Ophthalmic Nonsteroidal Anti-Inflammatory Drugs as a Therapy for Corneal Dystrophies Caused by SLC4A11 Mutation

Kumari Alka and Joseph R. Casey

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

PURPOSE. SLC4A11 is a plasma membrane protein of corneal endothelial cells. Some mutations of the SLC4A11 gene result in SLC4A11 protein misfolding and failure to mature to the plasma membrane. This gives rise to some cases of Fuchs’ endothelial corneal dystrophy (FECD) and congenital hereditary endothelial dystrophy (CHED). We screened ophthalmic nonsteroidal anti-inflammatory drugs (NSAIDs) for their ability to correct SLC4A11 folding defects.

METHODS. Five ophthalmic NSAIDs were tested for their therapeutic potential in some genetic corneal dystrophy patients. HEK293 cells expressing CHED and FECD-causing SLC4A11 mutants were grown on 96-well dishes in the absence or presence of NSAIDs. Ability of NSAIDs to correct mutant SLC4A11 cell-surface trafficking was assessed with a bioluminescence resonance energy transfer (BRET) assay and by confocal microscopy. The ability of mutant SLC4A11-expressing cells to mediate water flux (SLC4A11 mediates water flux across the corneal endothelial cell basolateral membrane as part of the endothelial water pump) was measured upon treatment with ophthalmic NSAIDs.

RESULTS. BRET-assays revealed significant rescue of SLC4A11 mutants to the cell surface by 4 of 5 NSAIDs tested. The NSAIDs, diclofenac and nepafenac, were effective in moving endoplasmic reticulum–retained missense mutant SLC4A11 to the cell surface, as measured by confocal immunofluorescence. Among intracellular-retained SLC4A11 mutants, 20 of 30 had significant restoration of cell surface abundance upon treatment with diclofenac. Diclofenac restored mutant SLC4A11 water flux activity to the level of wild-type SLC4A11 in some cases.

CONCLUSIONS. These results encourage testing diclofenac eye drops as a treatment for corneal dystrophy in patients whose disease is caused by some SLC4A11 missense mutations.

Keywords: Fuchs endothelial corneal dystrophy, congenital hereditary endothelial dystrophy, SLC4A11, nonsteroidal anti-inflammatory drug, corneal endothelial cell

Mutations of the gene encoding the corneal endothelial protein, SLC4A11, can cause blinding corneal dystrophies. Human cornea provides the outer protective surface of the eye. Stroma, the central and predominant corneal layer, is nearly acellular and comprises a highly ordered array of collagen fibrils. Posterior to the stroma is an extracellular matrix layer, Descemet’s membrane, to which corneal endothelial cells (CECs) attach. High solute levels in stroma lead to osmotic fluid accumulation, countered by the “pump” function of CECs, which reabsorb fluid from stroma into the aqueous humor.1 Defects in CECs cause corneal edema, which distorts the collagen fibril array, significantly clouding vision. This manuscript describes the use of nonsteroidal anti-inflammatory drugs (NSAIDs) as a corrector of defective SLC4A11.

SLC4A11 is a membrane transport protein of the CEC basolateral surface, facing Descemet’s membrane and the corneal stroma. SLC4A11 contributes to the water “pump” as a H+/OH transporter and by facilitating a water flux.2,3 SLC4A11 also transports ammonia,4,5 which may prevent toxic accumulation of this metabolite.6 SLC4A11 is the only defective gene found in patients with the rare recessive disease congenital hereditary endothelial dystrophy (CHED), which has onset at birth or early in life.7 Harboyan syndrome (HS), which presents as with CHED, but with addition of sensorineural deafness, is an alternate manifestation of CHED that is also caused by SLC4A11 mutations.8,9 SLC4A11 mutations may cause some cases of Fuchs’ endothelial corneal dystrophy (FECD),10 a dominantly inherited disease affecting 4% lifetime incidence of people over age 40, as quantified by the presence of guttae (hallmark bumps on Descemet’s membrane).11 When studied in model cells, the most common molecular phenotype of mutant membrane proteins is mild misfolding.12 This biosynthetic defect renders the protein incapable of transit from the endoplasmic reticulum (ER) site of synthesis to its normal cellular location. Note that SLC4A11 localization in corneas of endothelial dystrophy patients has not been examined; the conclusion of ER retention presently rests on data from cell culture models. Correcting such biosynthetic defects is an attractive therapeutic approach for some genetic diseases affecting membrane proteins, best exemplified by the efficacy of the drug, Lumacaftor, in rescuing to the cell-surface misfolded cystic fibrosis transmembrane conductance regulator (CFTR), the protein product of the cystic fibrosis (CF) gene.13 Here, we have identified members of a specific class of drugs,
Correction of SLC4A11 mutants by NSAIDs

NSAIDs, that hold promise to ameliorate fold defects in the membrane transport protein, SLC4A11, whose mutations cause blinding corneal dystrophies. 2-10,14-19

Most SLC4A11 mutations lead to biosynthetic defects marked by accumulation in the ER, and protein degradation before reaching the plasma membrane. 12 A small-scale drug screen showed that Glafenine, a disused NSAID, was able to move some SLC4A11 mutants to the cell surface, 20 suggesting that other NSAIDs might also have therapeutic potential. We thus set out to screen NSAIDs available as eye drops for their ability to correct the cell-surfacing trafficking of SLC4A11 proteins, using a bioluminescence resonance energy transfer (BRET) assay that enabled rapid analysis of SLC4A11 cell surface abundance. Confocal microscopy provided an independent measure of SLC4A11 cellular localization with and without drug treatment. We performed whole cell assays of SLC4A11-mediated water flow activity to measure functional rescue of corneal dystrophy mutants of SLC4A11. Together, the data support the therapeutic potential of NSAID eye drops, in some corneal dystrophy patients.

Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), calf serum (CS), and penicillin-streptomycin-glutamine, were from Life Technologies (Carlsbad, CA, USA). Cell culture dishes were from Sarstedt (Montreal, QC, Canada). Complete protease inhibitor tablets were from Roche Applied Sciences (Indianapolis, IN, USA). Poly-L-lysine, ketorolac tromethamine, nepafenac, bromfenac sodium, diclofenac, and flurbiprofen and 4-[6-diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich (Oakville, ON, Canada). Immobilon-P polyvinylidene difluoride was from Millipore (Billerica, MA, USA). Monoclonal antibodies against hemagglutinin epitope (hemagglutinin [HA]; clone 16B12) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Covance (Princeton, NJ, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin was from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA). Luminata Crescendo Western horseradish peroxidase Substrate Chemiluminescence reagent was from Millipore (Billerica, MA, USA). White opaque 96-well plates were from BD Falcon (Tewksbury, MA, USA). Coelenterazine-h was from Nanolight technology (Pinetop, AZ, USA). Pierce BCA Protein Assay Kit was from ThermoFisher Scientific (Rockford, IL, USA).

DNA Constructs

All SLC4A11 missense mutants were prepared as described. 12 pAMC1 encoding N-terminally HA-tagged human SLC4A11 in pcDNA3.1 20 or wild-type (WT) Luc-SLC4A11 with N-terminal luciferase tag 12 were used as template for mutagenesis experiments to create point mutants.

Tissue Culture

cDNA encoding N-terminally HA-tagged SLC4A11 WT, and SLC4A11 mutants were transiently transfected into HEK293 cells, using the calcium phosphate method. Cells were grown in DMEM supplemented with 5% (vol/vol) FBS, 5% (vol/vol) fetal CS, and 1% (vol/vol) penicillin-streptomycin-glutamine (DMEM), at 37°C in an air/CO2 incubator (19:1). Cells were harvested 40 to 48 hours post-transfection.

BRET Assay of Cell Surface Localization

This assay was a modified version of the basic BRET assay protocol reported. 12,22-23 HEK293 cells were transiently co-transfected in 100-mm dishes with 0.7 µg cDNA encoding N-terminal luciferase-tagged human SLC4A11 (splicing variant 2 of human SLC4A11 (891 amino acid, NCBI reference: NG_017072.1) and 4.9 µg cDNA encoding K-Ras-YFP (acceptor labeled protein). HEK293 cells transfected with 0.7 µg cDNA encoding Luc-SLC4A11 and 4.9 µg of pcDNA3.1 were included for each variant tested in this study (WT SLC4A11 and mutants W240S, R282P, E399K, T431I, G585D, G709E, E143K, R209W, S213L, S232N, R233C, C386R, G394R, T4501K, G417R, G418D, G464D, L473R, S540L, T584K, C611R, K655Q, R755W, R755W, S773L, T833M, R869C, L873P, S213P, R488K, and L843P). Twenty-four hours post-transfection, cells were rinsed with PBS (138 mM NaCl, 3 mM KCl, 8 mM sodium phosphate, pH 7.4), detached from the plate by incubation for 10 minutes at 37°C in 0.25% (wt/vol) trypsin/0.9 mM EDTA in DMEM and resuspended in DMEM containing serum, as above. HEK293 cells were seeded onto 96-well white opaque plates (5 × 10^4 cells/well of dish) in a total volume of 200 µl and transferred to a 37°C incubator, 5% CO2.

To assess the effect of drugs on the trafficking of SLC4A11, 50 µl of drug was added to each well post seeding, resulting in final concentration of the drug (see Table) in 0.2% DMSO (vol/vol). After 24 hours, medium was aspirated and each well was rinsed with 200 µl of PBS. Coelenterazine-h (100 µl of 5 µM in PBS/CMG, which is PBS containing 0.9 mM NaCl, 1 mM MgCl2 and 5.5 mM D-glucose) was added to each well and plates were incubated for 20 minutes at 37°C in the dark. Fluorescence emission at 480 and 540 nm were measured on a SynergyMX Reader (BioTek, Winooski, VT, USA) with an integration time of 2 s/well. BRET signal was calculated as:  

\[ \text{BRET} = \left( \frac{\text{Emission}_{540} \text{ (Luc-SLC4A11 and K-Ras co-transfected)}}{\text{Emission}_{480} \text{ (Luc-SLC4A11 alone transfected)}} \right) \times \left( \frac{\text{Emission}_{480} \text{ (Luc-SLC4A11 alone transfected)}}{\text{Emission}_{480} \text{ (Luc-SLC4A11 alone transfected)}} \right) \]

Confocal Immunofluorescence

HEK293 cells, grown on poly-L-lysine coated 18-mm circular glass cover slips, were transiently transfected with cDNA encoding SLC4A11 WT or SLC4A11 mutants E143K and G709E or pcDNA 3.1(+). SLC4A11 mutants carried a double HA tag insertion in extracellular loop 3, following amino acid 564, and have been previously reported as normal in functional activity and cell surface localization. 20 One hour post-transfection, cells were treated with 500 µl of drug to a final concentration as indicated in the Table in 0.2% DMSO (vol/vol), or DMSO alone as control. Forty-eight hours later, cells were washed in PBS, fixed with 4% paraformaldehyde in PBS for 10 minutes, followed by a quenching step with 100 mM glycine in PBS for 5 minutes at 20°C. Where indicated, cells were permeabilized with 0.2% Triton X-100 in PBS (15 minutes at 20°C). After three 5 minutes washes with PBS, nonspecific sites were blocked with 1% BSA in PBS for 30 minutes. Cells were incubated with polyclonal rabbit anti-HA (1:500; sc-8055; Santa Cruz Biotechnology) for 1 hour at 20°C, washed with PBS (three times, 5 minutes) and, thereafter, incubated with donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (1:500; 711-545-152; Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) for 1 hour at 20°C. Staining with DAPI (2 µg/ml in PBS for 1 hour at 20°C) was performed in the dark, followed by a washing step with PBS. Coverslips were mounted on a glass microscope slide, using Dako fluorescence mounting medium (Mississauga, ON, Canada), containing antifading agent and then visualized on a CSU 10 spinning disk confocal microscope.
describe previously. Cells were transfected with pcDNA plasmid constructs (WT, E143K, and G709E) in a 1:8 molar ratio. cDNA for SLC4A11 variants encoded a shortened version of splice variant 2 of human SLC4A11 (891 amino acid, NCBI reference: NG_017072.1) with an N-terminal HA tag, as previously described previously. One hour post transfection, cells were treated with the final concentration of drug in 0.2% DMSO (Table), and control cells received DMSO to a final concentration of 0.2% DMSO (vol/vol). Forty-eight hours later, cover slips were mounted in a 35-mm diameter Attofluor Cell Chamber (Molecular Probes, Ottawa, ON, Canada) and washed with PBS. During experiments, the chamber was perfused with isotonic 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, USA). The chamber was mounted on the stage of a Wave FX spinning disc confocal microscope (Quorum Technologies, Guelph, ON, Canada), with a Yokogawa CSU10 scanning head (Tokyo, Japan). The microscope has a motorized XY stage with Piezo Focus Drive (MS-4000 XYZ Automated Stage; ASI, Eugene, OR, USA) and a live-cell environment chamber (Chamilde, Seoul, Korea), set to 24°C during the experiment. Acquisition was performed with a Hamamatsu C9100-13 Digital Camera (EM-CCD: Chamilde) and a ×20 objective during excitation with a laser (Spectral Applied Research, Richmond Hill, ON, Canada) at 491 nm. GFP fluorescence, collected through a dichroic cube (Quorum Technologies) at wavelengths 520 to 540 nm, was acquired at 1 point s⁻¹ for 4 minutes. Quantitative image analysis was performed by selecting a region of interest for each HEK293 cell with Volocity 6.0 software (PerkinElmer, Waltham, MA, USA). The software was set to 24°C/C during the experiment. The acquisition was performed with a Hamamatsu C9100-13 Digital Camera (EM-CCD: Chamilde) and a ×20 objective during excitation with a laser (Spectral Applied Research, Richmond Hill, ON, Canada) at 491 nm. GFP fluorescence was collected through a dichroic cube (Quorum Technologies) at wavelengths 520 to 540 nm. The analysis was performed by selecting a region of interest for each HEK293 cell with Volocity 6.0 software (PerkinElmer, Waltham, MA, USA). The software was set to 24°C/C during the experiment.

Disk confocal scan-head (Yokogawa, Tokyo, Japan), using N-terminal HA tag, as previously described previously. One hour post transfection, cells were treated with the final concentration of drug in 0.2% DMSO (Table), and control cells received DMSO to a final concentration of 0.2% DMSO (vol/vol). Forty-eight hours later, cover slips were mounted in a 35-mm diameter Attofluor Cell Chamber (Molecular Probes, Ottawa, ON, Canada) and washed with PBS. During experiments, the chamber was perfused with isotonic 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, USA). The chamber was mounted on the stage of a Wave FX spinning disc confocal microscope (Quorum Technologies, Guelph, ON, Canada), with a Yokogawa CSU10 scanning head (Tokyo, Japan). The microscope has a motorized XY stage with Piezo Focus Drive (MS-4000 XYZ Automated Stage; ASI, Eugene, OR, USA) and a live-cell environment chamber (Chamilde, Seoul, Korea), set to 24°C during the experiment. Acquisition was performed with a Hamamatsu C9100-13 Digital Camera (EM-CCD: Chamilde) and a ×20 objective during excitation with a laser (Spectral Applied Research, Richmond Hill, ON, Canada) at 491 nm. GFP fluorescence was collected through a dichroic cube (Quorum Technologies) at wavelengths 520 to 540 nm. The analysis was performed by selecting a region of interest for each HEK293 cell with Volocity 6.0 software (PerkinElmer, Waltham, MA, USA). The software was set to 24°C/C during the experiment.

As originally described, 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; Clontech, Burlington, Ontario, Canada) and pcDNA 3.1 (empty vector) or the SLC4A11 plasmid constructs (WT, E143K and G709E) in a 1:8 molar ratio. cDNA for SLC4A11 variants encoded a shortened version of splice variant 2 of human SLC4A11 (891 amino acid, NCBI reference: NG_017072.1) with an N-terminal HA tag, as previously described previously. One hour post transfection, cells were treated with the final concentration of drug in 0.2% DMSO (Table), and control cells received DMSO to a final concentration of 0.2% DMSO (vol/vol). Forty-eight hours later, cover slips were mounted in a 35-mm diameter Attofluor Cell Chamber (Molecular Probes, Ottawa, ON, Canada) and washed with PBS. During experiments, the chamber was perfused with isotonic 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, USA). The chamber was mounted on the stage of a Wave FX spinning disc confocal microscope (Quorum Technologies, Guelph, ON, Canada), with a Yokogawa CSU10 scanning head (Tokyo, Japan). The microscope has a motorized XY stage with Piezo Focus Drive (MS-4000 XYZ Automated Stage; ASI, Eugene, OR, USA) and a live-cell environment chamber (Chamilde, Seoul, Korea), set to 24°C during the experiment. Acquisition was performed with a Hamamatsu C9100-13 Digital Camera (EM-CCD: Chamilde) and a ×20 objective during excitation with a laser (Spectral Applied Research, Richmond Hill, ON, Canada) at 491 nm. GFP fluorescence was collected through a dichroic cube (Quorum Technologies) at wavelengths 520 to 540 nm. The analysis was performed by selecting a region of interest for each HEK293 cell with Volocity 6.0 software (PerkinElmer, Waltham, MA, USA). The software was set to 24°C/C during the experiment. Acquisition was performed with a Hamamatsu C9100-13 Digital Camera (EM-CCD: Chamilde) and a ×20 objective during excitation with a laser (Spectral Applied Research, Richmond Hill, ON, Canada) at 491 nm. GFP fluorescence was collected through a dichroic cube (Quorum Technologies) at wavelengths 520 to 540 nm. The analysis was performed by selecting a region of interest for each HEK293 cell with Volocity 6.0 software (PerkinElmer, Waltham, MA, USA). The software was set to 24°C/C during the experiment.

**Assay of Osmotically Driven Water Flux**

As originally described, 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; Clontech, Burlington, Ontario, Canada) and pcDNA 3.1 (empty vector) or the SLC4A11 plasmid constructs (WT, E143K and G709E) in a 1:8 molar ratio. cDNA for SLC4A11 variants encoded a shortened version of splice variant 2 of human SLC4A11 (891 amino acid, NCBI reference: NG_017072.1) with an N-terminal HA tag, as previously described previously. One hour post transfection, cells were treated with the final concentration of drug in 0.2% DMSO (Table), and control cells received DMSO to a final concentration of 0.2% DMSO (vol/vol). Forty-eight hours later, cover slips were mounted in a 35-mm diameter Attofluor Cell Chamber (Molecular Probes, Ottawa, ON, Canada) and washed with PBS. During experiments, the chamber was perfused with isotonic 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, USA). The chamber was mounted on the stage of a Wave FX spinning disc confocal microscope (Quorum Technologies, Guelph, ON, Canada), with a Yokogawa CSU10 scanning head (Tokyo, Japan). The microscope has a motorized XY stage with Piezo Focus Drive (MS-4000 XYZ Automated Stage; ASI, Eugene, OR, USA) and a live-cell environment chamber (Chamilde, Seoul, Korea), set to 24°C during the experiment. Acquisition was performed with a Hamamatsu C9100-13 Digital Camera (EM-CCD: Chamilde) and a ×20 objective during excitation with a laser (Spectral Applied Research, Richmond Hill, ON, Canada) at 491 nm. GFP fluorescence was collected through a dichroic cube (Quorum Technologies) at wavelengths 520 to 540 nm. The analysis was performed by selecting a region of interest for each HEK293 cell with Volocity 6.0 software (PerkinElmer, Waltham, MA, USA). The software was set to 24°C/C during the experiment. Acquisition was performed with a Hamamatsu C9100-13 Digital Camera (EM-CCD: Chamilde) and a ×20 objective during excitation with a laser (Spectral Applied Research, Richmond Hill, ON, Canada) at 491 nm. GFP fluorescence was collected through a dichroic cube (Quorum Technologies) at wavelengths 520 to 540 nm. The analysis was performed by selecting a region of interest for each HEK293 cell with Volocity 6.0 software (PerkinElmer, Waltham, MA, USA). The software was set to 24°C/C during the experiment. Acquisition was performed with a Hamamatsu C9100-13 Digital Camera (EM-CCD: Chamilde) and a ×20 objective during excitation with a laser (Spectral Applied Research, Richmond Hill, ON, Canada) at 491 nm. GFP fluorescence was collected through a dichroic cube (Quorum Technologies) at wavelengths 520 to 540 nm. The analysis was performed by selecting a region of interest for each HEK293 cell with Volocity 6.0 software (PerkinElmer, Waltham, MA, USA). The software was set to 24°C/C during the experiment.
WT SLC4A11 levels. NSAIDs were dissolved in DMSO in these experiments. To assess any contribution of DMSO to the effects observed with NSAIDs, cell surface SLC4A11 abundance was also measured in untreated and DMSO-treated cells (Supplementary Fig. S1). No significant difference was observed, indicating that DMSO does not contribute to the observed changes of SLC4A11 cell surface abundance.

Confocal Immunofluorescence Microscopy of SLC4A11 Cell Surface Trafficking

To provide an additional approach to assess the effect of ophthalmic NSAIDs on plasma membrane trafficking of SLC4A11, a confocal immunofluorescence-based approach was employed (Figs. 2A, 2B). HEK293 cells were transiently transfected with cDNA encoding externally double HA-tagged SLC4A11 mutants. The tag was inserted into extracellular loop 3 of SLC4A11, following P564, and does not affect cell surface trafficking or water flux function of SLC4A11. Representative CHED (E143K) and FEDC (G709E) mutants were tested in this assay. Cells were treated with indicated ophthalmic NSAIDs in 0.2% DMSO (Table). Cells were probed for SLC4A11, using polyclonal rabbit anti-HA antibody. Intact cells revealed cell surface SLC4A11, whereas cells were also permeabilized with Triton X-100 to allow antibody access to SLC4A11 localized in the endoplasmic reticulum.

WT SLC4A11 staining was consistent with plasma membrane localization in nonpermeabilized cells, with additional intracellular staining upon permeabilization (Figs. 2A, 2B). Without drug treatment, SLC4A11 mutants E143K and G709E were not detected at the cell surface (left panels, Figs. 2A, 2B, respectively). Confocal immunofluorescence data were also quantified to assess cell surface abundance of SLC4A11 (Fig. 3). Bromfenac significantly increased cell surface abundance of E143K SLC4A11 and had a modest effect on G709E SLC4A11. In addition, flurbiprofen significantly increased cell surface processing of only G709E SLC4A11. Treatment with nepafenac and diclofenac markedly increased cell surface abundance of E143K and G709E SLC4A11 in nonpermeabilized cells.

Nepafenac and Diclofenac Restore Osmotically Driven Water Flux of SLC4A11 Mutants

Data to this point indicate that nepafenac and diclofenac increase cell surface trafficking of ER-retained SLC4A11 mutants. The effect is, however, of no therapeutic relevance unless the rescued mutants retain function. We thus assessed the effect of ophthalmic NSAIDs on osmotically driven water flux of two mutants (Fig. 4). SLC4A11 variants, co-expressed with enhanced green fluorescent protein (eGFP), were examined by confocal microscopy. Cells were exposed to hypotonic medium while the intensity of green fluorescence in a region of interest in the cell was monitored. Decreased green fluorescence arises upon water influx (Fig. 4A), which dilutes eGFP. Data are corrected for background rate of swelling in cells transfected with empty vector. Mutants E143K and G709E exhibited large, statistically significant increases in water flux activity upon treatment with nepafenac and diclofenac compared with untreated control (Figs. 4A, 4B). Nepafenac and diclofenac restored SLC4A11 mutant water flux activity to a level similar to WT SLC4A11 (Fig. 4B).

Identification of SLC4A11 Mutants That are Candidates for Folding Correction Therapy

With this observation, diclofenac holds promise to treat some corneal dystrophy patients. The potential patient cohort includes individuals whose mutant SLC4A11 is intracellularly retained. We recently examined all SLC4A11 mutants, and found that 6 of 18 FEDC mutants, 21 of 36 CHED mutants, and 6 of 6 HS mutants exhibited significant intracellular retention. We thus tested the efficacy of diclofenac in correcting the folding defect of the six intracellular-retained FEDC mutants.12
using BRET to assay SLC4A11 cell surface localization. Among these FECD mutants, five had significantly increased maturation when treated with 1 μM diclofenac (W240S, E399K, G583D, T434I, and G709E; Fig. 5). Diclofenac did not affect R282P. These data suggest that diclofenac holds promise of correcting the defect in 5 of 6 FECD missense mutants that are ER-retained.

Among 24 CHED- and HS-causing SLC4A11 mutants, 15 showed significant increase in cell surface abundance upon diclofenac treatment (Fig. 5). In general, a 100% to 500% relative increase in total abundance was observed upon treatment with 1 μM diclofenac, when compared with untreated (Fig. 5).

In a second analysis of the data, BRET measurements of cell surface SLC4A11 abundance with and without diclofenac
**Figure 3.** Quantification of SLC4A11 cell surface abundance following NSAID treatment. HEK293 cells were transiently transfected with cDNA encoding externally double HA-tagged (HA 564) WT-SLC4A11 or SLC4A11-E143K or SLC4A11-G709E. Cells were treated with ophthalmic NSAIDs or DMSO carrier alone (untreated) at concentrations indicated in Supplementary Table S1 in 0.2% DMSO right after transfection for 48 hours. Cells were probed for SLC4A11, using polyclonal rabbit anti-HA antibody. Donkey anti-rabbit IgG conjugated with Alexa Fluor 488 was secondary antibody, while nuclei were counterstained with DAPI. To quantify cell surface abundance of SLC4A11 variants, mean fluorescence intensities were determined using ImageJ software. Green fluorescence intensity was quantified at the plasma membrane of both untreated and drug treated cells (7–10 cells in each condition). Signal intensities were corrected for background by subtraction of intensity of region surrounding the cell. Fluorescence values are in arbitrary units. Asterisks indicate significant difference ($P < 0.05$) compared with untreated.

**Figure 4.** Water flux activity of SLC4A11 mutants. HEK293 cells, co-transfected with cDNA encoding eGFP and indicated SLC4A11 variants were treated with carrier (0.2% DMSO) (untreated) or 0.6 μM diclofenac or 64 μM nepafenac for 48 hours. (A) The level of green florescence was quantified in regions of interest in cells as medium was changed from iso-osmotic (black bar) to hypo-osmotic (open bar) Ringers’ buffer, using confocal microscopy. (B) The rate of fluorescence change upon switching to hypo-osmotic Ringer’s buffer was measured as a surrogate for the rate of cell swelling. Data were corrected for rates observed in vector-transfected cells and normalized to untreated WT-SLC4A11. Error bars indicate SD ($n=3–5$ experiments) of 10 to 20 cells/coverlip. Asterisks indicate significant difference ($P < 0.05$) compared with untreated.
treatment were plotted (Supplementary Fig. S2). Viewed in this way, there were highly significant rises in cell surface abundance for many SLC4A11 mutants. Indeed, T431I-SLC4A11 approached the level of cell surface abundance of WT-SLC4A11. While significant rises of cell surface abundance were observed, the range of cell surface abundance for mutants rose only to 12% to 84% of WT-SLC4A11, with many mutants in the 50% to 60% of WT range.

**DISCUSSION**

Rationale for this work came from a small-scale study that identified glafenine, a disused NSAID, as correcting some defective corneal-dystrophy–associated SLC4A11 mutants.20 Here, we screened available ophthalmic NSAIDs for their ability to correct cell surface trafficking defects in SLC4A11 mutants. Among five ophthalmic NSAIDs, nepafenac and diclofenac had the greatest ability to rescue the SLC4A11 intracellular retention phenotype, as assessed by a BRET-assay and confocal immunofluorescence. Diclofenac was superior in restoring SLC4A11 function (water flux assay). Earlier, 30 SLC4A11 mutations were identified as leading to intracellular SLC4A11 retention.12 We found that treatment with 1 μM diclofenac increased cell surface abundance of 20 of these mutant proteins. Some corneal dystrophy patients may therefore benefit by treatment with diclofenac (Voltaren Ophthalmic) eye drops. These patients are defined by carrying SLC4A11 mutations that are independent of COX2. Such an animal has not yet been reported but will provide a model to investigate NSAID therapy, because they do not express SLC4A11. An ideal model animal would express the equivalent of one of the intracellular-retained SLC4A11 mutants.12 Such an animal has not yet been reported but will be a welcome tool in developing therapeutics for corneal dystrophies. Another possible research direction could be the use of immortalized cultured human corneal endothelial cell line, for example HCEtT2I7 cells.30 To use such cells to screen possible SLC4A11 folding correctors, the endogenous SLC4A11 gene would need to be replaced with the gene-encoding folding defective, ER-retained SLC4A11 mutants.12

The degree of cell surface trafficking induced by NSAIDs varied depending on the specific mutation (Figs. 1, 3, 5, Supplementary Fig. S5). An earlier study examined the cell surface abundance of SLC4A11 mutants and the level of SLC4A11-mediated water flux function when CHED mutants were subjected to BRET-assay of cell surface localization. Data represent mean ± SD of percent relative change in the total cell surface abundance of treated versus untreated. Asterisks indicate significant difference (P < 0.05) comparing relative cell surface abundance of treated versus untreated.

**Figure 5.** Diclofenac efficacy in correcting folding defects in all identified mis-processing mutants of SLC4A11. HEK293 cells, transfected with cDNA encoding cell surface trafficking defective SLC4A11 mutants, were incubated with medium alone (untreated), or 1 μM diclofenac for 24 hours and subjected to BRET-assay of cell surface localization. Data represent mean ± SD of percent relative change in the total cell surface abundance of all identified mis-processing mutants of SLC4A11. FECD, cellular models, trafficking of mutant CFTR is increased by

ibuprofen.28 More recently, ibuprofen was found to increase the rate of microtubule formation in cells from CF patients.29 Another NSAID, celecoxib, increases trafficking through the ER of oligomeric proteins (like SLC4A11), in a mechanism independent of COX2, possibly modulating ER luminal chaperones.30 Together, NSAIDs appear beneficial to the maturation and targeting of some proteins, via mechanisms that are independent of COX2. Such a COX2-independent mechanism could explain the range of ophthalmic NSAID efficacy observed here, even though the drugs were tested at similar concentrations relative to their COX2 EC50. Finally, the increased level of apoptosis observed in cells expressing SLC4A1131 and elevated unfolded protein response12 in endothelium from explanted FECD corneas,32 are consistent with elevated ER stress in FECD. This raises the possibility that NSAIDs are beneficial by acting upon ER stress pathways.

Ophthalmic NSAIDs, in particular diclofenac, could be beneficial to corneal dystrophy patients carrying certain SLC4A11 mutations. Usually the step from an in vitro drug study, as performed here, to human testing would be to test in an animal model. Three lines of slc4a11 null mice have been developed that recapitulate the corneal defects found in patients.3,34,35 These mice are not, however, an appropriate model to investigate NSAID therapy, because they do not express SLC4A11. An ideal model animal would express the endogenous SLC4A11 gene would need to be replaced with the gene-encoding folding defective, ER-retained SLC4A11 mutants.12

The identification of the NSAID, glafenine, as an SLC4A11 corrector20 was inspired by the observation that this compound corrects trafficking of the 508del mutant of the CF protein, CFTR.26 Glafenine was noted to be a CFTR channel inhibitor, which may explain its interaction with the misfolded mutant CFTR.26 The NSAID, ibuprofen, is established as clinically beneficial for cystic fibrosis patients.27 and in cell culture models, trafficking of mutant CFTR is increased by
were expressed homozogously and FEDC mutants heterozygously, in a cell culture model. This work estimated that cells of CHED2 carriers, who are unaffected, would have approximately 60% of WT function, FEDC heterozygous cells 25% and CHED2 patients 5%. Thus, a therapy restoring the function of a CHED2 patient to 25% of WT levels could potentially delay disease for decades, as is seen in FEDC patients. While significant increases in FEDC expression were seen for diclofenac-treated FEDC mutants, only in the case of T431I was diclofenac able to push cell surface abundance past 60% of WT level (Supplementary Fig. S2). The lower level of correction seen in the other FEDC mutants might only lead to a degree of symptomatic relief and perhaps a delayed course of disease progression.

Experiments presented here analyzed the level of mutant SLC4A11 cell surface trafficking following ophthalmic NSAID treatment. Only in the case of E143K and G709E SLC4A11 was the effect of NSAIDs on SLC4A11 functional assessment. For these two mutants, a dramatic increase in SLC4A11 functional activity was observed, indicating that once trafficked to the cell surface, these mutants were sufficiently well folded to carry out their normal role in water flux. Thus, at least in some cases cell surface rescue may restore SLC4A11 functional activity to corneal endothelial cells in patients. A caveat to the present study, however, is that functional activity was not assessed for all SLC4A11 mutants following NSAID treatment. In the case of some mutants, cell surface trafficked protein may not be sufficiently well folded to be functionally competent.

NSAIDs varied in their ability to correct SLC4A11 trafficking and similarly NSAIDs differ in their reported adverse effects. NSAID eye drop preparations are commonly used by ophthalmologists and are well tolerated. In the context of corneal graft healing, some NSAIDs, in particular naproxen, slowed epithelial healing. Corneal dystrophy patients suffer pain and inflammation and NSAIDs would potentially provide additional beneficial symptomatic relief. Allergic contact dermatitis has been rarely reported associated with diclofenac eye drops. Drug delivery by eye drops would reduce systemic levels of NSAIDs to concentrations unlikely to cause adverse effects. Even without an animal model the transition toward clinical use of NSAIDs for some corneal dystrophy patients might be rapid.

The rare corneal dystrophies, CHED and HS, only arise from SLC4A11 mutations. Among 42 identified CHED and HS point mutations in SLC4A11, 24 give rise to ER-retained SLC4A11 protein. Thus, the majority of affected individuals could potentially benefit from NSAID therapy. This is especially promising as these diseases disproportionately burden poor countries where consanguineous marriages are culturally favored. Because the majority of CHED arises from ER-retained SLC4A11, it might be reasonable to attempt NSAID therapy in CHED patients without genetic testing to establish the genetic defect present in patients. Moreover, NSAID therapy is relatively inexpensive, thus representing an accessible therapy for disadvantaged populations.

FEDC has 4% lifetime incidence and accounts for approximately 25% of corneal transplants, making the disease clinically important. NSAID treatment of FEDC is unfortunately likely to be of narrower application. FEDC is genetically heterogeneous, with up to 70% of FEDC disease burden from mutations of transcription factor, TCF4. In these patients, poly-glutamine tract expansion may cause RNA toxicity and disease in affected patients. The FEDC patient population would need to be genetically screened to identify those carrying ER-retained mutants of SLC4A11. Only 6 of 18 FEDC-causing point mutants of SLC4A11 were ER-retained. Thus among FEDC patients with SLC4A11 mutations, approximately 50% would be candidates for NSAID therapy. Because FEDC is a common disease the number of candidate patients would be large, but genetic testing would be essential for this precision-medicine approach. The simplicity of treatment with existing ophthalmic eye drops, with low systemic drug travel does make this approach attractive. In FEDC patients the goal would be to reduce symptom severity and decrease the rate of corneal endothelial cell loss, to lengthen the interval between disease onset and corneal transplant.

Additional testing to develop an appropriate dosing regimen would be required to move ahead with NSAIDs in corneal dystrophy patients with SLC4A11 mutations. Here experiments were performed for 24 to 48 hours, using NSAIDs in the range for their pharmacologic effect on COX-2. Concentrations of NSAIDs are much higher in ophthalmic eye drops than the levels used in this study (Table), but testing would certainly need to be performed to establish the frequency of drop administration required to observe therapeutic symptom relief.

At present ophthalmic NSAIDS are used for a range of conditions. These include analectasia following corneal abrasion, sclerosis, and anterior chamber inflammation following cataract surgery. All of the ophthalmic NSAIDs used in this report were effective in reducing anterior chamber inflammation, with diclofenac revealed as most effective. In this context, it suggests that ophthalmic NSAIDS possess sufficient permeability to reach the corneal endothelium when applied topically.

**Conclusions**

Readily available ophthalmic NSAIDS, in particular diclofenac, effectively increase cell surface abundance of most ER-retained SLC4A11 mutants. Our data suggest that NSAID eye drops could be a useful therapy to treat a subset of FEDC patients identified by genetic testing, thus representing a personalized medicine strategy for this common disease. Among rare CHED patients, a sufficiently large fraction of patients might see increased SLC4A11 function upon NSAID treatment that such therapy might be implemented without genotyping. In both patient populations, wide use of NSAIDS will await appropriate clinical trial data.

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