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Inhibition of primary effusion lymphoma engraftment in SCID mice by morpholino oligomers against early lytic genes of Kaposi's sarcoma-associated herpesvirus

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Abstract

Background—Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with several malignant diseases, including Kaposi's sarcoma, primary effusion lymphoma (PEL) and multicentric Castleman's disease. The objectives of this study were to explore peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) against KSHV early lytic genes and assess their efficacy in SCID mice against PEL engraftment. PPMOs are short single-stranded DNA analogs that contain a backbone of morpholine rings and phosphorodiamidate linkages and have high delivery efficiency into cells.

Methods—PEL cells were treated with PPMOs against viral interferon regulatory factor 1 (vIRF-1) and expression of vIRF-1 was analyzed. The PPMOs against vIRF-1 and viral interleukin 6 (vIL-6) were evaluated against PEL cell engraftment in SCID mice. The PPMOs were administered into peritoneal cavities of SCID mice for ten doses at two-day intervals. At week 3 and 9 after BCBL-1 delivery, peritoneal lavage was collected and the ratio of PEL cells among total cells was determined by flow cytometry analysis.

Results—Treatment of PEL cells with PPMOs against vIRF-1 led to reduction of vIRF-1 expression in a dose-dependent manner. Reduction of vIRF-1 expression resulted in higher levels of cellular IRF-3 and STAT1 (signal transducers and activators of transcription 1). SCID mice receiving a PPMO against vIL-6 had no engraftment of PEL cells and remained healthy throughout the 120-day study.

Conclusion—This study showed that PPMOs can be effective antivirals against KSHV. Blocking expression of early lytic genes may lead to beneficial effect for control of KSHV-associated malignant diseases.

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Disclosure statement

There is no conflict of interest in this study.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is associated with several malignant diseases, including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) [1, 2]. Despite the use of highly active anti-retroviral therapy (HAART) that has reduced the incidence of KS, the disease remains the most common malignancy in patients with AIDS in developed countries [3]. Current anti-herpesvirus drugs, such as ganciclovir, cidofovir, and foscarnet, target DNA polymerase that are expressed during the late lytic phase of KSHV replication and have limited benefits against either KS or PEL [4, 5]. Specific anti-KSHV drugs are still much-needed for the treatment of KSHV-associated malignancies. In this study, peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) were explored to inhibit expression of KSHV early lytic genes and to suppress engraftment of PEL cells in mice with non-obese diabetic/severe combined immunodeficiency disease (NOD/SCID).

PPMOs are short single-stranded DNA analogs that contain a backbone of morpholine rings and phosphorodiamidate linkages [6]. PPMOs bind to complementary target mRNA by Watson-Crick base pairing and block translation through steric blockade. This is a distinctly different process from the RNase H-dependent mechanism induced by other antisense structural types, such as phosphorothioate DNA [6]. The sequence-specific antiviral efficacy of PPMOs has been documented against a number of RNA viruses in animal models, including Ebola Virus [7, 8], Cocksackievirus B3 [9], murine Coronavirus [10], Dengue virus [11], and West Nile virus [12, 13].

KSHV is a large, double-stranded DNA virus encoding over 80 genes [14]. Like other herpesviruses, KSHV displays a tightly regulated program of gene expression using two modes of infection: latent and lytic. The reactivation of KSHV lytic replication in PEL cells can be induced by chemicals, such as tetradecanoyl phorbol acetate (TPA) [15]. Lytic replication can be divided into immediate-early, early, and late stages, based on the expression time of viral genes after activation of lytic cycle. Two of the KSHV early lytic genes, viral interferon regulatory factor 1 (vIRF-1) and viral interleukin 6 (vIL-6), are among those that have been implicated in the pathogenesis of KSHV-associated malignant diseases.

The vIRF-1 is encoded by ORF-K9 and has homology to members of the cellular interferon regulatory factor family. The vIRF-1 interacts with IRF-1, IRF-3, p53, and CBP/p300 proteins to inhibit type I/II human interferon (IFN) signaling and leads to transformation of NIH3T3 cells, as well as induction of tumors in nude mice [16–19]. The IRF family members are transcription regulatory factors that bind to interferon-stimulated response elements (ISRE) to regulate interferon-responsive genes involved in pathogen response, cytokine signaling, cell growth regulation, and hematopoietic development [20]. The vIRF-1 protein directly interacts with these DNA binding proteins to interfere with their functions [21, 22]. vIRF-1 inhibits ATM/p53 signal transduction pathway by down-regulating the p53 total protein level [23]. These results suggest that vIRF-1 is an oncogenic protein and can potentially contribute to KSHV-driven malignancies.

ORF-K2 encodes vIL-6, which is expressed in only a few cells of KS lesions, but in a greater number of cells in PEL and MCD lesions, indicating a role of vIL-6 in KSHV-associated lymphoproliferative disorders [24–27]. vIL-6 is a homolog of human IL-6 (hIL-6), an important autocrine/paracrine growth factor and is notably expressed at a high level in some lymphomas [1, 2]. vIL-6 can stimulate all of the known hIL-6-induced signaling pathways [28, 29]. Treatment of BCBL-1 cells with a PPMO against vIL-6 leads to inhibition of vIL-6 expression and, as a result, slower cell growth [30]. Thus, vIL-6 is a multifunctional cytokine that can potentially contribute to KSHV-associated lymphoproliferative disorders.

Considering the important roles of vIRF-1 and vIL-6, we selected them as targets for development of anti-KSHV PPMOs. The PPMOs were tested in cell culture of PEL cells and in NOD/SCID mice engrafted with BCBL-1 cells. BCBL-1 cells were derived from a primary effusion lymphoma patient and were successfully engrafted into SCID mice [31]. In this study, we found that two anti-vIRF-1 PPMOs effectively inhibited vIRF-1 expression in PEL cells, as shown by Western blotting analysis. In NOD/SCID mice, administration of a PPMO against vIL-6 effectively blocked engraftment of BCBL-1 cells.

Materials and Methods

Cells and viruses

KSHV-infected BCBL-1 (from NIH AIDS Research & Reference Reagent Program) and BCP-1 (ATCC CRL-2294) cells were derived from body cavity-based lymphomas [15, 32]. Both cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. For induction of KSHV lytic replication, TPA (12-O-tetradecanoylphorbol 13-acetate) (Sigma, St Louis, MO) was added to the cell growth medium to a final concentration of 20 ng/mL.

PPMO design and synthesis

PPMOs against vIRF-1 were designed to be complementary to the 5' terminal region of vIRF-1 mRNA sequence. The sequences (5' to 3') of the anti-vIRF-1 PPMOs used in this study are KP1: GCCTGGGTCCATTGTCCCGC (underlined nucleotides corresponding to translation initiation codon AUG of vIRF-1 mRNA) and KP2: TAGCCGCAGAATTGCCTGGGT. KP1 is complementary to nucleotides 152–172 and KP2 complements nucleotides 1–22 of the vIRF-1 mRNA sequence (GenBank accession number AF145701). PPMOs were produced at AVI BioPharma Inc. (Corvallis, OR) and conjugated to arginine-rich P007 peptide, as previously described [30]. A scramble PPMO (hereinafter named CP1), having little agreement with KSHV or human mRNA sequences, was used as a negative control. ILP1, a PPMO against vIL-6, was described previously [30].

PPMO treatment of KSHV-infected PEL cells

Treatment of KSHV-infected BCBL-1 and BCP-1 cells was conducted as reported previously [30, 33]. Briefly, the cells were pelleted and resuspended in RPMI 1640 medium, and plated in a 12-well cell culture plate. PPMO was immediately added, mixed, and incubated for 4 h at 37°C. Growth medium was then added to a final volume of 1.5 mL per

well. For those cells to be induced with TPA, the medium was supplemented with TPA at 20 ng/mL. The cells were incubated for 48 h at 37°C and both cells and culture supernatant were then harvested for further analysis.

Western blotting analysis

Expression of KSHV proteins in KSHV-infected cells was detected by Western blotting analysis, as described previously [33]. Rabbit anti-vIRF-1 antibody (a gift from Dr. Margaret K. Offermann at Emory University, Atlanta, Georgia), goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) and chemiluminescence substrate were used in this assay. Chemiluminescence signals were collected by ChemiDoc XRS digital imaging system (Bio-Rad Laboratories, Hercules, CA). For normalization, β -tubulin also was detected on the same blot membrane after being stripped. Similarly, Western blotting analysis was conducted with rabbit anti-IRF3 and rabbit anti-STAT1 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Digital image analyses were conducted using Quantity One software (Version 4.6) (Bio-Rad).

Cell viability assay—The viability of BCBL-1 cells after PPMO- or mock-treatment was determined with CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega, Madison, WI) as described previously [30]. Briefly, BCBL-1 cells were treated with KP1 and CP1 as described above. TPA was not used in this assay. Normal BCBL-1 cells were included for comparison. At 48 h after the PPMO treatment, CellTiter-Glo reagent was added to the cells according to the manufacturer's instructions and the luminescence signal was then detected with a VICTOR3[™] Multilabel Counter (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA).

DNA isolation and Real-time PCR

Genomic DNA from cell culture supernatant was isolated using DNAzol (Invitrogen, Carlsbad, CA). Real-time PCR was conducted with a Chromo 4 Detector system and iQ SYBR Green Supermix (Bio-Rad), as described previously [30]. Primers from ORF73 were used to detect KSHV DNA. A plasmid pCDNA3-ORF73 was used to generate standard curve for quantification of the KSHV DNA copies in culture supernatant.

Engraftment of BCBL-1 cells to SCID mice and PPMO treatment

Six-week-old NOD/SCID female mice were purchased from Animal Production Program, NCI (Frederick, MD). The mice were housed in micro-isolator cages. All of the cages, food, water, and bedding were autoclaved prior to use. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee at the University of Maryland. Mice were monitored for clinical signs and ascites development, and euthanized if a humane endpoint was reached according to our protocol.

BCBL-1 cells were delivered to the NOD/SCID mice via intraperitoneal (i.p.) route at a dose of 1×10^7 cells per mouse, as described previously [31]. Peritoneal lavage of the mice was collected using 5 ml serum-free RPMI at 3–5 weeks post-inoculation. The BCBL-1 cells from *in vivo* propagation in SCID mice were used for the PPMO study in mice.

For PPMO study, 5 mice were used in each group. Mice in group 1 to 4 received 1 million BCBL-1 cells via i.p. injection. Group 1 did not receive PPMO treatment. Group 2 to 4 received treatment with KP1+KP2, ILP1, and CP1, respectively. Group 5 was used as a control that received no BCBL-1 cells or PPMO. In the groups that were given a PPMO treatment, the BCBL-1 cells were treated with PPMOs at a final concentration of 16 μ M for 2 h before being injected into SCID mice via i.p. PBS was used as mock treatment in a negative control group. The mice were injected with PPMOs for 9 times at two-day intervals at a dose of 5 mg/kg, which is about 100 μ g in 500 μ L volume per mouse. The mice were observed daily and subjected to peritoneal lavage collection at 3 and 9 weeks after BCBL-1 injection. To assess tumor cell burden, a fraction of lavage sample cells were stained with phycoerythrin (PE)-conjugated monoclonal antibody against human CD45, a leukocyte common antigen, and FITC-conjugated antibody against murine CD45.2 (BD Biosciences Pharmingen), as described previously [31]. Flow cytometry analysis was conducted to determine the ratio of BCBL-1 in the total number of cells from lavage samples.

Statistical analysis

A single factor ANOVA statistical analysis was used to analyze statistical differences between the treatment groups. A two-tailed P value of less than 0.05 was considered to be a significant difference.

Results

PPMO against ORF-K9 gene inhibits vIRF-1 expression in a transient transfection

The 5' untranslated region (UTR) of vIRF-1 mRNA is 159 nt long [34]. There is no internal ribosome entry site in the 5'UTR. The region around translation initiation codon of vIRF-1 transcript was initially selected as target for PPMO design. KP1, complementary to this region, was first designed and tested for its effect in inhibition of vIRF-1 expression. Later, another vIRF-1 PPMO, KP2, was designed to confirm the sequence-specific inhibition of KP1. KP2 is complementary to the 5' terminus of vIRF-1 mRNA, which is also an important region for ribosome binding and scanning in translation of the mRNA. A scramble PPMO (CP1), containing a random sequence, was included as a negative control. All the PPMOs were checked in NCBI Blast search to make sure no potential targets to cellular genes.

To test the effect of KP1 on target gene expression, we cloned ORF-K9 and 5' UTR of vIRF-1 mRNA to upstream of the GFP gene in a pEGFP-N1 vector, in order to express it as vIRF-GFP fusion protein. The resulting plasmid pEGFP-vIRF-1 was transiently transfected into HEK293 cells. Treatment of the cells with 16 μ M KP1 was conducted at 2 h after transfection and CP1 was included as a control. At 24 h post-transfection, the cells were observed under fluorescence microscopy. In comparison with CP1-treated cells, the KP1-treated cells had much fewer cells with green fluorescence (Figure 1A), indicating that KP1 suppressed the translation of target vIRF-GFP transcript. The CP1-treated cells had GFP-positive cells at a similar percentage to mock-treated cells. The result indicates that KP1-mediated inhibition of vIRF-GFP fusion protein expression is sequence-specific. We then tested the PPMOs against vIRF-1 in KSHV-infected PEL cells.

Suppression of vIRF-1 expression in KSHV-infected PEL cells

Immunofluorescence assay was initially conducted with rabbit anti-vIRF-1 antibody to detect vIRF-1 in BCBL-1 cells. After TPA induction, approximately 20% of the cells expressed vIRF-1, as expected. Fewer than 5% of the cells that were treated with 16 μ M KP1 were vIRF-1-positive. To assess vIRF-1 level, total proteins were separated by SDS-PAGE and detected by Western blotting analysis with rabbit anti-vIRF-1 antibody. The result showed that KP1 treatment led to reduction of vIRF-1 level in BCBL-1 cells (Figure 1B). Densitometry analysis of the digital blot image showed that treatment of BCBL-1 cells with KP1 at 16 μ M reduced vIRF-1 level by 80%, in comparison with mock-treated BCBL-1 cells after TPA induction (Figure 1B). BCBL-1 cells treated with CP1 had negligible effect on vIRF-1 protein level. These results suggest that KP1 inhibited vIRF-1 expression in BCBL-1 cells.

PPMO KP1 was also tested in BCP-1, another cell line of PEL, to make sure that the inhibition is not cell line-specific. The cells were treated with 16 μ M KP1 or CP1 and harvested 48 h post-treatment. Western blotting analysis showed that the KP1 treatment reduced vIRF-1 level in BCP-1 cells by 90% (Figure 1C), while the CP1 did not have an effect in comparison to mock-treated control.

PPMO KP2 complements to a site that is 152 nt upstream of the KP1 complementary site in vIRF-1 mRNA. Treatment of BCBL-1 cells with KP2 also reduced the vIRF-1 level by 80% in comparison to the mock-treated control (Figure 1D). The KP2 inhibitory effect was similar to the effect of KP1 and confirmed that the PPMO-mediated inhibition is sequence-specific. This indicates that the 5' UTR of vIRF-1 mRNA is a productive region for PPMO targeting.

We further tested dose-response effect of PPMO treatment in inhibition of vIRF-1 expression in BCBL-1 cells. KP1 was used as both KP1 and KP2 had similar effect. Along with incremental concentration of KP1 from 0.5 to 16 μ M, the vIRF-1 level in TPA-induced BCBL-1 cells decreased in a dose-dependent manner (Figure 1E). In contrast, treatment with CP1 did not have much effect on the expression of vIRF-1 in the TPA-induced BCBL-1 cells.

Cell culture supernatant was also harvested for DNA isolation and real-time PCR to measure KSHV DNA level. TPA induction led to an elevation of KSHV viral DNA level by an average of 39-fold. The KSHV DNA in the supernatant from KP1-treated cells was significantly reduced by 42% in comparison with CP1-treated control (supplemental figure 1). The result suggests KP1 treatment resulted in reduction of KSHV replication in TPA-induced BCBL-1 cells.

We conducted a cell viability assay to make sure that the PPMO did not have toxicity to the PEL cells at the concentration used. Cell viability assay did not detect any significant change in cell numbers of BCBL-1 cells after KP1 or CP1 treatment. The relative percentage of cell viability of BCBL-1 cells treated with KP1 was 97% in comparison with non-treated cells (supplemental figure 2). The result indicates that the PPMOs were not toxic to the PEL cells at the concentration used.

Elevation of IRF-3 and STAT1 in BCBL-1 cells by KP1 treatment

Since vIRF-1 interacts with cellular interferon regulatory factor 3, we speculated that suppression of vIRF-1 would have an effect on IRF-3 expression. Since KP1 and KP2 have similar effect on vIRF-1 expression, we used only KP1 for this experiment. We tested the IRF-3 protein level in BCBL-1 cells treated with 16 μ M KP1 or CP1. Mock-treated cells were also included for comparison. It was interesting to note that TPA induction led to reduction of IRF-3 level (Figure 2A). The cells treated with KP1 had a 6-fold higher IRF-3 level than CP1 treatment. The result suggests that the KP1 treatment suppressed vIRF-1 expression and relieved the inhibition of IRF-3 expression.

In interferon signaling pathway, STAT1, an important transcription factor, is needed for activating the expression of IFN-responsive genes. We speculated that KP1-mediated suppression of vIRF-1 would have an impact on STAT1. In BCBL-1 cells treated with KP1, the STAT1 level was higher than that in cells treated with CP1 (Figure 2B). The STAT1 band was 3-fold more intense in the KP1 lane than in the CP1 lane, which indicated that vIRF-1 suppression in the cells led to an increase in STAT1 expression in the cells induced by TPA. The expression level of STAT1 was not much altered by TPA induction.

PPMO Treatment reduces BCBL-1 engraftment in SCID mice

The SCID mouse model of PEL engraftment was used to assess the efficacy of the PPMOs *in vivo*. BCBL-1 cells propagated in intraperitoneal cavity of SCID mice were used in this experiment. When the BCBL-1 cells that were propagated *in vivo* were inoculated into naïve SCID mice, ascites and tumor development were much quicker than the mice inoculated with BCBL-1 cells cultured *in vitro*.

Since both KP1 and KP2 were effective in the inhibition of vIRF-1 expression in cell culture and target vIRF-1 mRNA, we combined both of them in one group in the mouse experiment for a more effective inhibition. ILP1, a PPMO against vIL-6 of KSHV, was also tested since it was shown to be effective in inhibiting vIL-6 expression and resulted in reduction of cell growth in culture [30]. A scramble PPMO CP1 was included as a negative control. Five mice were used in each group. BCBL-1 cells were incubated with PPMOs for 2 h before being injected into the peritoneal cavities of the mice. The PPMOs were administered 9 more times in two-day intervals. At week 3 after injection of BCBL-1 cells, peritoneal lavage was collected and cells were stained to determine the ratio of PEL cells in the lavage. The mice treated with ILP1 had significant average lower ratio of PEL cells, less than 2% of total cells (Figure 3), which is at the background level and is negligible. Two mice of the KP1+KP2 group had a low ratio of PEL cells, 3.94 and 7.01%, respectively (Table 1A), though the average ratio of PEL cells in this group was similar to those treated with scramble PPMO or untreated control. All five mice in the ILP1 group had a very low ratio (less than 10%) of PEL cells. All mice treated with CP1 and the mock-treated group had a relative high ratio (over 20%) of BCBL-1 cells, indicating efficient engraftment of the PEL cells.

At week 9 after injection of BCBL-1 cells, peritoneal lavage was collected again. All mice in the ILP1 group had negligible ratio (less than 3%) of PEL cells (Figure 3B and Table 1B) and all were healthy. The average ratios of BCBL-1 cells in the lavage of KP1+KP2, CP1,

and negative control groups were 45, 60, and 83%, respectively. One mouse in the KP1+KP2 group had negligible ratio, 1.98%, of BCBL-1 cells in its peritoneal lavage (Table 1B). Those mice that had a high ratio (over 20%) of BCBL-1 cells at week 3 still had similar or higher ratio of the cells at week 9.

All mice in CP1 control group receiving BCBL-1 cells were euthanized before or on day 77 as a humane endpoint was reached (Table 1C). The mice in the ILP1 group were still healthy when the study ended on day 120 after BCBL-1 cell injection. No ascites development was noticed in the mice of the ILP1 group. This result suggests that ILP1 suppressed BCBL-1 engraftment in the mice. The survival time of all mice of ILP1 group was much longer than others (Figure 4 and Table 1C). They remained healthy through the end of this study at day 120 after delivery of BCBL-1 cells. So did one mouse of KP1+KP2 group. All other mice died or were euthanized before day 77. One mouse in KP1+KP2 group that had negligible ratio, 7%, of BCBL-1 cells at week 3 died accidentally during handling on day 38. These data indicate that ILP1 inhibited BCBL-1 engraftment in SCID mice.

Discussion

In this study, we explored the use of PPMOs against early lytic genes vIRF-1 and vIL-6 and demonstrated that the anti-vIRF-1 PPMOs inhibited vIRF-1 expression in two KSHV-infected PEL cell lines, BCBL-1 and BCP-1. The PPMO ILP1 against vIL-6 protected SCID mice from PEL engraftment.

We designed and tested two PPMOs complementary to the 5'UTR and the translation initiation region of vIRF-1 mRNA. Both PPMOs worked efficiently in inhibiting vIRF-1 expression in BCBL-1 cells, indicating that the 5'UTR is critical for vIRF-1 translation. Our finding is consistent with previous publications on inhibition of vIRF-1 by antisense or ribozyme [35, 36]. The KSHV replication was also reduced in KP1-treated PEL cells. We speculated that the reduction of KSHV lytic replication might be a result of alleviation of vIRF-1 inhibition on cellular IFN signaling pathway. Our data on elevation of IRF-3 and STAT1 in KP1-treated cells also supports this speculation. BCBL-1 cells, after vIRF-1 inhibition, had higher levels of IRF-3 and STAT1, which play an important role in IFN-signaling and antiviral response. The elevation of the cellular genes in the IFN signaling pathway is encouraging data for further exploration of the PPMOs as a potential intervention of KSHV-associated malignant diseases.

We conducted a cell viability assay and did not detect any significant change in cell numbers two days after KP1 treatment of BCBL-1 cells. Since vIRF-1 is expressed in a low ratio of normal PEL cells, the suppression of vIRF-1 might not result in an immediate effect on cell growth.

Our earlier study showed that PPMOs can specifically suppress protein expression of KSHV RTA, LANA and vIL-6 [30, 33]. Application of an RTA-specific PPMO to BCBL-1 cells resulted in reduction of KSHV viral DNA levels in intracellular and culture supernatant samples. Blockage of vIL-6 expression resulted in slower growth of BCBL-1 cells. This study showed that vIRF-1 can be another productive target for inhibition of KSHV infection.

These results indicate that blocking expression of latent and early lytic genes is an effective strategy to inhibit KSHV infection and may lead to beneficial effects for control of KSHV-associated malignant diseases.

SCID mouse model of PEL represents an effective *in vivo* approach to assess the efficacy of the PPMOs in inhibiting the PEL engraftment. The PPMO ILP1 against vIL-6 blocked PEL development in SCID mice, which is consistent with its effective inhibition of vIL-6 expression and BCBL-1 growth [30]. The two PPMOs against vIRF-1 were less effective in SCID mice though two of the five mice had negligible engraftment of BCBL-1 cells, indicating that the dose of anti-vIRF-1 PPMOs was not sufficient to have an extending inhibitory effect on BCBL-1 cells in SCID mice. Another possible reason is that vIRF-1 expression might be dispensable in the PEL engraftment in SCID mice.

Current anti-herpesvirus drugs target the late lytic phase of herpesvirus replication, and it has been reported that cidofovir and foscarnet are the most efficient commercially available compounds against KSHV [37–39]. Cidofovir and foscarnet have no effect on the expression of latent or early lytic genes [40], as these drugs are inhibitors of the viral DNA polymerase and transcription of early lytic genes occurs before DNA replication in KSHV. Early lytic genes such as vIL-6 and vIRF-1 play important roles in KSHV replication and pathogenesis. Our results indicate that vIRF-1 and vIL-6 may be effective targets for antiviral therapy. Additionally, inhibition of the early lytic genes may have positive implications for managing KSHV-induced lymphoma development. Further studies to investigate the capacity of PPMOs to suppress KSHV tumorigenesis are needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Inhibition of vIRF-1 expression. A. Inhibition of vIRF-1 expression in HEK293 cells transiently transfected with pEGFP-vIRF-1 plasmid. At 2 h after transfection, the cells were treated with PPMO KP1 or CP1 at 16 μ M. GFP-positive cells were observed under fluorescence microscopy. Photos were taken at 200X magnification. Similar confluence of the cells among the treatments was observed under phase contrast. B. Western blotting analysis of vIRF-1 in BCBL-1 cells treated with KP1 or CP1 at 8 and 16 μ M. Around 30 μ g total proteins from whole cell lysate was loaded in each lane. The same blot was probed with

a β -tubulin antibody for assessment of protein loading. Mock-treated cells were also included for comparison. Graphic illustration of densitometry analysis of the digital image is shown on the right. The vIRF-1 level is shown as a relative percentage of the mock-treated control. C. vIRF-1 in BCP-1 cells detected by Western blot. The cells were treated with KP1 or CP1 at 16 μ M. The figure on the right is the graphic illustration of densitometry analysis of the digital image. D. Inhibition of vIRF-1 expression in BCBL-1 cells by both KP1 and KP2 at 16 μ M. Graphic illustration of densitometry analysis of the digital image is shown on the right. E. Dose-responsive inhibition of vIRF-1 expression in BCBL-1 cells by KP1 treatment detected by Western blot. The BCBL-1 cells were induced with TPA. Graphic illustration of densitometry analysis of the digital image is shown on the right.

Fig. 2.

Modulation of IRF-3 and STAT1 expression in BCBL-1 cells by KP1 treatment. A. Western blot of IRF3 level in BCBL-1 cells treated with KP1 or CP1 at 16 μ M. Mock-treated cells were also included for comparison. Relative expression level in fold in comparison with normal BCBL-1 cells is shown below the blot image. B. Western blot of STAT1 in BCBL-1 cells after KP1 or CP1 treatment at 16 μ M. Relative expression level in fold in comparison with normal BCBL-1 cells is shown below the blot image.

Fig. 3.

Average ratio of BCBL-1 cells in peritoneal lavage of PPMO-treated SCID mice analyzed by flow cytometry. The percentage of BCBL-1 cells among total cells from peritoneal lavage of each mouse was used to calculate the average ratio in each group. A. Samples were collected at week 3 after BCBL-1 delivery. B. Samples collected at week 9 after BCBL-1 delivery. Dead mice were not counted in calculation of the average ratio of BCBL-1 cells in each group. Error bars represent variations among the five mice in each group. Significant differences between groups of ILP1 and CP1 are denoted by “*”, which indicates $P < 0.01$.

Fig. 4.

Survival curve of PPMO-treated SCID mice that were engrafted with BCBL-1 cells. One mouse in mock-treatment group (filled diamond), one mouse in CP1 group (cross out), and 2 mice in KP1+KP2 group (filled square) died before or during week 8. All five mice in ILP1 group (filled triangle) and one mouse in KP1+KP2 group were still healthy by week 17. All other mice were euthanized in week 11 due to poor health conditions. Observation ended at week 17.

Table 1

PPMO treatment of SCID mice with BCBL-1 engraftment

A. Percentage of BCBL-1 cells in SCID mice at week 3^a				
Mouse	Mock	KP1+KP2	ILP1	CP1
1	59.15	3.94	8.65	58.64
2	60.55	7.01	0.79	45.25
3	42.81	60.66	0.02	46.18
4	73.26	57.37	0.00	31.26
5	24.36	72.60	0.00	31.44

B. Percentage of BCBL-1 cells in SCID mice at week 9^a				
Mouse	Mock	KP1+KP2	ILP1	CP1
1	Dead	1.98	0.14	63.19
2	86.97	Dead	0.41	79.00
3	65.44	66.61	2.82	Dead
4	90.22	Dead	0.61	66.16
5	88.67	65.68	0.08	31.34

C. Total days alive for the mice after BCBL-1 delivery^b				
Mouse	Mock	KP1+KP2	ILP1	CP1
1	21 ^c	120	120	77
2	77	38 ^c	120	77
3	70	77	120	64
4	77	64	120	77
5	77	77	120	77

^a Peritoneal lavage was collected at week 3 and 9 after BCBL-1 delivery. To assess tumor cell burden, a fraction of each lavage was stained with PE-CD45 and FITC-CD45.2, and analyzed by flow cytometry analysis, as described in the Methods. Percentage of BCBL-1 (PE-positive) in total cells is shown. Dead mice were not counted for calculation of average percentage of BCBL-1 cells.

^b Euthanization was conducted on and before day 77. In this study, observation ended at day 120 after BCBL-1 delivery.

^c Accidentally died during handling.