Inhibition of Ataxia Telangiectasia Mutated (ATM) Kinase Suppresses Herpes Simplex Virus Type 1 (HSV-1) Keratitis

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PURPOSE. Herpes keratitis (HK) remains the leading cause of cornea-derived blindness in the developed world, despite the availability of effective antiviral drugs. Treatment toxicity and the emergence of drug resistance highlight the need for additional therapeutic approaches. This study examined ataxia telangiectasia mutated (ATM), an apical kinase in the host DNA damage response, as a potential new target for the treatment of HK.

METHODS. Small molecule inhibitor of ATM (KU-55933) was used to treat herpes simplex virus type 1 (HSV-1) infection in three experimental models: (1) in vitro—cultured human corneal epithelial cells, hTCEpi, (2) ex vivo—organotypically explanted human and rabbit corneas, and (3) in vivo—corneal infection in young C57BL/6J mice. Infection productivity was assayed by plaque assay, real-time PCR, Western blot, and disease scoring.

RESULTS. Robust ATM activation was detected in HSV-1-infected human corneal epithelial cells. Inhibition of ATM greatly suppressed viral replication in cultured cells and in explanted human and rabbit corneas, and reduced the severity of stromal keratitis in mice. The antiviral effect of KU-55933 in combination with acyclovir was additive, and KU-55933 suppressed replication of a drug-resistant HSV-1 strain. KU-55933 caused minimal toxicity, as monitored by clonogenic survival assay and fluorescein staining.

CONCLUSIONS. This study identifies ATM as a potential target for the treatment of HK. ATM inhibition by KU-55933 reduces epithelial infection and stromal disease severity without producing appreciable toxicity. These findings warrant further investigations into the DNA damage response as an area for therapeutic intervention in herpetic ocular diseases.

Keywords: ataxia telangiectasia mutated (ATM), herpes simplex keratitis, DNA damage response, HSV-1, cornea
complex network of proteins responsible for the maintenance of genomic integrity of the cell. Sensor proteins of the DDR respond to DNA lesions and promote their repair by facilitating the assembly of repair proteins at the damaged DNA loci. Simultaneously, the DDR induces transient cell cycle arrest to prevent the lesion from being passed on to the daughter cells. The DDR also induces transcriptional changes to optimize the cellular response to the incurred lesion. In the case of overwhelming or irreparable damage, the DDR promotes apoptosis of the affected cell.

Three main sensor kinases serve as the apical proteins in the DDR—ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related), and DNA-PK (DNA-dependent protein kinase). All three of these kinases are known to be manipulated by many viruses, including herpesviruses. Herpes simplex virus type 1 infection induces the activation of ATM23 while shutting off ATR activity25 and degrading DNA-PK.26 Activation of ATM and other downstream DDR proteins by HSV-1 has been well characterized.27 Importantly, Lilley et al.24 have shown that HSV-1 infection in fibroblasts isolated from ataxia telangiectasia patients is strongly suppressed compared to matched normal fibroblasts.

In this report, we show that ATM is a significant participant in HSV-1 infection of corneal epithelium. ATM is rapidly activated in response to infection, and inhibition of its kinase activity with a small molecule inhibitor, KU-55933,28 greatly reduces replication of the virus and the cytopathic effect produced in the infected cells. The antiviral activity of KU-55933 is demonstrated in the human and rabbit corneal explant models, as well as in the mouse model of ocular HSV-1 keratitis. In cultured cells, KU-55933 exhibits an additive effect when coadministered with acyclovir. Importantly, KU-55933 effectively suppresses replication of a drug-resistant HSV-1 strain harboring a TK mutation. Our results demonstrate that ATM is a potential therapeutic target for the treatment of HSV-1 keratitis. These findings warrant further investigation into the mechanisms underlying inhibition of HSV-1 replication, as well as the use of ATM inhibitors in ocular herpesvirus infections.

**Materials and Methods**

**Cells and Viruses**

All cells were cultured at 37°C and 5% CO₂ and supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. Human corneal epithelial cells immortalized with hTERT (hTCepi,29 a kind gift from James Jester at the University of California-Irvine) were grown in complete keratinocyte growth medium 2 (KGM-2; Lonza, Basel, Switzerland). African green monkey kidney fibroblasts (CV-1; American Type Culture Collection, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% FBS. The KOS strain of HSV-1 was used in the in vitro and ex vivo infections, whereas McKrae strain was used for in vivo mouse experiments (both strains were a kind gift from Stephen Jennings at Drexel University College of Medicine), and TK mutant dl52tk strain30 was used in the drug resistance experiments (a kind gift from Donald Coen at Harvard Medical School). All viral stocks were titered on CV-1 monolayers.

**Infection and Treatments of Cultured Cells**

Subconfluent monolayers of cells were grown in six-well plates. Drug treatments were administered 45 minutes prior to infection and continued for the entire duration of each experiment. Unless indicated otherwise, KU-55933 (Batch No. 5, 99.7% purity; Tocris Bioscience, Bristol, UK) was used at 10 μM final concentration, phosphonoacetic acid (PAA) at 400 μg/mL (Sigma-Aldrich, St. Louis, MO), and acyclovir at 50 μg/mL (Sigma-Aldrich). KU-55933 was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in both KU-55933 and mock treatment in the in vitro and the ex vivo experiments was 0.1%. Infections with KOS strain of HSV-1 were carried out in six-well plates in a 200 μL inoculum volume at 37°C for 1 hour with intermittent rocking. The cells were then rinsed and overlaid with fresh medium.

**Corneal Explant Model**

Human corneas were obtained from the Lions Eye Bank of Delaware Valley. Experimentation using human corneas was approved by the Drexel University College of Medicine Institutional Review Board and adhered to the tenets of the Declaration of Helsinki. Rabbit corneas were excised from intact fresh eyeballs of young (8-12 weeks) albino rabbits (Pel-Freez Biologicals, Rogers, AR). Protocol established by Alekseev et al.31 for ex vivo corneal culture, infection, and treatment was followed closely. Briefly, corneoscleral buttons were excised and rinsed in PBS containing 200 U/mL penicillin and 200 μg/mL streptomycin. The endothelial concavity was filled with culture medium containing 1% low melting temperature agarose. The corneas were cultured epithelial side up in MEM medium supplemented with nonessential amino acids (1×), 2 mM L-glutamate, 200 U/mL penicillin, and 200 μg/mL streptomycin. The next day, they were infected with 1×10⁶ plaque forming units (PFU)/cornea of strain KOS HSV-1 for 1 hour, rinsed, and overlaid with fresh medium. Drug treatments were administered at the same concentrations as for cultured cells. For KU-55933 bioavailability assessment, corneas were treated with bleomycin (200 μg/mL) for 1 hour. The epithelial cell layer was collected by scraping to isolate DNA or protein. For immunohistochemistry studies, corneas were flash frozen in optimal cutting temperature (OCT) compound, sectioned, and immunostained using standard protocols. Treatment toxicity was assessed by briefly staining the cornea with fluorescein (1% wt/vol in PBS) and imaging the epithelial defects with 405-nm wavelength blue light (LDP LLC, Carlstadt, NJ).

**Mouse Ocular Infection and Treatments**

Animal studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Drexel University College of Medicine Institutional Animal Use & Care Committee. Four-week-old female C57BL/6j mice were anesthetized with isoflurane, and their left eyes were scarified in a 4° cross hatch pattern with a 28-gauge needle. McKrae strain HSV-1 was applied in 1 μL inoculum volume at 8×10³ PFU/eye and the eyelid gently massaged. The infection was allowed to develop for 24 hours, at which point treatments were initiated. KU-55933 was delivered to the corneas dissolved in PBS to a concentration of 200 μM. Control treatments constituted an equivalent amount of DMSO (0.2% vol/vol) in PBS drops. Treatments were administered every 4 hours for 1 full day and then every 8 hours for the remainder of the experiment.

**Disease Scoring**

Ocular disease severity was assessed at every 24-hour period postinfection. Two disease parameters were scored based on a number scale used by Jose et al.32 Briefly, stromal keratitis was scored as 1+, cloudiness, some iris detail visible; 2+, iris detail obscured; 3+, cornea totally opaque; and 4+, corneal perforation. Blepharitis was scored as 1+, puffy eyelids; 2+, puffy eyelids with some crusting; 3+, eye swollen shut with...
severe crusting; and 4+, eye completely swollen shut and crusted over.

**Viral Genome Replication and Transcription**

Viral genome replication and transcription were measured by quantitative PCR (qPCR). Total DNA and RNA from infected cells were isolated using the DNeasy Blood & Tissue Kit and the RNeasy Mini Kit, respectively (QIAGEN, Hilden, Germany). RNA was converted to cDNA using qScript (Quanta BioSciences, Gaithersburg, MD). Real-time qPCR was performed with SYBR Green (Bio-Rad, Hercules, CA). Target primers for UL30 (DNA polymerase catalytic subunit) and reference primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to measure genome replication. Transcription of the three gene families was measured with primers for RL2 (ICP0), UL30 (DNA polymerase catalytic subunit), and UL44 (gC), with reference primers for the 18S ribosomal RNA (rRNA). All primer sequences are listed in the Table.

**Immunocytochemistry and Immunohistochemistry**

For immunocytochemistry analysis, cells were grown on cover slips and infected as indicated. Cells were fixed in 3% paraformaldehyde/2% sucrose solution for 10 minutes and permeabilized with 0.5% Triton X-100 for 5 minutes. For immunohistochemistry, corneas were flash frozen in OCT compound and sectioned at 10-μm thickness. Indirect immunofluorescence was performed with primary antibodies against ICP8 (rabbit polyclonal; a kind gift from Bill Ruyechan at State University of New York at Buffalo), pATM S1981 (mouse monoclonal; Rockland, Gilbertsville, PA), and cleaved caspase 3 (rabbit polyclonal; Cell Signaling, Danvers, MA). Nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich).

**Western Blot**

Standard protocol was followed for Western blot analysis. Cell lysates were collected in 200 μL Laemmli buffer, vortexed, and boiled at 95°C for 5 minutes. Protein concentrations were measured by bicinchoninic acid (BCA) assay. SDS–PAGE was followed by transfer onto a polyvinylidene difluoride (PVDF) membrane, which was then blocked in 5% BSA. Primary antibodies against the following proteins were used: ICP0 (mouse monoclonal; Virusys Corporation, Taneytown, MD), ICP4 and nucleolin (both mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA), ICP8 (rabbit polyclonal; a kind gift from Bill Ruyechan at State University of New York at Buffalo), glycoprotein B and C (mouse monoclonal and rabbit polyclonal, respectively; kind gifts from Roselyn Eisenberg at University of Pennsylvania), ATM and pATM S1981 (mouse monoclonal and rabbit polyclonal, respectively; kind gifts from Roselyn Eisenberg at University of Pennsylvania), ATM and pATM S1981 (mouse monoclonal and rabbit polyclonal, respectively; Cell Signaling). Blots were stained with Hoechst 33258 (Sigma-Aldrich).

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F, forward primer; R, reverse primer; bp, base pairs.
secondary antibodies and visualized with the Odyssey near-infrared system (LI-COR, Lincoln, NE).

**Colony Survival Assay**

hTCEpi cells were treated with KU-55933 (10 μM) or DMSO for 24 hours, trypsinized, counted, and plated into 6-cm dishes at 50 cells/dish. After 2 weeks, colonies were fixed with 10% buffered formalin, stained with 0.01% crystal violet, rinsed, and counted.

**Statistical Analysis**

Statistical significance was determined using Student’s t-test and is indicated as ns (P > 0.05), *(P < 0.05), ***(P < 0.01), or ***(P < 0.001).

**RESULTS**

**HSV-1 Induces ATM Activation in Corneal Epithelial Cells**

We first investigated whether HSV-1 infection of human corneal epithelial cells induces the activation of ATM. A time course of protein lysates from infected hTCEpi cells was analyzed by Western blot with antibodies against known phosphorylation targets of ATM—Ser1981 of ATM (autophosphorylation) and Thr68 of Chk2. Consistent with studies in HeLa cells,24 ATM activity was observed as early as 1 hour post infection (hpi) and plateaued at a peak level between 4 and 6 hpi (Fig. 1A). Indirect immunofluorescence with pATM-specific antibodies demonstrated the expected pattern of ATM activation, which closely correlated with the known viral replication compartment dynamics 27 (Fig. 1B). Diffuse weak pATM gradually concentrated in numerous small foci, which further coalesced to form larger areas, eventually taking over the entire nucleus by 5 hpi. The timing of maximum ATM activation detected by Western blot corresponded to the pan-nuclear stage of pATM staining.

**ATM Inhibition Suppresses HSV-1 Infection in Corneal Epithelial Cells**

Experiments with ATM-deficient fibroblasts24 and ATM-inhibited HEK293 cells38 have demonstrated a reduction in viral yields from infected monolayers. We used a highly specific small molecule inhibitor of ATM, KU-55933, to examine the effects of ATM inhibition on HSV-1 infection specifically in human corneal epithelial cells. KU-55933 prevented the cytopathic effect of HSV-1, which was otherwise pronounced in the mock treatment (Fig. 2A). Plaque assays revealed a potent inhibition (greater than 10,000-fold at 20 hpi) of infectious particle...
**FIGURE 3.** ATM inhibition reduces accumulation of viral transcripts and proteins in vitro. hTCEpi cells were infected at MOI 0.1 in the presence of ATM inhibitor (KU-55933, 10 μM). Mock treatment (DMSO) and viral replication inhibitor (PAA, 400 μg/mL) were used as negative and positive controls, respectively. Under these experimental conditions, PAA contains activities that were found to inhibit several stages of HSV-1 gene expression. Cells were collected for protein lysates or RNA isolation at 16 hpi. (A) Transcripts from all three HSV-1 gene families were detected with primers for ICP0 (immediate early), DNA polymerase (early), and glycoprotein C (true late). Bars represent relative ΔΔC(t) values ± SEM. (B) Viral protein accumulation was assayed by Western blot with antibodies against ICP0 and ICP4 (immediate early), ICP8 (early), glycoprotein B (leaky late), and glycoprotein C (true late). Control lysates were collected from cells that were neither infected nor treated. Nucleolin is a loading control. n = 2 for all.

**FIGURE 4.** ATM inhibition suppresses HSV-1 replication in explanted human and rabbit corneas. (A) Schematic representation of the ex vivo culture method of explanted corneoscleral buttons. Reprinted with permission from Alekseev O, Tran AH, Azizkhan-Clifford J. Ex vivo organotypic corneal model of acute epithelial herpes simplex virus type 1 infection. J Vis Exp. 2012;69:e3631, doi:10.3791/3631. (B) Ex vivo human corneas were pretreated for 1 hour with ATM inhibitor (KU-55933, 10 μM) or DMSO, followed by administration of bleomycin (200 μg/mL) for an additional hour. The epithelial layers were collected for protein lysates and analyzed by Western blot with antibodies against pATM (Ser1981) and total ATM. Each lysate was collected from three pooled corneas. (C, D) Human and rabbit corneas were infected with 1 × 10^4 PFU/cornea. Treatments were applied at 1 hpi: ATM inhibitor (KU-55933, 10 μM) and mock treatment (DMSO). (C) PAA (400 μg/mL) was included as a positive control. Under these experimental conditions, PAA contains activities that were found to inhibit several stages of HSV-1 gene expression. DNA was isolated from the epithelial layers at 48 hpi and analyzed by qPCR with primers for HSV-1 DNA polymerase and GAPDH. Bars represent relative ΔΔC(t) values ± SEM. n = 6 for each treatment. (D) Alternatively, human corneas were processed for indirect immunofluorescence staining for cleaved caspase-3. Counterstain is Hoechst 33258.
production associated with KU-55933 treatment of infected hTCEpi cells (Fig. 2B). The effect of ATM inhibition on viral genome replication was monitored by qPCR using primers against the viral genome. We observed a sharp reduction in viral genome replication throughout the course of infection in cells with inhibited ATM activity (Fig. 2C).

The inhibition of genome replication was associated with reduced accumulation of viral transcripts in the infected monolayers. Levels of viral transcripts from all three kinetic families—immediate early, early, and late—were reduced, as measured by qRT-PCR with primers against ICP0, DNA polymerase, and glycoprotein C, respectively (Fig. 3A). This reduction was accompanied by a pronounced decrease in the levels of viral proteins necessary for successful progression of the viral life cycle (Fig. 3B).

ATM Inhibition Suppresses HSV-1 in Explanted Corneas

In order to study the antiviral effect of ATM inhibition in a more physiologically relevant model of epithelial herpes keratitis, we developed an ex vivo model of corneal infection.\(^1\) Intact corneoscleral buttons from humans and rabbits were infected and treated with KU-55933 in tissue culture (Fig. 4A). The bioavailability of KU-55933 in human corneal explants was evaluated by measuring its activity in the context of DNA damage induced by bleomycin, a known double strand break-inducing agent.\(^{39}\) Corneas damaged with bleomycin exhibited a high level of pATM, which was completely eliminated by pretreatment with KU-55933, demonstrating good penetration and activity of this inhibitor in the epithelial layers of an intact cornea (Fig. 4B). Consistent with our in vitro findings, viral genome replication in the epithelium of human and rabbit corneas was greatly reduced due to ATM inhibition (Fig. 4C). This effect was more pronounced in human corneas, likely due to the human specificity of the chemical structure of KU-55933. In addition, we observed a reduction in cleaved caspase-3 staining, a marker of apoptosis, in the epithelium of ATM-inhibited corneas compared to mock-treated controls (Fig. 4D).

KU-55933 Reduces Disease Severity in the Mouse Model of Herpes Keratitis

Our in vitro (Figs. 1, 2) and ex vivo (Fig. 3) experiments demonstrate a pronounced reduction of viral replication in cells with inhibited ATM activity. While these data may relate well to epithelial keratitis, they do not necessarily predict an effect on stromal keratitis, a more severe form of herpetic corneal infection that is characterized by lymphocytic invasion of the stroma.\(^1\) We used the mouse model of ocular HSV-1 infection to evaluate the effect of KU-55933 on the development of stromal disease. To increase the clinical relevance of our findings, mouse corneas were infected with McKrae strain, an ocular isolate of HSV-1, and infection was allowed to take place for a full day before initiation of treatments (Fig. 5A). KU-55933 treatments resulted in a notable and statistically significant reduction in stromal disease severity (Fig. 5B). For example, by day 5 postinfection, all of the control mice developed corneal perforation, while KU-55933-treated mice, on average, had only corneal opacity. Differences in the blepharitis score between the two groups were not statistically significant (Fig. 5C). The strong neurovirulence of the McKrae strain\(^{40}\) necessitated that the animals be euthanized before the resolution of disease.

KU-55933 Exhibits Low Toxicity in Corneal Epithelium

The toxicity of ATM inhibition with KU-55933 in hTCEpi cells was assessed using the clonal survival assay, which revealed a roughly 70% survival of cells continuously treated with KU-55933 for 24 hours compared to the mock-treated controls (Fig. 6A); however, the difference was not statistically significant. In addition, toxicity assessment was performed in explanted human corneas by fluorescein staining. No epithelial defects were detected after 30 hours of continuous treatment with KU-55933 (10 \(\mu\)M), while treatment with doxorubicin, a known proapoptotic agent,\(^{41}\) produced severe toxicity to the corneas (Fig. 6B). To assess the potential toxicity of prolonged KU-55933 treatment, fluorescein staining was similarly used on mouse corneas treated with 200 \(\mu\)M KU-55933 at the same schedule as outlined in Figure 5A. Despite the frequent administration of KU-55933 for a total of 4 days, the corneas...
exhibited no epithelial ulceration or any other visually detectible abnormalities (Fig. 6C).

Combination Treatments With KU-55933 and Acyclovir

We addressed the potential for using KU-55933 in combination with established antiviral agents. A range of combined low concentrations of KU-55933 and acyclovir was used to treat infected hTCEpi monolayers. Quantitative PCR analysis of viral genome replication demonstrated an enhanced antiviral effect of the combined treatment as compared to the individual drugs alone. The addition of KU-55933 effectively shifted the acyclovir dose–response curve to the left (Fig. 7A). Acyclovir had a similar effect on the KU-55933 dose–response curve (Fig. 7B).

Inhibition of Drug-Resistant HSV-1 by KU-55933

We examined the antiviral activity of KU-55933 against a drug-resistant strain of HSV-1, \( \text{dl}_{\text{sptk}} \). This strain harbors a mutation in the TK gene, which confers resistance against all antiviral agents that undergo activating phosphorylation catalyzed by this protein. \( \text{dl}_{\text{sptk}} \) infection in hTCEpi cells was largely unresponsive to acyclovir treatment; however, KU-55933 was able to markedly suppress genome replication of the \( \text{dl}_{\text{sptk}} \) strain (Fig. 8). The inhibitory effect of KU-55933 on \( \text{dl}_{\text{sptk}} \) infection was as potent as its effect on KOS infection.

DISCUSSION

The work presented here examines for the first time the concept of interfering with the host DDR in order to suppress corneal herpesvirus infection. Rapidly expanding knowledge of the molecular mechanisms underlying virus–host interactions has allowed for a fundamentally new perspective on designing antiviral therapies. The traditional approach of inhibiting critical viral proteins, such as DNA polymerase, has proven very successful, but it does have clear limitations. Analogous to antibiotic drugs, antiviral compounds that specifically target a viral factor leave room for mutation-driven resistance.

**FIGURE 6.** KU-55933 exhibits low toxicity in corneal epithelium. (A) The toxicity of ATM inhibition in hTCEpi cells was assessed by colony survival assay after a 24-hour treatment with KU-55933 (10 \( \mu \)M). Bars represent average colony survival \( \pm \) SEM. \( n = 3 \). (B) Ex vivo human corneas were treated with KU-55933 (10 \( \mu \)M) continually for 30 hours, and the epithelial toxicity was assessed by fluorescein staining. Toxic treatment with doxorubicin (100 \( \mu \)M) for 30 hours served as a positive control for detection of damage by staining. \( n = 2 \). (C) The eyes of uninfected healthy mice were treated with 200 \( \mu \)M KU-55933 administered at the same frequency and duration (4 days) as in the mouse ocular infection experiments (Fig. 5A). At the end of the experiment, the treated corneas were assessed for toxicity by fluorescein staining. A mouse cornea de-epithelialized as a consequence of untreated HSV-1 infection served as a positive staining control. \( n = 2 \).

**FIGURE 7.** ATM inhibition enhances the antiviral activity of acyclovir. hTCEpi cells were infected at MOI 0.1 in the presence of 16 different dose combinations of KU-55933 (0, 2, 4, and 7 \( \mu \)M) and acyclovir (0, 0.2, 0.5, and 1.5 \( \mu \)g/mL). Total DNA was collected at 16 hpi for analysis by qPCR with primers for HSV-1 polymerase and GAPDH. Viral genome replication was calculated using the \( \Delta \Delta C(t) \) method. Data are representative of at least two independent experiments. The same data set was plotted in two different ways to highlight (A) the effect of KU-55933 on the acyclovir dose–response curve and (B) the effect of acyclovir on the KU-55933 dose–response curve.
development of resistance. This is a well-recognized emerging clinical problem, particularly in immunosuppressed populations,\(^\text{17}\) the most common mechanism of resistance to nucleoside analogues (~95%) is mutation of the viral TK gene.\(^\text{42}\) By contrast, disruption of a critical virus–host interaction via inhibition of a host factor suppresses viral replication without the risk of rapid development of mutation-based resistance. Another potential niche for ATM inhibitors in the treatment of herpes keratitis is in combination therapy with established antiviral agents. The diversification of targeted pathways accomplished by combination therapy has the 2-fold advantage of preventing resistance and allowing for a reduction in drug dosage, with a consequent attenuation of side effect severity of each individual drug. Our experiments with resistant infection (Fig. 8) and combination treatments (Fig. 7) demonstrate that inhibition of ATM could offer these advantages in the treatment of herpes keratitis. An intriguing possibility that remains to be addressed in future studies is that ATM inhibition could have an antiviral effect not only in HSV-1 infection, but also in the context of the greater herpesvirus family, which includes such ocular pathogens as herpes simplex virus type 2, varicella zoster virus, and cytomegalovirus.\(^\text{43}\)

The molecular mechanisms involved in the ATM inhibition phenotype are currently under investigation. It is unknown how HSV-1 infection results in such rapid and robust activation of the DDR. The genome of HSV-1 is a linear double-stranded DNA molecule known to contain gaps and nicks in the sugar backbone structure.\(^\text{44}\) These defects, along with the DNA ends, may be perceived as damage by the cell and thereby trigger DDR activation. The physical disruption of nuclear architecture in response to the injection of the viral genome from its pressurized capsid\(^\text{45}\) could also explain DDR activation, since ATM is known to be activated by chromatim disassembly even in the absence of DNA damage.\(^\text{46}\) Alternatively, since activation of ATM is a critical event in the viral life cycle (Figs. 2–5), the virus may have a specialized mechanism in place to facilitate this activation. This could be dependent on the initial events during infection, such as increased calcium flux,\(^\text{47}\) or directly attributed to a viral protein, as is the case in human papilloma virus infection.\(^\text{48}\)

Also unknown are the downstream effects of DDR activation responsible for promoting HSV-1 replication. Li et al.\(^\text{49}\) identify cell cycle arrest at the G2/M checkpoint mediated by ATM signaling through its downstream target, Chk2.\(^\text{49}\) Alternatively, elevation of ATM activity may be necessary to achieve a highly recombinogenic environment within the nucleus. The viral genome forms complex branched intermediate structures in the process of replication, and it has been proposed that DNA recombination and repair are necessary for processing of these intermediates to allow for viral genome replication.\(^\text{50}\) Another possibility is the potential mediation of this effect through the Sp1 transcription factor. Sp1 is known to be phosphorylated by ATM in response to HSV-1 infection or DNA damage,\(^\text{51,52}\) which is particularly interesting since Sp1 binding sites are widespread in the promoters of HSV-1 genes. Phosphorylation by ATM has been shown to alter the transcriptional activity of Sp1,\(^\text{53}\) which could in turn affect the progress of infection. It is also noteworthy that ATM signaling through Chk2 has been shown to directly phosphorylate promyelocytic leukemia (PML) protein and contribute to PML nuclear body dynamics.\(^\text{53}\) Promyelocytic leukemia nuclear bodies are the intrinsic antiviral defense system of the cell and are known to be dispersed by HSV-1 immediate early protein ICP0 to achieve productive infection.\(^\text{54}\) Thus, activation of the DDR may enhance infection by promoting ICP0-mediated degradation of PML.

It is worth noting that treatment with PAA performed under our experimental conditions appears to have inhibitory activity on viral gene expression in addition to blocking genome replication. Since the in vitro infections were carried out at a multiplicity of infection (MOI) 0.1 for as long as 16 to 20 hours, this effect is partially produced by the inhibition of a few rounds of re-infection. However, given the strong transcript and protein reduction (Fig. 3), it is very likely that there is additional activity affecting several stages of HSV-1 gene expression.

The toxicity that may arise as a consequence of topical inhibitors of DDR proteins is an important issue to be addressed. Our examination of the corneal toxicity of KU-55933 has revealed a generally favorable toxicity profile in cultured cells, which were able to survive and proliferate well for 2 full weeks following a 24-hour treatment (Fig. 6A). In line with this result, explanted human corneas did not develop any surface defects following a continuous 24-hour treatment with KU-55933 (Fig. 6B). Importantly, mice that had received prolonged topical KU-55933 treatment for 4 full days (every 4 hours for the first day and every 8 hours for the next 3 days) did not show epithelial abnormalities by fluorescein staining. The low toxicity profile of this particular inhibitor is not surprising, given its high selectivity and potency.\(^\text{55}\) Moreover, it is logical to expect low corneal toxicity of ATM inhibition, based on the complete absence of corneal involvement in ataxia telangiectasia (AT) patients. Since the initial description of AT in 1941, the only reported ocular surface feature of this disease has been the gradual appearance of conjunctival telangiectasias (part of a systemic dermal phenotype).\(^\text{56,57}\)

Considering the function of ATM in the DDR, potential prooncogenic effects of ATM inhibition are of therapeutic concern. However, it is worth noting that ATM is necessary only for the repair of an estimated 10% to 15% of all double-strand DNA breaks in the cell.\(^\text{58}\) In addition, primary neoplasms of the cornea are known to be extremely rare.\(^\text{59}\) While extensive studies will be required to properly evaluate the adverse effects of ATM inhibition, this information, along with our preliminary toxicity assessment, suggests that ATM
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inhibitors may be sufficiently safe for topical application to the cornea.
In summary, our work highlights the DDR as a promising area that should be further explored for potential antiviral targets in the treatment of herpes keratitis. ATM inhibitors could potentially be used in combination therapy to reduce the toxicity of topical antivirals, and as standalones therapy against drug-resistant HSV-1 strains. Development of ATM inhibitors is of great interest in the field of cancer chemotherapeutics; even though no compounds are currently approved for clinical use, high-quality ATM inhibitors will likely become available in the near future. Elucidation of the precise molecular mechanisms behind ATM activation by HSV-1 will allow for more focused targeting of the relevant pathways in corneal epithelium.

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