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PLL-g-PEG Polymer Inhibits Antibody-Drug Conjugate Uptake into Human Corneal Epithelial Cells *In Vitro*

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Abstract

Purpose: Antibody-drug conjugates (ADCs) are a relatively recent advance in the delivery of chemotherapeutics that improve targeting of cytotoxic agents. However, despite their antitumor activity, severe ocular adverse effects, including vision loss, have been reported for several ADCs. The nonspecific uptake of ADCs into human corneal epithelial cells (HCECs) and their precursors via macropinocytosis has been proposed to be the primary mechanism of ocular toxicity. In this study, we evaluated the ability of a novel polymer, poly(L-lysine)–graft–poly(ethylene glycol) (PLL-g-PEG), to decrease the ADC rituximab-mc monomethylauristatin F (MMAF) (RIX) uptake into human corneal epithelial (HCE-T) cells.

Methods: HCE-T cells were exposed to increasing concentrations of RIX to determine inhibition of cell proliferation. HCE-T cells were treated with PLL-g-PEG, the macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA), or vehicle. After 30 min of incubation, RIX was added. ADC was detected by fluorescent anti-human immunoglobulin G and fluorescently conjugated dextran as viewed by microscopy.

Results: RIX caused dose-dependent inhibition of HCE-T cell proliferation. EIPA significantly reduced RIX uptake and decreased macropinocytosis as assessed by direct quantification of RIX using a fluorescently conjugated anti-human antibody as well as quantification of macropinocytosis using fluorescently conjugated dextran. PLL-g-PEG resulted in a dose-dependent inhibition of RIX uptake with half-maximal inhibitory concentrations of 0.022%–0.023% PLL-g-PEG.

Conclusion: The data show PLL-g-PEG to be a potent inhibitor of RIX uptake by corneal epithelial cells and support its use as a novel therapeutic approach for the prevention of ocular adverse events associated with ADC therapy.

Keywords: ocular surface disease, antibody-drug conjugate, macropinocytosis, cornea

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Introduction

Antibody-drug conjugates (ADCs) are a relatively recent advance in the delivery of chemotherapeutics that improve targeting of cytotoxic agents. By linking a cytotoxin to an antibody that detects an epitope highly expressed on tumor cells, ADCs offer the selective targeting of the chemotherapeutic payload directly to the tumor (for review, see Khongorzul et al.[1]). ADCs are one of the fastest growing classes of oncology drugs and excellent outcomes have been described in various clinical trials of the currently approved ADCs for clinical use.[2]

However, despite their targeted antitumor activity, severe ocular adverse effects have been reported for several ADCs. These adverse events include blurred vision, keratitis, dry eye, microcyst-like epithelial changes, and, in the most severe cases, central corneal ulceration.[3,4] For some of these agents, ocular toxicity has been identified in >50% of treated patients.[5] Dosage modifications, treatment interruption, or treatment discontinuation are currently the only means of mitigating ocular toxicity.[5] Nonspecific ADC uptake into human corneal epithelial cells (HCECs) and their precursors via macropinocytosis has been proposed to be the primary mechanism of ocular toxicity.[6]

Macropinocytosis is a cellular uptake mechanism in which a cell engulfs and takes up large amounts of fluid from the extracellular space through actin-dependent membrane protrusion and retraction (for review, see Kay[7]). Internalization of ADCs via macropinocytosis leads to accumulation in the macropinosome where the ADC is cleaved, releasing the cytotoxic payload. Positive surface charges on ADCs have been shown to increase ADC uptake by macropinocytosis given the electrostatic interactions with the negatively charged cell surfaces.[6]

The present study is based on the rationale that acutely preventing ADC uptake into affected corneal cell types can mitigate toxicity, increase patient comfort and compliance, and eliminate the need for treatment interruption. In this study, we investigated the ability of poly(L-lysine)–graft–poly(ethylene glycol) (PLL-g-PEG) polymer to prevent the ADC rituximab-mc Monomethylauristatin F (MMAF) (RIX) uptake into human corneal epithelial (HCE-T) cells.

PLL-g-PEG is a water-soluble, polycationic graft copolymer composed of poly(ethylene glycol) side chains grafted to a poly(L-lysine) backbone.[8] The poly(L-lysine) backbone carries multiple positive charges allowing it to electrostatically adsorb from aqueous solution onto negatively charged cell surfaces such as the corneal epithelium.[9] We have previously reported the clinical safety and efficacy of topically administered PLL-g-PEG in stabilizing the tear film, as a polymer excipient in artificial tear eye drops.[9] If indeed effective, PLL-g-PEG would allow rapid translation to the clinic to counteract ADC-mediated corneal toxicity.

RIX is a chimeric molecule that comprised human immunoglobulin G (IgG) 1 and kappa-chain constant regions together with heavy- and light-chain variable regions from a mouse antibody to CD20 (MS4A1). The CD20 antigen is highly expressed on the surface of both normal B lymphocytes and B cell lymphomas, thereby mediating both complement-dependent and antibody-dependent cell-mediated cytotoxicity.[10,11] Importantly, we also demonstrated that corneal epithelial cells do not express CD20.

Corneal toxicity secondary to ADC treatment presents an urgent and unmet clinical need.[5,12–14] Ocular toxicity-related ADC treatment modifications (e.g., dose delays, dose holds, dose reductions, and discontinuations) may compromise cancer treatment response rates, add logistical challenges to the clinical management of malignancies, and cause psychiatric stress to patients and caregivers. The research presented herein offers a comprehensive *in vitro* analysis supporting the use of PLL-g-PEG as a novel therapeutic approach for preventing ADC uptake into corneal cells.

Methods

Culture of HCECs

The HCE-T cell line[15] was obtained under Material Transfer Agreement from RIKEN BioResource Center/RIKEN Cell Bank (RBRC-RCB2280, Lot 005) and cultured according to the supplier's instructions. Briefly, HCE-T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 medium supplemented with 5% fetal bovine serum, 1% penicillin–streptomycin, 5 μg/mL insulin, 10 ng/mL human epidermal growth factor, 0.5% dimethyl sulfoxide (DMSO) on T-25 cell culture flasks at 37°C in 5% CO_2/95% humidity, as described by us previously.[16–18] For experiments, cell culture plastics were coated with poly(L-lysine) (PLL; 50 μg/mL for 1 h at 37°C) to mitigate the binding of PLL-g-PEG to the cell culture growth surface.[19]

Culture of primary HCECs

Primary HCECs (pHCECs) were obtained under Material Transfer Agreement from American Type Culture Collection (ATCC) (Catalog PCS-700-010, Lot 80331223) and cultured according to the supplier's instructions. Briefly, pHCECs were cultured in Corneal Epithelial Cell Basal Medium (ATCC PCS-700-030) plus one Corneal Epithelial Cell Growth Kit (ATCC PCS-700-040) that contains 5 μg/mL apotransferrin, 1.0 μM epinephrine, 0.4% Extract P, 100 ng/mL hydrocortisone hemisuccinate, 6 mM L-glutamine, 5 μg/mL recombinant human insulin, 0.2% CE growth factor, and 0.1% penicillin–streptomycin on T-25 cell culture flasks at 37°C in 5% CO_2/95% humidity.

Test articles and key chemicals and reagents

PLL-g-PEG polymer (Lot# KS-I-97, 2.94% solution) was provided by Calm Water Therapeutics LLC. PLL-g-PEG solution (2.94%) was treated with an antibiotic–antimycotic solution to generate a final stock solution of a concentration of 2.9%. There is inherent polydispersity associated with the manufacturing of PLL-g-PEG; for the material used in the following experiments, the approximate molarity of the stock solution was 430 μM.

Rituximab-mc MMAF (RIX, 3.3 mg/ml, lot #A-MAL-000028) was custom synthesized by Aji Bio-Pharma.

The macropinocytosis inhibitor, 5-(N-ethyl-N-isopropyl) amiloride (EIPA), was sourced from Sigma Aldrich, dissolved in DMSO, and used as positive control for RIX uptake experiments. Texas Red-conjugated Dextran (10,000 MW, ThermoFisher Scientific) was used as a surrogate molecule to quantify its uptake via macropinocytosis into HCE-T cells.

Gene expression studies

HCE-T cells and pHCECs were grown to confluency in T-75 flasks, and total RNA was isolated using the total RNA Purification Plus Kit (Norgen Biotek), as per the manufacturer's instructions. RNA purity and concentration were assessed using a Cytation 5 plate reader with nucleic acid module (BioTek). cDNA synthesis was performed using the High Capacity cDNA reverse transcription Kit (ThermoFisher Scientific), according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed using TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific) and gene-specific TaqMan® Gene Expression Assays (FAM; *MS4A1*: Hs00544819_m1; FAM and *GAPDH*: 4326317E; VIC, primer limited) in an Aria MX Real-time PCR system (Agilent).

Quantification of cell viability and proliferation

HCE-T cells were seeded on PLL-coated (not PLL-g-PEG) 96-well plates. HCE-T cells were exposed to a dose range of RIX for either 4 hours (h) as previously described[6] (seeded at 16,000 cells per well and grown for 3 days; 1–22.8 μM RIX) or 96 h (seeded at 500 cells per well, 0.001–0.1 μM RIX, media, and RIX refreshed at 48 h) allowing for sufficient time to detect differences in cell proliferation because of RIX. Methodology followed standard approaches to ADC cytotoxicity assays.[20]

HCE-T cell viability and proliferation as a surrogate of RIX cytotoxicity were quantified using resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one), as described previously.[17] Briefly, resazurin was added and incubated at 37°C for 2 h. Formation of the fluorescent metabolite, resorufin (7-hydroxyphenoxazin-3-one), was measured at 560 nm (excitation) and 590 nm (emission) using a Cytation 3 multimode plate reader. For each experiment, the assay was conducted in quadruplicate. Proliferation was quantified as percentage relative to control cells.

RIX uptake assay

Uptake of RIX was performed essentially as described previously.[6] Briefly, HCE-T cells were seeded (40,000 cells/well) on PLL-coated 8-well chamber slides and cultured for 48 h before treatment. HCE-T cells were pretreated for 30 min with EIPA (188 μM), PLL-g-PEG (0.0625%–1.25%), or the corresponding vehicle (DMSO or water, H_2O). Subsequently, RIX was added to the media to a final concentration of 75 nM either with or without 0.5 mg/mL Dextran–Texas Red and incubated for an additional 3.5 h. The final EIPA concentration was 150 μM and PLL-g-PEG (0.05%–1%, equal to ~7.4–148 μM). HCE-T cells were washed with sterile Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) and fixed in 4% paraformaldehyde for 20 min. HCE-T cells were then permeabilized and blocked with DPBS supplemented with 0.1% Triton X-100 and 10% (v/v) goat serum for 15 min. Cell surface-bound and internalized cytosolic RIX was detected by immunocytochemistry using an Alexa Fluor 488-labeled goat anti-human IgG antibody (ThermoFisher Scientific) at 4°C overnight. Nuclei were visualized with 4′,6-diamidino-2-phenylindole dihydrochloride, and coverslips were mounted using Fluoroshield (ThermoFisher Scientific) for imaging. Images were acquired using a THUNDER Imager 3D Tissue (Leica Micrsystems). Cellular fluorescence was quantified in FIJI/ImageJ software (National Institutes of Health) using standardized settings for background subtraction across all images. Images were analyzed by an investigator blinded to the experimental condition.

Statistical analysis

Data were graphed and analyzed using Prism version 9.5.0 (GraphPad, Inc.). RIX cytotoxicity, proliferation data, and RIX uptake data were fitted with a four-parameter dose–response curve with a variable slope to obtain the half-maximal inhibitory concentration (IC_{50}). Fluorescence data were tested for normality using the Kolmogorov–Smirnov test and subsequently analyzed using one-way analysis of variance followed by Dunnett's multiple comparisons test and compared with the vehicle group. Differences were considered statistically significant at $P < 0.05$.

Results

RIX uptake into corneal epithelial cells is mediated by macropinocytosis and inhibition of cellular viability and proliferation

ADCs have been reported to exhibit corneal toxicity that is mediated by their nonspecific cellular uptake through macropinocytosis.[6] To validate HCE-T cells as a model system to study the mechanism of RIX uptake and the ability of PLL-g-PEG to mitigate RIX toxicity, we first quantified the expression of *MS4A1*, the gene encoding the cellular CD20 target of RIX using qPCR. There was no detectable expression of *MS4A1* in HCE-T cells (Supplementary Fig. S1A). Furthermore, we confirmed lack of *MS4A1* expression in pHCECs (Supplementary Fig. S1B), validating HCE-T cells as a suitable model for evaluating the effect of RIX in corneal epithelial cells.

Subsequently, we tested the effects of RIX on HCE-T cells after a 4 h and 96 h exposure. RIX resulted in a significant inhibition of cellular viability and proliferation. The IC_{50} was 15.5 μM with a 95% confidence interval of 14.8–16.1 μM after a 4 h cell viability assay (Fig. 1A) and 33.8 nM with a 95% confidence interval of 21.3–49.8 nM after a 96 h cell proliferation assay (Fig. 1B). For subsequent experiments quantifying RIX uptake, we selected 2 times the IC_{50} concentration of the 96 h time point, that is, 75 nM.

We next sought to quantify RIX uptake using two separate methods, by labeling RIX using a fluorescently conjugated anti-human IgG antibody and by using Texas Red-conjugated Dextran as a surrogate marker for macropinocytosis.[6]

Exposure to RIX (75 nM) resulted in strong immunofluorescence in HCE-T cells, suggestive of RIX uptake into HCE-T cells. Background-corrected cellular immunofluorescence was 45% lower in the presence of the macropinocytosis inhibitor, EIPA ($n = 10$), compared with vehicle (DMSO; $n = 8$; $P < 0.001$; Fig. 2A, B), confirming uptake of RIX by macropinocytosis. PLL-g-PEG (1%) reduced RIX uptake by 18% ($n = 6$–8, $P < 0.001$; Fig. 2A, B).

To eliminate the possibility that RIX uptake was influenced by the concomitant exposure to Dextran–Texas Red, we performed a parallel experiment without dextran and quantified RIX uptake by anti-human IgG fluorescence. EIPA ($n = 9$) reduced fluorescence by 43% compared with vehicle (DMSO; $n = 7$; $P < 0.001$; Supplementary Fig. S2), whereas PLL-g-PEG (1%; $n = 8$) reduced fluorescence by 27% compared with

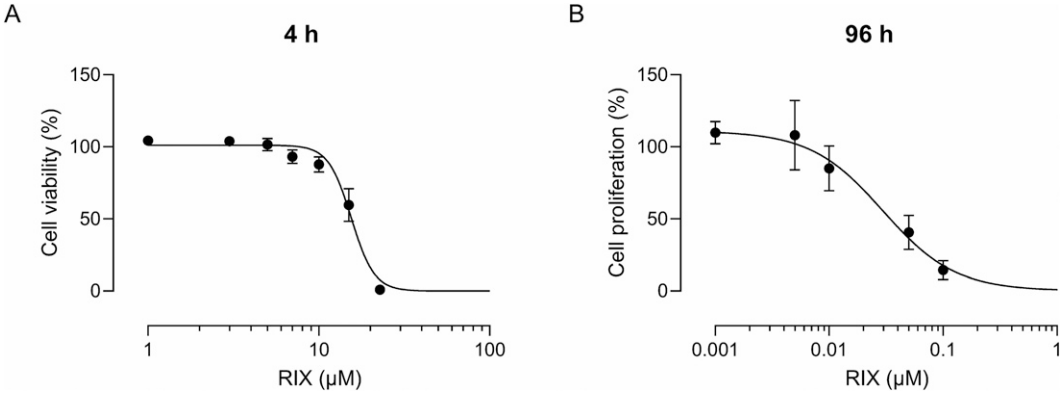

FIG. 1. Effect of RIX on the viability and proliferation of HCE-T cells. **(A)** RIX resulted in a significant inhibition of cellular viability. The half-maximal inhibitory concentration was 15.5 μM with a 95% confidence interval of 14.8 to 16.1 μM after 4 h of exposure. **(B)** Exposure to RIX for 96 h resulted in a half-maximal inhibitory concentration of 33.8 nM with a 95% confidence interval of 21.3 to 49.8 nM. HCE-T, human corneal epithelial; RIX, rituximab-mc MMAF.

vehicle (1% H_2O; $n = 6$; $P < 0.001$; Supplementary Fig. S2). These results suggest that the presence of Dextran–Texas Red does not alter RIX uptake or the ability to detect RIX uptake using anti-human IgG.

Texas Red fluorescence decreased by 46% in the presence of EIPA ($n = 10$) when compared with vehicle (DMSO; $n = 8$; $P < 0.001$; Fig. 2C, D). PLL-g-PEG (1%; $n = 8$) reduced Texas Red fluorescence by 43% when compared with vehicle (H_2O; $n = 6$; $P < 0.001$; Fig. 2C, D). Taken together, these data suggest that RIX uptake in HCE-T cells can be quantified using direct immunocytochemistry or fluorescently labeled Dextran as a surrogate marker as previously shown for other ADCs and fluorescently labeled Dextran in pHCECs.[6,21]

PLL-g-PEG dose-dependently prevents RIX uptake into corneal epithelial cells

Following the validation of the experimental assay, we set out to determine the dose–response relationship for PLL-g-PEG in preventing RIX uptake.

Uptake of RIX (75 nM) resulted in a 62 ± 5% increase in immunofluorescence in vehicle-treated HCE-T cells compared with cells without RIX ($n = 18$, $P < 0.001$; Fig. 3A, B). Increasing doses of PLL-g-PEG (0.05%–1%; $n = 16$–18 per concentration; $P < 0.001$ compared with vehicle; Fig. 3A, B) decreased immunofluorescence dose-dependently to baseline levels, preventing RIX uptake at a concentration of 1% PLL-g-PEG. We further assessed the dose dependency of PLL-g-PEG in preventing Dextran–Texas Red uptake in the presence of RIX. Presence of RIX (75 nM) did not alter Texas Red fluorescence. In contrast, PLL-g-PEG (0.05%–1%; $n = 18$ per concentration; $P < 0.0001$ compared with vehicle; Fig. 3C, D) exhibited a dose-dependent decrease in fluorescence. In a separate experiment, we confirmed the dose-dependent effect of RIX (75 nM) uptake by anti-human IgG and Dextran–Texas Red quantification (Fig. 4A–D).

To estimate the half maximal concentrations of PLL-g-PEG in preventing RIX (75 nM) uptake, we fitted immunofluorescence data using a nonlinear four-parameter equation. The derived IC_{50} values were 0.023% PLL-g-PEG for detection by anti-human IgG ($R^2 = 0.73$; Fig. 5A) and 0.022% PLL-g-PEG for detection by Dextran–Texas Red ($R^2 = 0.51$; Fig. 5B).

Discussion

There is a significant unmet clinical need to mitigate corneal epithelial changes associated with select approved ADCs. Furthermore, corneal adverse events are likely to be encountered with additional ADCs currently in preclinical and clinical development, emphasizing the potential widespread benefit from an effective corneal toxicity mitigation strategy. In this study, we report the favorable, dose-dependent effect of preventing ADC uptake by PLL-g-PEG into corneal epithelial cells, representing an important advance for the mitigation of ocular adverse events associated with ADC therapy. To our knowledge, this is the first report of a universal approach, relying on a safe, topically applied, multifunctional graft copolymer, to mitigate the nonspecific corneal ADC uptake and associated toxicity.

ADC-associated corneal epithelial changes typically manifest as one or more of the following: microcyst-like epithelial findings, superficial punctate keratopathy, alterations in corneal thickness reduced corneal sensation, changes to the corneal sub-basal plexus, subepithelial haze, and whorl-like findings, suggestive of limbal stem cell dysfunction.[3–5,14,22] In rare cases, infectious corneal ulcers can develop.[23] These ADC-associated ocular adverse events commonly lead to ocular discomfort and decreased visual acuity. However, even without symptoms, the presence of corneal epithelial changes often lead to dose holds, dose reductions, and dose delays.[24,25] These dosing changes may lead to decreased therapeutic efficacy in a life-threatening condition. Currently, there are no proven effective therapies leaving clinicians only supportive management approaches.[25] Preservative-free artificial tears and/or compresses are typically started at the time of ADC therapy initiation and continued throughout the treatment period.[25] Topical steroids have also been evaluated, and, in a randomized controlled substudy of the DRiving Excellence in Approaches to Multiple Myeloma-2 (DREAMM2) trial, their use was not found to be beneficial.[22,26,27] There exists the potential for ADC-specific interventions, but these are not generalizable solutions. For example, there is a recently completed study (no results available at time of publication) evaluating sodium thiosulfate eye drops to reduce ocular toxicity in cancer patients treated with the ADC SYD985, which addresses the toxicity associated with a duocarmazine payload (Clinical trial identifier, NCT04983238). Other approaches,

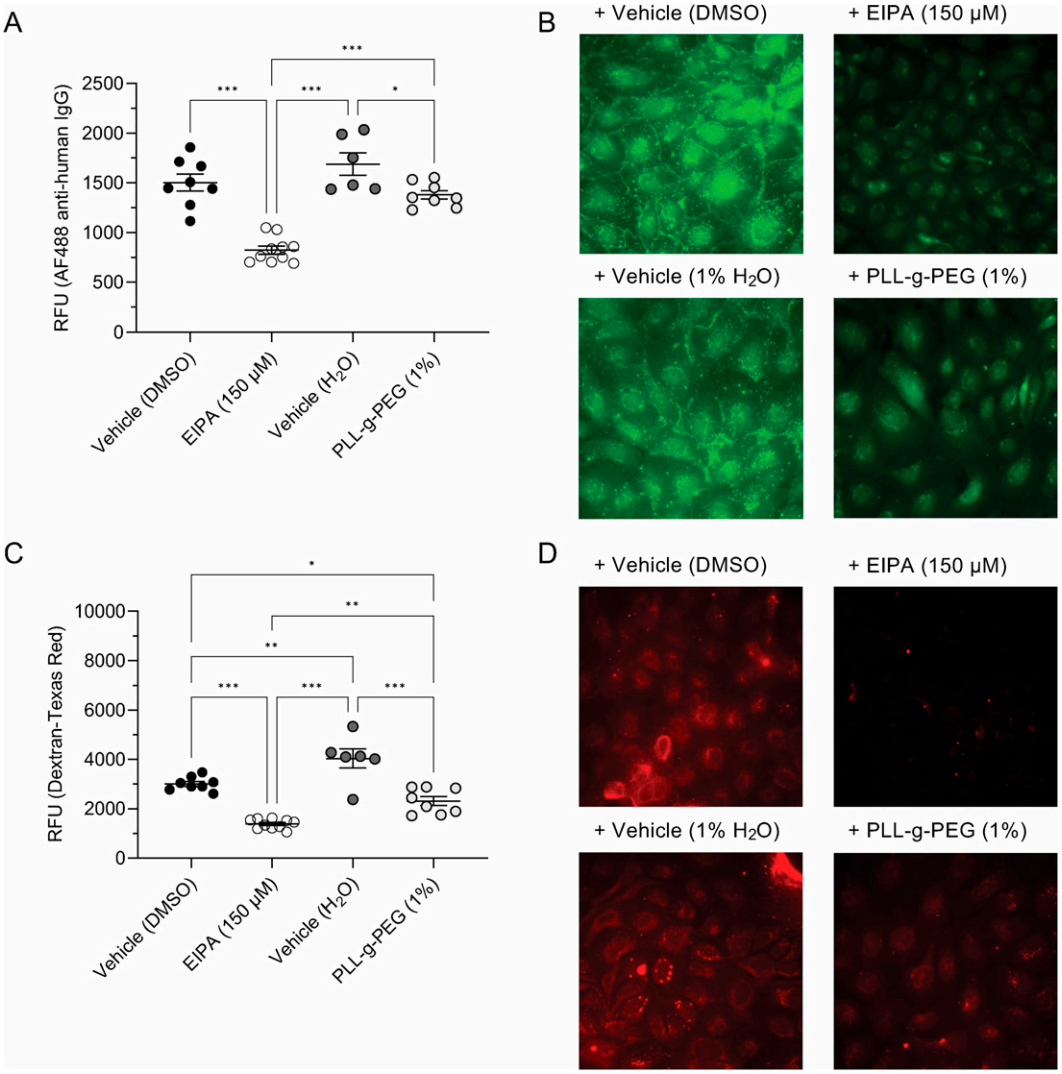

FIG. 2. RIX uptake into HCE-T cells is mediated by macropinocytosis. **(A)** RIX was detected using a fluorescently labeled anti-human IgG antibody. Exposure to RIX (75 nM) resulted in strong immunofluorescence in HCE-T cells, suggestive of RIX uptake, which was significantly decreased by 45% in the presence of the prototypic macropinocytosis inhibitor, EIPA, compared with DMSO vehicle. PLL-g-PEG (1%) reduced RIX uptake by 18% compared with the matched H_2O vehicle. **(B)** Representative images of RIX (75 nM) detection by immunocytochemistry. **(C)** Similar results were obtained by quantification of macropinocytosis using the surrogate substrate, Dextran–Texas Red, together with RIX, resulting in a 46% reduction in the presence of EIPA. PLL-g-PEG (1%) reduced Texas Red fluorescence by 43%. **(D)** Representative images of Dextran–Texas Red fluorescence. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. RFU is relative fluorescence unit. DMSO, dimethyl sulfoxide; EIPA, 5-(N-ethyl-N-isopropyl) amiloride; IgG, immunoglobin G; PLL-g-PEG, poly(L-lysine)–graft–poly(ethylene glycol).

such as autologous serum eye drops or episomal or secretome treatment, are currently experimental. ADC-associated adverse events may also contribute to increased neuropsychiatric comorbidities, thus negatively impacting treatment outcomes, highlighting the urgent unmet clinical need for mitigation strategies with ADC treatment paradigms.

It is generally accepted that corneal toxicity associated with ADC therapy results from the nonspecific internalization of the ADC mediated by macropinocytosis.[6] In this study, we show that PLL-g-PEG exhibits dose-dependent inhibition of macropinocytosis-mediated ADC uptake into corneal epithelial cells. Specifically, we used HCE-T cells as a model for corneal epithelial cells to study macropinocytosis-mediated ADC uptake. HCE-T cells are widely used for ocular surface pharmacological studies and for their ability to form an epithelial barrier with properties similar to the corneal epithelial surface *in vivo*.[28] HCE-T cell-model barriers are extensively relied upon in preclinical drug discovery.[29] Although it is recognized that transformed cells acquire a less differentiated phenotype, we confirmed that HCE-T cells have active macropinocytosis pathways resulting in similar ADC uptake as previously demonstrated in pHCECs.[30–32] Importantly, we here confirmed that both HCE-T cells and pHCECs lack the expression of the molecular target of RIX, CD20 encoded by the *MS4A1* gene, demonstrating that the cellular uptake of RIX into corneal epithelial cells is a result of receptor-independent internalization. As such, the data derived from HCE-T cells are consistent with previous findings on the uptake of different ADCs and serve as critical feasibility data supporting the therapeutic potential of PLL-g-PEG.

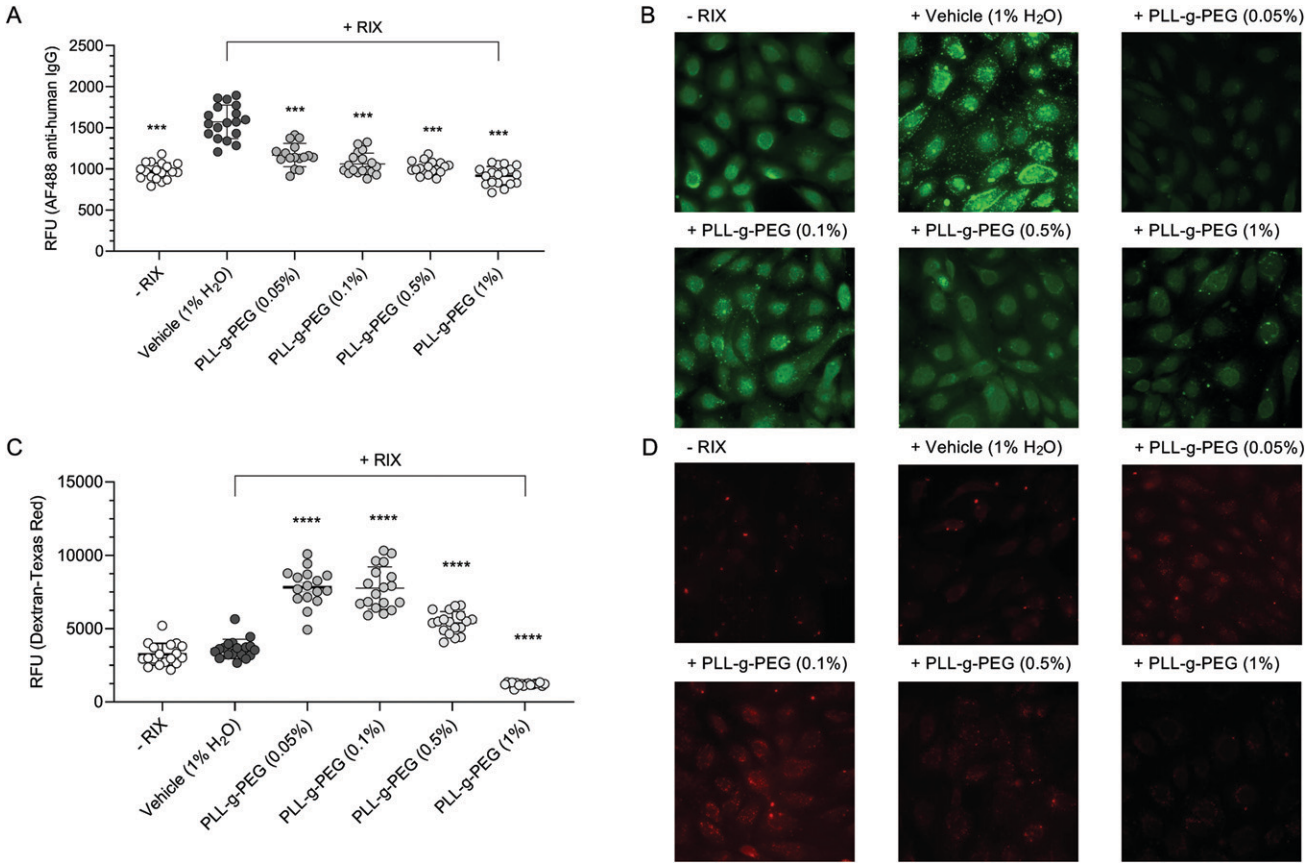

FIG. 3. PLL-g-PEG prevents ADC uptake in a dose-dependent manner. **(A)** Exposure to RIX (75 nM) resulted in a 62 ± 5% increase in immunofluorescence in vehicle-treated HCE-T cells compared with cells without RIX. Increasing doses of PLL-g-PEG (0.05%–1%) resulted in significantly decreased immunofluorescence, preventing RIX uptake at a concentration of 1% PLL-g-PEG. **(B)** Representative images of RIX (75 nM) detection by immunocytochemistry. **(C)** Using Dextran–Texas Red as a surrogate to quantify RIX uptake, we observed a similar dose-dependent reduction in fluorescence. **(D)** Representative images of Dextran–Texas Red fluorescence. ***$P < 0.001$, ****$P < 0.0001$. ADC, antibody-drug conjugate.

In this study, we demonstrated the efficacy of PLL-g-PEG in reducing RIX uptake in 3 separate experiments (Figs. 2–4). We used two distinct methodologies to assess the effects of PLL-g-PEG on macropinocytosis-mediated ADC uptake, immunocytochemical detection of human IgG, and quantification of fluorescently labeled Dextran as a surrogate marker.[6] Dextran uptake is an established method for evaluating macropinocytosis,[21] and we used this marker to confirm that study results were driven by inhibiting large-molecule (RIX approx. 145 kDa) uptake by macropinocytosis. We used a 10 kDa Texas Red-conjugated Dextran, which has shown to be a marker for both macro- and micropinocytosis, and both processes may be taking place concurrently in the Dextran results presented.[33] Notably, we confirmed the dose-dependent effects of PLL-g-PEG using two experimental strategies. Interestingly, in Fig. 3C, D, we noted that the fluorescence of Dextran–Texas Red was higher in PLL-g-PEG, 0.05%, than the vehicle. This may be due to biological variability in the assay or because of the concurrent micropinocytosis processes taking place with the small-molecular-weight Dextran. For this reason, we have included data from a repeated experiment to confirm PLL-g-PEG's dose-dependent response (Fig. 4). Estimates of the half-maximal concentration of PLL-g-PEG in inhibiting RIX uptake were similar between the two methods (0.023% and 0.022%, respectively), confirming the activity of PLL-g-PEG as well as the suitability of the assays to study macropinocytosis in corneal epithelial cells.

It is well established that PLL-g-PEG inhibits cellular proliferation of cells grown in monolayer culture on coated surfaces.[34,35] It is important to note that a monolayer cell culture system does not offer translatability to assess the potential negative impact of PLL-g-PEG administered *in vivo*, where cell proliferation and migration occur in a three-dimensional microenvironment that is not appropriately modeled by a coated vessel surface. Clinical data from multiple studies support the safety of PLL-g-PEG in topical ophthalmic use.[9,36]

The specific mechanism of action of PLL-g-PEG in inhibiting ADC uptake is not known; however, given its molecular structure, it is likely that PLL-g-PEG interferes with the interactions of the ADC and the cell surface. Specifically, its positive charge makes PLL-g-PEG likely to adhere to the negatively charged corneal epithelium, thereby disrupting the electrostatic interactions between positively charged RIX and the corneal surface. This robust electrostatic binding and interaction are critical to the efficacious properties of PLL-g-PEG, given that topically applied drugs typically wash away in approximately 10 min.[37,38] *In vitro* studies using physiological solutions have demonstrated that PLL-g-PEG has prolonged adherence to negatively charged surfaces for at least 24 h, making it uniquely suited for mitigating ADC toxicity

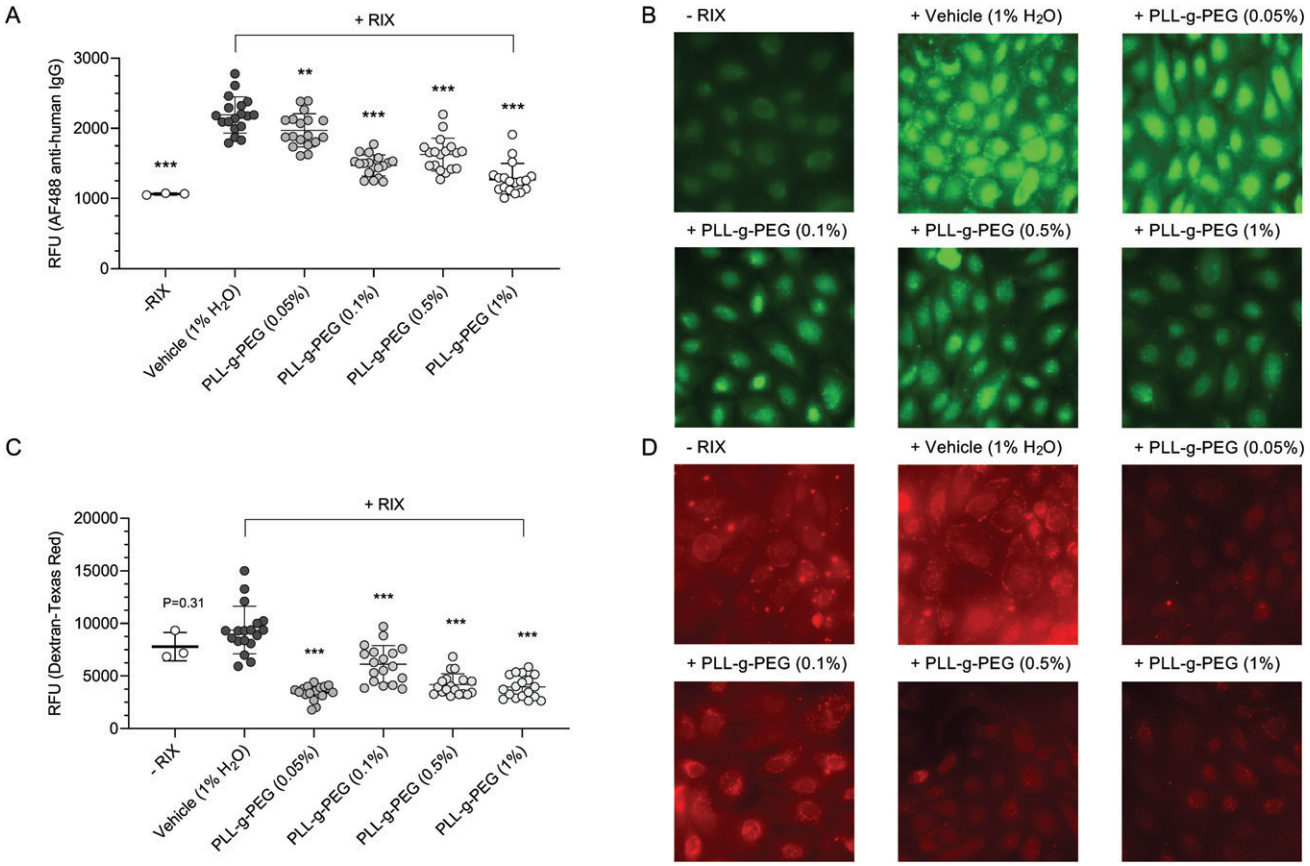

FIG. 4. PLL-g-PEG prevents RIX uptake in a dose-dependent manner. **(A)** In a separate experiment using the same experimental design in Fig. 3, increasing doses of PLL-g-PEG (0.05%–1%) resulted in significantly decreased immunofluorescence, preventing RIX (75 nM) uptake at a concentration of 1% PLL-g-PEG. **(B)** Representative images of RIX (75 nM) detection by immunocytochemistry. **(C)** A similar dose-dependent reduction in fluorescence was observed using Dextran–Texas Red as a surrogate to quantify RIX uptake. **(D)** Representative images of Dextran–Texas Red fluorescence. $^{**}P < 0.005$, $^{***}P < 0.001$.

that is mediated by off-target uptake into anterior segment tissues and occurs over a prolonged period.[35,39] Additional mechanisms such as steric interference and possibly direct complexation with the ADC cannot be excluded.

PLL-g-PEG-containing artificial tears have been studied clinically; in two, randomized, controlled, clinical trials, PLLg-PEG-containing eye drops have been shown to be well tolerated and to improve the performance of demulcents for at least 2 h.[9,36] The *in vitro* results presented herein further support the feasibility and translatability of using PLL-g-PEG to mitigate ADC toxicity. The paucity of *in vivo* models that mimic the clinical keratopathy and toxicity to the ocular surface associated with ADC therapy poses challenges for preclinical evaluation of drug candidates.[40] However, cellular models, such as HCE-T cells and pHCECs, offer an opportunity for the mechanistic evaluation of ADC-mediated effects on macropinocytosis and relevant cell types. Excellent tolerability and performance of PLL-g-PEG in humans demonstrate

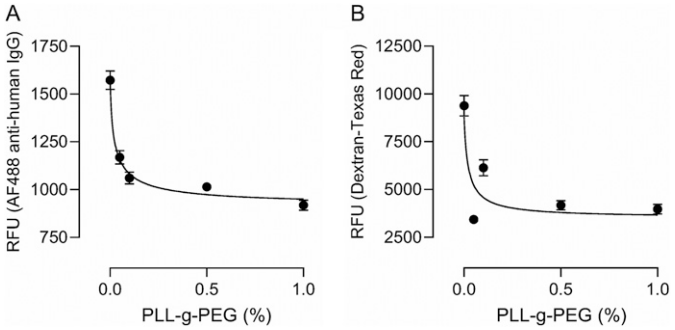

FIG. 5. Identification of half-maximal concentrations of PLL-g-PEG that inhibit RIX uptake. **(A)** Immunofluorescence data were fitted using a nonlinear four-parameter equation. The derived IC_{50} value for quantification by immunocytochemistry was 0.023% PLL-g-PEG ($R^2 = 0.73$). **(B)** Similarly, the derived IC_{50} value for quantification by Dextran–Texas Red was 0.022% PLL-g-PEG ($R^2 = 0.51$). IC_{50}, half-maximal inhibitory concentration.

that its unique physiochemical properties exert potent clinical efficacy that is minimally impaired by extrinsic factors such as tear turnover and blinking rate.[9,36] The efficacy of PLL-g-PEG in preventing ADC uptake *in vitro* provides strong evidence supporting a direct path to a formalized clinical study for topical self-administration concurrent with intravenous ADC treatment.

Conclusions

The novel polymer, PLL-g-PEG, limits ADC uptake into corneal epithelial cells in a dose-dependent manner. Topically instilled PLL-g-PEG has the potential to be an important component of ocular safety management in patients undergoing ADC therapy and addresses this significant unmet clinical need.

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Authors' Contributions

Conceptualization: D.K., M.M., and S.K.; methodology: S.D.O., J.J.H., and S.K.; formal analysis: D.K. and S.K.; investigation: S.I., S.D.O., A.K.G., and J.J.H.; data curation: D.K., S.I., A.K.G., S.K., and J.J.H.; writing—original draft preparation: S.I. and S.K.; writing—reviewing and editing: D.K., S.I., S.D.O., A.K.G., J.J.H., M.M., and S.K.; supervision: D.K., J.J.H., and S.K.; project administration: D.K. and S.K.; funding acquisition: D.K., M.M., and S.K. All authors have read and agreed to the published version of the article.

Author Disclosure Statement

D.K. and M.M. hold equity ownership in Calm Water Therapeutics LLC and are inventors on a patent related to this article. D.K. has a consulting relationship with GSK. J.J.H. is an employee of Experimentica Ltd.; J.J.H. holds equity ownership in Experimentica Ltd.; A.K.G. and S.K. are inventors on a patent application on drug targets for dry eye disease, unrelated to this article. A.K.G. holds equity ownership in eyeNOS, Inc. S.K. holds equity ownership in K&P Scientific LLC. S.D.O. has a client relationship with Astellas Pharma Inc.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

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