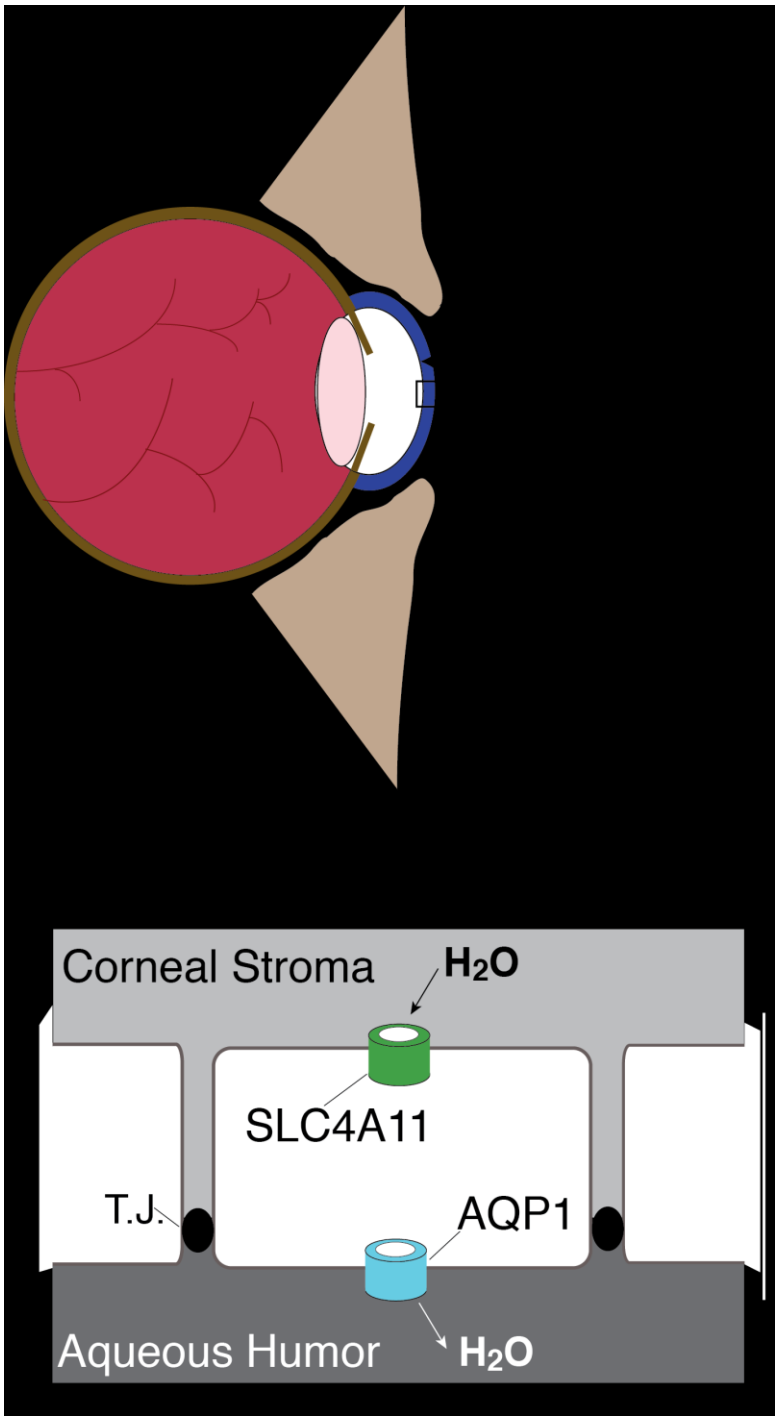

Figure 1.4 Model of SLC4A11 and AQP1 in corneal endothelial cells. SLC4A11 is localized to the basolateral surface whereas AQP1 is localized to the apical surface. SLC4A11 and AQP1 work cooperatively to move water across the endothelial cells into the aqueous humor. T.J. denotes tight junctions between cells.

spliceform are identical. The second spliceform has been the focus of the majority of studies involving SLC4A11 (66). Recently, RT-PCR analysis revealed that the spliceform 3 was the dominant spliceform in human corneal endothelial cells (66). However, whether this translates to protein expression in endothelial cells is yet to be determined. Unpublished data from our lab indicates the presence of both spliceform 2 and 3 in whole cornea samples. Additionally, bioinformatic analysis revealed that the expected methionine for the translation of SLC4A11 is poorly conserved among close relatives of *Homo sapiens*. Another methionine, 35 amino acids downstream, is much more highly conserved among other mammals. Our data indicate the presence of the shortened SLC4A11 in human corneal lysates and the absence of the long protein. The second, more highly conserved methionine is common to all three spliceforms of SLC4A11. If SLC4A11 is only expressed as the shortened protein, the expression of any particular spliceform at the RNA level may not affect SLC4A11 expressed at the protein level.

1.5.3 Loss of SLC4A11 Function in Disease

The corneal stroma is primarily an extracellular matrix comprised of lamellar arrangement of collagen fibrils (1). Individual collagen fibrils are separated by a matrix of proteoglycans (1). This high concentration of proteins gives rise to an osmotic gradient between the aqueous humor and stroma. In normal function, water moves osmotically from the aqueous humor, through the leaky barriers of the endothelial cell layer into the stroma (Fig. 1.1). SCL4A11 is involved in the movement of water from the stroma back to the aqueous humor (46). When SLC4A11 is mutated, it is retained in the ER. Mutations of SLC4A11 cause cases of CHED, HS and FECD (44, 45, 52-54, 60, 61, 65). These diseases have also been linked to

mutations of *COL8A2* (68), *LOXHD1* (69), *ZEB1* (11) and the transcriptional repressor TCF4 (70, 71).

The loss of functional SLC4A11 at the basolateral surface of the corneal endothelial cells disrupts the movement of water from the stroma causing edema in the stroma and a loss of corneal transparency (1). The depletion of SLC4A11 has been implicated in degeneration and apoptosis of corneal endothelial cells (72). Prior work in our lab has shown that mutant SLC4A11 does not induce apoptosis in HEK293 cells on its own (67). Instead, a recent study showed that HEK293 cells transfected with mutant SLC4A11 had decreased expression of antioxidant proteins rendering the cells more susceptible to oxidative stress (73).

Disease causing mutations of SLC4A11 display two molecular phenotypes: catalytically inactive and ER-retained (46). In both cases, SLC4A11 is unable to perform its role in water movement across the basolateral surface of the endothelial layer, resulting in development of symptoms of CHED, HS and FECD. Currently, there are 57 identified disease causing point mutations (45). Some ER-retained mutants of SLC4A11 could be rescued to the cell surface in HEK293 cells cultured at 30°C (43). Moreover, the rescued protein displayed functional activity upon rescue (67).

1.5.4 Oligomerization of SLC4A11

CHED, HS and FECD are caused by mutations in SLC4A11 leading to retention in the ER or catalytic inactivity, yet these diseases differ in their age of onset. CHED and HS are inherited in an autosomal recessive pattern and affected individuals experience onset of symptoms in the first decade of life (2, 60). Conversely, individuals who are heterozygous for a single CHED

mutation do not experience onset of symptoms. SLC4 family members express as dimers and SLC4A11 has been shown to express as dimers as well (43). The same study showed that CHED mutant monomers are capable of forming dimers with WT monomers, resulting in a dimer that traffics to the plasma membrane to a level of approximately 50% that of WT/WT dimers (43). Individuals suffering from FECD do not experience symptoms until the fourth decade of life (2). FECD monomers dimerized with WT monomers traffic to the plasma membrane at a rate of approximately 25% that of WT/WT dimers (43). FECD is inherited dominantly (6, 45) and the low rate of processing to the plasma membrane may explain late onset of symptoms in FECD.

Further study of the functional ability of heterozygous dimers found that CHED/WT dimers displayed approximately 60% of the functional ability of WT/WT dimers (67). This is in support of the fact that mutations of CHED are recessive (44, 53, 54) and that individuals who are carriers of these disease alleles do not experience symptoms. This also suggests that 60% of the functional ability of WT/WT dimers is needed to prevent onset of symptoms of these corneal dystrophies (67). FECD/WT dimers displayed approximately 25% of the functional ability of WT/WT dimers suggesting that only 25% of functional activity of WT/WT is needed to delay onset of the symptoms of corneal dystrophies (67). The results of these studies indicate that a treatment strategy aimed at rescuing mutant SLC4A11 from the ER would only need to achieve 25% of the functional ability of WT/WT dimers to delay the onset of disease symptoms and 60% to prevent the symptoms.

1.6 Cystic Fibrosis as a Model for Rescue from the ER

The link between SLC4A11 and endothelial corneal dystrophies has only recently been identified. One way to identify chemical therapies for these diseases is to look to other, more studied diseases for inspiration. A number of diseases are caused by ER retention of membrane proteins. Considerable research has focused on other diseases caused by misfolding mutations of membrane proteins. Perhaps the best studied of these diseases is cystic fibrosis (CF). CF is caused by mutations in the CF transmembrane conductance regulator (CFTR). CFTR is an ATP regulated anion channel located on the apical membrane of secretory epithelia of the airway, pancreas and intestines. Disease causing CFTR mutations result in viscous secretions that would otherwise be fluid (74). A result of this is chronic bacterial infections in the airway that can lead to death (75). There are currently >1900 known mutations possible to the CFTR gene (available at http://www.genet.sickkids.on.ca/cftr/app). The most common of these is a deletion of phenylalanine at amino acid 508 (F508del), which impairs the folding and trafficking of CFTR protein resulting in ER retention (76, 77). Several studies have screened potential correcting molecules for F508del in cellular models (78-80). Some small molecule correctors have been successful in rescuing F508del CFTR to the plasma membrane and are now used clinically (81, 82). Some of these molecules, such as VRT-325 and Corr-4a, have been created that are thought to directly interact with the CFTR protein (83).

There have also been screens of other readily available molecules (i.e. 4-phenylbutyrate, glafenine, ouabain, carbamazepine, curcumin, MG-132, etc.) that have yielded positive results with F508del CFTR (84-87). Some of these molecules have been traditionally used in other

capacities in human health and so are more likely to be approved relatively easily for human testing. The mechanisms of many of these molecules to restore CFTR to the cell membrane remain unknown. These seemingly unrelated compounds may have effects elsewhere in the cell that could not only restore misfolded proteins for CF but other diseases as well.

In addition to correcting the trafficking defect of CFTR, a subset of molecules has also shown efficacy to correct the trafficking of other ER-retained membrane proteins that are seemingly unrelated to CFTR. The sildenafil analog, KM11060, rescues the trafficking of F508del CFTR (88). In addition, KM11060 also rescues the trafficking defect in select mutants of the cardiac ion channel, hERG, the sulfonylurea receptor SUR1 and the vasopressin receptor V2R (87). Compounds that show the ability to rescue multiple mutant ER-retained membrane proteins represent very promising candidates as therapeutics for diseases caused by ER-retained membrane proteins.

1.7 Thesis Objectives

The objective of this thesis was to develop a high throughput screening assay for the detection of the level of SLC4A11 at the plasma membrane. SLC4A11 has only recently come into significant study but since its mutations cause endothelial corneal dystrophies, a large body of work has been produced in a short time. This thesis will draw on the literature to selectively screen certain mutants for rescue. Additionally, a small screen of molecules revealed a potential corrector for the trafficking defect of mutant SLC4A11.

Experimental materials and methods are detailed in Chapter 2. Chapter 3 discusses the development of a high throughput assay and the identification of a potential correcting

molecule for mutant SLC4A11. Chapter 4 summarizes the key findings of this thesis and provides future directions for moving toward a clinical treatment of these corneal endothelial dystrophies.

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Chapter 2: Materials and Methods

2.1 Materials

Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Q5® Site Directed Mutagenesis Kit was from New England Biolabs (Ipswich, MA). Dulbecco's Modified Eagle's Medium, fetal bovine serum (FBS), calf serum (CS), penicillin-streptomycin-glutamine (PSG), Geneticin and Amplex® UltraRed Reagent were from Life Technologies (Carlsbad, CA). Cell culture dishes were from Sarstedt (Montreal, QC). Complete protease inhibitor tablets were from Roche Applied Science (Indianapolis, IN). Immobilized Streptavidin Sepharose resin, sulfo-NHS-SS-biotin, glass coverslips and 10% formalin in phosphate buffer were from Thermo Fisher Scientific (Ottawa, ON). Poly-L-lysine was from Sigma–Aldrich (Oakville, ON). Hydrogen peroxide was from Ricca Chemical Company (Arlington, TX). Immobilon-P PVDF was from Millipore (Billerica, MA). Monoclonal antibodies against HA epitope (clone 16B12) and GAPDH were from Covance (Princeton, NJ) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin was from GE Healthcare Bio-Sciences Corp. (Piscattaway, NJ). Luminata TM Crescendo Western HRP Substrate chemiluminescence reagent was from Millipore (Billerica, MA). All compounds for screening were from Sigma-Aldrich (Oakville, ON), Thermo Fisher Scientific (Ottawa, ON) or Cayman

2.2 Methods

2.2.1 DNA constructs

The eukaryotic expression construct (pSKL1) for splicing variant 2 of human SLC4A11, encoding an 891 amino acid protein (NCBI reference: NG_017072.1) with an N-terminal

Hemagglutinin tag (HA-tagged) was described previously (1). A shortened version of this construct removing the first 35 amino acids of the protein and the HA tag, resulting in an expression construct (pAMC1) encoding an 856 amino acid protein, was created using the Q5® site directed mutagenesis kit. All subsequent modifications to pAMC1 were carried out with the Q5® Kit. Double HA-epitope tags were inserted at cDNA positions encoding amino acid 495 or 529 (amino acid 530 or 564 in NCBI reference: NG_017072.1, respectively), using HA tagged pAMC1 as template. For ease of reference, numbering conventions for point mutations and HA tags are the same as in the 891 amino acid protein. Expression constructs for the point mutations E143K (c.427G>A (p.Glu143Lys)), A269V (c.806>T (p.Ala269Val)) and G709E (c.2126G>A (p.Gly709Glu)) were created, using HA tagged pAMC1 as template. Expression constructs were confirmed by DNA sequencing (Institute of Biomolecular Design, Department of Biochemistry, University of Alberta).

2.2.2 Tissue culture

HEK293 cells were grown in complete DMEM (cDMEM, supplemented with 5% (v/v) FBS, 5% (v/v) CS and 1% (v/v) penicillin/streptomycin/glutamine) maintained at 37 °C in an air/5% CO_2 environment. HEK293 cells were transiently transfected, using the calcium phosphate method (2). All experiments using transiently transfected cells were carried out 48 h post-transfection. For experiments requiring treatment of cells, compounds made up in DMEM and DMSO solution were added 16-24 h before cell harvesting. To make stably transfected cell lines, HEK293 cells were transfected and grown in cDMEM/G (cDMEM, containing 0.75 mg/ml geneticin). Cell lines were monoclonally selected.

2.2.3 Cell lysis

HEK293 cells were solubilized in IPB (1% (v/v) IGEPAL CA-630, 5 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate (w/v), 10 mM Tris-HCl, pH 7.5), containing complete, EDTA-free, protease inhibitors (Roche Applied Science, IN, USA) and incubated for 20 min on ice. Samples were centrifuged at 13,400 g for 10 min and the resulting supernatant was isolated and stored at -20 ˚C.

2.2.4 Poly-L-Lysine coating of culture dishes and coverslips

96 well plates (Sarstedt, QC) or 25 mm round glass coverslips (Thermo, ON) in a 100 mm dish (Sarstedt, QC) were coated with poly-L-lysine (Sigma-Aldrich, ON), using sterile solutions in a cell culture hood. NaOH (150 µl per well of 5 M) was added to 96 well plates, which were incubated for 15 min. NaOH was then removed and wells were washed with H_2O. Ethanol (150 µl per well of 95%) was added for 5 min. Ethanol was removed and wells were rinsed with H_2O. Wells were washed twice with PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and then 100 µl per well of 1 mg/ml poly-L-lysine in PBS was added for 15 min. After 15 min the plates with poly-L-lysine solution remaining in wells were left overnight under ultraviolet light. Any remaining solution was removed the next day and the plates were stored at 22 °C. Before use, plates were rinsed with PBS to remove dried salts.

2.2.5 Amplex Red High Throughput Assay

This assay is a modified version of one previously described (3, 4). Stably transfected cells were grown in T75 flasks to a confluency of approximately 90%. Growth medium was

removed and the cells were rinsed with PBS. Cells were removed from the flask using 5 ml of 0.5 mM EDTA in PBS per flask and transferred to a 15 ml conical tube. Cells were centrifuged at 1000 g (2481 rpm, Beckman GS-6K centrifuge) for 5 min. The 0.5 mM EDTA in PBS was removed and the cell pellet resuspended in 5 ml cDMEM/G. Cells were counted with a hemocytometer and plated onto a poly-L-lysine coated 96 well plate at a cell density of 2.2 x 10^5 cells per well in 150 µl with cDMEM/G/well and the cells were placed in a 37 °C incubator for 3 h to allow the cells to settle on the plate. After 3 h, cells were treated with compounds for screening and returned to the 37 °C incubator for a period of 18-24 h. Compounds for testing were added to each well of the plate in 50 µl of cDMEM/G, containing 0.4 or 0.8% (v/v) DMSO, where indicated.

Using a multi-channel pipetter, cells were rinsed with 200 µl/well of PBSCM (PBS, containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$). Cells were then fixed with 10% formalin in phosphate buffer for 10 min at 22 °C and then quenched with 150 µl/well of 50 mM NH_4Cl in PBS for 10 min at 22 °C. Cells were then blocked with 150 µl/well of PBS-B (1% (w/v) BSA in PBS) for 5 min at 22 °C and then incubated with monoclonal mouse anti-HA antibody (16B12) at 1:1000 dilution in PBS-B for 1 h at 22 °C. Cells were rinsed three times with 200 µl/well PBS and incubated with sheep anti-mouse IgG conjugated to horseradish peroxidase (NXA931) at 1:1500 dilution in PBS-B for 1 h at 22 °C. Cells were rinsed three times with 200 µl/well PBS. Amplex UltraRed stock solution was made to 10 mM in DMSO and frozen at -20 °C. Amplex Red working solution (200 µl/well of 50 µM Amplex UltraRed, 0.0068% H_2O_2 in PBS) was added. Plates were placed on ice and covered with aluminum foil for 10 min after which, 150 µl of the resulting solution was collected and dispensed into a black opaque 96 well plate (Sigma-Aldrich, Greiner

CELLSTAR®). After a 5 s shaking step, fluorescence of the solution was read in a SynergyMX Plate Reader (BioTek, VT) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm at 22 ˚C.

2.2.6 Immunoblotting

Cell lysates were prepared in 2x SDS-PAGE sample buffer (10% (v/v) glycerol, 2% (w/v) SDS, 0.5% (w/v) bromophenol blue, 75 mM Tris, pH 6.8). Before electrophoresis, lysates were made to 1% (v/v) 2-mercaptoethanol and heated for 5 min at 65 °C. Samples were then resolved by SDS-PAGE on 7.5% (w/v) acrylamide gels (5). Proteins were electrotransferred onto Immobilon-P PVDF membranes (Millipore, MA). Mouse anti-HA, or mouse anti-GAPDH were used at 1:2,000 or 1:4,000 dilution, respectively in TBS-TM (5% skim milk powder in TBS-T: 0.1% (v/v) Tween-20, 0.15 M NaCl, 50 mM Tris, pH 7.5). After incubation for one hour at 22 °C with sheep anti-mouse HRP-conjugated secondary antibody at 1:4,000 dilution in TBSTM, immunoblots were developed, using Luminata TM Crescendo Western HRP Substrate chemiluminescence reagent and visualized, using an ImageQuant LAS 4000 (GE Healthcare Life Sciences, ON). Densitometry was performed, using ImageQuant TL 1D software, v8.1 (GE Healthcare Life Sciences, ON).

2.2.7 Cell surface biotinylation assay

Transfected or stable cells were rinsed with PBS and washed with 4 °C borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM $CaCl_2$, 10 mM boric acid, pH 9.0). Cells were then incubated with Sulpho-NHS-SS biotin (0.5 mg/ml in borate buffer) for 30 min on ice. Cells were incubated with quenching buffer (192 mM glycine, 25 mM Tris, pH 8.3) three times for 5 min. Cells were

solubilized in 500 µl IPB buffer, containing protease inhibitors for 20 min on ice. Samples were centrifuged at 13,400 g for 10 min at 22 °C. Supernatant was recovered and split into two equal fractions. One fraction was reserved for later SDS-PAGE analysis (total protein, T). Immobilized streptavidin Sepharose (100 µl of 50% suspension) was added to the other fraction of lysate and placed on a rotator and left to incubate at 4 °C overnight. After incubation, the sample was centrifuged at 9800 g for 2 min and the supernatant collected (unbound protein, U). Both U and T fractions were processed for SDS-PAGE analysis and immunoblotting and densitometry as described. The formula (U-T)/T x 100% was used to determine percentage of protein biotinylated.

2.2.8 Osmotically-driven water flux assay

HEK293 cells were grown on poly-L-lysine-coated 25 mm round glass coverslips and co-transfected with cDNA encoding enhanced green fluorescent protein (GFP) (peGFP-C1 vector; Clontech, CA) and, pcDNA 3.1 (empty vector) or the indicated SLC4A11 plasmid constructs in a 1:8 molar ratio. Twenty-four h post-transfection, cells were treated with 800 µl 67.5 µM glafenine in 2.7% DMSO (v/v) resulting in a final concentration of 5 µM glafenine in 0.2% DMSO (v/v). Untreated cells received DMSO to a final concentration of 0.2%. Fourty-eight h later, coverslips were mounted in a 35 mm diameter Attofluor Cell Chamber (Molecular Probes) and washed with PBS. During experiments, the chamber was perfused with isotonic MBSS buffer (90 mM NaCl, 5.4 mM KCl, 0.4 mM $MgCl_2$, 0.4 mM $MgSO_4$, 3.3 mM $NaHCO_3$, 2 mM $CaCl_2$, 5.5 mM glucose, 100 mM D-mannitol and 10 mM HEPES, pH7.4, 300 mOsm/kg) and then with hypotonic (200 mOsm/kg) MBSS buffer, pH 7.4 (same composition as previous but lacking D-mannitol).

Solution osmolarity was measured using an osmometer (Advance Instruments Inc., Norwood, MA). The chamber was mounted on the stage of a Wave FX Spinning Disc Confocal Microscope (Quorum Technologies, ON), with a Yokogawa CSU10 scanning head. The microscope has a motorized XY stage with Piezo Focus Drive (ASI, MS-4000 XYZ Automated Stage) and a live cell environment chamber (Chamlide, Korea), set to 24 °C during the duration of the experiment. Acquisition was performed with a Hamamatsu C9100–13 Digital Camera (EM-CCD) and a 20x objective during excitation with a laser (Spectral Applied Research, ON) at 491 nm. GFP fluorescence, collected though a dichroic cube (Quorum Technologies, ON) at wavelengths 520–540 nm, was acquired at 1 point sec^{-1} for 4 min. Quantitative image analysis was performed by selecting a region of interest for each HEK293 cell with Volocity 6.0 software (PerkinElmer, ON). Following the switch to hypotonic MBSS buffer, the rate of fluorescence change was determined from the initial 15 s of linear fluorescence change.

2.2.9 Statistical analysis

Values are expressed as mean ± standard error of measurement. Statistical analyses were performed, using Prism software (Graphpad v5). Groups were compared with one-way ANOVA and unpaired *t*-test with $P < 0.05$ considered significant.

2.3 References

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Chapter 3: Identification of Chemical Correctors for the Trafficking Defect in Mutant SLC4A11

3.1 Introduction

Approximately 93,000 single amino acid mutations have been identified that cause human disease (1). The majority of these affect protein folding and trafficking (2), including 1900 mutations in the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene (3). The most common is deletion of phenylalanine 508 (F508del), which impairs the folding and trafficking of CFTR protein, resulting in endoplasmic reticulum (ER)-retention (4, 5). Amongst genetic diseases of membrane proteins, the most common phenotype is protein ER-retention, secondary to protein misfolding. Potential small molecules correcting F508del folding have been identified to rescue F508del CFTR to the plasma membrane (6-8) including one that has entered recent clinical use (9, 10).

Small molecule folding correctors are thus proven to be effective therapeutics for ER-retained membrane proteins. Development of assays amenable to high throughput drug screening are the essential first step toward identification of folding corrector drugs. Here we have targeted for folding correction an integral membrane protein, SLC4A11, whose mutations cause ER-retention or catalytic inactivity (11, 12). SLC4A11 is an attractive therapeutic target as its mutations cause blindness because of corneal defects. The relative accessibility of the cornea opens the possibility to apply small molecule folding correctors as eye drops, meaning that the treatment approach is relatively facile and treatment could be targeted to the site of disease.

In human cornea, high solute concentration of the stromal layer creates an osmotic gradient that draws water from the aqueous humor (13). This osmotic gradient is opposed by water reabsorption into the aqueous humor by the endothelial monolayer. Posterior

endothelial corneal dystrophies develop when this reabsorption is interrupted (13), giving rise to an accumulation of fluid in the stroma and abnormal formations on the endothelial layer (gutatta). The edematous corneas develop a ground-glass appearance, leading to vision loss and eventual blindness.

Congenital Hereditary Endothelial Dystrophy (CHED; MIM 217700) (14) Harboyan Syndrome (HS; MIM 217400) (15) and Fuchs' Endothelial Corneal Dystrophy (FECD; MIM 136800) (16) are three forms of genetic corneal blindness that arise in some cases from mutations in the integral membrane protein, SLC4A11 (11, 17, 18). HS patients present with sensorineuronal hearing loss in addition to progressive blindness (13). CHED and HS are recessive, whereas FECD is dominant with a 4% prevalence in North America (13). These diseases have also been linked to mutations of *COL8A2* (19), *LOXHD1* (20), *ZEB1* (21) and the transcriptional regulator, TCF4 (22, 23). The only effective treatment for these diseases is corneal transplant (13), with FECD alone accounting for 10-25% of all corneal transplants in North America (13). Tissue availability and compatibility with the recipient are major limitations of this treatment strategy. Alternative treatments could have a significant impact on not only quality of life of those suffering from these diseases, but also make available corneal tissues to treat other diseases.

SLC4A11 is a member of the SLC4 family of bicarbonate transporters, but does not transport bicarbonate (24). Plant SLC4A11 orthologs are established borate transporters (25). Human SLC4A11 was originally reported to be a Na^+-coupled borate transporter (24), but other groups have not been able to replicate this finding (26, 27). Instead, SLC4A11 has been found to facilitate Na^+/OH^- transport (28), NH_3^+/H^+ co-transport (29), and electrogenic $H^+(OH^-)$

permeation (30). Human SLC4A11 mediates water movement when expressed in *X. laevis* oocytes and HEK293 cells (31). Depletion of SLC4A11 leads to degeneration and apoptosis of corneal endothelial cells (28). Mutant SLC4A11 does not induce apoptosis in HEK293 cells on its own (32). One report found that HEK293 cells transfected with mutant SLC4A11 decrease expression of antioxidant proteins, rendering the cells more susceptible to oxidative stress (33). Finally, three independent *slc4a11*$^{-/-}$ mouse lines replicate the corneal edema found in human disease (34-36), indicating that loss of SLC4A11 function causes corneal disease.

Disease causing mutations of SLC4A11 display two molecular phenotypes: catalytically inactive and ER-retained (31). In both cases, SLC4A11 is unable to perform its role in transport across the basolateral surface of the endothelial layer, resulting in development of symptoms of CHED, HS and FECD. Approximately 60 disease-causing point mutations of human SLC4A11 have been identified (11, 12, 17, 18, 37-50). Some ER-retained mutants of SLC4A11 could be rescued to the cell surface in cells cultured at 30 ˚C, suggesting a temperature-sensitive folding defect (51). Moreover, the rescued protein displayed functional activity upon rescue (32), suggesting that rescue from the ER is a promising therapeutic approach.

CHED, HS and FECD differ in their genetics of inheritance and age of onset, which in part is explained by SLC4A11 dimerization. CHED and HS show autosomal recessive inheritance with symptom onset in the first decade of life (13, 18). SLC4A11 is dimeric and CHED mutant/wildtype (WT) heterodimers traffic to the cell surface, enabling sufficient traffic of WT protein to the cell surface to prevent disease symptoms in carriers (51). The recessive ER-retention phenotype of CHED SLC4A11 explains this disease's inheritance. Individuals with FECD do not experience symptoms until the fourth or fifth decade of life (13). FECD SLC4A11/WT

heterodimers do not traffic to the cell surface, indicating that the FECD protein phenotype is dominant over WT (51). Examination of the level of cell surface transport activity in cells mimicking CHED carrier (unaffected), CHED affected (early onset symptoms) and FECD affected (late onset symptoms) states revealed that 25% of the functional activity of unaffected individuals will delay the onset of disease symptoms and 60% will prevent corneal dystrophy symptoms (32).

In this study, we developed an assay amenable to high throughput screening to identify small molecule correctors of SLC4A11 misprocessing defect. This screen was modified from one used to identify correctors of F508del CFTR (52, 53). To assess further the ability of the technique to identify SLC4A11 therapeutics, we performed a small scale screen of potential correctors. One candidate compound was characterized and found to show a promising ability to rescue SLC4A11 cell surface functional activity.

3.2 Results

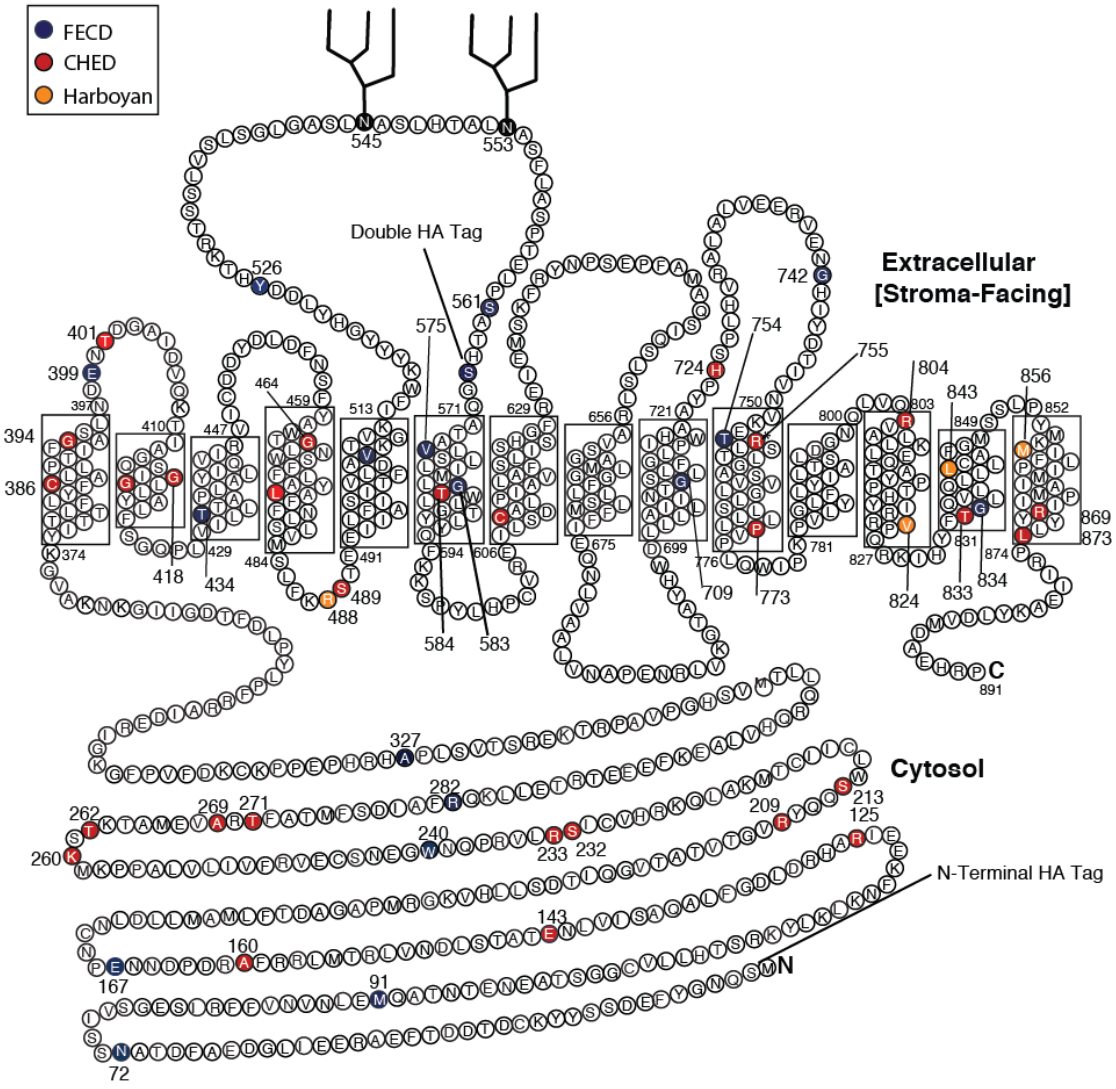
3.2.1 Amplex red high throughput assay

A chemical therapy to treat corneal endothelial dystrophies associated with missense mutations of SLC4A11 requires rescue of the ER-retained, mutant SLC4A11 protein to the plasma membrane. Accurate measurement of the level of SLC4A11 at the cell surface is needed to identify suitable chemical correctors. Additionally, the method to screen compounds should be high throughput to enable screening many compounds at once. Existing techniques such as cell surface biotinylation (54) can measure the level of protein at the plasma membrane, but are time and material intensive. An easily accessible, reliable and high throughput method to detect SLC4A11 at the cell surface is needed. We developed a high throughput method to identify HA-tagged SLC4A11 at the plasma membrane, which can be applied to any epitope-tagged plasma membrane protein. This assay was inspired by an assay to measure movement of CFTR-F508del to the cell surface (53).

The first step was to insert an epitope tag into an extracellular position, allowing detection of protein at the cell surface in intact cells. Positions for epitope insertion were selected based on proteolytic analysis of the extracellular loops (55). Double hemagglutinin epitope (HA) tags were inserted into extracellular loop three at amino acid 530 or 564 (Fig. 3.1A).

To assess SLC4A11 function, HEK293 cells were co-transfected with cDNA encoding GFP and cDNA encoding SLC4A11 constructs. Cells were perfused with iso-osmotic medium and then hypo-osmotic medium. GFP fluorescence was monitored

A


FECD
CHED
Harboyan
Double HA Tag
Extracellular
[Stroma-Facing]
Cytosol
N-Terminal HA Tag
N
C

B

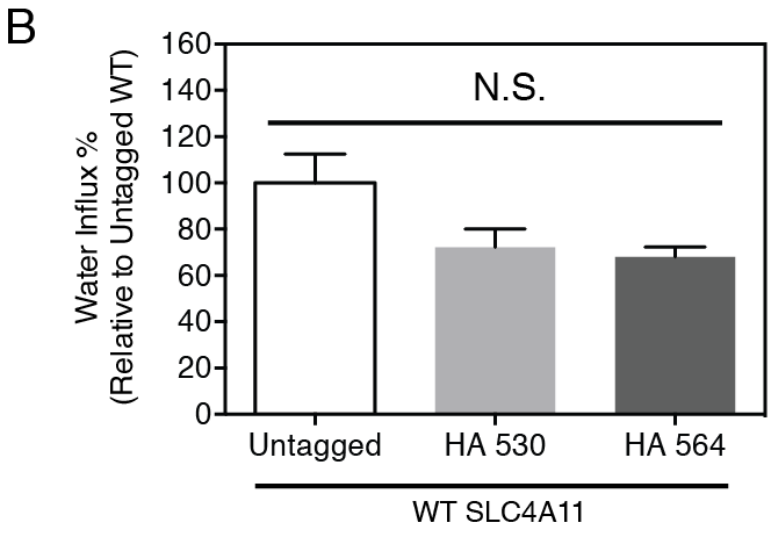

Water Influx %
(Relative to Untagged WT)
N.S.
Untagged
HA 530
HA 564
WT SLC4A11

Figure 3.1 SLC4A11 topology model and characterization of epitope-tagged SLC4A11. A) SLC4A11 topology model established by *in situ* limited proteolysis and immunolocalization of the C-terminus (55). Mutations associated with FECD (blue), CHED2 (red) and HS (orange) are highlighted. Position of double HA epitope tag is indicated following H564. Structures at amino acids 545 and 553 mark glycosylation sites. B) Osmotically driven water influx activity of HEK293 cells transiently co-transfected with cDNA encoding GFP and WT SLC4A11 and SLC4A11 with double HA tag insertion following H530, or H564, or vector. GFP fluorescence was monitored in a region of interest in cells subjected to a shift to hypo-osmotic medium. The initial rate of fluorescence decrease following shift to hypo-osmotic medium (a surrogate for cell swelling) was measured as the rate of cell swelling. Data were corrected for the rate in vector transfected cells (41% ± 3% relative to non-corrected WT) and normalized to WT SLC4A11. Data represent the mean ± SEM water flux of 3–6 independent experiments of 10-20 cells per coverslip. N.S.: not significant.

as a measure of cytosolic GFP concentration (31). The rate of fluorescence change, which is a surrogate for cell volume change, was measured and corrected for the rate found in vector-transfected cells (Fig. 3.1B) (31). Water fluxes of cells expressing WT and HA-tagged SLC4A11 were not significantly different (Fig. 3.1B). These data reveal that insertion of a double HA-tag did not alter functional activity of the two mutants, indicating that their folding is not compromised by epitope tag insertion.

HEK293 cells, stably expressing HA-tagged SLC4A11 variants, were established for the assay. The first had an N-terminal HA tag (on the cytoplasmic domain), allowing it to be used as a control for detection of accessible cytosolic epitopes (Fig. 3.1A). The second had the double HA-tag following H564, to detect cell surface protein. The remaining three cell lines express the SLC4A11 disease alleles A269V (CHED), E143K (CHED) and G709E (FECD) (12, 39, 41), along with a double HA tag after H564. These three mutations were chosen because culture of these mutants in cells at 30 ˚C induced partial rescue to the cell surface, suggesting that these proteins had the potential to be cell-surface rescued by small molecules (55).

In this high throughput assay (HTA) cells stably expressing SLC4A11 variants are cultured in 96 well dishes. Incubation with anti-HA antibody allows binding of cell surface HA epitopes. The primary antibody is detected by a secondary antibody, conjugated to horse radish peroxidase (HRP). Amplex red reagent, in the presence of H_2O_2, is cleaved by HRP to produce resorufin, a highly fluorescent red compound (Fig. 3.2) (56). Red fluorescence is then quantified on a 96 well plate fluorescence reader.

We first assessed whether the HTA can differentiate between cell surface and ER-retained SLC4A11. The maximum resorufin fluorescence was found for cells

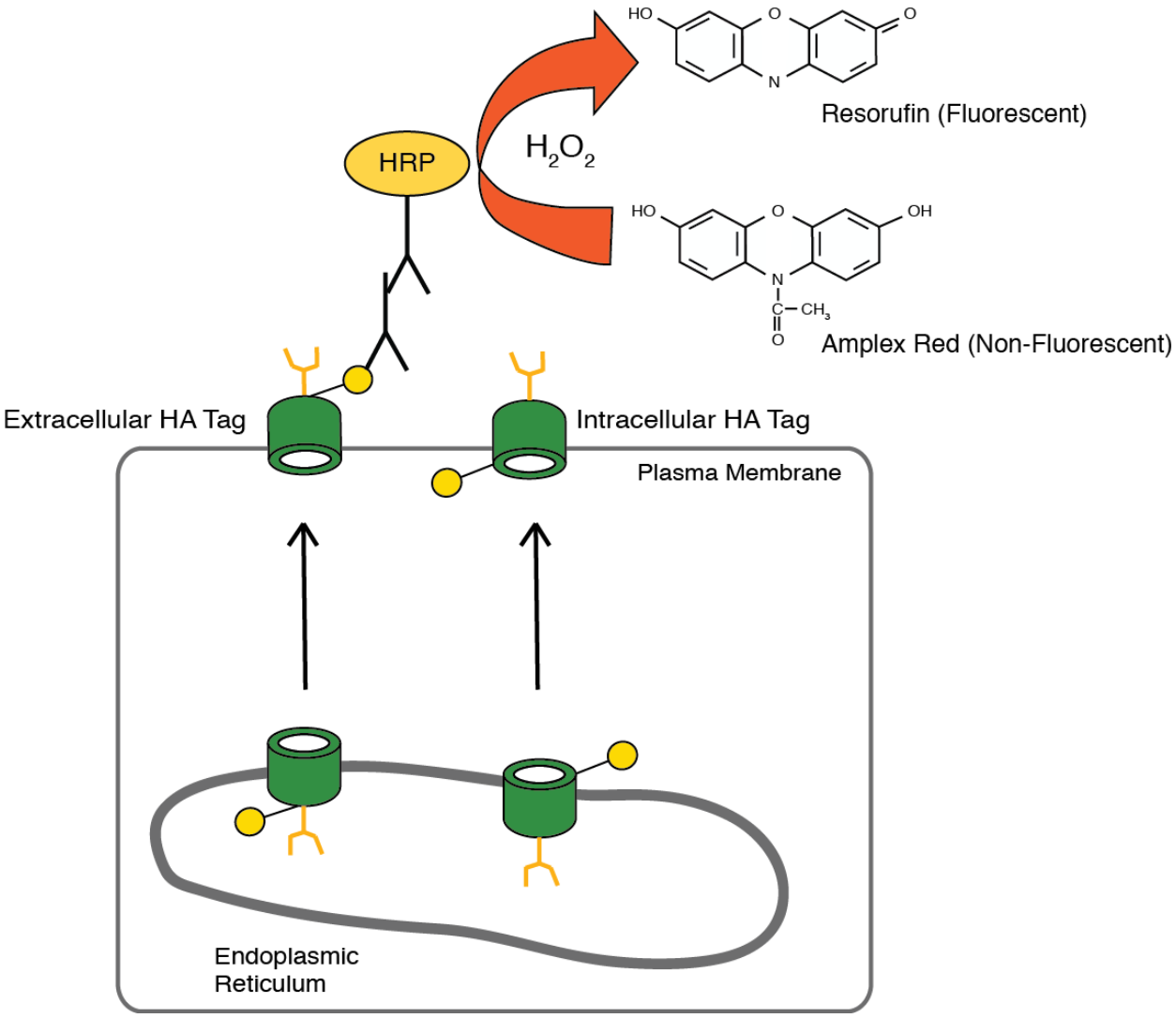


Figure 3.2 Diagramatic representation of Amplex Red High throughput Assay (HTA). HA epitope (yellow circle) can be placed at the cytoplasmic N-terminus of SLC4A11 (right) or in an extracellular loop (left). With either tag, WT SLC4A11 traffics to the plasma membrane. Only protein with an extracellular (external) HA tag is detected by a mouse anti-HA antibody (black Y) and is in turn detected by a sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP). Amplex red, in the presence of hydrogen peroxide and HRP is cleaved to produce resorufin, a highly fluorescent red product.

expressing WT SLC4A11 with extracellular HA tag, a value that was used to normalize the fluorescence level for the other variants for screening. Cells stably expressing SLC4A11 were created for use with the HTA (Fig 3.3A). Cells expressing N-terminally (internal) HA-tagged WT SLC4A11 had a fluorescence signal of 19% ± 3% of the externally HA-tagged WT (HA 564) (Fig. 3.3B). Since the HA tag of the N-terminally tagged WT is not present at the cell surface, this represents non-specific background of the assay. The A269V line gave a fluorescent signal of 62% ± 13% relative to externally tagged WT. A269V SLC4A11 partially traffics to the plasma membrane at levels approximately 50% of WT (55). E143K and G709E mutant lines both gave fluorescent signals of 24% ± 3%, which is indistinguishable from N-terminal HA tag WT. This was expected as both mutants are ER-retained (12, 51, 55).

To assess the validity of the cell surface processing measurements determined by the HTA, we also measured cell surface expression, using cell surface biotinylation. In these assays cells were treated with membrane-impermeant biotinylating reagent, and the fraction of biotinylated protein (extracellular-accessible) was determined (Fig. 3.3C). Cytosolic GAPDH served as a negative control for the assay and its level of biotinylation was subtracted from the values for SLC4A11 variants. Relative to WT SLC4A11, 190% ± 2%, 54% ± 3%, 0% ± 8% and 0% ± 7% protein was biotinylated for External Tag WT, A269V, E143K and G709E respectively.

To compare the level of surface SLC4A11 determined by the HTA and cell surface biotinylation, the background value was subtracted from the data in each assay. That is, in the HTA (Fig. 3.3B) fluorescence observed for cells expressing N-terminally HA-tagged WT SLC4a11 was subtracted and in cell surface biotinylation, the level of biotinylation by cytosolic

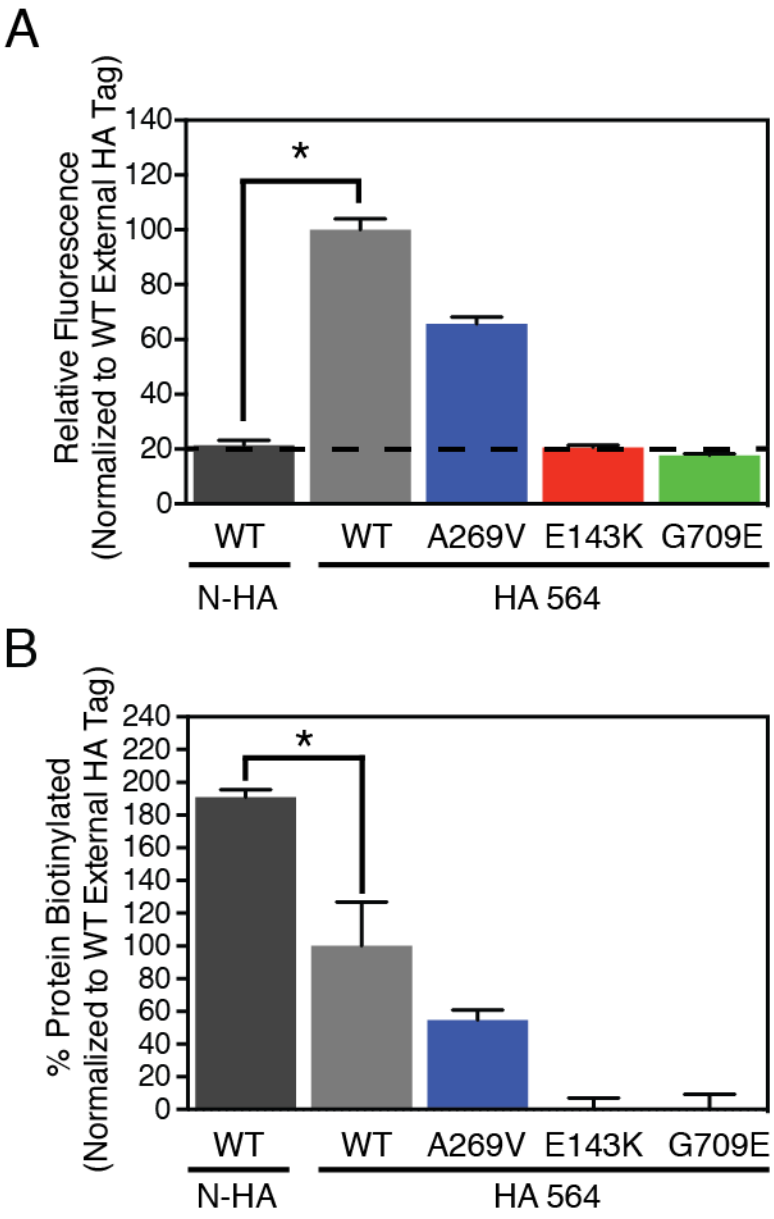


Figure 3.3 Comparison of cell surface SLC4A11 level as measured by high throughput assay (HTA) and cell surface biotinylation. A) In the HTA protocol, HEK293 cells stably expressing the indicated SLC4A11 variants (with an N-terminal cytosolic HA tag (N-HA), or with an external double HA tag (HA 564)) and processed through the HTA. Cells were incubated with amplex red reagent, which in the presence of HRP/H_2O_2, is converted to the fluorescent red product, resorufin. Red fluorescence in each well was quantified on a 96 well plate fluorescence reader.

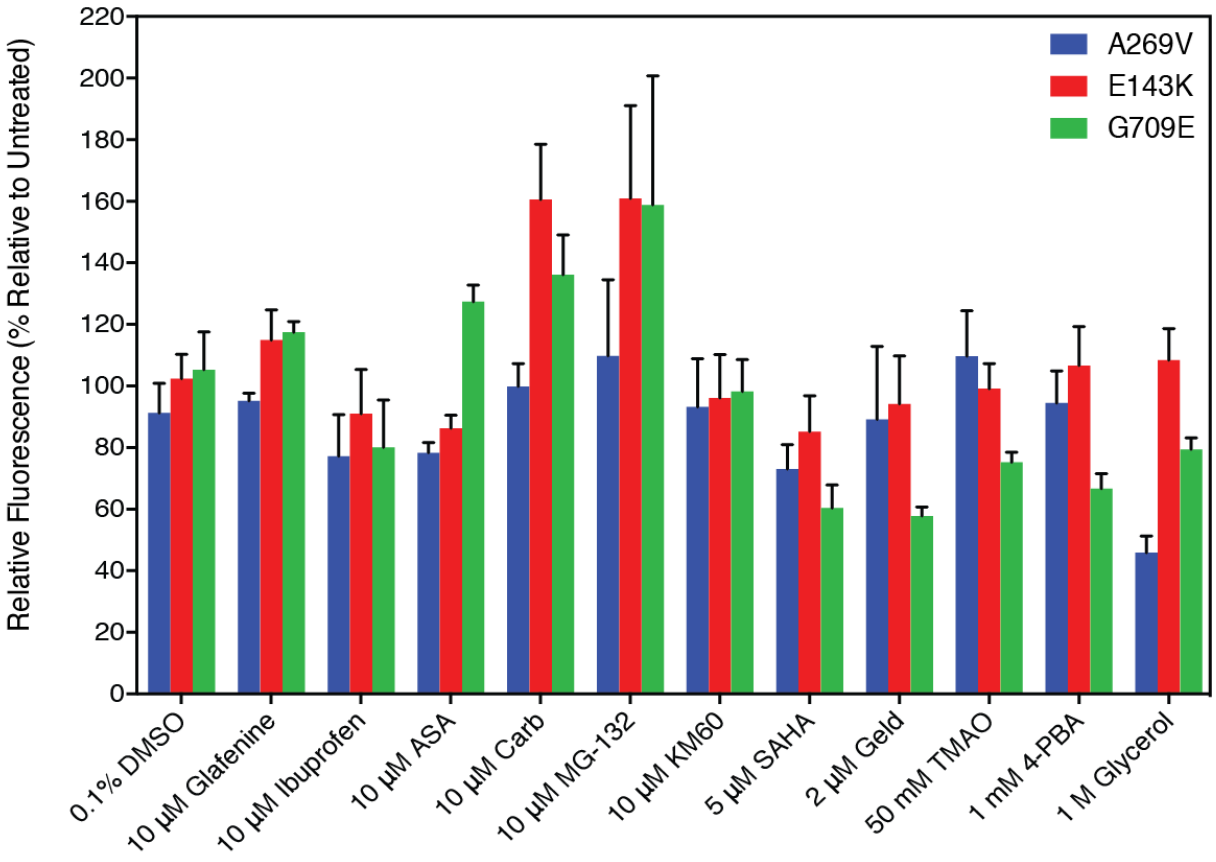
Data were normalized to HA 564 WT. *: significant difference ($P < 0.05$). Data represent the mean ± SEM of fluorescence relative to HA 564 WT (n = 8 wells). Dashed line indicates the fluorescence level of cells expressing N-terminally tagged SLC4A11, representing the background level of the assay. B) Cell surface biotinylation assay of cells stably expressing the indicated SLC4A11 variants. HEK293 cells were incubated with membrane-impermeant biotinylating reagent. Cell lysates were incubated with streptavidin Sepharose to remove the biotinylated proteins. The fraction of biotinylated protein, representing cell surface protein, was quantified. The level of biotinylation of GAPDH (6% ± 11%), representing the background of the assay, was subtracted from all values. Data represent the mean ± SEM of % total protein biotinylated (n = 3).

GAPDH was subtracted. Relative cell surface processing values (Fig. 3.3B, C) were compared for the two techniques. In each case (each of the extracellular tagged WT, A269V, E143K and G709E) the relative cell surface level obtained for each variant was not significantly different when data from the two methods were compared. The N-terminal HA-tagged WT stable cell line displays a significantly higher biotinylation compared to HA 564 WT cell line, suggesting that the extracellular tag affected processing efficiency (Fig. 3.3C). Together these results indicate that the HTA reliably quantifies the fraction of SLC4A11 at the cell surface.

3.2.2 Small-scale screen of folding corrector drugs

We next examined the ability of the HTA to identify chemical correctors of mutant SLC4A11 misfolding. We thus carried out a small screen, focused on compounds effective in rescue of ER-retained CFTR mutants (57-65). Concentrations of each of the compounds screened were selected on the basis of the effective concentrations for CFTR rescue. Acetylsalicylic acid has not been previously examined in the literature. We chose to test acetylsalicylic acid because it is a non-steroidal anti-inflammatory drug (NSAID) and other NSAIDs rescue some ER-retained proteins to the plasma membrane (57, 61, 65). To emulate a large scale screen, compounds were initially screened with four replicates on a single plate (Fig. 3.4). Because this was a small-scale screen, compounds were added manually, using a multi-channel pipetter, whereas a large screen would make use of robotic plating. A269V, E143K and G709E mutant stable cells were treated with each of 11 different small molecules, and with solvent carrier (0.1% or 0.2% DMSO) (Fig. 3.4). DMSO was used at two concentrations since

A


Relative Fluorescence (% Relative to Untreated)
220
200
180
160
140
120
100
80
60
40
20
0
A269V
E143K
G709E
0.1% DMSO
10 µM Glafenine
10 µM Ibuprofen
10 µM ASA
10 µM Carb
10 µM MG-132
10 µM KM60
5 µM SAHA
2 µM Geld
50 mM TMAO
1 mM 4-PBA
1 M Glycerol

B

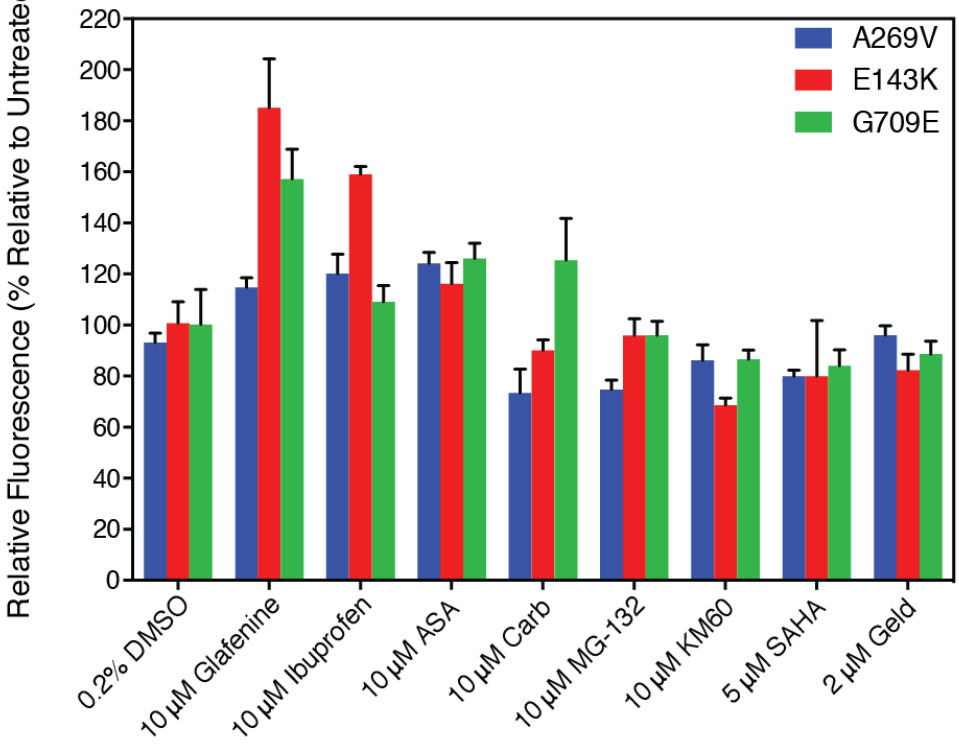

Relative Fluorescence (% Relative to Untreated)
220
200
180
160
140
120
100
80
60
40
20
0
A269V
E143K
G709E
0.2% DMSO
10 µM Glafenine
10 µM Ibuprofen
10 µM ASA
10 µM Carb
10 µM MG-132
10 µM KM60
5 µM SAHA
2 µM Geld

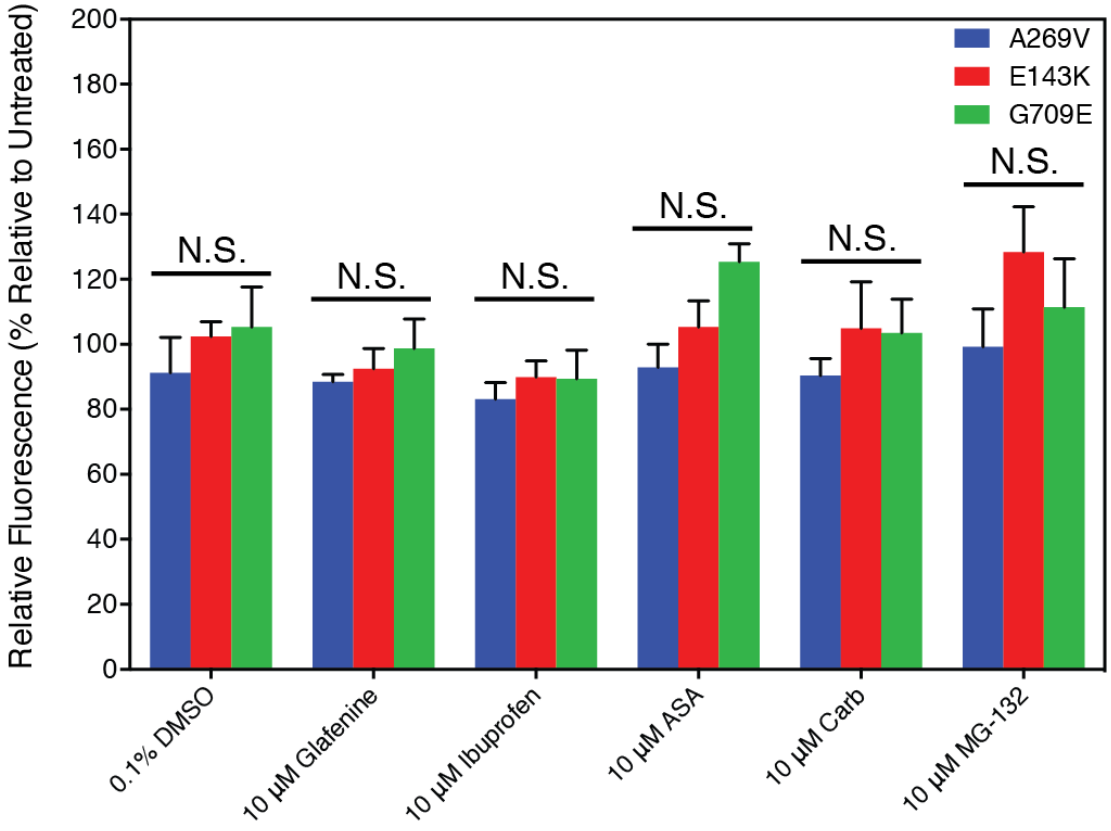
Figure 3.4 A small-scale screen of potential SLC4A11 folding corrector compounds. In the HTA assay protocol, cells stably expressing the indicated SLC4A11 mutants were treated with compounds at indicated concentrations for a period of 18-24 hours. Assays were carried out in the presence of A) 0.1% DMSO (except TMAO, 4-PBA and glycerol, which were screened in the absence of DMSO) or B) 0.2% DMSO. Red fluorescence arising from amplex red conversion to resorufin, was measured on a 96 well plate reader. Data represent the mean ± SEM of fluorescence relative to the level found for each untreated variant, (n = 4). *: significant difference ($P < 0.05$). ASA – acetylsalicylic acid, Carb – carbamazepine, Geld – geldanamycin.

high concentrations of DMSO act to assist folding of some misfolded proteins (66). We wanted to determine whether there might be a synergistic effect of low concentration DMSO and other small molecules. DMSO at 0.1% and 0.2% did not significantly affect cell surface levels of SLC4A11 mutants, as assessed by the HTA (Fig. 3.4A, B).

In this initial screen, most compounds gave results clustering around 100% of untreated cell surface expression (Fig. 3.4). Five compounds, glafenine, ibuprofen, acetylsalicylic acid, carbamazepine and MG-132, stood out as leading to mutant SLC4A11 cell surface abundance about 1.5 fold higher than in untreated cells. Reflecting the limitations of this HTA, these compounds did not show significant difference from untreated controls for each mutant when subjected to screening on a single plate. These three compounds were subjected to additional screening to determine whether their effect on cell surface processing was significant (Fig. 3.5).

Glafenine (10 μM) in the presence of 0.2% DMSO led to a significant increase in relative fluorescence for all three mutants tested, compared to 0.2% DMSO-treated cells (Fig. 3.5). Ibuprofen (10 μM) in 0.2% DMSO only had a significant effect on A269V and E143K. Acetylsalicylic acid (10 μM) in 0.2% DMSO only had a significant effect on A269V. Most compounds screened had no significant effect on HTA-measured cell surface processing for any of the mutants (Fig. 3.4 and 3.5). These results show that the HTA can discriminate between compounds that affect the cell surface level of SLC4A11 mutants from those that do not. Because glafenine treatment gives rise to an increase in relative fluorescence for all three of the mutants, we moved forward with this compound for further experiments.

A


Relative Fluorescence (% Relative to Untreated)
200
180
160
140
120
100
80
60
40
20
0
A269V
E143K
G709E
N.S.
N.S.
N.S.
N.S.
N.S.
N.S.
0.1% DMSO
10 µM Glafenine
10 µM Ibuprofen
10 µM ASA
10 µM Carb
10 µM MG-132

B

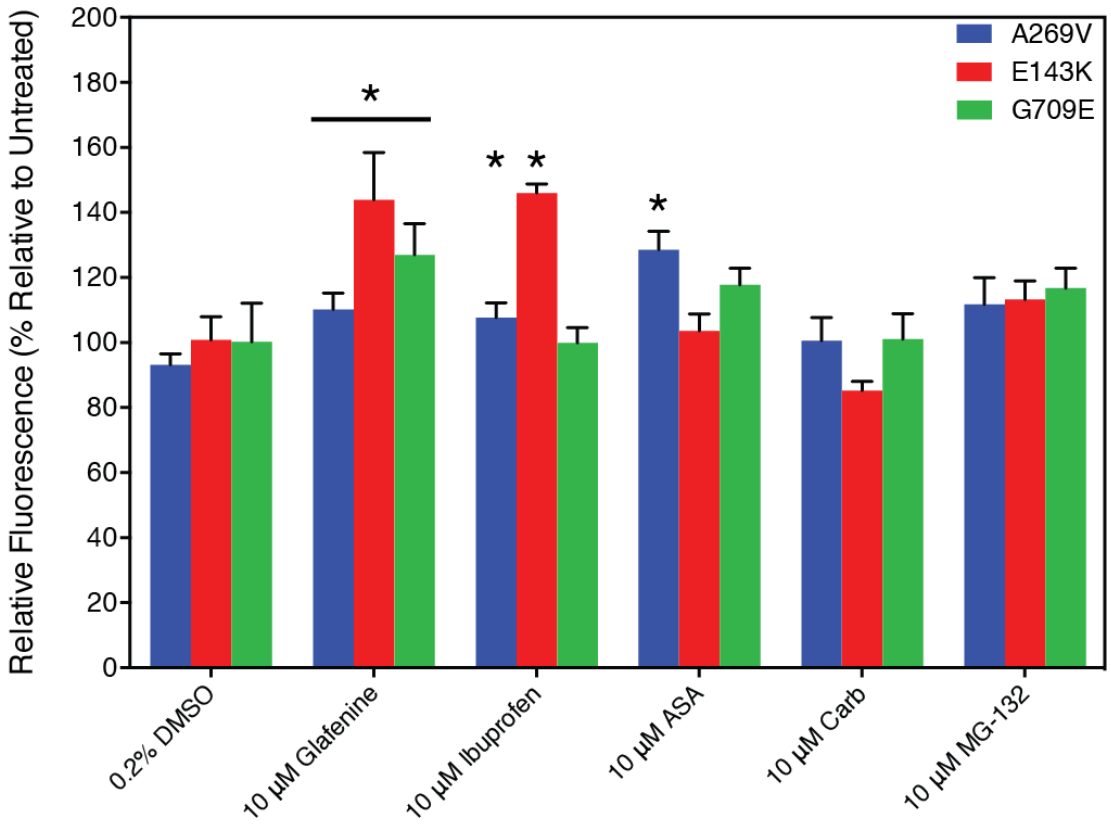

Relative Fluorescence (% Relative to Untreated)
200
180
160
140
120
100
80
60
40
20
0
A269V
E143K
G709E
*
* *
*
0.2% DMSO
10 µM Glafenine
10 µM Ibuprofen
10 µM ASA
10 µM Carb
10 µM MG-132

Figure 3.5 High-repetition screening of compounds identified in preliminary screen. HTA was used to assess the level of SLC4A11 cell surface abundance in the presence of A) 0.1% or B) 0.2% DMSO. Data represent the mean ± SEM of fluorescence relative to the level found for each untreated variant, (n = 12). *: significant difference ($P < 0.05$).

3.2.3 Dose-response to glafenine of G709E-SLC4A11 trafficking

The HTA suggested that glafenine, when applied in 0.2% DMSO was effective at increasing the cell surface abundance of ER-retained SLC4A11 mutants. To further probe the effect of glafenine on mutant SLC4A11, we established the relationship for the compound's effects. We tested G709E-SLC4A11 as this mutant is profoundly affected, showing nearly complete ER-retention (Fig. 3.3). Stable cells expressing the G709E mutant were treated with 0-20 µM glafenine, and the amount of fluorescence produced in the HTA was measured. The dose-response curve displayed classical saturating kinetics, revealing that G709E-SLC4A11 was rescued with an EC_{50} of 1.5 ± 0.7 µM (Fig. 3.6).

3.2.4 Effect of glafenine on SLC4A11 cell surface trafficking

To assess further the effect of glafenine on mutant SLC4A11, we measured cell surface processing, using the cell surface biotinylation assay (Fig. 3.7). For this experiment, we used transiently transfected cells rather than stable cell lines, to provide an assessment in a different cell context. Transfected cells were treated with or without 5 µM glafenine with 0.2% DMSO 24 h prior to biotinylation. Cells not treated with glafenine were incubated with 0.2% DMSO solvent carrier. For all three mutants, a significant difference of biotinylation was found between the untreated and treated cohorts (Fig. 3.7). Interestingly, 5 µM glafenine did not significantly alter WT SLC4A11 cell surface processing. Additionally, 27% ± 5% and 26% ± 7% of E143K and G709E SLC4A11, respectively, was at the cell surface. This is similar to the cell surface processing values determined in the HTA in the presence of 10 µM glafenine (39% ±

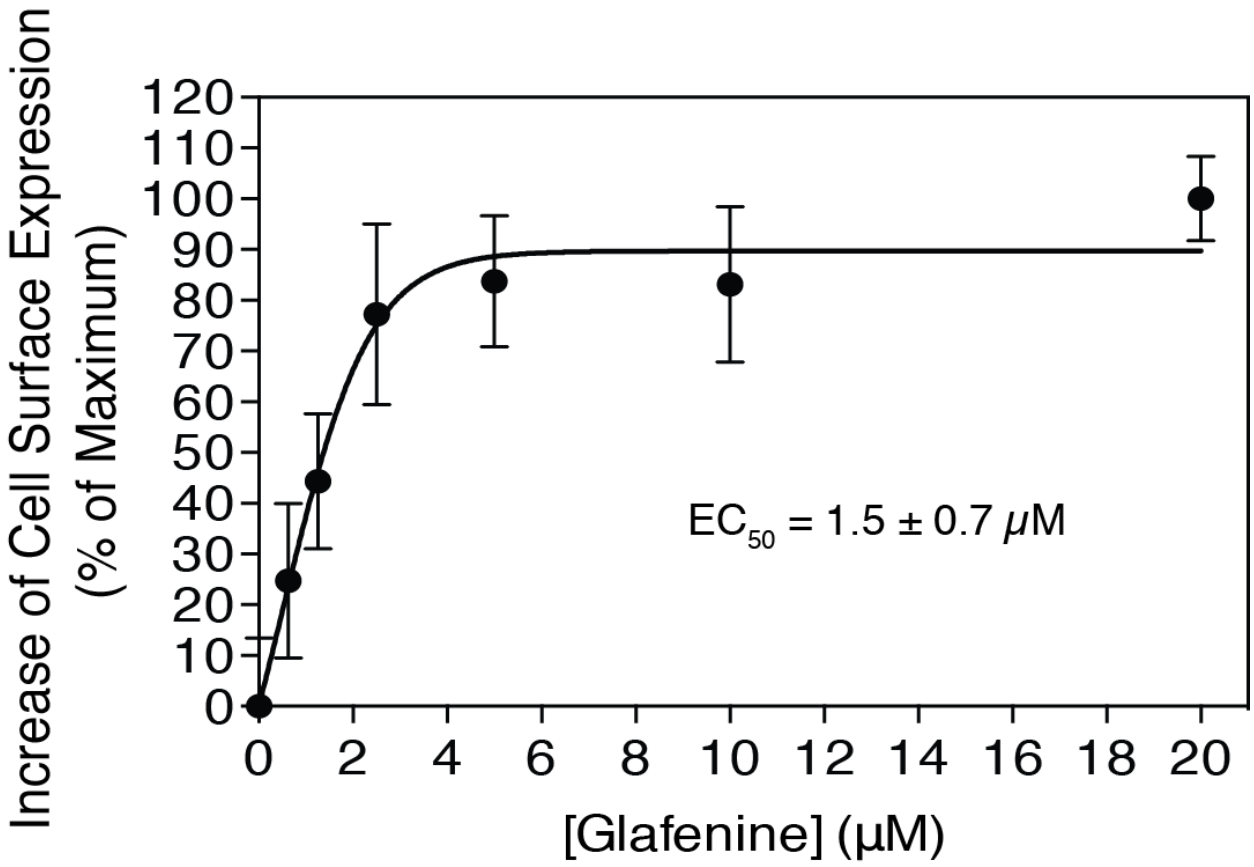


Figure 3.6 Correction of G709E-SLC4A11 trafficking by glafenine. Cells stably expressing G709E-SLC4A11 were subjected to the HTA protocol on 96 well plates in the presence of indicated concentrations of glafenine and 0.2% DMSO. Red fluorescence arising from amplex red conversion to resorufin, was measured in each well. Fluorescence values of cells treated with 0 µM glafenine were subtracted from each value and data were normalized to the maximum red fluorescence observed. Data represent the mean ± SEM fluorescence (n = 8). EC_{50} was calculated to be 1.5 ± 0.7 µM glafenine.

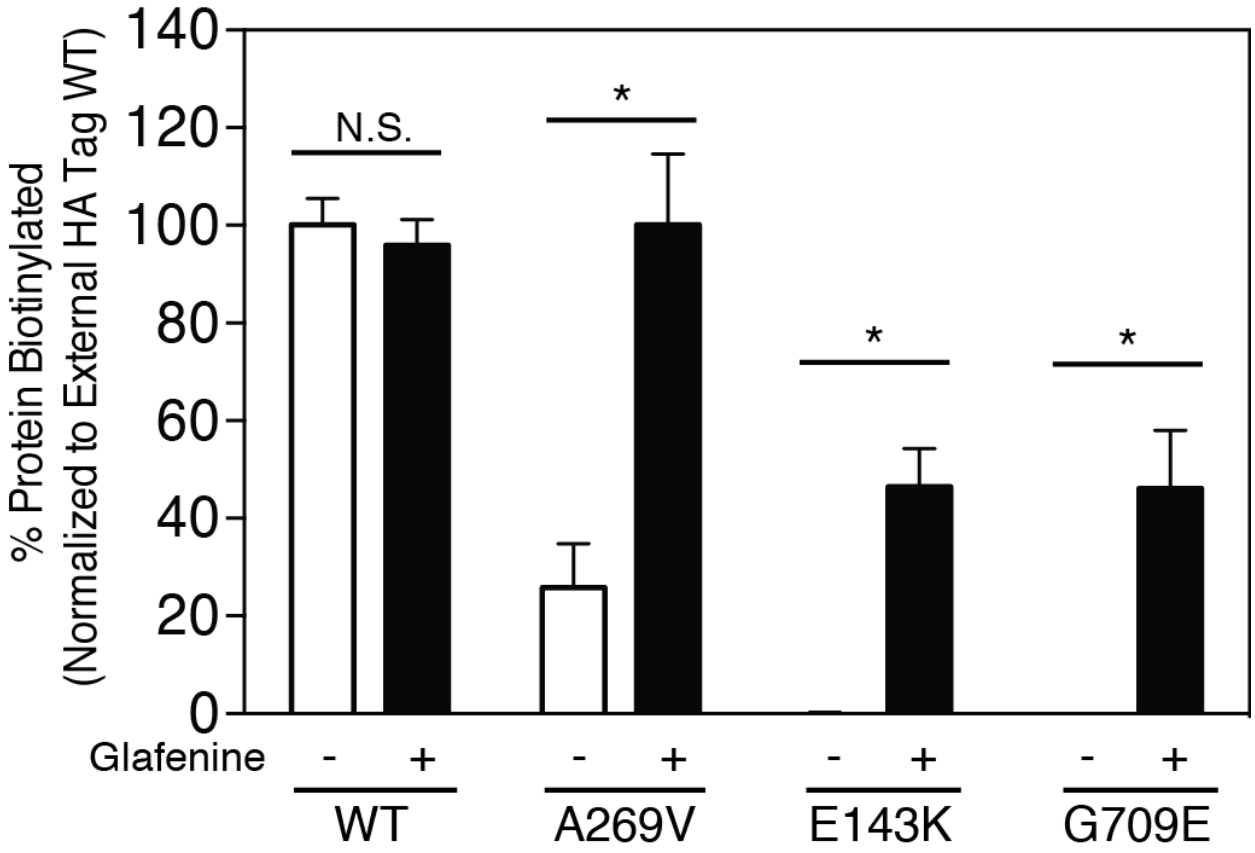


Figure 3.7 Effect of glafenine on mutant SLC4A11 cell surface processing efficiency**.** Cells were transiently transfected with vector or cDNA encoding the indicated SLC4A11 type. Twenty-four hours post transfection, cells were treated with 0.2% DMSO (-) or 5 µM glafenine and 0.2% DMSO (+). Cells were subjected to cell surface biotinylation assays 48 hours post transfection. Samples have been corrected for GAPDH biotinylation which represents the background of the assay. N.S., no significant difference. *: significant difference ($P < 0.05$, $n = 3$-4).

3% and 34% ± 2% respectively) (Fig. 3.5). These data confirm that glafenine in the presence of 0.2% DMSO increased the level of cell surface processing of three ER-retained SLC4A11 mutants.

3.2.5 Glafenine restores mutant SLC4A11 transport function

Glafenine rescued SLC4A11 to the plasma membrane, but the results have little therapeutic significance unless the rescued protein is functional. We thus assessed the effect of glafenine on osmotically-driven water flux in HEK293 cells expressing the three mutants (Fig. 3.8). A269V, E143K and G709E SLC4A11 mutants without glafenine treatment showed a low water flux activity, 16% ± 1%, 13% ± 1% and 12% ± 1%, relative to WT SLC4A11 respectively. Glafenine treatment induced a significant increase in water flux activity for all three mutants (67% ± 8%, 87% ± 6% and 114% ± 11%, relative to WT SLC4A11 respectively). Glafenine did not affect water flux in cells expressing WT SLC4A11. This is consistent with the findings of the cell surface biotinylation assay (Fig. 3.7), where glafenine treatment did not significantly affect WT SLC4A11. Furthermore, cells expressing the mutants had their water flux increase to a level not significantly different to WT SLC4A11. These data indicate that these mutants retain functional activity and, if rescued to the plasma membrane, are as effective as WT SLC4A11 in terms of functional activity. Glafenine treatment may represent a feasible pharmacological therapeutic for these mutations of SLC4A11.

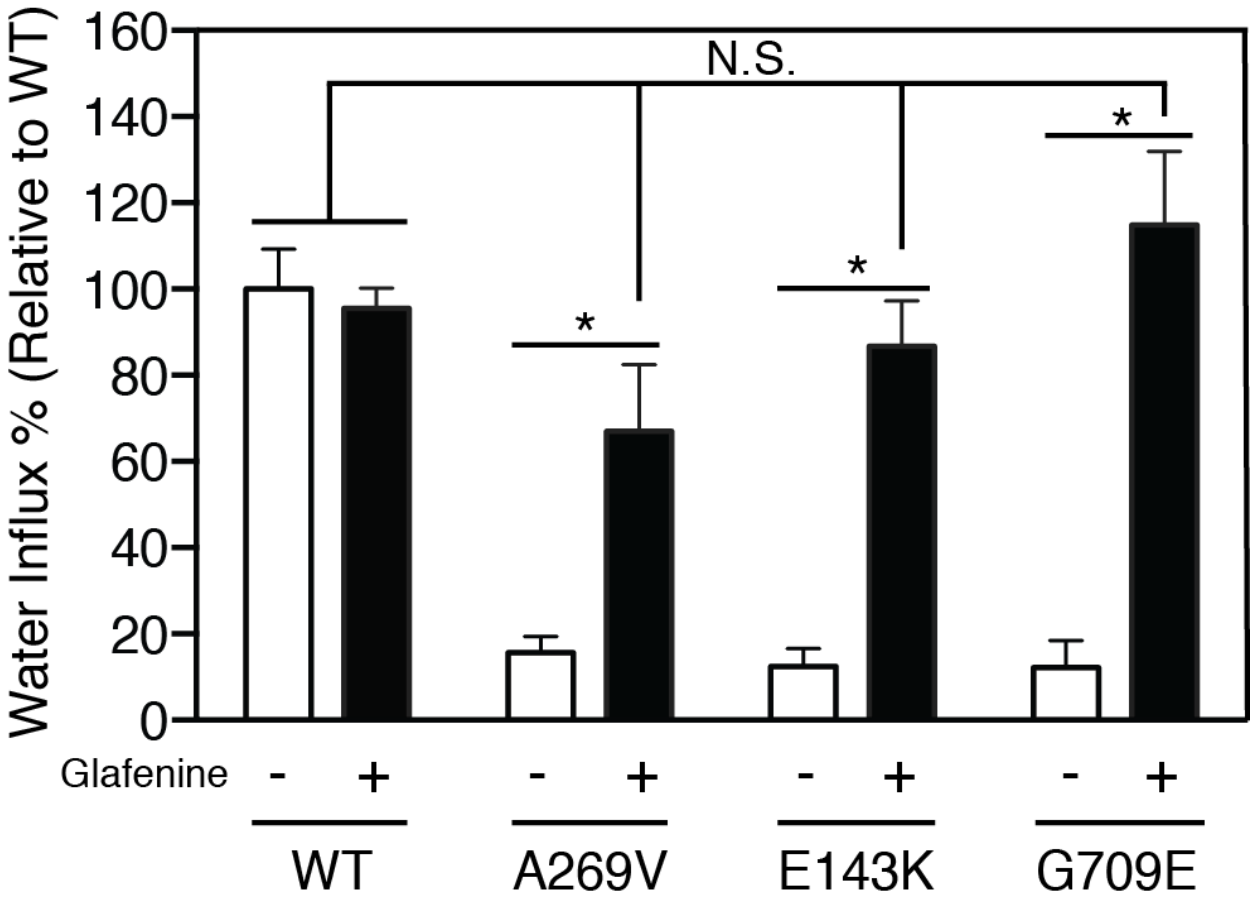


Figure 3.8 Effect of glafenine on osmotically-driven water flux by ER-retained mutant SLC4A11. HEK293 cells were transiently co-transfected cDNA encoding external H564 HA-tagged SLC4A11- WT, A269V, E143K, G709E or vector, along with GFP CDNA. The level of green florescence was quantified in regions of interest in cells as medium was changed from iso-osmotic to hypo-osmotic. The rate of fluorescence change upon switching to hypo-osmotic medium was measured as a surrogate for the rate of cell swelling. Data were corrected for rates observed in vector transfected cells and normalized to WT SLC4A11. Data represent the mean ± SEM of 3-5 independent experiments of 10-20 cells per coverslip. *: significant difference in water flux ($P < 0.05$). N.S., no significant difference compared to WT SLC4A11 without glafenine treatment.

3.3 Discussion

The goal of this study was to develop a high throughput assay (HTA) able to screen for compounds that increase the processing of ER-retained SLC4A11 mutants to the cell surface. The assay, working in a 96 well format, provided reliable measurements of surface abundance of SLC4A11. An initial small screen with potential correcting molecules identified glafenine as increasing the level of mutant SLC4A11 at the plasma membrane. Independent assessment by cell surface biotinylation revealed that glafenine does increase mutant processing to the cell surface. Glafenine increased the water flux activity of cells expressing SLC4A11 mutants to levels found in cells expressing WT-SLC4A11. The efficacy of glafenine suggests that corrector drugs with potential efficacy in treating corneal dystrophies caused by SLC4A11 mutants can be identified, using the HTA described here.

Although there are other methods to measure protein abundance at the plasma membrane, including cell surface biotinylation, flow cytometry or confocal microscopy based immunofluorescence, these are relatively time and material intensive. Development of high throughput assays to detect protein at the cell surface has been of recent interest to many different groups (67-70). We developed an assay using amplex red and commonly available equipment to detect fluorescence. This assay includes elements of an assay developed to assess F508del CFTR cell surface processing (52, 53). We modified the assay moving from a 24 well format to 96 wells, enabling a higher level of assay throughput. We found that the cells needed to grow to almost 100% confluency on 96 well plates, otherwise they would detach from the wells during the multiple washes of the cells and the experiment would fail. We also coated the

96 well plates with poly-L-lysine to promote cell adhesion. As another step to reduce cell loss, we fixed the cells, using formalin. We also found that deplating the stable cell lines with trypsin adversely affected our results because of the proteolytic sites located on the extracellular loop where the HA epitope is located (55). Instead, we deplated cells, using 0.5 mM EDTA in PBS. Finally, we used a 1% BSA in PBS solution instead of DMEM-HEPES in immune steps of the protocol. Together these modifications provide an assay amenable to high throughput screening of SLC4A11 folding correctors.

The data indicate that the HTA provides reliable data, reflecting the relative cell surface abundance of SLC4A11 variants. We were concerned that the epitope insertion required for the assay might affect SLC4A11 folding. Transport function was unaffected by the epitope insertion, as measured in water flux assays in transiently-transfected cells indicating no effect on cell surface processing and folding. In contrast, cell surface biotinylation of stably-transfected cells suggested a reduced cell surface processing of the H564 HA insertion mutant, relative to the N-terminally tagged protein. While this suggests that the epitope insertion had an effect, we were reassured that cell surface biotinylation of H564 HA tagged proteins (Fig. 3.3B) was not different from that reported for untagged versions (51, 55).

Cell surface biotinylation data correlated well with HTA data, indicating that the HTA represents the relative cell surface abundance of SLC4A11 variants. We found a similar protein biotinylation pattern across the mutant types compared to the relative fluorescence from the HTA. Cells that express an extracellularly inaccessible, cytosolic HA tag (N-Terminal HA Tag WT), or that express extracellularly tagged HA-SLC4A11 that is ER-retained (E143K and G709E) produce approximately 20% fluorescence relative to the external HA Tag WT. This background

fluorescence may arise from non-HRP catalyzed amplex red conversion, or reflect some degree of cell lysis, allowing antibody access to intracellular HA-epitopes.

Assay capacity is a significant issue as we look ahead to screens of larger compound libraries. We found that at least four replicates in a single dish were needed to produce a reliable result. In a 96 well format, this could translate to 22 unique conditions per plate, assuming that both the positive and negative controls are also performed in four replicates. Although we could detect a difference in fluorescence for cells treated with compounds in four replicates, further replicates were needed to lessen standard error to produce statistically significant results. The assay could be further refined by use of larger format (e.g. 128 or 256 well) plates. One major limitation of the HTA is the skill and reproducibility of the person performing the assay. This limitation could be overcome with robotic automation, especially if larger format plates are used.

Currently, the only permanent treatment strategy for corneal endothelial dystrophies is corneal transplant. Corneal transplant is complicated by tissue availability, recipient rejection and the high amount of skill and resources required to perform the transplant. Additional treatment options for corneal dystrophies caused by SLC4A11 mutants are much needed. In this study, we identified glafenine combined with 0.2% DMSO as a potential pharmacological corrector for the trafficking defect of the A269V, E143K and G709E mutations of SLC4A11 (Fig. 3.5). Our screen also identified ibuprofen and acetylsalicylic acid as correcting the defect of some SLC4A11 mutants. It remains to be determined if other mutations of SLC4A11 will respond in a similar fashion to these drugs as those studied here.

We decided to further characterize glafenine as it was the only compound that was identified as effective in rescuing all three mutants screened. Glafenine is a non-steroidal anti-inflammatory drug (NSAID), used as an analgesic mainly in Europe in the 1970's. Its use was discontinued because it was implicated in anaphylaxis, gastrointestinal injury and kidney failure (71, 72). Recently, glafenine was found to rescue the F508del mutation of CFTR and the G601S mutation of the hERG K^+ channel to the plasma membrane (57, 61). The mechanism of rescue by glafenine for these unrelated proteins is unknown. Most NSAIDs work by inhibition of one or both of the cyclooxygenase (COX) isoforms (73, 74). Both COX-1 and COX-2 are integral membrane proteins (75), but any relationship between inhibition of these enzymes and the rescue of mutant membrane proteins is unclear. We determined that the EC_{50} of glafenine in SLC4A11 rescue was 1.5 µM (Fig. 3.6), but could not find literature identifying the EC_{50} of glafenine as a COX inhibitor. Clinically, glafenine can achieve plasma concentrations up to 10 µM (61, 76).

Our initial HTA screen of compounds included the NSAIDs acetylsalicylic acid and ibuprofen. When used with 0.2% DMSO, acetylsalicylic acid had a significant effect on A269V mutant (Fig. 3.5). Ibuprofen with 0.2% DMSO had a significant effect on A269V and E143K mutants (Fig. 3.5). Ibuprofen is effective in rescuing F508del CFTR and is proposed to do so through inhibition of COX-1 (65). Knockdown of COX-1 in HEK293 cells expressing F508del CFTR is effective in rescuing the mutant protein, however, knockdown of COX-2 does not have a significant effect on F508del CFTR (65). Rescue of mutant CFTR, and now SLC4A11, with the use of NSAIDs suggests that prostaglandin synthesis plays a role in ER-retention of mutant

membrane proteins. Further probing of this mechanism could lead to a novel treatment strategy for diseases involving ER-retained membrane proteins.

Despite rescue of mutant SLC4A11 to the plasma membrane, these results are only clinically relevant if the mutant proteins remain functional once at the plasma membrane. Prior work showed that E143K forms heterodimers with R125H-SLC4A11 monomer and displays a functional activity of approximately 40% that of WT SLC4A11 (32). R125H is a catalytically inactive SLC4A11 mutant that traffics to the plasma membrane just as WT SLC4A11, and which brings E143K to the cell surface in heterodimers. This showed that if E143K was trafficked to the plasma membrane, it would be functional. Furthermore, to delay onset of symptoms associated with corneal dystrophies, 25% of the function of WT SLC4A11 is required and to completely avoid symptoms, 60% of WT SLC4A11 function is required (32). Our *in vitro* investigations revealed that the 60% benchmark can be exceeded for all three SLC4A11 mutants treated with glafenine (Fig. 3.8). Treatment with glafenine could, thus, represent an effective pharmacological therapy. Interestingly, the G709E mutation after treatment with glafenine produced a higher, albeit not significantly different, level of water influx than the WT (Fig. 3.8).

This study leads to three major conclusions. First, the HTA we developed is an effective and reliable method to determine the abundance of SLC4A11 at the plasma membrane. Second, glafenine in 0.2% (v/v) DMSO corrects the trafficking defect for A269V, E143K and G709E mutations of SLC4A11. Third, mutant SLC4A11 remains functional once localized to the plasma membrane and this functionality may be of sufficient level to prevent symptoms of corneal dystrophies. Here we presented a paradigm for the high throughput identification and

in vitro testing of potential SLC4A11 folding correctors. These data suggest that glafenine is a potential pharmacological therapeutic for some corneal dystrophy-causing mutants of SLC4A11.

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Chapter 4: Summary and Future Directions

4.1 Summary

The first objective of this thesis was to develop a high throughput screening assay for the detection of SLC4A11 at the plasma membrane. We developed our assay using another designed for the detection of mutant CFTR (1). We introduced some changes for our uses. First, we developed stable cell lines expressing HA epitope tagged SLC4A11. Second, we modified the assay to work in a 96 well format. Third, we coated the 96 well plates with poly-L-lysine to combat cell loss when performing the assay. Finally, we found that culturing our cells with trypsin could adversely affect our results as there are proteolytic sites on the extracellular loop containing the HA tag. We validated the assay using cell surface biotinylation and found that both techniques produced similar results.

The second objective of this thesis was to perform a small scale screen of compounds shown to have rescuing effects on other mutant membrane proteins using our assay. We found that a variety of compounds had a small effect on certain mutants of SLC4A11 but these were not statistically significant. However, we found that the NSAIDs which have been shown to rescue mutant CFTR (2, 3), significantly increased the amount of mutant SLC4A11 at the plasma membrane. In particular, glafenine had a significant effect on all three mutants screened. Interestingly, glafenine did not seem to have a significant effect on the trafficking of wild type SLC4A11. Furthermore, functional analysis of rescued mutant SLC4A11 showed that the rescued SLC4A11 displayed a similar level of water flux compared to wild type.

Overall, these results hold great promise in developing a pharmacological treatment for the corneal endothelial dystrophies associated with mutations of SLC4A11. Glafenine has been

used traditionally as an analgesic but has fallen out of use because it has been implicated in renal failure and high incidences of anaphylaxis. If glafenine is administered topically to a relatively superficial area of the body such as the cornea, glafenine could avoid the primary circulatory system and thereby avoid many of its adverse effects.

4.2 Future Directions

The rescuing effect of NSAIDs on certain mutants of SLC4A11 has been established *in vitro*. The next step would be to examine the effects of NSAIDs on mutant SLC4A11 in an animal model. A mouse model with a CHED mutation has been developed in our lab. Although mouse and human SLC4A11 differ slightly in structure, the mouse model displays the CHED phenotype in the same manner that has been reported for humans. Currently, there are 57 known point mutations associated with CHED, HS and FECD. In addition to *in vivo* study, further *in vitro* study of NSAIDs on other mutations of SLC4A11 is needed to identify which mutants can be rescued by NSAIDs.

In this thesis, we show that three NSAIDs are able to correct the trafficking defect of mutant SLC4A11. Other studies have shown that NSAIDs are capable of rescuing other mutant membrane proteins to the plasma membrane (2, 4) such as CFTR. These proteins are not closely related other than the fact that they are membrane proteins. A number of diseases are caused by trafficking defects in mutant membrane proteins causing ER-retention. Perhaps these NSAIDs may have a similar rescuing effect on a number of mutant membrane proteins. Screening of these mutant membrane proteins with NSAIDs is needed. NSAIDs work through inhibition of one or both COX enzymes, however if there is a connection between COX

inhibition and the rescue of mutant membrane proteins, it is unclear. Further study on the mechanism by which these NSAIDs are rescuing mutant membrane proteins is needed.

The Amplex Red Assay is a powerful tool for quantifying protein at the plasma membrane. It can be further improved by scaling the experiment to even smaller replicates than 96 wells. A major limitation of the assay is the reproducibility of the technician performing the experiments. This limitation could be overcome by automation of the assay. This would also allow the technician to perform even more replicates by freeing up their time by not performing the assay manually. Finally, there are large libraries of compounds available for screening and we have only examined a select few in this study. Further screening of compounds and mutants is needed.

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