NC-1059: A Channel-Forming Peptide That Modulates Drug Delivery across In Vitro Corneal Epithelium

Jesica Martin,[1] *Pradeep Malreddy,*[1] *Takeo Iwamoto,*[2] *Lisa C. Freeman,*[1] *Harriet J. Davidson,*[3] *John M. Tomich,*[2] *and Bruce D. Schultz*[1]

Purpose. The goal of this study was to determine whether a synthetic peptide, NC-1059, can modulate the corneal epithelium to increase the permeation of therapeutic agents across this barrier.

Methods. An in vitro system employing transformed human corneal epithelial (THCE) cells was optimized for this study. Culture conditions were identified to promote formation of a confluent monolayer that rapidly develops a substantial transepithelial electrical resistance. Electrical parameters were measured with a modified Ussing flux chamber, and solute flux was quantified with fluorescently labeled compounds.

Results. NC-1059 causes a concentration-dependent increase in short-circuit current and an increase in transepithelial electrical conductance when assessed in modified Ussing chambers. The effect of NC-1059 on transepithelial electrical resistance was reversible. To test for paracellular permeability and size exclusion, FITC-labeled dextran ranging in size from 10 to 70 kDa was used. Dextran permeated the corneal cell monolayer in the presence, but not the absence, of NC-1059. Fluorescein sodium and carboxyfluorescein were then used as low molecular weight markers with similar NC-1059–modulated kinetics being observed. Maximum permeation for the fluorescein derivatives occurred 30 to 90 minutes after a 5-minute NC-1059 exposure. A prototypical drug, methotrexate, also exhibited increased permeation in the presence of NC-1059.

Conclusions. NC-1059 enhances drug permeation across cultured corneal epithelial cell monolayers by transiently affecting the paracellular pathway. Thus, NC-1059 is a lead compound for development of cotherapeutic agents to enhance access and effectiveness of ophthalmic compounds. (*Invest Ophthalmol Vis Sci.* 2009;50:3337–3345) DOI:10.1167/iovs.08-3053

Drug delivery of ocular therapeutics presents several challenges. There are many difficulties in attaining and sustaining adequate therapeutic levels in the eye while avoiding systemic toxicity. Although there are several routes of administration available for the eye (e.g., topical, subconjunctival, retrobulbar, intracameral, and systemic), topical application of therapeutic agents to the eye offers several advantages including localized drug effects with limited entry into systemic circulation, better accessibility than can be achieved by systemic delivery, avoidance of first-pass hepatic metabolism, convenience, and simplicity. Despite the benefits of topical application, many factors limit the bioavailability of therapeutic agents. For instance, the maximum volume that can be contained within the conjunctival cul-de-sac and precorneal tear film is approximately 30 μL, and the solutes are eliminated rapidly from the precorneal area by lacrimal secretions. The cornea is the outermost transparent portion of the eye and provides the primary barrier through which ocular absorption must occur.[1] The anatomic structure of the cornea results in inefficient delivery of drugs to the deeper structures of the eye due to the relatively small surface area and its intrinsic nature as a barrier. Three main strategies are used to increase the bioavailability of ophthalmic drugs including increasing the contact time of the drug with the cornea, improving drug penetration characteristics, and enhancement of the corneal permeability (reviewed in Ref. 2).

Corneal epithelial cells constitute the major rate-limiting barrier to drug absorption after topical application. There are two major routes for the movement of compounds through the corneal epithelium: transcellular and paracellular. Since epithelial cell membranes are barriers rich in lipid, lipophilic agents show the best intrinsic penetration through the transcellular pathway. In the absence of an active transport process, however, hydrophilic compounds are limited substantially to permeation through the paracellular pathway where movement is blocked by tight junctions that join adjacent cells near their mucosal surface. The tight junctions define the apical and basolateral membrane components and they serve as a semipermeable barrier to the flow of solutes through the paracellular pathway. In some tissues, the permeation rates across the tight junctions change in conjunction with various events such as leukocyte transmigration and nutrient uptake in the intestine.[3,4] Tight junctions are also regulated by hormones in some tissues, such as transforming growth factor, glucocorticoids, and progesterone in mammary epithelium.[5,6] Thus, therapeutic modulation of corneal tight junctions may allow alteration of the paracellular pathway that would enhance ophthalmic drug concentrations.

A synthetic channel-forming peptide, designated NC-1059, has been shown to transiently modulate the barrier function of tight junctions in Madin-Darby canine kidney (MDCK) epithelial cell monolayers.[7] This functionality was also observed with epithelial cells derived from the urogenital duct, gut, and airway of other species.[8] In initial studies that targeted the development of a synthetic ion channel, the peptide used was based on the second transmembrane segment of the glycine receptor α-subunit (M2GlyR). This peptide forms a selective pathway in epithelial cells that allows for anion secretion,[9] but exhibits relatively low bioavailability due to solution aggregation. Modification of the M2GlyR peptide increased aqueous solubility while maintaining the channel-forming properties as well as anion selectivity.[10,11] A peptide was then designed in which the transmembrane segment had an N-terminal tetra-lysine seg-

From the Departments of [1]Anatomy and Physiology, [2]Biochemistry, and [3]Clinical Sciences, Kansas State University, Manhattan, Kansas.

This article represents contribution no. 09-138-J from the Kansas Agricultural Experiment Station.

Supported by National Institutes of Health Grants GM 074096, EY 015606, T32-RR 017497, and P20-RR 017686.

Submitted for publication October 23, 2008; revised December 26, 2008; accepted May 7, 2009.

Disclosure: **J. Martin**, None; **P. Malreddy**, None; **T. Iwamoto**, Nacelle Therapeutics (I, P); **L.C. Freeman**, None; **H.J. Davidson**, None; **J.M. Tomich**, Nacelle Therapeutics (I, P); **B.D. Schultz**, Nacelle Therapeutics (C, P)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Bruce D. Schultz, Department of Anatomy and Physiology, Kansas State University, 1600 Denison Avenue, Coles Hall 228, Manhattan, KS 66506; bschultz@vet.ksu.edu.

ment and a central leucine that was flanked by a palindrome that is based on the N-terminal portion and was designated NC-1059 (KKKK-AARVGLGITTVLVTTIGLGVRAA). NC-1059 elicited an increase in short circuit current (I_{sc}, a sensitive indicator of net ion transport), remains monomeric in aqueous solution,[10] and exhibits no obvious indication of cytotoxicity.[7] Thus, NC-1059 represents a possible means of manipulating the epithelial barrier of the cornea at the tight junctions.

Studies that assess ophthalmic bioavailability can be conducted in a variety of assay systems. Historically, drug permeation studies have been conducted in vivo with the use of rabbits. However, an in vitro system has many advantages over conventional techniques including the opportunity to use tissues derived from humans and the opportunity to minimize time-consuming, expensive, and controversial animal studies.[12] THCE cells[13] provide a model system to asses drug permeation, as has been reported recently.[12] These cells have been used to assess tight junctions,[14,15] to characterize corneal wound healing,[16–18] and to test for cytotoxicity.[19] Additional electrophysiological studies verified that THCE cells provided a viable option for the assessment of drug permeation across a confluent epithelial layer.

The goal of this study was to determine whether a synthetic channel-forming peptide, NC-1059, can modulate the barrier function of corneal epithelium in vitro using THCE cells. After establishing critical basal parameters for the cultured corneal epithelial cells, experiments were conducted to test for an NC-1059-associated enhancement in gradient driven drug permeation across confluent monolayers. The outcomes show that methotrexate and surrogate drugs of various sizes and chemistries more readily gain access across the corneal epithelial barrier in the presence of or after brief exposure to NC-1059.

Methods

Peptide Synthesis

The peptides used in this study were generated by solid-phase synthesis with 9-fluorenylmethoxycarbonyl chemistry, as described in detail previously.[20] The peptides were purified and characterized by reversed-phase HPLC and matrix-assisted laser desorption time-of-flight mass spectroscopy, respectively.

Cell Culture

THCE cells were obtained from Kaoru Araki-Sasaki (Ehime University, Japan) and Fu-Shin Yu (Medical College of Georgia, Augusta, GA). The cultures were maintained in defined keratinocyte-serum free medium (DK-SFM; Invitrogen Corp., Carlsbad, CA), supplied as a kit with all components being used according to the manufacturer's directions, in a 5% CO_2, humidified environment at 37°C. At 80% confluence, THCE cells were washed with phosphate-buffered saline (PBS; composition in mM: 140 NaCl, 2 KCl, 1.5 KH_2PO_4, and 15 Na_2HPO_4) and dissociated with 0.05% (wt/vol) trypsin and 0.47 mM ethylenediamine tetra-acetic acid (Invitrogen Corp., Carlsbad, CA). After 8 minutes of incubation, 0.2 mL of fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 0.5 mL DK-SFM were added to neutralize the trypsin. Cells were dislodged from the substrate and suspended by pipetting. Cells were pelleted by centrifugation, the supernatant was removed and the pellet was resuspended in 6 mL fresh DK-SFM. One milliliter was transferred to a fresh tissue culture flask containing 3 mL DK-SFM (1:6 split ratio). Cells were also seeded onto permeable supports, 0.33, 1.13, or 4.67cm^2 (Transwell or Snapwell; Corning-Costar, Acton, MA) with a density of $\sim 5 \times 10^5$ cells/cm^2 and incubated in DK-SFM with the medium refreshed every other day. Cells were allowed to attach and form monolayers for 5 to 10 days before experimentation although assays were conducted typically 6 days after seeding.

Immunofluorescence Labeling/Hematoxylin and Eosin Staining

THCE monolayers were cultured on filters (Transwell; Corning-Costar) and fixed in 10% buffered neutral formalin (Fisher, Pittsburgh, PA). Monolayers were washed briefly with 0.02% Triton X-100 in PBS, blocked with 3% goat serum (Invitrogen Corp.) and 1% bovine serum albumin (Sigma-Aldrich, Inc., St. Louis, MO) in PBS for 20 minutes and then exposed to rabbit antibody raised against an occludin epitope in a 1:100 dilution (rabbit anti-occludin antibody; Zymed, South San Francisco, CA) for 2 hours at room temperature. Monolayers were washed three times with PBS and exposed for 1 hour at room temperature to goat anti-rabbit IgG secondary antibody that was conjugated to Alexa 488 (Molecular Probes, Eugene, OR). After three washes with PBS for 5 minutes each at room temperature, the monolayers were mounted on glass slides (Vectashield Mounting Medium; Vector Laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole (DAPI) as a nuclear stain. Labeling was then examined and photographed under a light microscope. A portion of each filter was processed identically in parallel with the exception that exposure to primary antibody was omitted.

In a separate set of experiments, THCE cell monolayers were cultured on permeable supports, fixed in 10% neutral buffered formalin, and processed by a commercial diagnostic laboratory (Department of Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, KS) to visualize cell structures after staining with hematoxylin and eosin (H&E).

Electrophysiological Measurements

THCE cells were seeded on permeable supports (Snapwell; Corning-Costar) and allowed to grow as confluent monolayers. On day 6 after seeding (unless indicated otherwise), the monolayers were mounted in modified Ussing flux chambers (model P2300; Physiologic Instruments, San Diego, CA). Chambers were maintained at 37°C in symmetrical Ringer's solution (in mM: 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, and 1.2 $CaCl_2$), which was made fresh daily. Hemichambers were bubbled continuously with 5% CO_2 to 95% O_2 to provide aeration, pH control, and mix the fluid. After mounting, the transepithelial electrical potential for each monolayer was recorded and then clamped to zero with a multichannel voltage clamp (VCCMC8, Physiologic Instruments) and I_{sc} was monitored continuously. Data were acquired at 0.1 to 1 Hz (Acquire and Analyze software, ver. 2.3.159; Physiologic Systems, on an Intel-based computer, Intel, Mountain View, CA). Monolayers were exposed periodically to a 1 mV bipolar pulse of 100-ms duration. Resultant current deflections were used by the software program, with Ohm's law, to determine the transepithelial electrical resistance (R_{te}) and its inverse, transepithelial electrical conductance (g_{te}), which are sensitive indicators of epithelial restrictions to the flow of small, charged solutes.

FITC-Dextran Permeability

Confluent THCE monolayers grown on tissue culture inserts were used to assess the permeability to various sizes of fluorescein isothiocyanate (FITC)–conjugated dextran (Sigma-Aldrich, Inc.). Tissue culture medium was removed and the cells were washed with warmed Ringer's solution before being exposed to one of three treatments: (1) Ringer's solution in both the apical and basolateral compartments; (2) Ringer's solution in the apical and basolateral compartments with NC-1059 (100 μM) in the apical compartment; or (3) hypotonic Ringer's solution (diluted 1:1 with distilled water) in the apical and basolateral compartments with EDTA (5 mM) in the apical compartment. FITC-dextran (25 μg/μL; nominally 10 kDa (2.5 μM), 20 kDa (1.25 μM), 40 kDa (0.63 μM), or 70 kDa (0.36 μM) was added to the apical compartment of each well and incubated at 37°C for 60 minutes. The solution from the basolateral side was then sampled, and the fluorescence immediately determined (485 nm excitation, 527 nm emission; Fluoroskan Ascent FL; Thermo Fisher Scientific, Waltham, MA). Standard curves were

obtained by serial dilution (2.5 μg/μL to 25 pg/μL) of the FITC-dextran. Data were plotted with commercial software (SigmaPlot, ver. 6.00; SPSS Inc., Chicago, IL) and, after an initial "fit by eye" to determine limits of the linear portion of the relationship for regression analysis (typically between 0.25 and 25 pg/μL), fitted by a zero-order polynomial. All experimental samples were within the linear portion of the standard curve, and the regression relationship was used to quantify the amount of fluorescent marker in the sample.

Fluorescein Permeability

The permeabilities of the monolayers to sodium fluorescein and 5(6)-carboxyfluorescein (Sigma-Aldrich, Inc.) were assessed in monolayers grown on permeable tissue culture supports. Before treatment, the medium was removed and the monolayers were washed with warmed Ringer's solution. This experiment included one of the following three treatments in symmetrical Ringer's solution: (1) Either sodium fluorescein or carboxyfluorescein was added at to the apical compartment at time 0; (2) either sodium fluorescein or carboxyfluorescein was added to the apical compartment with concurrent apical exposure to NC-1059 (100 μM); or (3) the apical compartment included NC-1059 (100 μM) for 5 minutes. The apical solution was then removed and replenished with fresh Ringer's solution. Sodium fluorescein or carboxyfluorescein was added to the apical compartment immediately, 30, 60, or 90 minutes after the removal of NC-1059. On fluorescein addition in all conditions, monolayers were incubated at 37°C for 60 minutes before sampling from the basolateral side for immediate fluorescence quantification and permeation determination as described earlier, with the exception that the linear range of the standard curve was typically between 3.76 ng/μL and 0.376 pg/μL.

Methotrexate Permeability

THCE cell monolayers grown on tissue culture inserts were used to assess the permeability to a representative clinical compound, methotrexate (MTX). Before treatment, the medium was removed and monolayers were washed with warmed Ringer's solution. Experiments to assess MTX permeation contained one of the following four treatments in symmetrical Ringer's solution: (1) MTX was then added to the apical compartment; (2) hypotonic Ringer's solution (diluted 1:1 with distilled water) was placed in the apical and basolateral compartments and MTX was added to the apical compartment with EDTA (5 mM); (3) MTX was added to the apical compartment with NC-1059 (100 μM); or (4) the apical compartment included NC-1059 (100 μM) for 5 minutes, after which the apical solution was removed and replenished with fresh Ringer's solution containing MTX. Samples (100 μL) were removed (with replacement by an equal volume of Ringer's solution) from the basolateral side 15 minutes, 1 hour, 2 hours, and 4 hours after the addition of MTX. Samples were dried and reconstituted in water (10 μL).

A separate set of similar paired experiments was conducted to test for effects of benzalkonium chloride (BAC) on MTX permeation. Treatments 1 to 3 are identical to those just listed. In the fourth treatment, however, Ringer's solution was placed in the apical and basolateral compartments, and MTX was added to the apical compartment immediately after apical exposure to 0.01% BAC (Sigma-Aldrich, Inc.). Sampling procedures were identical to those listed.

A rapid, sensitive, and simple high-performance liquid chromatographic (HPLC) method was used for the determination of the amount of MTX in samples from the basolateral compartment. The HPLC system, (1100 series, Agilent Technologies, Waldbronn, Germany) was fitted with a binary pump (G1312A), diode array (G1315B), and multiple-wavelength detectors and an online electron spray ionization (ESI)-ion trap (IT) mass spectrometer (Esquire 3000 Plus; all from Bruker Daltonics Inc., Billerica, MA). A reversed-phase column (MaxRP, 4 mm × 2 mm × 2 μm Synergi; Phenomenex, Torrance, CA) was used. Eluents containing different ratios of acetonitrile to water were used to determine the optimal mobile phase. The MTX was eluted at retention times of 3.2 and 9.6 minutes with a linear 10-minute gradient from 7% to 90% acetonitrile in water containing 0.1% formic acid. The flow rate was 0.2 mL/min. Each of the sample and standard injection volume was 2 μL. The UV absorbance at 215 nm was monitored. For the quantification of MTX in Ringer's solution samples, standard solutions (3.125–62.5 μM) were used for daily calibrations before analysis. Quantification was achieved by measurement of the peak-area of each compound, and the lower detection limit was 0.5 picomoles (S/N = 2) for MTX. Separated peak purity was analyzed with an online ESI-IT mass spectrometer. Positive-ion electrospray mass spectra were obtained in the following conditions: nitrogen gas was used as the nebulizing (20 psi) and drying gas (6 L/min) while the source temperature was maintained at 350°C. Capillary voltages were −4.1 kV. Spectra were acquired in multichannel-acquisition mode from scans encompassing m/z 50 to 1000 at 7.3 ms/scan (and averaging five such scans per spectrum).

Statistical Analysis

All results are presented as the mean and associated SEM. Where appropriate, a one-way ANOVA was performed to assess the likelihood of population differences. A probability of a type I error of <5% was considered statistically significant. Fitting of defined functions to data sets was conducted by using commercial software (SigmaPlot, ver. 6.00; SPSS Inc., Chicago, IL).

Results

Epithelial Characteristics of THCE Cells

THCE cells cultured on permeable supports were evaluated first to test for the presence of epithelial characteristics. Cultured cells were processed by standard techniques, stained with H&E and assessed by light microscopy. As shown in Figure 1A, THCE cells formed a confluent layer

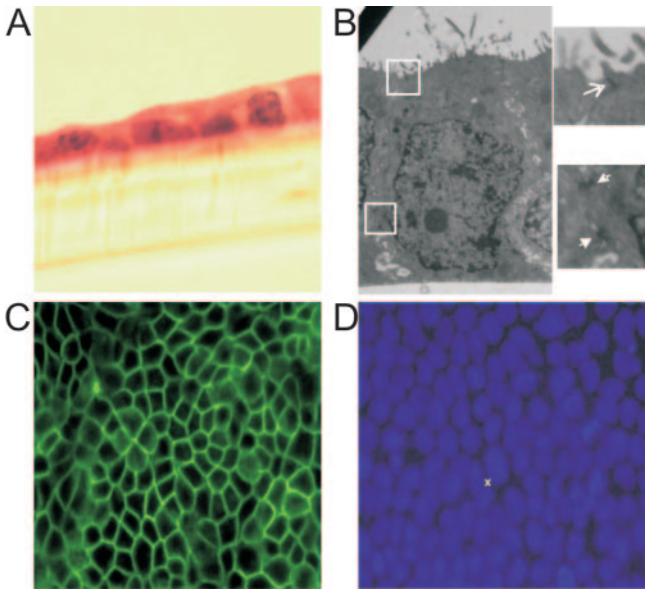

Figure 1. THCE cells exhibit epithelial characteristics when microscopically evaluated. (**A**) H&E stain of a cell monolayer on the permeable tissue culture support. (**B**) Transmission electron micrograph showing the cross-sectional profile of a THCE cell on the tissue culture support (*bottom*). Microvilli are present at the apical membrane. *Boxed areas* are enlarged on the *right* to show electron dense regions that are consistent with apical tight junctions (*top*; *arrow*) and desmosomes (*bottom*; *arrowheads*). (**C**, **D**) Indirect immunocytochemistry demonstrating occludin immunoreactivity in THCE monolayers in the presence (**C**) or absence (**D**) of primary antibody. Presence of cells confirmed by DAPI staining (**D**). Images acquired with standard light microscopy.

that exhibits cuboidal epithelial morphology, consistent with observations reported previously,[21] although there was no evidence for multiple cell layers. Electron microscopy revealed the presence of microvilli on the apical cell membrane (Fig. 1B) and electron dense regions consistent with the presence of tight junctions (top inset) and desmosomes (bottom inset). Immunochemical techniques were used to test for the expression and distribution of occludin, a protein that is present at tight junctions. Antioccludin immunoreactivity was observed as a reticular pattern that fully circumscribes all cells in the cultured monolayer (Fig. 1C). This pattern was consistent with the expected localization of occludin at cell margins, where it contributes to the tight junctions and indicates that THCE cells were capable of forming an epithelial barrier that may be expected to be electrically resistive. The pattern of occludin reactivity seen was similar to the pattern of expression of other tight junction associated proteins, specifically ZO-1 and -2 reported previously with the same cell type.[14,15,21] Alexa-488 fluorescence associated with the secondary antibody was not observed when the primary antibody was omitted from the assay protocol, although the presence of cells was clearly demonstrated with nuclear staining by DAPI (Fig. 1D).

Electrophysiological techniques were also used to evaluate the epithelial nature of the cultured cells. R_{te} was determined by applying Ohm's law with the observed change in I_{sc} that results from exposing the epithelial monolayer to a bipolar voltage pulse. THCE monolayers exhibited an initial R_{te} (454 ± 16 Ω cm^2; n = 42), which indicates the ability to separate fluid compartments of differing compositions. The experiments detailed herein demonstrate that, when compared with filter supports without cells, THCE cell monolayers reduced gradient-driven flux from the apical to the basolateral compartment by greater than 99% for solutes ranging in size from 0.3 to 70 kDa. Taken together, these results indicate that THCE cells formed a resistive monolayer and function as an epithelial barrier after being grown in culture for only 5 to 10 days.

NC-1059 and Concentration-Dependent Increases in I_{sc} and g_{te}

Results from a typical experiment to test for effects of NC-1059 on THCE monolayers are presented in Figures 2A and 2B. THCE monolayers responded after apical exposure to 100 μM NC-1059 with changes in I_{sc} that were characterized by a rapid increase in I_{sc} from a baseline of less than 1.5 μA/cm^2 to a peak of greater than 19 μA/cm^2 in less than 10 minutes, which declined toward pretreatment values over the next hour of continual monitoring (Fig. 2A). This increase in I_{sc} is interpreted to indicate net anion secretion. Results presented in Figure 2B demonstrated that apical exposure to NC-1059 was associated with a substantial and transient increase in g_{te}, although the time course for this effect was decidedly different from that for changes in I_{sc}. In this experiment, g_{te} increased from a baseline of less than 3 mS/cm^2 to a peak value of greater than 70 mS/cm^2, before declining. The change in g_{te} developed more slowly than the change in I_{sc} and required greater than 15 minutes to achieve a maximum, which suggests that a distinct underlying mechanism accounts for the outcome. Data from Figures 2A and 2B and 8 to 11 additional observations for each concentration are summarized in Figures 2C–E. Results in Figure 2C showed that paired monolayers exposed to greater concentrations of NC-1059 exhibited a greater maximum change in I_{sc}. The data represented in Figure 2C were fitted by a modified Hill equation: $I_{sc} = I_{sc\text{-}max}[x^n/(k_{app}{}^n + x^n)]$, where I_{sc} is observed, $I_{sc\text{-}max}$ is the derived maximum change in I_{sc},

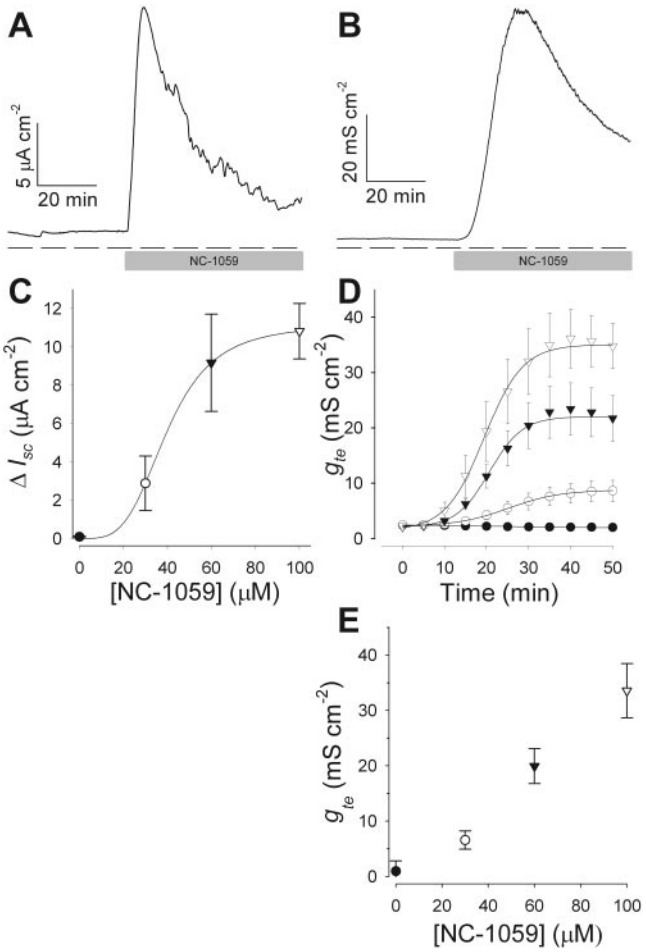

FIGURE 2. NC-1059 causes a concentration and time dependent increase in I_{sc} and g_{te}. (**A**, **B**) Representative experiment showing effect of NC-1059 on I_{sc} (**A**) and g_{te} (**B**) across THCE monolayer. *Dashed line*: zero current or conductance. (**C**) Change of I_{sc} plotted as a function of peptide concentration. *Solid line*: best fit of a modified Hill equation to the data set as described in the text. (**D**) Time-dependent increase in g_{te} at each NC-1059 concentration: vehicle (●), 30 μM (○), 60 μM (▼), and 100 μM (▽). (**E**) Change of g_{te} plotted as a function of peptide concentration. Data in (**C–E**) are the mean ± SEM of 9 to 12 observations at each concentration.

k_{app} represents the peptide concentration at half $I_{sc\text{-}max}$ (the apparent dissociation constant, K_D), x represents the peptide concentration, and n is the Hill coefficient. The results indicate a maximum NC-1059-induced current of 11.2 ± 2.3 μA/cm^2, and k_{app} of 39.8 ± 9.9 μM. The $I_{sc\text{-}max}$ was in the same range and the k_{app} was similar to that reported previously for other cell types.[7,8] The Hill coefficient of 3.7 ± 2.4 was similar to that reported previously[7] and reflected a complex reaction scheme, which suggests the oligomerization of monomers in the membrane to form a functional channel.

The concentration dependence of the effect of NC-1059 on g_{te} (Figs. 2D, 2E) appeared to have fundamental differences from the effect on I_{sc}. Concentration dependence was observed, although there was not a clear indication that the effect was saturating at the greatest concentration used in these studies (100 μM). Significant and long-lasting effects were observed with as little as 30 μM NC-1059 and stepwise increments in g_{te} were observed at 60 and 100 μM. Results summarized in Figure 2D show that g_{te} increased to a plateau before beginning to decline and that the NC-1059-induced increase in g_{te} was dependent on concentration. Data in Figure 2D were

fitted by a logistic equation as reported previously,[8] to predict the maximum change in g_{te} and the time course of this change.

$$g_{te} = g_{teo} + \frac{g_{te\text{-}max}}{1 + e^{-[(t-t_0)/b]}}$$

where g_{teo} is the initial g_{te}, whereas $g_{te\text{-}max}$ represents the maximum change in g_{te}, t_0 represents the time to reach $g_{te\text{-}max}/2$, and b is inversely proportional to the rate of increase at t_0. g_{teo} was constrained to be positive in this analysis, and $g_{te\text{-}max}$ demonstrates concentration dependence with a maximum value of 33.6 ± 4.6 mS/cm^2, this value directly correlates with data gathered previously on MDCK cells (33.5 ± 2.2 mS/cm^2)[7] and in other cell types.[8] There was no distinct concentration dependence observed for t_0 or b and the rate of increase for both varied over a narrow range. Figure 2E represents the summarized maximum g_{te} that was measured after exposure to each concentration of NC-1059. As mentioned earlier, there was no apparent indication of saturation within the concentration range tested. It is important to note that, at 100 μM, the conductance observed approached the maximum observable conductance of the recording system, such that additional concentrations were not tested.

Effect of NC-1059 on g_{te}

Experiments were performed to determine whether the effects of NC-1059 on THCE monolayers are both transient and repeatable. On day 5 after seeding, sets of six monolayers were placed in Ussing chambers and apically exposed to either NC-1059 (100 μM; two chambers) or a retroinversed M2GlyR sequence (KKKK-ARSGSSQTMTLVTTIGLGVRAA designated NC-1076; 100 μM; four chambers) that does not affect g_{te}, the responses were recorded and the monolayers were recovered. Each of the monolayers was returned to the incubator and the media refreshed daily before any further experiments. Two days later, the monolayers that had been exposed to NC-1059 were again exposed, and two of the four chambers that were exposed previously to NC-1076 were exposed to NC-1059 for the first time while the remaining two monolayers were exposed again to NC-1076. The monolayers were recovered again. On day 9 after seeding all the chambers were exposed to NC-1059, two chambers for the first time and four chambers for the second or third time. Data from four to six separate experiments on each day are summarized in Figure 3; all monolayers exposed to NC-1059 for the first time resulted in a substantial increase in g_{te}, regardless of which day the first exposure occurred. Monolayers exposed to NC-1076 showed no change from baseline g_{te}. During the 2 days after exposure to NC-1059, g_{te} of THCE cell monolayers returned toward baseline as has been reported for other cell types.[7,8] Monolayers exposed to NC-1059 for the second or third time showed a peptide-induced increase in g_{te}, although not to the extent as seen with the first exposure. Results presented in Figure 3 demonstrate that changes in g_{te} induced by NC-1059 were reversible, although only partially repeatable with this cell line. This finding differs from data obtained previously in experiments with MDCK cells that were both fully reversible and repeatable.[7]

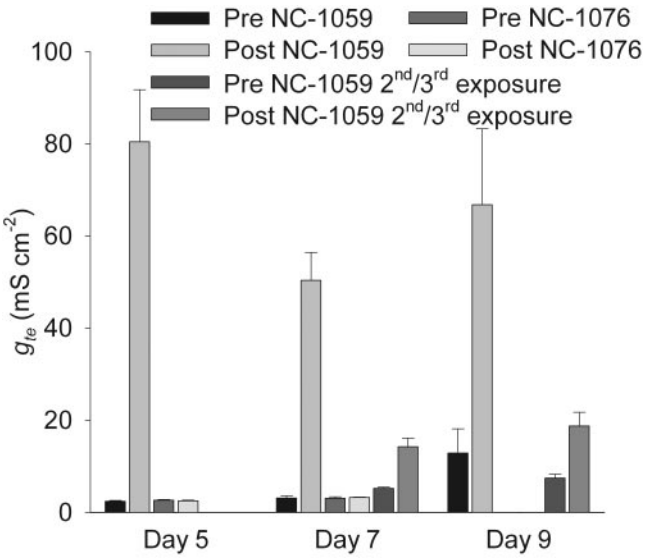

FIGURE 3. Effect of NC-1059 on g_{te} is reversible and partially repeatable within two days. Average g_{te} was determined for 5 minutes before exposure to either 100 μM NC-1059 or NC-1076, an inactive peptide analogue. Maximum g_{te} was then determined after exposure. After each experiment, the monolayers were recovered and again exposed to either NC-1059 or NC-1076 on days 7 and 9. Summary of data from four experiments with six monolayers in each experiment.

Effect of NC-1059 on Permeation of FITC-Dextran Conjugate

The electrophysiological data just presented suggest that NC-1059 affects the integrity or barrier function of corneal epithelial monolayers at the paracellular pathway. To test this hypothesis further, experiments were conducted to assess transepithelial permeation of large solutes in the absence and presence of NC-1059. Dextran, ranging in mass from 10 to 70 kDa that was conjugated to FITC, was placed on the apical aspect of the THCE monolayers in the absence or presence of NC-1059 or EDTA. After 1 hour, the basolateral medium was analyzed for the amount of FITC-dextran that had permeated the monolayer in each condition. Results presented in Figure 4 demonstrate that the culture supports were freely permeant to all masses of dextran tested and that the permeation was reduced to less than 1% by confluent THCE cell monolayers. Dextran permeation across the monolayers was enhanced two- to threefold by concurrent exposure to NC-1059 (Fig. 4, inset), although the maximum flux remained below 1.5% of that observed in the absence of cells. EDTA in hypotonic medium was used in paired experiments as a condition that is known to disrupt tight junctions. This treatment resulted in a 15- to 35-fold increase in dextran permeation compared with vehicle-treated monolayers that, for the 10-kDa dextran allowed for a total flux that was more than 10% of that observed in the absence of cells. These results demonstrate that NC-1059-stimulated changes in g_{te} are mirrored by and probably result partially from an increase in the permeability of tight junctions to both charged and uncharged solutes, which can be in excess of 10 kDa.

Effect of NC-1059 on the Permeation of Sodium Fluorescein and Carboxyfluorescein

Experiments designed to determine whether NC-1059 proportionately increases the permeation of low-molecular-weight compounds were conducted. Paired sets of experiments were performed to assess permeation of sodium fluorescein, a hydrophobic compound, and carboxyfluorescein, a hydrophilic compound, across THCE monolayers in the absence of, in the presence of, and after apical exposure to NC-1059. Experiments similar to those for dextran permeation were conducted in which either of the fluorescent dyes was placed in contact with the apical aspect of an epithelial monolayer for 60 minutes and the appearance of marker in the basolateral compartment quantified. Results presented in Figure 5 show that the

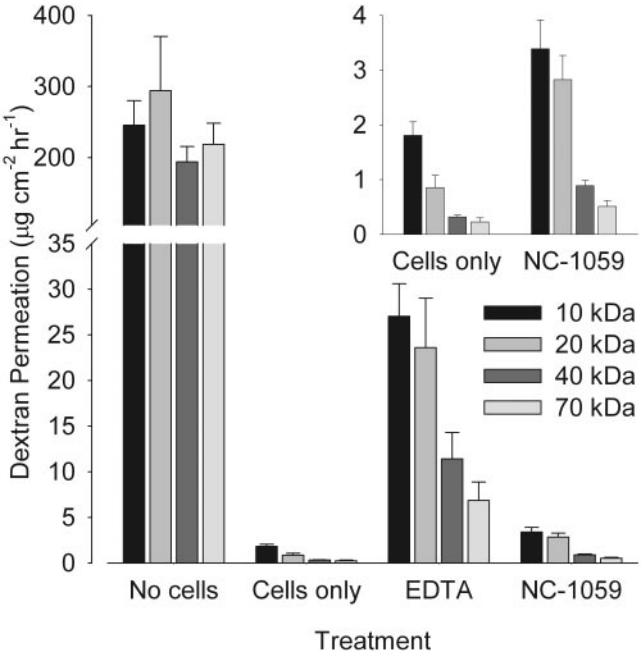

FIGURE 4. Exposure to NC-1059 increases dextran permeation across THCE monolayers. Permeation across the tissue culture support (No cells) was approximately 100-fold higher than in the presence of cells. Application of EDTA (5 μM) in hypotonic Ringer's (50% dilution with water) allows permeation for all sizes of FITC-dextran tested. Each assay was performed in triplicate, and the results are summarized from four experiments. *Inset*: Data presented on an expanded scale.

culture supports were freely permeable to both sodium fluorescein and carboxyfluorescein (Figs. 5A, 5B, respectively; no cells) and that THCE cell monolayers formed a barrier that reduced diffusional flux by greater than 96% (cells only). Exposure to the fluorescent dyes in the presence of NC-1059 (concurrent) enhanced dye permeation by 1.8- to 2.6-fold. Paired experiments were conducted to test for the duration over which marker permeation could be enhanced after a short exposure to NC-1059. Thus, monolayers were exposed to NC-1059 for 5 minutes; the apical medium was removed; and, after the monolayer was rinsed one time, the medium was replaced by Ringer's solution. Fluorescent dyes were added to the apical compartment immediately or 30, 60, or 90 minutes later and net flux determined over the subsequent 60-minute period. The results demonstrate that flux was enhanced modestly, when compared with concurrent exposure, and the maximum flux was observed for the period starting 30 minutes after exposure to NC-1059 and extending to 90 minutes after NC-1059 exposure. Ninety minutes after NC-1059 washout, the barrier function of THCE monolayers, as assessed by carboxyfluorescein permeation, was indistinguishable from untreated controls. However, sodium fluorescein permeation was still increased over that of the control. These results demonstrate that either concurrent treatment with NC-1059 or minimal exposure (5 minutes) to NC-1059 will result in a transient increase of permeation for carboxyfluorescein as well as sodium fluorescein across THCE monolayers.

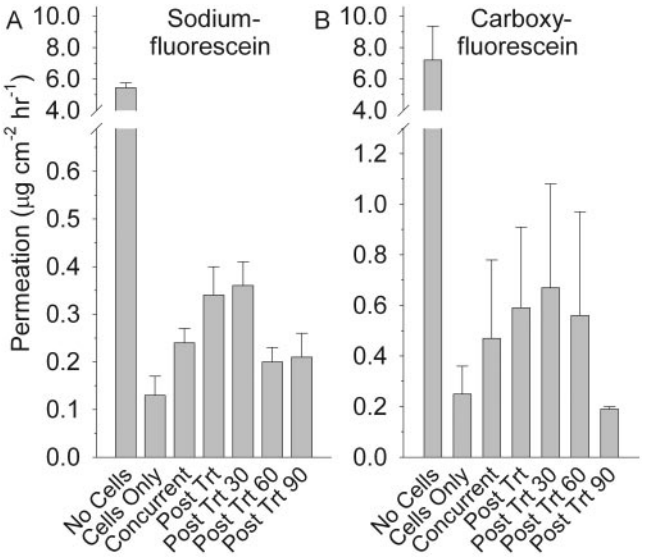

FIGURE 5. NC-1059 increases the permeation of both sodium fluorescein (**A**) and carboxyfluorescein (**B**) across cultured THCE monolayers. Culture supports (No Cells) are freely permeable to the fluorescein derivatives and are reduced more than 25-fold by the cultured THCE cells (Cells Only). Fluorescein permeation was assessed for 1 hour with concurrent exposure or starting immediately, 30, 60, or 90 minutes after a 5-minute exposure to NC-1059. The results are summarized from four similar experiments.

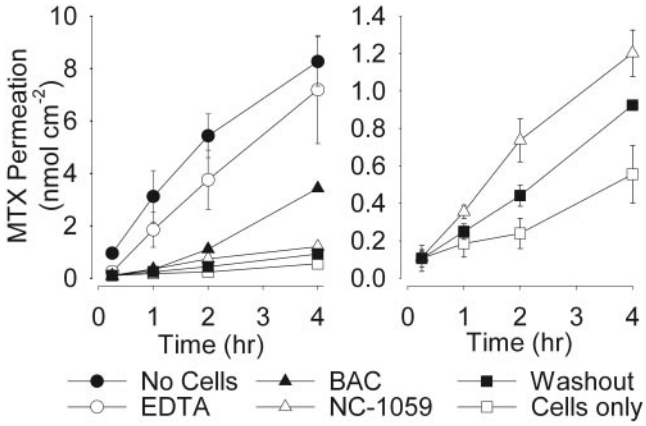

FIGURE 6. NC-1059 increases the permeation of methotrexate (MTX) across THCE monolayers. The treatments are defined as follows: No Cells, tissue culture support; EDTA (5 mM) in hypotonic Ringer's solution; BAC (0.01%); NC-1059, (100 μM) exposure throughout the assay period; Washout, NC-1059 exposure (100 μM) for 5 minutes before assay; and Cells only, vehicle exposure. Each assay was performed in triplicate, and the results are summarized from four experiments.

Effect of NC-1059 on Methotrexate Permeation

Experiments were conducted to determine the effects of NC-1059 on the permeation of a prototypical drug, methotrexate, across THCE monolayers. Experiments were preformed to assess the permeation of methotrexate across THCE monolayers in the absence or presence of NC-1059, EDTA, and BAC, and after apical exposure of NC-1059 (Fig. 6). The results show that methotrexate continued to diffuse across the tissue culture support for the duration of the experimental protocol (4 hours) and that the THCE monolayer reduced permeation across the culture support by more than 16-fold. Exposure to EDTA for the duration of the protocol resulted in an outcome that was indistinguishable from the tissue culture support alone. Of importance, NC-1059 enhanced methotrexate permeation by ~2.5-fold over the 4-hour sampling period. After a 5-minute exposure to NC-1059, methotrexate permeation also increased approximately 1.8-fold over that of the vehicle control. BAC was used in this experiment because it is a corneal

permeation enhancer and a preservative that is used in current ophthalmic compounds even though it has been shown to have toxic effects on cells by damaging the corneal epithelium.[22,23] In this experiment, BAC increased the amount of methotrexate that permeated the epithelial layer by 7.8-fold compared with that of the vehicle control and by 3.2-fold relative to that in the presence of NC-1059. These results demonstrate that NC-1059 is able to promote increased permeation of methotrexate as a prototypical therapeutic agent across cultured THCE cells in addition to enhancing the permeation of salts as measured by g_{te}, other low-molecular-weight compounds exemplified by fluorescein derivatives, and larger uncharged solutes represented by dextrans.

Discussion

In this study, we determined that a synthetic peptide, NC-1059, modifies the barrier function of THCE cells grown as a monolayer culture. Initially, experiments were conducted to verify that THCE cells cultured for fewer than 10 days form an epithelial barrier in culture that is capable of separating fluid compartments with distinctly different compositions. After the utility of this in vitro system was demonstrated, experiments were conducted to show that NC-1059 caused a concentration-dependent enhancement of g_{te} that is paralleled by an increase in passive, gradient-driven permeation rates for either large uncharged solutes (dextran), low-molecular-weight markers (fluorescein), or a prototypical low-molecular-weight drug (methotrexate). Of importance, the results showed that short-term (5 minutes) exposure to NC-1059 enhanced permeation of fluorescein for 60 to 90 minutes, after which no effect on permeation rates was observed. The rate of methotrexate permeation was likewise enhanced over the first 2 hours after NC-1059 exposure. Equally important is the observation that g_{te}, the most sensitive indicator of epithelial barrier integrity, returned to pretreatment values and became responsive to repeated exposure. Taken together, these results indicate that NC-1059 enhanced drug permeation across a model corneal epithelium by transiently affecting the paracellular pathway.

This study identified THCE cells cultured on permeable supports for ~1 week as a robust in vitro system to evaluate corneal drug permeation. Many human corneal cell culture models exist today including primary cell cultures, immortalized cell lines,[12,13,24–26] and whole corneal models.[27–29] At this time, whole corneal models are being developed and show great promise as tools for drug-permeation studies, but currently data on the barrier function of these models are sparse. Immortalized cell lines offer many advantages over primary corneal cells including minimizing the expense in culturing and maintaining the cells, absence of individual-to-individual variation, control of experimental design, and the opportunity to use tissue initially derived from humans. Immortalized cell lines also offer the opportunity for rapid screening before ex vivo experiments. Many of the corneal epithelial models that exist[24–26] were developed for use with in vitro toxicity testing and cell physiology, therefore the barrier function of these cell lines has not been examined, and it is not known whether these cell models could play a role in drug-permeation studies. Transepithelial electrical resistance has long been used as a gold standard to quantify barrier integrity. An early report indicated that freshly excised rabbit cornea exhibited resistances of 5,000 to 15,000 Ω cm^2.[30] More recent reports, however, indicate values in the range of 800 to 1000 Ω cm^2 for rabbit cornea and approximately 500 Ω cm^2 for human cornea.[12,31] In an elegant and systematic study, Becker et al.[12] compared both drug permeation and R_{te} across numerous corneal models and reported that THCE cells exhibited barrier parameters that were similar to freshly excised human corneas and to cultured human corneal epithelial cells obtained from a commercial source.[12] Various culture conditions that promote barrier function for this cell line have been identified and the system has been used by others to examine drug permeation.[32–34] Becker et al. reported that THCE cells could be cultured in standard culture medium and that the apical fluid could be removed allowing for an apical air interface with a resulting multilayer architecture.[12] Experiments conducted in the present study identified culture conditions that decreased further the time needed to reach the maximum R_{te}, which was similar to the value reported by Becker et al.[12] Thus, THCE cells are an ideal system for permeation studies, and although they differ in this case, from the intact cornea by being only a single cell layer thick, the superficial surface of the corneal epithelium contributes over half of the total electrical resistance of the entire cornea,[35] making this system the best experimental model for this study. Experiments conducted in this study further validate this assumption, in that THCE monolayers are able to form a resistive monolayer and function as an epithelial barrier separating distinct fluid compartments.

For adequate vision, the eye must uphold specialized barriers that regulate the uptake of materials into the eye. These barriers also inhibit the access to deeper eye tissues of therapeutic compounds resulting in poor drug permeation and potential treatment failure and hinder the development of new therapeutic agents for ocular tissues. Bioavailability is limited by the volume that can be held by the human eye (~30 μL) and the rapid rate of tear turnover (~1 μL/min).[36] Thus, only 1% to 5% of an eye drop–instilled dose is delivered to anterior sections of the eye.[2,36] To overcome the problem of insufficient drug delivery through the cornea, new advances in ocular drug delivery are needed.

Numerous attempts at improving ocular bioavailability of topically applied drugs have been reported. Penetration enhancers such as surfactants,[37,38] bile salts,[39,40] chelators,[41] and preservatives[40,42] have been used to promote corneal permeation of ophthalmic agents. However, these enhancers generally exhibit their effects by inducing morphologic changes in the corneal structure[38,43,44] and occasionally lead to adverse effects such as irritation, hypersensitivity, cellular damage,[45,46] and reduction in corneal wound healing.[47] Clearly, new technologies are needed to optimize ocular drug delivery.

Reversibly modulating the tight junctions of epithelial barriers for therapeutic considerations provides many benefits. One of the techniques currently undergoing investigation is the zonula occludens toxin (ZOT) elaborated from *Vibrio cholerae*[48] and the biologically active component of ZOT, ΔG.[49] ZOT has been shown to reversibly open the tight junctions between intestinal epithelial cells and bovine brain microvessel endothelial cells resulting in enhanced permeability of compounds[50,51] and early studies suggest that ΔG has similar effects although it must be protected from enzymatic degradation.[52] ZOT and ΔG provide a lead structure to act as an absorption enhancer for therapeutic agents although a major limitation is that the ZOT "receptor" has only been located in the intestine, nasal epithelium, heart, and the blood–brain barrier endothelium. This differs from NC-1059 in which the effects have been observed with epithelia from a variety of tissues[8] and which is now shown to be effective with corneal epithelial cells.

In this study, NC-1059 induced a concentration-dependent increase in I_{sc} across THCE monolayers with a concurrent and delayed transient increase in g_{te}. Peak g_{te} was observed ~15 minutes after NC-1059 exposure. Previous studies suggested that the magnitude of increase in g_{te} after the addition of NC-1059 is due to an opening of the paracellular pathway, as well as the introduction of an ion channel into the apical

membrane.[7] This was confirmed in the present study by the use of FITC-dextran. After the addition of NC-1059, there was an increase in the amount of permeation seen for all sizes of FITC-dextran used compared with the control, although the increment for 40- and 70-kDa dextran was modest. Nonetheless, THCE cells do not show the same degree of size exclusion for FITC-dextran as reported previously with MDCK cells.[7] EDTA enhanced the corneal permeation for all sizes of FITC-dextran to a much greater extent and is believed to cause structural damage to the epithelial cell layer, which accounts for the greater effect that was observed with this chelating agent.

Sodium fluorescein and carboxyfluorescein were used to test the effects of NC-1059 on permeation of low-molecular-weight compounds as labeled surrogates for drugs. Data suggest that the permeation of both sodium fluorescein and carboxyfluorescein increases over a 30-minute period whether in the presence of NC-1059 or after a 5-minute exposure. Ninety minutes after NC-1059 washout, the barrier function of THCE monolayers, as assessed by fluorescein permeation, is indistinguishable from untreated controls. These results indicate that NC-1059 does not function by exerting a toxic effect on THCE monolayers since the cells are able to regain the functional barrier in a short time frame. The practical inference from these observations is that a single dose of NC-1059 might be used to "prime" an eye for the uptake of a therapeutic agent that is administered 15 to 30 minutes later. NC-1059 would need to stay in contact with the eye for 5 minutes or less, which is consistent with tear turnover rate. A second drug applied 15 to 30 minutes later would be expected to achieve two- to threefold greater uptake into the eye, which ultimately might reduce the number of times that the therapeutic drug must be applied.

Both FITC-dextran and fluorescein derivatives were used as surrogate drugs to look at the affect NC-1059 had on the permeation across THCE monolayers. A prototypical drug was then selected to look at the effect of NC-1059 on its permeation. In recent years methotrexate has been used experimentally as a treatment for systemic lymphoma with ocular involvement,[53–55] chronic uveitis,[56] and corneal angiogenesis.[57] The outcomes suggest that topical application of methotrexate would be beneficial for the treatment of many ocular diseases. The permeation of methotrexate in the presence of NC-1059, EDTA, and BAC was determined. NC-1059 enhanced the permeation of methotrexate with the most robust difference relative to untreated controls observed in the first 2 hours. The permeation rate in control conditions began to approach the permeation rate of NC-1059 treated monolayers during the second half of the 4-hour monitoring period. Both EDTA and BAC also increased the permeation rate of methotrexate, 20 and 6 times that of the control, respectively, but both of these compounds have deleterious effects on the corneal epithelium. The quaternary ammonium compound BAC is one of the most frequently used preservatives in ophthalmic formulations. It can alter corneal permeability; however, the BAC damages the corneal epithelium, resulting in the loss of the protective barrier.[22,23,58] Damage to the cornea has been shown to increase with increased concentrations and exposure time of BAC.[22] A perceived benefit with NC-1059 is that the epithelial barrier is fully recovered within 90 minutes.

Although first synthesized to form anion-selective channels in epithelial monolayers as a potential therapeutic for cystic fibrosis, NC-1059 has also been shown to modulate epithelial tight junctions.[7,8] This characteristic of NC-1059 provides the potential to increase the permeation of therapeutic agents across epithelial monolayers via modulation of the tight junctions. In this study, NC-1059 had a significant effect on the corneal permeability to surrogate compounds such as FITC-dextran, sodium fluorescein, and carboxyfluorescein. Permeation of methotrexate, a prototypical drug, was also greater for monolayers that were concurrently or previously exposed to NC-1059. Thus, NC-1059 should be considered a leading compound for development of co-therapeutic agents to enhance access and effectiveness of ophthalmic compounds.

Acknowledgments

The authors thank Ryan Carlin, Rebecca Quesnell, Lloyd Willard, and Suma Somasekharan for technical support.

References

1. Doane MG, Jensen AD, Dohlman CH. Penetration routes of topically applied eye medications. *Am J Ophthalmol.* 1978;85:383–386.
2. Sultana Y, Jain R, Aqil M, Ali A. Review of ocular drug delivery. *Curr Drug Deliv.* 2006;3:207–217.
3. Edens HA, Parkos CA. Modulation of epithelial and endothelial paracellular permeability by leukocytes. *Adv Drug Deliv Rev.* 2000;41:315–328.
4. Nusrat A, Turner JR, Madara JL. Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. *Am J Physiol Gastrointest Liver Physiol.* 2000;279:G851–G857.
5. Woo PL, Cha HH, Singer KL, Firestone GL. Antagonistic regulation of tight junction dynamics by glucocorticoids and transforming growth factor-beta in mouse mammary epithelial cells. *J Biol Chem.* 1996;271:404–412.
6. Nguyen DA, Parlow AF, Neville MC. Hormonal regulation of tight junction closure in the mouse mammary epithelium during the transition from pregnancy to lactation. *J Endocrinol.* 2001;170:347–356.
7. Broughman JR, Brandt RM, Hastings C, Iwamoto T, Tomich JM, Schultz BD. Channel-forming peptide modulates transepithelial electrical conductance and solute permeability. *Am J Physiol.* 2004;286:C1312–C1323.
8. Somasekharan S, Brandt R, Iwamoto T, Tomich JM, Schultz BD. Epithelial barrier modulation by a channel forming peptide. *J Membr Biol.* 2008;222:17–30.
9. Reddy GL, Iwamoto T, Tomich JM, Montal M. Synthetic peptides and four-helix bundle proteins as model systems for the pore-forming structure of channel proteins. II. Transmembrane segment M2 of the brain glycine receptor is a plausible candidate for the pore-lining structure. *J Biol Chem.* 1993;268:14608–14615.
10. Broughman JR, Shank LP, Takeguchi W, et al. Distinct structural elements that direct solution aggregation and membrane assembly in the channel-forming peptide M2GlyR. *Biochemistry.* 2002;41:7350–7358.
11. Wallace DP, Tomich JM, Iwamoto T, Henderson K, Grantham JJ, Sullivan LP. A synthetic peptide derived from glycine-gated Cl- channel induces transepithelial Cl- and fluid secretion. *Am J Physiol.* 1997;272:C1672–C1679.
12. Becker U, Ehrhardt C, Schneider M, et al. A comparative evaluation of corneal epithelial cell cultures for assessing ocular permeability. *Altern Lab Anim.* 2008;36:33–44.
13. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci.* 1995;36:614–621.
14. Ryeom SW, Paul D, Goodenough DA. Truncation mutants of the tight junction protein ZO-1 disrupt corneal epithelial cell morphology. *Mol Biol Cell.* 2000;11:1687–1696.
15. Yi X, Wang Y, Yu FS. Corneal epithelial tight junctions and their response to lipopolysaccharide challenge. *Invest Ophthalmol Vis Sci.* 2000;41:4093–4100.
16. Filenius S, Tervo T, Virtanen I. Production of fibronectin and tenascin isoforms and their role in the adhesion of human immortalized corneal epithelial cells. *Invest Ophthalmol Vis Sci.* 2003;44:3317–3325.
17. Xu KP, Yin J, Yu FS. SRC-family tyrosine kinases in wound- and ligand-induced epidermal growth factor receptor activation in hu-

man corneal epithelial cells. *Invest Ophthalmol Vis Sci.* 2006;47: 2832–2839.
18. Xu KP, Yin J, Yu FS. Lysophosphatidic acid promoting corneal epithelial wound healing by transactivation of epidermal growth factor receptor. *Invest Ophthalmol Vis Sci.* 2007;48:636–643.
19. Huhtala A, Alajuuma P, Burgalassi S, et al. A collaborative evaluation of the cytotoxicity of two surfactants by using the human corneal epithelial cell line and the WST-1 test. *J Ocul Pharmacol Ther.* 2003;19:11–21.
20. Tomich JM, Wallace D, Henderson K, et al. Aqueous solubilization of transmembrane peptide sequences with retention of membrane insertion and function. *Biophys J.* 1998;74:256–267.
21. Wang Y, Zhang J, Yi XJ, Yu FS. Activation of ERK1/2 MAP kinase pathway induces tight junction disruption in human corneal epithelial cells. *Exp Eye Res.* 2004;78:125–136.
22. Cha SH, Lee JS, Oum BS, Kim CD. Corneal epithelial cellular dysfunction from benzalkonium chloride (BAC) in vitro. *Clin Exp Ophthalmol.* 2004;32:180–184.
23. Lemp MA, Zimmerman LE. Toxic endothelial degeneration in ocular surface disease treated with topical medications containing benzalkonium chloride. *Am J Ophthalmol.* 1988;105:670–673.
24. Kahn CR, Young E, Lee IH, Rhim JS. Human corneal epithelial primary cultures and cell lines with extended life span: in vitro model for ocular studies. *Invest Ophthalmol Vis Sci.* 1993;34: 3429–3441.
25. Mohan RR, Possin DE, Sinha S, Wilson SE. Development of genetically engineered tet HPV16–E6/E7 transduced human corneal epithelial clones having tight regulation of proliferation and normal differentiation. *Exp Eye Res.* 2003;77:395–407.
26. Offord EA, Sharif NA, Mace K, et al. Immortalized human corneal epithelial cells for ocular toxicity and inflammation studies. *Invest Ophthalmol Vis Sci.* 1999;40:1091–1101.
27. Griffith M, Osborne R, Munger R, et al. Functional human corneal equivalents constructed from cell lines. *Science.* 1999;286:2169–2172.
28. Reichl S, Bednarz J, Muller-Goymann CC. Human corneal equivalent as cell culture model for in vitro drug permeation studies. *Br J Ophthalmol.* 2004;88:560–565.
29. Zorn-Kruppa M, Tykhonova S, Belge G, Bednarz J, Diehl HA, Engelke M. A human corneal equivalent constructed from SV40-immortalised corneal cell lines. *Altern Lab Anim.* 2005;33:37–45.
30. Klyce SD, Neufeld AH, Zadunaisky JA. The activation of chloride transport by epinephrine and Db cyclic-AMP in the cornea of the rabbit. *Invest Ophthalmol.* 1973;12:127–139.
31. Nakamura T, Yamada M, Teshima M, et al. Electrophysiological characterization of tight junctional pathway of rabbit cornea treated with ophthalmic ingredients. *Biol Pharm Bull.* 2007;30: 2360–2364.
32. Ranta VP, Laavola M, Toropainen E, Vellonen KS, Talvitie A, Urtti A. Ocular pharmacokinetic modeling using corneal absorption and desorption rates from in vitro permeation experiments with cultured corneal epithelial cells. *Pharm Res.* 2003;20:1409–1416.
33. Toropainen E, Ranta VP, Talvitie A, Suhonen P, Urtti A. Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest Ophthalmol Vis Sci.* 2001;42:2942–2948.
34. Toropainen E, Ranta VP, Vellonen KS, et al. Paracellular and passive transcellular permeability in immortalized human corneal epithelial cell culture model. *Eur J Pharm Sci.* 2003;20:99–106.
35. Klyce SD, Crosson CE. Transport processes across the rabbit corneal epithelium: a review. *Curr Eye Res.* 1985;4:323–331.
36. Kaur IP, Garg A, Singla AK, Aggarwal D. Vesicular systems in ocular drug delivery: an overview. *Int J Pharm.* 2004;269:1–14.
37. Marsh RJ, Maurice DM. The influence of non-ionic detergents and other surfactants on human corneal permeability. *Exp Eye Res.* 1971;11:43–48.
38. Green K, Tonjum A. Influence of various agents on corneal permeability. *Am J Ophthalmol.* 1971;72:897–905.
39. Morimoto K, Nakai T, Morisaka K. Evaluation of permeability enhancement of hydrophilic compounds and macromolecular compounds by bile salts through rabbit corneas in-vitro. *J Pharm Pharmacol.* 1987;39:124–126.
40. Sasaki H, Nagano T, Yamamura K, Nishida K, Nakamura J. Ophthalmic preservatives as absorption promoters for ocular drug delivery. *J Pharm Pharmacol.* 1995;47:703–707.
41. Madhu C, Rix PJ, Shackleton MJ, Nguyen TG, Tang-Liu DD. Effect of benzalkonium chloride/EDTA on the ocular bioavailability of ketorolac tromethamine following ocular instillation to normal and de-epithelialized corneas of rabbits. *J Pharm Sci.* 1996;85:415–418.
42. Ashton P, Diepold R, Platzer A, Lee VH. The effect of chlorhexidine acetate on the corneal penetration of sorbitol from an arnolol formulation in the albino rabbit. *J Ocul Pharmacol.* 1990;6:37–42.
43. Green K, Tonjum AM. The effect of benzalkonium chloride on the electropotential of the rabbit cornea. *Acta Ophthalmol (Copenh).* 1975;53:348–357.
44. Monti D, Chetoni P, Burgalassi S, Najarro M, Saettone MF. Increased corneal hydration induced by potential ocular penetration enhancers: assessment by differential scanning calorimetry (DSC) and by desiccation. *Int J Pharm.* 2002;232:139–147.
45. De Saint Jean M, Debbasch C, Brignole F, Rat P, Warnet JM, Baudouin C. Toxicity of preserved and unpreserved antiglaucoma topical drugs in an in vitro model of conjunctival cells. *Curr Eye Res.* 2000;20:85–94.
46. Rojanasakul Y, Liaw J, Robinson J. Mechanisms of action of some penetration enhancers in the cornea: Laser scanning confocal microscopic and electrophysiological studies. *Int J Pharm.* 1990; 66:131–142.
47. Green K, Johnson RE, Chapman JM, Nelson E, Cheeks L. Preservative effects on the healing rate of rabbit corneal epithelium. *Lens Eye Toxic Res.* 1989;6:37–41.
48. Fasano A, Baudry B, Pumplin DW, et al. Vibrio cholerae produces a second enterotoxin, which affects intestinal tight junctions. *Proc Natl Acad Sci USA.* 1991;88:5242–5246.
49. Di Pierro M, Lu R, Uzzau S, et al. Zonula occludens toxin structure-function analysis. Identification of the fragment biologically active on tight junctions and of the zonulin receptor binding domain. *J Biol Chem.* 2001;276:19160–19165.
50. Cox DS, Raje S, Gao H, Salama NN, Eddington ND. Enhanced permeability of molecular weight markers and poorly bioavailable compounds across Caco-2 cell monolayers using the absorption enhancer, zonula occludens toxin. *Pharm Res.* 2002;19:1680–1688.
51. Karyekar CS, Fasano A, Raje S, Lu R, Dowling TC, Eddington ND. Zonula occludens toxin increases the permeability of molecular weight markers and chemotherapeutic agents across the bovine brain microvessel endothelial cells. *J Pharm Sci.* 2003;92:414–423.
52. Salama NN, Fasano A, Thakar M, Eddington ND. The impact of DeltaG on the oral bioavailability of low bioavailable therapeutic agents. *J Pharmacol Exp Ther.* 2005;312:199–205.
53. Batchelor TT, Kolak G, Ciordia R, Foster CS, Henson JW. High-dose methotrexate for intraocular lymphoma. *Clin Cancer Res.* 2003;9: 711–715.
54. Smith JR, Rosenbaum JT, Wilson DJ, et al. Role of intravitreal methotrexate in the management of primary central nervous system lymphoma with ocular involvement. *Ophthalmology.* 2002; 109:1709–1716.
55. Tourville E, Tardif Y. Intravitreal methotrexate injections for intraocular involvement in primary central nervous system lymphoma. *Can J Ophthalmol.* 2003;38:598–601.
56. Kaplan-Messas A, Barkana Y, Avni I, Neumann R. Methotrexate as a first-line corticosteroid-sparing therapy in a cohort of uveitis and scleritis. *Ocul Immunol Inflamm.* 2003;11:131–139.
57. Joussen AM, Kruse FE, Volcker HE, Kirchhof B. Topical application of methotrexate for inhibition of corneal angiogenesis. *Graefes Arch Clin Exp Ophthalmol.* 1999;237:920–927.
58. Berdy GJ, Abelson MB, Smith LM, George MA. Preservative-free artificial tear preparations. Assessment of corneal epithelial toxic effects. *Arch Ophthalmol.* 1992;110:528–532.