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Inhibition of HSV-1 ocular infection with morpholino oligomers targeting ICP0 and ICP27

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ABSTRACT

Alternative therapies are needed for HSV-1 infections in patients refractory to treatment with Acyclovir (ACV) and its derivatives. Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) are single-stranded DNA analogues that enter cells readily and reduce target gene expression through steric blockage of complementary RNA. When applied before or soon after infection PPMO targeting the translation-start-site regions of HSV-1 ICP0 or ICP27 mRNA reduced HSV-1 plaque formation by 70–98% *in vitro*. The ICP0 PPMO also reduced ACV-resistant HSV-1 (strain 615.9) plaque formation by 70–90%, while an equivalent dose of ACV produced only 40–50% inhibition when the treatment was applied between 1 and 3 hpi. Seven daily topical treatments of 100 μg ICP0 PPMO caused no gross or microscopic damage to the corneas of uninfected mice. Topical application of 10 μg ICP0 PPMO to the eyes of HSV-1 infected mice reduced the incidence of eye disease by 37.5–50% compared to controls. This study demonstrates that topically applied PPMO holds promise as an antiviral drug candidate against HSV-1 ocular infection.

1. Introduction

Herpes simplex virus type 1 (HSV-1) is a member of the *Alphaherpesvirinae* subfamily of the *Herpesviridae*. HSV-1 infection is extremely common in humans. Serology data indicate that HSV-1 is widespread, with seropositivity at 45–98% worldwide and 57–85% in the United States (Brugha et al., 1997; Bunzli et al., 2004; Corey and Spear, 1986; Fatahzadeh and Schwartz, 2007; Miller and Ship, 1977; Nahmias et al., 1990; Roizman and Pellette, 2001; Spruance, 1992; Xu et al., 2006). Primary infection occurs through a break in the mucous membranes of the mouth, throat, eye or genitalia, or via minor abrasions in the skin. Initial infection of HSV-1 is usually asymptomatic, although there may be minor local vesicular lesions. During primary infection, HSV-1 enters nerve endings near the site of infection, then travels along nerve axons via retrograde transportation to sensory nerve or dorsal root ganglia, where it establishes life-long latent infection.

A unique feature of alphaherpesviruses is reactivation from latent infection (latency) (Roizman et al., 1993) following exposure to stressors and/or immune suppression (Minarovits et al., 2007; Roizman and Pellette, 2001). Recurrent corneal disease from HSV-1 reactivation can lead to corneal scarring, thinning, stromal opacity and neovascularization and, eventually, blindness (Toma et al., 2008). In spite of intensive antiviral and anti-inflammatory therapy, a significant percentage of patients do not respond to chemotherapy for herpetic necrotizing stromal keratitis. In addition, there is increasing evidence that HSV may damage the nerve cells in which it hides between outbreaks, possibly contributing to neurological diseases (Cohrs et al., 2004; Steiner et al., 2007). There is no treatment that can prevent the establishment or persistence of latent infection. Reactivation of HSV-1 infections is controlled clinically with long-term administration (e.g. 18 months) of acyclovir or its derivatives. Antiviral drugs can quell symptoms resulting from reactivation outbreaks but cannot eliminate latent virus. Furthermore, long-term usage of antiviral drugs, such as Acyclovir (ACV), Valcyclovir (VCV), and Famcyclovir (FCV), can lead to the development of drug-resistant viruses (Mori et al., 1994; Reyes et al., 2003; Ziyaeyan et al., 2007). The most frequently observed drug-resistant strains contain mutations in the gene for thymidine kinase (TK). TK is required to efficiently convert ACV or its derivative into acylo-guanosine monophosphate (acyclo-GMP) which can then be phosphorylated by cellular kinases into acyclo-guanosine triphosphate (acyclo-GTP), a potent inhibitor of HSV DNA polymerase.

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Mutations in the coding sequence of TK can indirectly affect the efficacy of ACV as an inhibitor of virus replication. Drug-resistant strains are found to occur most often in immunocompromised patients. Five to ten percent of patients with AIDS are estimated to develop ACV-resistant HSV-1 isolates, as are 18% of bone marrow transplant recipients (Longerich et al., 2005; Pillay, 1998; Pillay and Schinazi, 1998). Alternative anti-HSV therapies are therefore much needed.

Replication of alphaherpesviruses is coordinately and sequentially regulated. Transcription of the HSV-1 genome proceeds in a coordinated cascade-like fashion within the infected cell nucleus (Dulbecco and Ginsberg, 1988; Roizman and Pellette, 2001; Roizman et al., 1993; Sandri-Goldin, 2006). In general, the alphaherpesviral genes are grouped into three temporal classes: immediate-early (IE), early (E) and late (L), based on both transcriptional and post-transcriptional regulation (Dulbecco and Ginsberg, 1988; Roizman and Pellette, 2001). The IE genes are the first to be expressed and are essential for E and L gene expression (Jean et al., 1974; Peyman et al., 1995; Rice and Knipe, 1988; Roizman and Pellette, 2001; Sedlackova and Rice, 2008). The development of a drug capable of inhibiting IE gene expression could potentially reduce virus replication early in the infection process and is an appealing strategy for controlling HSV reactivation. ICP0, ICP4 and ICP27 are nonstructural IE genes that are essential for HSV-1 replication, and abrogation of their expression will interfere with the viral replication cycle (Cai and Schaffer, 1991; Chen and Silverstein, 1992; Clusel et al., 1995; Draper et al., 1990; Kulka et al., 1993; McCarthy et al., 1989; Peyman et al., 1995).

Various antisense-mediated strategies have been used successfully to address viral infections. MicroRNA (Sall et al., 2008; Scaria et al., 2007), amphipathic DNA polymers (Guzman et al., 2007), antisense oligonucleotides (Galletti et al., 2007; Li et al., 2008; Shoji et al., 1996, 1998; Van Aerschot, 2006; Wasmuth et al., 2003), modified antisense oligonucleotides (Fujihara et al., 1989; Gao et al., 1990; Larsson et al., 1986; Pyles et al., 2002; Smith et al., 1986; Yoo et al., 2002) and siRNA (Bagasra, 2005; Palliser et al., 2006; Shim et al., 2009; Wu et al., 2009; Zhang et al., 2008) have shown efficacy in the prevention or treatment of pathogenic virus infections. However, poor delivery, stability and pharmacokinetic properties *in vivo* have hampered the clinical development of nucleic acid based compounds. Phosphorodiamidate morpholino oligomers (PMO) are single-stranded nucleic acid analogues with a modified backbone composed of nitrogen-containing morpholine rings with phosphorodiamidate intersubunit linkages (Summerton and Weller, 1997). PMO are water soluble, resistant to cellular nucleases, and typically synthesized to a length of 20–25 bases (Summerton and Weller, 1997). PMO can hybridize to a complementary sequence in target mRNA and can reduce mRNA translation through steric blocking (Stein et al., 1997). Conjugation of an arginine-rich cell-penetrating peptide (CPP) to the 5′ end of a PMO, creating a peptide-conjugated PMO (PPMO), has been shown to enhance uptake into cells (Deas et al., 2005; Moulton et al., 2004). PPMO have been shown to inhibit infections by a number of RNA viruses in a sequence-specific and dose-dependent manner in both cell cultures and mouse models (Burrer et al., 2007; Deas et al., 2007; Smith et al., 20s08; Stein, 2008; Yuan et al., 2006). PPMO have also been shown to specifically reduce gene expression of human herpes virus type-8 *in vitro* (Patel et al., 2008; Zhang et al., 2007). In this study, we investigated the antiviral activities of five PPMO targeted against three HSV-1 IE genes: *ICP0*, *ICP4*, and *ICP27*. Four of the PPMO were designed to target mRNA in the AUG translation-start-site region, or upstream thereof, while the other PPMO targeted an exon/intron splice junction region of ICP0 pre-mRNA. In cell culture evaluations, PPMO targeted against sequences in the 5′ UTR of the ICP0 or ICP27 mRNA proved to be the most efficacious. The ICP0 PPMO was able to inhibit the replication of several HSV-1 strains, including an ACV-resistant strain, and had considerable efficacy against HSV-1 infection in a mouse model of ocular herpes infection.

Table 1
PPMO sequences (3′–5′) and target genes of HSV-1.

P7-PMO	Gene target region	P7-PMO sequence
ICP0-1	ICPO-preAUG	5′-GGTCGTATGCGGCTGGAGGGTCGC-3′
ICP0-EX	ICPO-exon	5′-CAGACATGGCGCCCGGCCCCTCAC-3′
ICP27-1	ICP27-preAUG	5′-GGTGTCGGATATGGCCTCTGGTGG-3′
ICP27-2	ICP27-AUG	5′-GTCGCCATGACCGGGCTGTCGCT-3′
ICP4	ICP4-AUG	5′-GTTCTCCGACGCCATCGCCGATGC-3′

2. Material and methods

2.1. Cells and viruses

Rabbit skin (RS) cells and Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), penicillin (100 U/ml), streptomycin (100 μg/ml) (Sigma–Aldrich, Inc.), and gentamicin (10 μg/ml) (Invitrogen) at 37 °C with 5% CO_2 in a humidified incubator. HSV-1 strains McKrae and 17+ have been previously described (Perng et al., 1994). HSV-1 294.1 and 615.9 are genetically related clinical isolates that are acyclovir susceptible and resistant, respectively (Griffiths et al., 2003), and were obtained from Dr. Don Cohen (Harvard University). The quantity of virus in the inoculum used in the experiments of this study was determined by standard plaque assay using RS cells.

2.2. PPMO synthesis

All PMO were synthesized at AVI BioPharma, Inc. (Corvallis, OR), as previously described (Summerton and Weller, 1997). The CPP $(RXR)_4XB$ (where R stands for arginine, X for 6-aminohexanoic acid and B for beta-alanine) was covalently conjugated to the 5′ end of each PMO, producing PPMO, as previously described (Abes et al., 2006). PPMO were designed to be complementary to sequences in the HSV-1 strain 17+ genome (GenBank accession #X14112). PPMO sequences and their target locations are described in Table 1. A schematic representation of the PPMO target locations relative to the HSV-1 genome is provided in Fig. 1B. A PPMO of random sequence containing 50% G/C content (named DSscr) was synthesized in the same manner as the antisense PPMO, and used as a negative control throughout this study. All PPMO sequences were screened with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against primate and murine mRNA sequences, and the negative control PPMO was also screened against all HSV sequences, to preclude unintentional hybridization events. Prior to use, lyophilized PPMOs were resuspended in sterilized distilled water to a concentration of 2 mM and stored at 4 or −80 °C.

2.3. In vitro cytotoxicity assay

Ninety-six-well plates were seeded with RS cells or Vero cells and grown overnight at 37 °C and 5% CO_2 until approximately 80% confluent. The cells were washed once with phosphate-buffered saline (PBS) and treated with indicated concentrations of PPMO diluted in 100 μl DMEM (Invitrogen) without serum or antibiotics. The PPMO-treated cells were then incubated at 37 °C and 5% CO_2 in a humidified incubator for 2 h, at which point the treatment-containing medium was removed, the cells were washed once with 1× PBS, then replenished with DMEM containing 5% FBS (Invitrogen) and antibiotics (as described above), and further incubated for 24 h. After incubation, 20 μl CellTiter Blue reagent (Promega) was added to each well, mixed thoroughly, and allowed to incu-

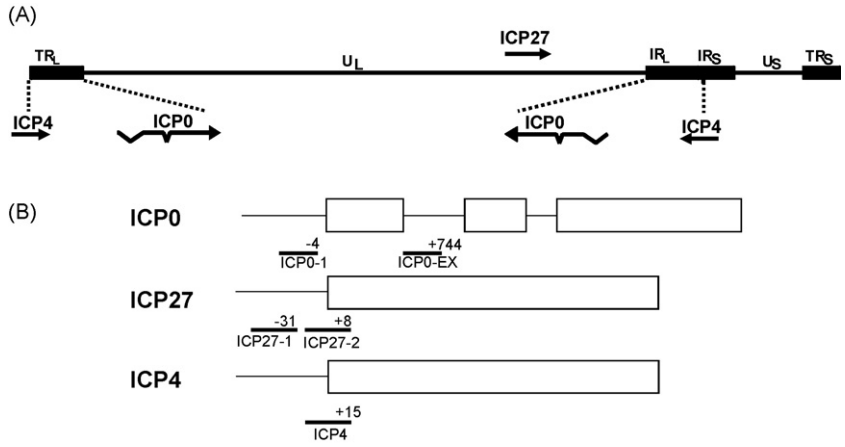

Fig. 1. Schematic of the HSV genome and location of PPMO targets. (A) Structure of the HSV-1 genome and the location of the targeted genes. TR_L and IR_L indicate the viral long repeats (terminal and internal). IR_S and TR_S indicate the viral short repeats. U_L and U_S indicate the long and short unique regions. The dashed lines indicate that the region of the TR_L and IR_L are expanded below with the TR_L inverted relative to the IR_L so that both TR_L and IR_L regions can be represented by a single image in the subsequent schematics. (B) Approximate location of PPMO target sites in relation to HSV-1 ORFs. Protein coding regions are indicated with rectangular boxes, and non-coding sequence with thin lines. PPMO are named and indicated below their targets by bold lines. The number above the PPMO represents the position of the 3′-terminal nucleotide in relation to the A of the AUG start-site codon of the appropriate ORF.

bate at 37 °C for 3 h. Fluorescence at 560 nm/590 nm was read on a microplate fluorescence reader (BioTek) and recorded. Wells containing each PPMO at each concentration in media without cells served as a blank to ensure that the PPMO itself was not registering fluorescence.

2.4. Plaque reduction assay with PPMO treatment before virus infection

RS or Vero cells were seeded in 6-well tissue culture plates at approximately 1×10^6 cells/well on the day before drug treatment. Following a 1× PBS wash, 0.5 ml of a solution containing 0, 10 or 20 μM PPMO in DMEM media without serum and antibiotics was added to three wells on each plate. After a 2 h exposure, the cells were washed twice with 1× PBS to remove PPMO treatment medium. Cells were then infected with HSV-1 (McKrae) at 200–300 plaque-forming units (PFU)/well. Following a 1 h viral absorption, 3 ml methylcellulose overlay media (DMEM containing 3% methylcellulose, 5% FBS and antibiotics as described above) was added to each well. Plates were incubated at 37 °C with 5% CO_2 in a humidified incubator. Plaques were visualized at 3–4 days post-infection (dpi) by staining with 1% crystal violet in 20% methanol.

2.5. Plaque reduction assay with PPMO treatment after HSV-1 infection

Six-well plates of RS cells were prepared as above. The cells were infected with HSV-1 at 200–300 PFU/well. Following 1 h virus absorption, all the wells were supplemented with medium containing 5% FBS and antibiotics as described above. At 1, 3, 6, or 12 h post-infection (hpi), the culture media was removed and the cells in each well were treated with 0.5 ml of 0, 10 or 20 μM PPMO diluted in PBS without Ca^{2+} and Mg^{2+} (Invitrogen), and incubated at 37 °C with 5% CO_2 in a humidified incubator for 2 h. Following the PPMO treatment, the cells were washed once with 1× PBS and overlaid with 3 ml methylcellulose overlay media as above. Plaques were visualized at 3–4 dpi by staining with 1% crystal violet in 20% methanol.

2.6. Virus growth assay

Twelve-well plates were prepared the day before infection by seeding each well with approximately 2.5×10^5 Vero cells. The cells were washed once with 1× PBS and treated with 0.25 ml of PPMO at 20 μM or DMEM without serum or antibiotics. Cells were incubated at 37 °C with 5% CO_2 in a humidified incubator for 2 h, and the treatment media was then removed. The cells were washed twice with 1× PBS and infected with HSV-1 at a target input of 1000 PFU/well. The inoculums were removed after a 1 h absorption period, and the cells were washed once with PBS. Infected cells were then maintained in DMEM supplemented with 5% FBS and antibiotics as described above. At 24 hpi, infected cells in the media were subjected to three freeze/thaw cycles and briefly centrifuged to remove large cellular debris. The supernatants were then collected, and the total amount of infectious virus was determined by a standard plaque assay on RS cells. The quantity of virus in the initial inoculum was also determined by a standard plaque assay.

2.7. Semi-quantitative RT-PCR

Six-well plates of RS cells were prepared the day before infection, as above. The cells were washed once with 1× PBS and treated with 0.5 ml of 20 μM ICP0-1, ICP27-1 or DSscr PPMO, or DMEM only, without serum or antibiotics. Treated cells were incubated at 37 °C with 5% CO_2 in a humidified incubator for 2 h, after which the treatment media was removed. The cells were washed twice with 1× PBS and infected with HSV-1 (McKrae) at a multiplicity of infection (MOI) of 2. Infected cells were maintained in DMEM supplemented with 5% FBS and antibiotics as described above. At the indicated time points, total cellular RNA was harvested by using TriZol in accordance with manufacturer's instructions. The isolated total RNA was resuspended in 50% formamide. Primers were designed to amplify portions of the ICP0, ICP27 and host GADPH mRNAs. Primers 5′-CCCCTAGATGCGTGTGAGTAA-3′ and 5′-ACAGACCCCCAACACCTACA-3′ were specific for ICP0; 5′-AAGATGTGCATCCACCACAA-3′ and 5′-CAAACACGAAGGATGCA-ATG-3′ were specific for ICP27; and 5′-GAGCTGAACGGGAA-ACTCAC-3′ and 5′-CCCTGTTGCTGTAGCCAAAT-3′ were specific for GADPH. RT-PCR was performed using the iScript RT-PCR Kit for Probes (BioRad) in accordance with manufacturer's instructions with 1 μg total RNA as templates. The reverse transcription step was carried out in a volume of 25 μl at 50 °C for 45 min followed by 94 °C for 2 min and 26 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s and finished with a extension step of 72 °C for 5 min. Aliquots of these reactions were separated on a 4% agarose gel in 1× TAE (40 mM Tris–HCl [pH 7.4], 40 mM acetic acid, 1 mM EDTA).

2.8. Western blot

Cells were infected similarly as described above for the relative quantitative RT-PCR. At the indicated time points, the cells were lysed in 100 μl Laemmli sample buffer (BioRad) with 5% of 2-mercaptoethanol, and boiled for 10 min. Twenty microliter aliquots were separated on a 7.5% (for ICP27 detection) or 4–15% (for ICP0 and ICP4 detection) Tris–HCl Ready Gel (BioRad) and transferred to a PVDF membrane. Membranes were blocked for 2 h in 1× TBS [0.02 M Tris–HCl, 0.14 M NaCl, pH 7.6] with 5% nonfat dry milk (w/v) and 0.1% Tween-20. The mouse α-ICP27 monoclonal antibody (Virusys) was diluted 1:1000 and the rabbit α-actin monoclonal antibody (Sigma) was diluted 1:2000 in the same buffer as used for blocking, while the mouse α-ICP0 or ICP4 monoclonal antibody (Virusys) was diluted 1:1000 in 1× TBS with 5% BSA (w/v) and 0.1% Tween-20. The membrane was incubated with diluted primary antibody overnight at room temperature and washed four times with TTBS (1× TBS, 0.1% Tween-20). The goat α-mouse HRP conjugated antibody (Chemicon) was diluted 1:1000 and the goat α-rabbit AP conjugated antibody (Upstate) was diluted 1:2000 in the milk-based buffer and incubated with the appropriate membrane for 1 h. The membranes were then washed three times with 1× TBS and developed using DAB2 Component Substrate (BioFX Lab) for the HRP conjugates or Western Blue Stabilized Substrate for AP conjugates (Promega).

2.9. Evaluation of PPMO toxicity to uninfected mice eyes

Eight-week-old Swiss Webster female mice were acquired from Simonsen Laboratories, Inc., and maintained and handled under veterinary supervision in accordance with National Institutes of Health guidelines and the Oregon State University Animal Care and Use Committee (IACUC). Four groups of mice (containing three mice per group) received 10 μl of 100 μM (10 μg), 200 μM (20 μg), or 1 mM (100 μg) ICP0-1 PPMO diluted in sterile 1× PBS, or 1× PBS alone, by direct administration to the eye via a pipettor. Each group of mice received the same dose of PPMO daily for 7 days. The overall appearance and behavior of the mice was observed daily, as well as the condition of their eyes and body temperatures. On day 7 post-treatment, the mice were euthanized, and each eye was examined microscopically following fixation in 10% neutral buffered formalin, routine processing into paraffin blocks, and staining of cut sections with hematoxylin and eosin.

2.10. Protection of mouse eye against HSV-1 by PPMO

Eight-week-old Swiss Webster female mice were obtained and maintained as above. Viral infections were carried out as previously described (Perng et al., 1994), but without corneal scarification. Nine groups of infected mice (8 mice per group) were treated with 10 μl of PBS, or 100 μM of ICP0-1 or DSscr diluted in PBS, at 4, 24, and 72 hpi with 2×10^3 PFU/eye HSV-1, respectively. Two groups of uninfected mice (8 mice per group) were mock-treated with PBS (control) or not treated at all (untreated). In addition to the initial treatment, each treatment group received the same dose of PPMO or PBS daily for an additional 4 days. All groups were monitored daily for signs of eye diseases. On day 7 post-infection, mice showing the worst eye disease, from Mock 72 h, DSscr 72 h, and ICP0-1 72 h groups, were euthanized and each eye was examined microscopically following fixation in 10% neutral buffered formalin, routine processing into paraffin blocks, and staining of cut sections with hematoxylin and eosin.

2.11. Statistics

All statistical analyses were performed using GraphPad Prism, version 4 for Windows (GraphPad Software, San Diego, California). Plaque reduction results were analyzed by two-way ANOVA with Bonferroni post-test.

3. Results

3.1. PPMO design

Previous studies have shown that the region immediately 5′ of and spanning the translation-start-site of viral (Enterlein et al., 2006; Neuman et al., 2005; Stein, 2008) or cellular (Eisen and Smith, 2008) mRNA frequently constitutes an effective region for targeting with PMO technology. We therefore designed 4 of the 5 anti-HSV-1 PPMO to be complementary in sequence to such regions (see Table 1 and Fig. 1B). ICP0 pre-mRNA is composed of 2 introns and 3 exons. The fifth PPMO was designed to target the 5′-terminal sequence of the first intron of ICP0, in an effort to interfere with the splicing reactions of mRNA maturation via steric blocking (Aartsma-Rus and van Ommen, 2007). The *ICP0* and *ICP4* genes are in the inverted repeat regions of the HSV-1 genome and are present as two identical copies (Fig. 1A).

3.2. Cytotoxicity assay of PPMOs in vitro

To investigate PPMO-associated cytotoxicity, uninfected RS cells and Vero cells were treated with the PPMO used in this study, then subjected to a cell-viability assay. Fig. 2 shows viability results from both types of cells treated with ICP0-1, ICP0-Ex, ICP27-1, ICP27-2, and Vero cells additionally treated with ICP4, VP16-1 and VP16-2, over a concentration range from 10 to 40 μM. No significant difference in viability was apparent between mock-treated cells and those treated with up to 20 μM of DSscr, ICP0-1, ICP0-Ex, or ICP27-1. The ICP27-2 PPMO produced moderate toxicity at 20 μM. At 40 μM, ICP0-1, ICP0-Ex and ICP27-1 PPMOs reduced viability of both RS and Vero cells by 10–20% compared to mock-treatment, and these differences were statistically significant ($p < 0.001$ and $p < 0.01$, respectively). Pre-treatment for 2 or 4 h with 40 μM of either ICP27-2 or ICP4 PPMO reduced Vero cell viability by 40–50% compared to mock-treated cells. DSscr produced little toxicity to RS and Vero cells under any of the conditions used in this study. All PPMO were used at 20 μM or less for the remainder of the cell culture experiments in this study.

3.3. Plaque reduction assay with PPMO treatment before virus infection

Dose–response activity of each PPMO against HSV-1 (McKrae) was evaluated in RS cells by standard plaque reduction assay. Cells were treated with PPMO for 2 h, then infected with 200–300 PFU/well of HSV-1 and plaque formations were evaluated at 3–4 dpi. Similar results were obtained in Vero cells, and results with RS cells are presented here. As shown in Fig. 3, ICP0-1 was the most effective PPMO against HSV-1 replication *in vitro*, inhibiting plaque formation by 82% at 10 μM and 98% at 20 μM, compared to mock-treatments. The ICP0-EX reduced plaque formation by 49% at 10 μM, and 70% at 20 μM. The ICP27-1 showed intermediate efficacy, providing 47 and 88% inhibition in plaque formation at 10 and 20 μM, respectively. ICP27-2 did not provide high protection against HSV-1 infection and inhibited plaque formation by only 31% at 20 μM. The ICP4-1 PPMO did not produce significant inhibition of viral replication either, with only 15% and 28% reduction in plaque formation when present at 10 and 20 μM, respectively (Fig. 3). However, immunoblotting indicated that PPMO treatment produced a considerable reduction of ICP4 protein expression at later time points (Fig. 8). Together, these data suggest that ICP4 protein expression soon after infection is important in the HSV-1 replication cycle. The DSscr PPMO

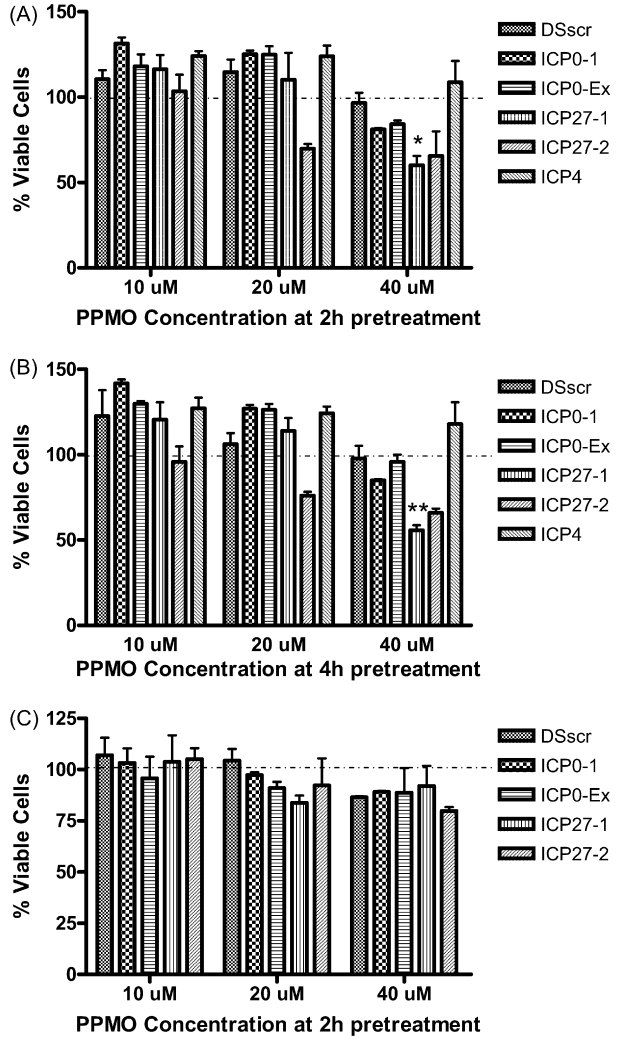

Fig. 2. PPMO cytotoxicity assay *in vitro*. Vero cells were incubated with the indicated concentration of PPMO in serum free DMEM for 2 h (A), 4 h (B). (C) Rabbit skin (RS) cells were incubated with the indicated concentration of PPMO in serum free DMEM for 2 h. The treatment was then removed and the cells further incubated for 24 h in DMEM with 5% serum and antibiotic as described in Section 2. Cell viability was evaluated with CellTiter Blue reagent (Promega, Madison, WI), which measures metabolic activity, and expressed as a percentage of the mock-treated control ($n = 3$). Significant statistical difference from the mock-treated control is marked with $^{**}p < 0.01$ (two-way ANOVA with Bonferroni post-test).

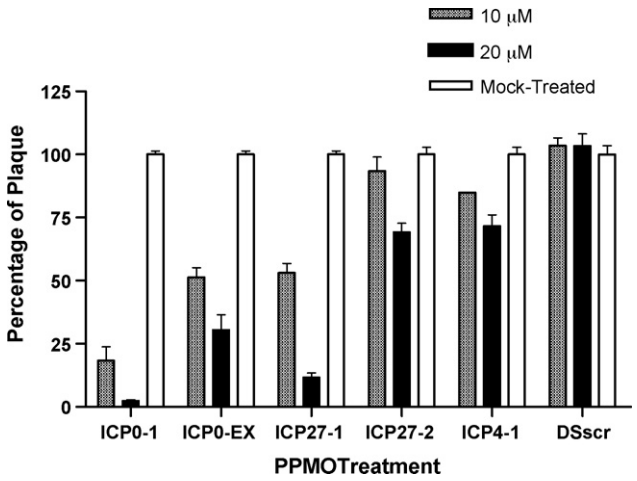

Fig. 3. Plaque reduction results from cells treated with PPMO prior to HSV-1 infection. RS cells were treated with the indicated PPMO at the indicated concentrations for 2 h, then infected with approximately 250 PFU/well of HSV-1 (McKrae). Plaque formation was quantified at 3–4 dpi and expressed as a percentage of the mock-treated control ($n = 3$–4).

had no inhibitory effect and produced nearly identical results to mock-treatment (Fig. 3). Because of the high antiviral efficacy of ICP0-1 and ICP27-1, these two PPMO were chosen for further study.

3.4. Effect of post-infection PPMO treatment on HSV-1 replication

To determine the efficacy of PPMO treatment that commenced after HSV-1 infection, RS cells were treated with PPMO for 2 h starting at 1, 3, 6, or 12 h post-infection (hpi), and plaque formation was evaluated as above at 4 day post-infection (dpi). As shown in Fig. 4, when treatment began at 1 hpi, 10 and 20 μM ICP0-1 PPMO treatment inhibited plaque formation by 86 and 95%, respectively. When treatment began at 3 hpi, they inhibited only 37 and 66%, respectively. The difference in plaque reduction between 10 μM ICP0-1 and DSscr treatment was statistically significant when treatment began at 1, 3 or 6 hpi ($p < 0.01$). As evaluated by our statistical analysis, all PPMO at a dose of 10 μM were ineffective when applied at or after 12 hpi. However, the 20% difference in plaque reduction between 20 μM ICP0-1 and DSscr treatment started at 12 h was statistically significant ($p < 0.05$). DSscr- and mock-treated samples produced nearly identical results. These results show that the antiviral effect of PPMO diminishes with time lapsed between infection and treatment application.

3.5. Evaluation of ICP0-1 PPMO activity against various strains of HSV-1

The *ICP0* gene is highly conserved between strains of HSV-1. To investigate if the ICP0-1 PPMO could produce antiviral activity against various strains of HSV-1, we evaluated its activity against three different strains of HSV-1 (17+, McKrae, and 294.1). RS cells were treated with 20 μM ICP0-1 for 2 h before inoculation with the various strains, and the level of virus replication was measured at 24 hpi. As shown in Fig. 5, ICP0-1 inhibited the replication of all three viruses by around or over one log, compared to mock or

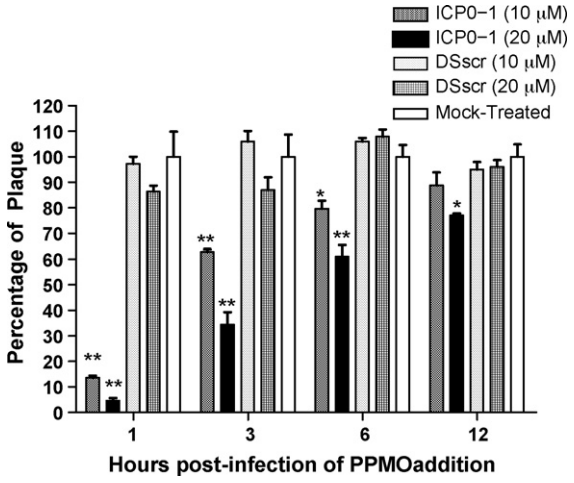

Fig. 4. Plaque reduction results from cells treated with PPMO after HSV-1 infection. RS cells were infected with HSV-1 at 200–300 PFU/well, then treated with the indicated concentration of PPMO for 2 h starting at 1, 3, 6, and 12 hpi. Plaque formation was quantified at 3 dpi and expressed as a percentage of the mock-treated control ($n = 3$). $^{**}p < 0.001$, $^{*}p < 0.05$ (two-way ANOVA with Bonferroni post-test). The p values represent reduction in plaque formation comparing to mock-treated controls.

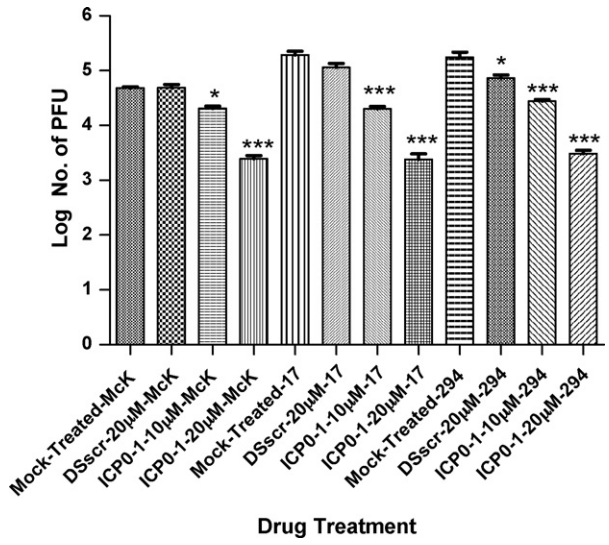

Fig. 5. Comparative activity of ICP0-1 against various HSV-1 strains. RS cells were treated with the indicated concentration of PPMO for 2 h, then infected with approximately 1000 PFU/well of HSV-1 strains. McKrae, 294.1, C: 17+. Viral titer was determined at 24 hpi using a standard plaque assay. McK:McKrae, 17:17+, 294:294.1. The values represent the mean of 3 independent experiments. Significant statistical difference from the mock-treated control is marked with $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ (one-way ANOVA with Bonferroni post-test).

DSscr-treated controls. These data indicate that ICP0-1 was capable of significantly and specifically inhibiting the replication of several strains of HSV-1.

3.6. Evaluation of the ICP0-1 PPMO antiviral activity against an acyclovir-resistant strain

To determine the efficacy of ICP0-1 PPMO against an acyclovir-resistant strain of HSV-1, Vero cells were infected with strains 615.9, 294.1 or McKrae, treated with ICP0-1 PPMO for 2 h starting at 1, 3, or 12 hpi, then incubated for 3 days. As shown in Fig. 6, treatment of cells with 20 μM ICP0-1 starting at 1 hpi reduced plaque formation by 96% for 615.9, 93% for McKrae and 75% for 294.1 (Fig. 6A). The slightly lower level of inhibition by ICP0-1 against the 294.1 strain was likely because a higher amount of initial input virus was used than with the McKrae and 615.9 strains. Corresponding treatment with 10 μg/ml (22 μM) ACV reduced viral plaque formation by 41% for 615.9 and 100% for McKrae and 294.1. Treatment with 20 μM ICP0-1 starting at 3 hpi decreased plaque formation by about 73% for 615.9, 65% for McKrea and 56% for 294.1, respectively, while corresponding treatment with 22 μM ACV reduced viral plaque formation by 50% for 615.9 and 100% for McKrae and 294.1. The differences in plaque reduction observed between 20 μM ICP0-1 and 22 μM ACV at both 1 and 3 hpi, are statistically significant ($p < 0.001$) (Fig. 6A and B). Similar to the experiment depicted in Fig. 4, the efficacy of ICP0-1 PPMO against ACV-resistant virus diminished with time lapse between infection and treatment application. With ACV-sensitive HSV-1 strains, we found that ACV was more effective than PPMO, if the treatment began at 12 h post-infection (Fig. 6C). This finding may reflect the different mechanisms of action by which the two compounds, function, with PPMO being a translation blocker and therefore active earlier in the replicative cycle than ACV, an inhibitor of viral DNA polymerase (Crumpacker and Schaffer, 2002). Overall, however, at most time points tested, the ICP0-1 PPMO generated considerably better suppression of 615.9 replications than ACV.

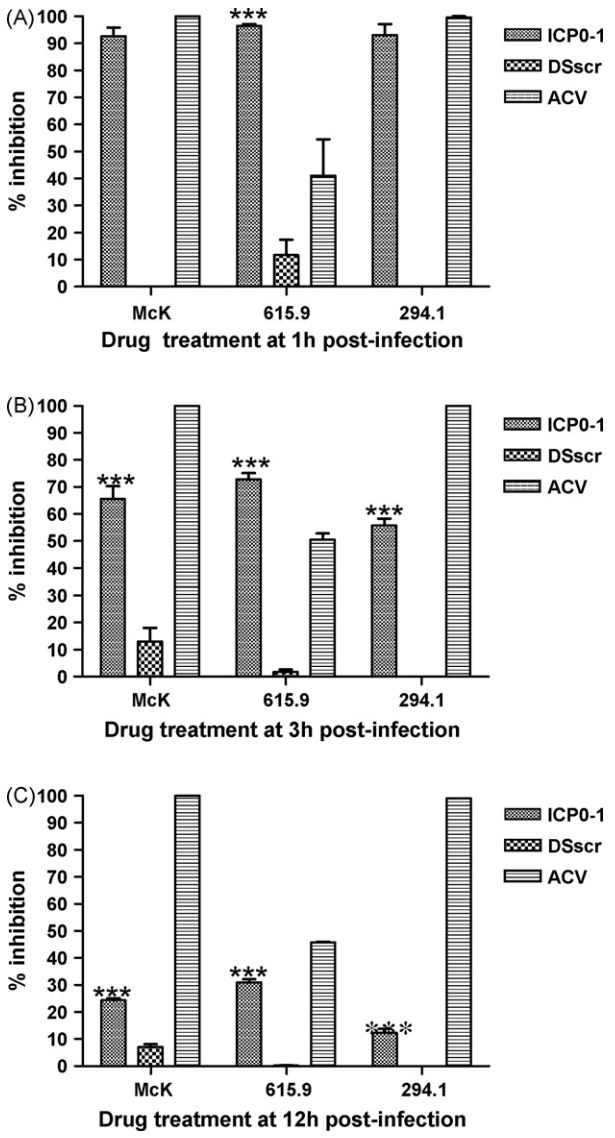

Fig. 6. Antiviral activity of ICP0-1 PPMO against an ACV-resistant strain. Vero cells were infected with 200–300 PFU/well of HSV-1 615.9, McKrae and 294.1, and then treated with the indicated concentration of PPMO or ACV for 2 h starting at 1 hpi (A), 3 hpi (B) and 12 hpi (C). Plaque formation was quantified at 3 dpi and expressed as a percentage of the mock-treated control ($n = 3$). Significant statistical difference ($p < 0.001$) between 20 μM ICP0-1 and 22 μM ACV is marked with symbol '$^{***\prime}$' (two-way ANOVA with Bonferroni post-test).

3.7. The effect of PPMO on target gene expression

To investigate the mechanism of action of HSV-1-specific PPMO, the transcription and translation of targeted genes were examined after PPMO treatment and HSV-1 infection. Cells were treated with ICP0-1, ICP27-1, or ICP4 PPMO for 2 h prior to infection with HSV-1. Following virus infection, total cellular RNA and protein were harvested at 0, 1, 2, 4, 8, 12, and 24 hpi. The mRNA expression of ICP0 or ICP27 was monitored by semi-quantitative RT-PCR. As shown in Fig. 7, levels of cellular GAPDH transcript were constant through time for both PPMO- and mock-treated cells, indicating that the treatments did not have off-target effects. Both ICP0 and ICP27 transcripts showed a time-dependent increase in mRNA level that was consistent across treatments (Fig. 7). In contrast, Western blot analysis showed that the expression of ICP0 and ICP27 protein was delayed by about 12 h in cells treated with ICP0-1 or ICP27-1 PPMO, respectively (Fig. 8). ICP4 PPMO treatment did not appear

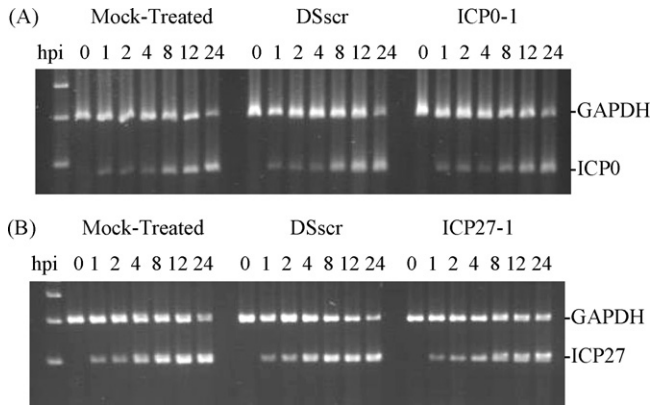

Fig. 7. Effect of PPMO on target gene transcription. RS cells were treated with 20 μM DSscr, ICP0-1 or ICP27-1 PPMO, or DMEM for 2 h, then infected with HSV-1 McKrae at 2 MOI. At the indicated times post-infection, cells were harvested, total RNA extracted, and 1 μg RNA was reverse transcribed and PCR amplified with primers specific for cellular GAPDH and (A) ICP0 or (B) ICP27.

to prevent early ICP4 translation (Fig. 8), which may explain why ICP4 PPMO had low inhibition against HSV-1 replication (Fig. 3). DSscr- and mock-treated control samples showed nearly identical protein expression. Levels of the host cellular protein actin were unchanged over time and across treatments. Since the target of each PPMO occurs as both genomic DNA and as mRNA in HSV-1 infected cells, it is noteworthy that our results indicate that PPMO specifically inhibited the translation, but not the transcription, of the HSV-1 targeted genes.

3.8. PPMO toxicity in uninfected mice

To help establish a maximum tolerable dose, and to investigate the effects of PPMO topically applied to the eyes of mice, three groups of uninfected mice (3 per group) were treated topically with 10 μl of either 100 μM (1 μg/μl), 200 μM (2 μg/μl) or 1 mM (10 μg/μl) ICP0-1 PPMO daily for 7 days. On day 7 post-treatment, no gross lesions were observed and microscopic examination revealed no histopathology in the eyes from all members of the three groups. As shown in Fig. 9, the histology of eyes treated with ICP0-1 PPMO at the various doses appears similar to that of a mock-treated eye. Furthermore, PPMO treatment had no effect on body weight, temperature, appearance or overall behavior of the mice over the 7-day observation period (data not shown).

3.9. Effect of PPMO against HSV infection in mice

To evaluate whether ICP0-1 PPMO could specifically prevent HSV-1 infection in the eye, nine groups of mice (8 per group) were inoculated with 2×10^3 PFU HSV-1/eye, then treated with 10 μl of PBS, or PBS containing 100 μM of ICP0-1 or DSscr starting at 4, 24, or 72 hpi. The three ICP0-treatment groups are referred to as ICP0 4 h, ICP0 24 h, and ICP0 72 h; the three PBS-treatment groups as mock 4 h, mock 24 h, and mock 72 h; and the three DSscr-treatment groups as DSscr 4 h, DSscr 24 h, and DSscr 72 h. Each group of mice also received the same treatment daily for an additional 4 days after the initial treatment. Two groups of uninfected mice were also included; one uninfected group was treated with 10 μl PBS (referred to as 'control') while the other was left untreated (referred to as 'untreated'). The uninfected mice, both control and untreated groups, showed no abnormalities throughout the experiment, and the descriptions below refer only to infected mice. No eye disease was observed in the first 2 dpi. Starting on 3 dpi, 1–2 mice in each of the mock- and DSscr-treated groups exhibited eye disease, whereas all of the ICP0-1 treated mice in the ICP0 4 h and ICP0 24 h group appeared normal at 6 dpi. By day 7, 25–50% of the mock or DSscr-treated groups had developed eye disease, while none of the ICP0-4 treated group and only 12.5% (one of eight) of the mice in the ICP0-4 or ICP0-72 treated groups had developed eye disease (Table 2). By day 8, 50 and 62.5% of the mock or DSscr-treated groups, respectively, had developed eye disease. The eye disease observed in mice of the ICP0-72 treated group was clearly less severe than that present in the mock- and DSscr-treated mice (Fig. 10A). At 7 dpi, one death occurred in the mock 72 h and one in the DSscr 4 h-treated groups. On day 7 pi, eyes from all the treatment groups were examined histologically. All of the Mock- and DSscr-treated mice exhibited inflammation, while mice from the ICP0-treated groups showed only mild corneal thickening. Fig. 10B shows eye histology from Mock 72 h-, DSscr 72 h-, and ICP0 72 h-treated mice on day 7 pi. The Mock 72 h eyes had severe ulcerative conjunctivitis and suppurative keratitis with suppurative anterior uveitis. The DSscr 72 h

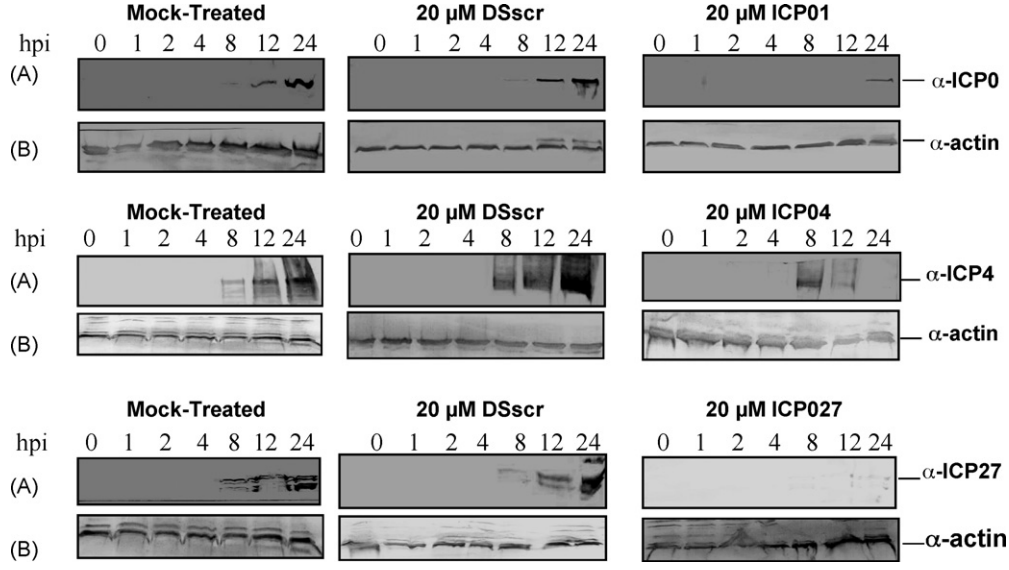

Fig. 8. Effect of PPMO on the translation of targeted mRNA. RS cells were treated with 20 μM DSscr, ICP0-1, ICP27-1, ICP4, or DMEM (vehicle) for 2 h, then infected with HSV-1 McKrae at an MOI of two. At the indicated times post-infection, cells were harvested, lysed in sample buffer, separated on 4–15% SDS for ICP0 and ICP4, or 7.5% SDS PAGE gels for ICP27 PAGE gels and probed with antibodies against ICP0, ICP27, or ICP respectively (A) and actin (B).

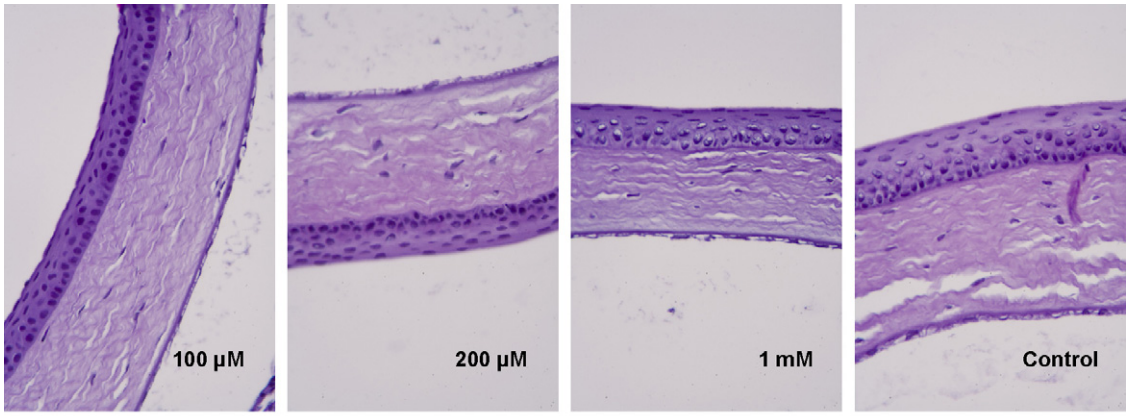

Fig. 9. Histology of mouse corneas. Sections are from uninfected mouse eye treated with ICP0-1 PPMO at 100 μM (A), 200 μM (B), 1 mM (C), or PBS (D) daily for seven days. Histology sections were stained with hematoxylin and eosin.

eyes displayed somewhat milder lesions than the Mock 72 mice, but showed anterior uveitis plus corneal erosion and ulceration with neutrophils in the edematous stroma. We observed thickened corneas and stromal edema, but no ulcers, in the ICP0-1 72 h eyes. Thus, our results indicate that PPMO administration as late as 72 hpi was effective at protecting mice from HSV-induced eye disease. Overall, these data demonstrate that ICP0-1 PPMO can reduce eye disease and prevent death associated with HSV-1 infection in mice.

4. Discussion

The experimental results described here demonstrate that PPMO targeting HSV-1 mRNAs of IE genes, such as *ICP0* and *ICP27*, can inhibit viral replication in cell cultures and in the eyes of mice. Each evaluation of antiviral efficacy by PPMO in this study included efforts to control for non-sequence-specific effects. A negative control PPMO (DSscr) was included in all experiments. Cell-viability assays performed on uninfected cells showed little or no cytotoxic effects by the PPMO at up to 20 μM concentration, under the same cell culture conditions in which the PPMO demonstrated high antiviral activity. Ocular administration of PPMO doses far greater than those used in antiviral trials produced no signs of ill effects in uninfected mice.

The two most effective PPMO in this study, ICP0-1 and ICP27-1, were designed against sequence upstream of the AUG translation-start-site of their respective target mRNAs. Previous studies with RNA viruses have shown that PPMO targeted to the 5′-UTR and AUG-region of viral mRNA can interfere with the translation process (Deas et al., 2005; Holden et al., 2006; Stein, 2008; van den Born et al., 2005). Our finding that the ICP0-1 and ICP27-1 PPMO did not affect the transcription of their target genes (Fig. 7), but did delay and reduce protein production from the respective target gene mRNAs (Fig. 8), likewise supports that translation–inhibition was the mechanism of action of these PPMOs. In this study, PPMO targeting sequences upstream of the AUG initiation site produced a higher protection than those targeting the AUG-region itself. For instance, at 20 μM, ICP27-1 inhibited HSV-1 plaque formation by 88%, while ICP27-2 reduced HSV-1 plaque formation by only 31% (Fig. 3). It is possible that the HSV-1 sequence targeted by ICP27-1 was somehow more accessible to the PPMO than the sequence targeted by the ICP27-2 PPMO. No sequence heterogeneity was revealed by comparative sequence analysis of the ICP0 and ICP27 PPMO target sites for all available HSV-1 strains in GenBank.

Notably, the ICP0-EX PPMO, which targets the 5′-most exon/intron splice junction region of the ICP0 pre-mRNA, also significantly inhibited viral replication, although to a lesser extent than the ICP0-1 and ICP27-1 mRNA-directed PPMO. Since the ICP0-EX targets exclusively intronic sequence, it is highly unlikely that this PPMO worked by interfering in the process of translation. PMO technology has been shown to be capable of altering the splicing of eukaryotic pre-mRNAs (Giles et al., 1999; McClorey et al., 2006). The result here constitutes the first report of inhibition of viral replication by a PPMO designed to interfere with the splicing process. However, because the level of inhibition by ICP0-EX was comparatively low, we pursued the more highly antiviral ICP0-1 and ICP27-1 PPMO in this study.

Reducing ICP27 and ICP0 protein levels was clearly detrimental to viral replication, and these results are consistent with the current view of ICP27 and ICP0 as necessary for optimal viral replication at low MOI in cell cultures (Sandri-Goldin, 2006). Although ICP0 was not found to be essential for virus replication at high MOIs (Cai and Schaffer, 1992), we found that PPMO targeting ICP0 mRNA reduced virus replication by 3–4 logs (when measured at 24 hpi) when the infection dose was low, at 0.1 MOI (Fig. 5). Similar results with antisense oligonucleotides directed against the ICP0 (or IE 110) have been previously reported (Peyman et al., 1995). Oddly, although ICP4 is considered to be an essential protein for the HSV-1 life cycle, (Clusel et al., 1995; Panagiotidis et al., 1997; Samaniego et al., 1995) the ICP4 PPMO did not produce high protection against HSV-1 infection (Fig. 3). Our data shows that treatment with the ICP4 PPMO resulted in a reduction of ICP4 expression at 12 and 24 h post-infection, but not at 8 h post-infection (Fig. 8). We conclude that the level of ICP4 expression that occurred under PPMO treatment was sufficient for HSV-1 replication in our system.

Table 2
No. of mice with eye disease and death.

Group[a]	D1[b]	D3[b]	D4[b]	D6[b]	D7[b]	D8[b]	D9[b]	D10[b]
Mock 4	0	0	1	3	4	4	4	4
Mock 24	0	0	0	2	4	5	5	4
Mock 72	0	2	2	4	4/1[c]	5	5	3
DSscr 4	0	2	2	3	3/1[c]	5	5	3
DSscr 24	0	2	2	2	3	4	4	3
DSscr 72	0	2	2	3	2	4	4	3
ICP0 4	0	0	0	0	1	1	1	0
ICP0 24	0	1	0	0	0	0	0	0
ICP0 72	0	1	1	1	1	0	0	0
Control	0	0	0	0	0	0	0	0
Untreated	0	0	0	0	0	0	0	0

[a] Represents 8 mice per group.
[b] Days of post-infection.
[c] Represents number of dead animals.

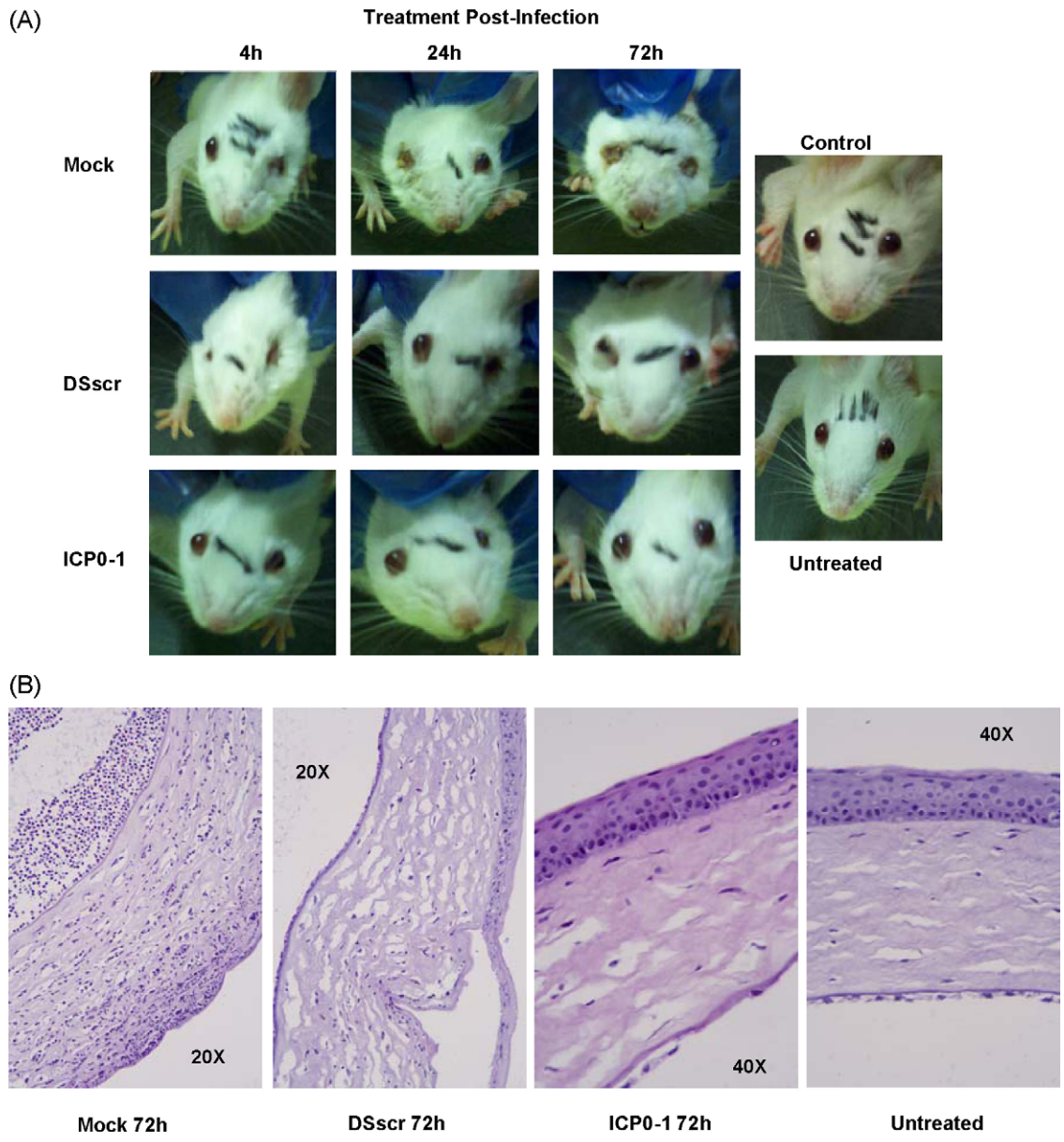

Fig. 10. Gross and histologic effects of ICP0-1 PPMO in HSV-1 infected mice. Mice ($n = 8$) were infected with HSV-1 at 2×10^3 PFU/eye and treated with 10 μl/eye of PBS containing 100 μM ICP0-1 or DSscr PPMO, or with PBS alone (mock), with the first treatment at the indicated hours post-infection. Each group also received additional treatments daily on each of the 4 days following the first treatment. Uninfected mice either untreated (untreated) or PBS-treated (control) as above were also included. (A) Pictures of representative mouse eyes at 10 dpi from each of the groups receiving an initial treatment starting at 4, 24, and 72 hpi. (B) Histology of corneas from infected and uninfected mouse eyes. Cornea sections are from groups receiving an initial treatment starting at 72 hpi. Histology sections were stained with hematoxylin and eosin.

Overall, ICP0-1 was the most effective PPMO against both ACV-sensitive and ACV-resistant HSV-1 (Figs. 3–6). Treatment with ICP0-1 shortly after infection dramatically reduced the incidence of HSV-1 induced eye disease. Importantly, the ICP0-1 was also able to prevent death associated with 2×10^3 PFU/eye infection in mice. When a higher inoculation dose of 2×10^4 PFU/eye was used, we found that dosing with 10 μl of 100 μM ICP0-1 PPMO (per eye) was not nearly as effective at suppressing viral replication as it had been against the lower inoculation dose (data not shown). These results suggest that PPMO antiviral activity can be overwhelmed by a high inoculation dose. However, 2×10^3 PFU/eye is already 4–8 fold higher than the amount of virus that is reactivated in the rabbit eye during a typical recurrent infection (Ling Jin, unpublished data) and therefore has considerable relevance to a real-life infectious dose in humans. Notably, the ICP0-1 PPMO reduced the incidence of eye disease even when first applied at 72 hpi (Table 2; Fig. 10), suggesting that PPMO can be used effectively after infection to prevent virus replication and reduce local inflammatory damage.

Our results strongly suggest that topically applied ICP0-1 PPMO was able to suppress HSV-1 replication in mouse eyes. In an experiment similar to the one which produced Fig. 10, groups of mice ($n = 4$) were infected with a similar dose of HSV-1 (2×10^3 PFU/eye), treated with PPMO for 2 h before HSV-1 infection (Mock 2 h, DSscr 2 h, and ICP0 2 h), and virus replication was monitored by eye swabs on day 1, 3, 5, 7 and 10 pi. The results indicated no virus replication in mouse eyes treated with ICP0-1, in contrast to considerable virus propagation in mock- and DSscr-treated mouse eyes (Fig. 11). However, the eye swabbing procedure caused eye irritation in some uninfected control animals, complicating interpretation of the overall results. In the ICP0-treated mice and uninfected controls, 30% of mice developed mild diseases, while in the untreated mice and DSscr-treated mice, 100% of mice developed eye diseases.

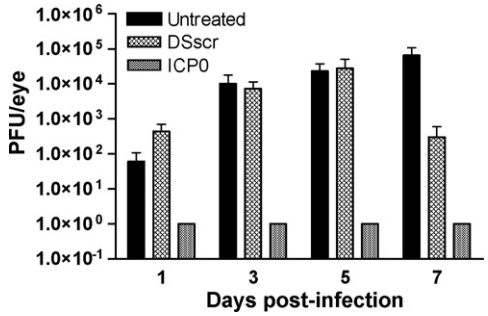

Fig. 11. Effect of PPMO against HSV-1 replication in the eye. Mouse eyes were treated with 10 μl/eye of 100 μM ICP0-1 or DSscr PPMO, or vehicle alone at 1 h before infection with 10^3 PFU/eye HSV-1 (McKrae). Each group received the same treatment daily for an additional 4 days following infection. Mouse tear swabs were taken at indicated time points and virus titers were determined by plaque assay in RS cells. The limit of detectable virus is indicated as 1 in this graph.

In the infected, untreated- and DSscr-treated group, 50% of mice died between day 7 and 9 pi. We therefore evaluated PPMO efficacy against HSV-1 infection in this experiment by only clinical and histological criteria. The results shown in Fig. 10 strongly suggest that ICP0-1 PPMO can block HSV-1 replication, as no inflammation, except corneal thickening and edema was observed in the ICP0-1 treated eyes. Furthermore, the symptoms observed in the eyes of ICP0-1 treated mice likely did not result directly from exposure to PPMO, but from cleaning the eye with moistened swabs as part of the application procedure.

In summary, our results demonstrate that ICP0-1 and ICP27 PPMO can effectively prevent HSV-1-induced eye disease in mice, and that this oligomer warrants consideration for further development as a treatment for recurrent HSV-1 disease in humans.

Acknowledgments

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