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(54) Title: MORPHOLINO ANTISENSE DRUGS FOR HUMAN HERPESVIRUS 8

(57) Abstract: The present invention provides antisense oligomers useful for the treatment or prevention of HHV8 viral infections. The antisense oligomers are directed to bind to a targeted HHV8 gene, which would inhibit the replication or tranformation of HHV8. The oligomers may bind to HHV8 genes, such as RTA, vIRF-1, v-Cyclin, LANA, vGCR, or vFLIP.

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MORPHOLINO ANTISENSE DRUGS FOR HUMAN HERPESVIRUS 8

RELATED APPLICATIONS

This application claims priority to U.S. Serial No. 60/586,291, filed on July 9, 2004, which is hereby incorporated by reference in its entirety.

GOVERNMENT SUPPORT

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BACKGROUND OF THE INVENTION

Kaposi's sarcoma (KS) is a skin tumor and recognized as the most common malignancy among patients with AIDS. Primary effusion lymphoma (PEL), also called body cavity-based lymphoma (BCBL), is characterized by malignant effusions in the pleural or abdominal cavity (1). (References are listed in numerical order at the end of the specification, and are all incorporated by reference in their entireties for all purposes.) PEL occurs predominantly in patients with AIDS, but occasionally in other patients. The development of KS and PEL has been associated with a herpesvirus, named Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8), identified in 1994 in a KS lesion from a patient with AIDS (2,3). For the purposes of this application, KSHV and HHV8 constitute the same virus. Epidemiological studies have also found that HHV8 is associated with the development of a subset of MCD, another lymphoproliferative disorder described as angiofollicular lymphoid hyperplasia (4). A cohort study found that HHV8-associated non-Hodgkin's lymphoma in HIV-infected patients with MCD is 15-fold what is expected in the general HIV-positive population (5), though the

widespread use of HAART reduces the incidence of opportunistic infections and KS in patients with AIDS. Lymphomas and MCD remain fatal malignancies for the majority of those patients with these diseases (5-7). Conventional cytotoxic chemotherapy only achieves negligible short-term remission (8) and new options for treatment are urgently needed.

Like other herpesviruses, HHV8 displays a tightly regulated program of gene expression with two modes of infection: latent and lytic. In latent infection, the HHV8 genome persists in host cells with limited gene expression, including major latency-associated nuclear antigen (LANA) encoded by ORF73, a homologue of cyclin D (v-Cyclin) encoded by ORF72, a homologue of FLACE inhibitory protein (vFLIP) encoded by ORF71, and LANA2 by ORFK10.5 (9,10). When latency is disrupted, the virus can switch to a lytic life cycle. The reactivation of virus replication in primary effusion lymphoma cells can be induced by various chemicals, such as phorbol ester tetradecanoyl phorbol acetate (TPA) (11). In lytic infection, infectious virions are produced and host cells are destroyed. Lytic infection can be divided into immediate-early, early and late stages. The viral genes that are expressed during immediate-early and early stages encode regulatory proteins altering the expression of viral and cellular genes during virus infection and reactivation. A key immediate-early protein is a replication and transcriptional activator (RTA) encoded by HHV8 ORF50, which activates viral lytic gene expression (12).

Unlike other herpesviruses, the HHV8 genome contains ORFs homologous to cellular genes (13). These viral gene products play a variety of roles in HHV8-associated pathogenesis, by disrupting cellular signal transduction pathways, cell growth, apoptosis, and cell cycle control. For example, HHV8 encodes a homologue to human interleukin-6 (vIL-6) in ORFK2, macrophage inflammatory proteins in ORFs K4 and K6, a homologue of interferon regulatory factor (vIRF-1) in ORFK9, v-Cyclin in ORF72, a homologue of G-

protein-coupled receptor (vGCR) in ORF74 and vFLIP in ORF71. These viral gene products are either capable of causing transformation, indicative of a role in HHV8-associated malignancies, or playing important roles in virus replication and infection.

There is a need to develop antiviral oligomers specific for the HHV8 viral targets, which are clinically efficacious. The invention is directed to these and other important ends.

SUMMARY OF THE INVENTION

One aspect of the present invention is an antisense antiviral oligomer having at least 10 nucleotides, which specifically binds to a portion of targeted RNA expressed from the human herpesvirus 8 (HHV8). The HHV8 RNA is selected from genes whose gene products affect the replication or transformation of HHV8. The HHV8 genes may be selected from RTA, vIL-6, vIRF-1, v-Cyclin, LANA, vGCR, and vFLIP gene. The antisense oligomer of the present invention is designed to be effective in reducing the expression of the targeted gene.

An aspect of the invention includes antisense oligomers having a nucleic acid sequence effective to reduce expression of the RTA gene selected from the group consisting of SEQ ID NOS: 2, 3, 4, and 26. Another aspect of the invention includes antisense oligomers having a nucleic acid sequence effective to reduce expression of the vIL-6 gene selected from the group consisting of SEQ ID NOS: 6-8, 34, and 36-38. Yet another aspect of the invention includes antisense oligomers having a nucleic acid sequence effective to reduce expression of the vIRF-1 gene selected from the group consisting of SEQ ID NOS: 10-12. The antisense oligomer may alternatively be a nucleic acid sequence effective to reduce expression of the vCyclin gene selected from the group consisting of SEQ ID NOS: 14-16. In another embodiment, the antisense oligomer may be the nucleic acid sequence

effective to reduce expression of the LANA gene selected from the group consisting of SEQ ID NOS: 18, 20, 21, 29, and 30. Another aspect includes an antisense oligomer having the nucleic acid sequence effective to reduce expression of the vGCR gene selected from the group consisting of SEQ ID NOS: 23-25. A further aspect includes an antisense oligomer having the nucleic acid sequence effective to reduce expression of the vFLIP gene selected from the group consisting of SEQ ID NOS: 39-41.

In another embodiment of the invention, the antisense oligomer comprises a morpholine ring and a phosphorodiamidate linkage.

Another aspect of the invention includes compositions comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the HHV8 RTA gene, wherein said oligomer is effective for reducing the expression of said RTA gene, and a pharmaceutically acceptable carrier. The oligomer can be selected from the group consisting of SEQ ID NOS: 2, 3, 4, and 26.

Another aspect of the invention includes compositions comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the HHV8 vIL-6 gene, wherein said oligomer is effective for reducing the expression of said vIL-6 gene, and a pharmaceutically acceptable carrier. The oligomer can be selected from the group consisting of SEQ ID NOS: 6-8, 34, and 36-38.

An aspect of the invention includes compositions comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the HHV8 vIRF-1 gene, wherein said oligomer is effective for reducing the expression of said vIRF-1 gene, and a pharmaceutically acceptable carrier. The oligomer can be selected from the group consisting of SEQ ID NOS: 10-12.

In another embodiment, the invention includes compositions comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA

expressed from the human herpesvirus 8 v-Cyclin gene, wherein said oligomer is effective for reducing the expression of said v-Cyclin gene, and a pharmaceutically acceptable carrier. The oligomer can be selected from the group consisting of SEQ ID NOS: 14-16.

Another embodiment includes compositions comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 LANA gene, wherein said oligomer is effective for reducing the expression of said LANA gene, and a pharmaceutically acceptable carrier. The oligomer can be selected from the group consisting of SEQ ID NOS: 18, 20, 21, 29, and 30.

Another embodiment includes composition comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 vGCR gene, wherein said oligomer is effective for reducing the expression of said vGCR gene, and a pharmaceutically acceptable carrier is further embodied. The oligomer can be selected from the group consisting of SEQ ID NOS: 23-25.

Another embodiment includes compositions comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 vFLIP gene, wherein said oligomer is effective for reducing the expression of said vFLIP gene, and a pharmaceutically acceptable carrier. The oligomer can be selected from the group consisting of SEQ ID NOS: 39-41.

The present invention further includes methods. For example, the method for inhibiting the expression of a HHV8 gene is provided herein. The HHV8 gene may be selected from the group consisting of the RTA, vIL-6, vIRF-1, v-Cyclin, LANA, vGCR, and vFLIP gene. The method includes an antisense antiviral oligomer having at least 10 nucleotides, which specifically binds to a portion of RNA of the HHV8 gene, wherein said oligomer is effective for reducing the expression of said gene, under conditions sufficient for the antisense antiviral oligomer to bind to the HHV8 gene.

Another aspect of the invention includes reducing expression of the RTA gene using an antisense oligomer selected from the group consisting of SEQ ID NOS: 2, 3, 4, and 26; reducing expression of the vIL-6 gene using an antisense oligomer selected from the group consisting of SEQ ID NOS: 6-8, 34, and 36-38; reducing expression of the vIRF-1 gene using an antisense oligomer selected from the group consisting of SEQ ID NOS: 10-12; reducing expression of the vCyclin gene using an antisense oligomer selected from the group consisting of SEQ ID NOS: 14-16; reducing expression of the LANA gene using an antisense oligomer selected from the group consisting of SEQ ID NOS: 18, 20, 21, 29, and 30; reducing expression of the vGCR gene using an antisense oligomer selected from the group consisting of SEQ ID NOS: 23-25; and reducing expression of the vFLIP gene using an antisense oligomer selected from the group consisting of SEQ ID NOS: 39-41.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the structure of P4-PMO and positions of PMOs corresponding to KSHV immediately early (IE) and latent transcripts. A) A morpholine ring and a phosphorodiamidate linkage in PMO replace the deoxyribose and phosphodiester bond of DNA, respectively. "B" represents the bases A, G, C, or T. The peptide $R_5F_2R_4$, designated P4, when present, was covalently linked to the 5' end of PMO. B) Positions of PMOs against KSHV RTA and LANA transcripts. The numbers above the lines of transcripts indicate nucleotide positions based on Russo et al. (13). The arrows and the numbers in bold font above arrow indicate open reading frames. The sizes of the latent transcripts are indicated above each line on the right. There are splicing events in both IE (83) and latent transcripts (62, 76).

FIG. 2 shows PMO uptake assay in KSHV-infected BCBL-1 cells. A) BCBL-1 cells were treated for 4h with 8 μM P4-PMO-F1 and photographed under fluorescent microscopy. B) The same observing field as (A) was photographed under phase-contrast. C) and D) Flow cytometry analysis of BCBL-1 cells treated as above with P4-PMO-Fl (C), or mock-treated (D). The scale on the X and Y axes represents fluorescence intensity. Only green fluorescence on the X-axis was measured as this experiment used a single dye. E) Percentage of fluorescence positive cells in different treatments as in (A) analyzed by Flow cytometry. F) Percentage of fluorescence positive cells in BCBL-1 cells treated as in (A) and cultured in growth medium for 24, 48 and 72 h before harvested for flow cytometry analysis. Note the PMO uptake and stable presence in BCBL-1 cells at all three time points.

FIG. 3 shows a cell-free luciferase reporter assay. PMO was added to block cell-free translation of RNA from a reporter construct containing RP1 target sequences upstream of and in-frame with firefly luciferase coding sequence. CP1 was included as a negative control. Luciferase activity in the presence of two compounds is compared, and graphed as the percentage of mock-treated reactions, which is set as 100%. The average of three tests is shown and the error bar represents variation among the experiments. Luciferase activity in the presence of PMO is compared with the activity in the absence of PMO, which is set as 100%. The average of three tests is shown and the error bar represents variation among the experiments.

FIG. 4 shows an immunofluorescence assay with anti-RTA antibody showing RP1 inhibition of RTA protein expression in BCBL-1 cells. Cells were treated with RP1 or CP1 (16 μM). TPA induction control (TPA + No PMO) and no-induction control (No TPA or PMO) were included. The cell nuclei were counterstained blue by 4',6'-diamidino-2-phenylindole (DAPI) in mounting solution (Molecule Probes). The lower image panel was taken from the same

observed fields as the upper panel, but with a DAPI filter to show all cells. Note the reduction in RTApositive cells in RP1-treated sample (TPA + RP1) in comparison with samples CP1-treated (TPA + CP1) or mock-treated (TPA + No PMO).

FIG. 5 shows a Western-blot analysis showing dose-responsive inhibition of RTA expression in RP1- treated BCBL-1 cells. Cells were treated with RP1 or CP1 at concentrations ranging from 0.125 μM to 16 μM. A polyclonal antibody to RTA was used to detect RTA protein expression and the same blot was incubated with an anti-tubulin antibody for normalization. RTA signal after TPA induction in BCBL-1 cells was reduced with increasing RP1 concentration (A), while the CP1 did not cause an observable change (B). Mock-treated cells were included in the experiment for comparison. Quantitative image analysis confirmed the dose-responsive inhibition of RTA expression by RP1 (C). RTA expression in cells treated with PMO is shown as relative percentage relative to that of the mock-treated control. Treatment of BCBL-1 cells with mRP1 yielded no observable change in RTA expression detected by Western-blot analysis (D). This experiment was repeated three times and had similar results, and a representative experiment is shown.

FIG. 6 shows a Western-blot analysis of inhibition of KSHV lytic gene expression in RP1-treated BCBL-1 cells. Expression of vIL-6, vIRF-1-1, and a glycoprotein encoded by ORF-K8.1A in TPA-induced BCBL-1 cells was reduced with increasing RP1 concentration (A). The CP1 did not cause an observable change (B). Quantitative image analysis confirms the dose-responsive inhibition of vIL-6 (C) and vIRF-1-1 (D) in the cells treated with RP1 in comparison with mock- treated cells.

FIG. 7 shows flow cytometry analysis of reduction of the number of RTA-positive cells after RP1 treatment. TPA-induced BCBL-1 cells were stained as in IFA after fixation with 1%

paraformaldehyde. The number of RTA-positive cells after PMO treatment was compared with that in the mock-treated control (No PMO) and shown as relative percentages. The average of three tests is shown and the error bar represents variation among experiments. The cells treated with RP1 had a significantly low number of RTA-positive cells ($P<0.001$) in comparison with the cells CP1-treated or mock-treated.

FIG. 8 shows an immunofluorescence assay with anti-LANA antibodies shows LP2 or LP3 inhibit LANA protein expression in BCBL-1 cells (A). BCBL-1 cells were treated with LP2, LP3, or CP1 (16μM) three times at intervals of 2 days as described in text. The cell nuclei were counterstained to blue by DAPI in mounting solution (Molecule Probes). The lower image panel was taken with a DAPI filter from the same observed fields as the upper panel. Note that LANA-staining is much weaker in cells treated with LP2 or LP3 than in cells treated with CP1 or mock treatment. Total LANA protein expression was also evaluated by Western-blot analysis (B). Total proteins from all three treatments were collected separately and used for Western-blot analysis. The number above each lane represents the number of PMO applications at two-day intervals to BCBL-1 cells. This experiment was repeated three times and results from a representative experiment are shown. Quantitative image analysis confirmed the inhibition by LP2 or LP3 after the second and third application of PMO (C). LANA protein expression in cells treated with LP2 or LP3 is shown as a percentage relative to the mock-treatment control.

FIG. 9 shows an immunofluorescence assay (IFA) of BCBL-1 cells treated with vIL-6 PMO at indicated concentrations. Images were detected with vIL-6 polyclonal antibody. TPA- and TPA+ are untreated cell controls representing latent and lytic replication of KSHV, respectively.

FIG. 10 shows that PMO targeting vIL-6 reduces vIL-6 protein expression in BCBL-1 cells. (A) Western-blot analysis of the lysates from the cells with vIL-6 antibody. The membrane was also probed with a monoclonal antibody against tubulin for normalization. (B) Conversion of the digital image data from (A) to show the dose-response inhibition of the ILP1 PMO against vIL-6 in BCBL-1 cells.

FIG. 11 shows that PMO targeting vIL-6 reduces vIL-6 expression in BC-1 cells. (A) Western-blot analysis of the lysates from the cells with vIL-6 antibody. The membrane was also probed with a monoclonal antibody against tubulin for normalization. (B) Conversion of the digital image data from (A) to show the dose-response inhibition of the ILP1 PMO against vIL-6 in BC-1 cells.

FIG. 12 shows that treatment of BCBL-1 cells with ILP1 PMO against vIL-6 reduces expression of hIL-6, detected by ELISA with specific antibodies against hIL-6. The cell culture supernatant from the cells after PMO treatment was subjected to ELISA. Supernatant from cells mock-treated or no-TPA was included as controls. Relative fold induction of hIL-6 is shown in comparison with mock-treated cells (TPA+). The control PMO (Cont) did not inhibit the expression of hIL-6, as expected.

DETAILED DESCRIPTION OF THE INVENTION

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to specific embodiment and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alteration and further modifications of the invention, and such further applications of the principles of the invention as illustrated herein, being contemplated as would normally occur to one skilled in the art to which the invention relates.

All terms as used herein are defined according to the ordinary meanings they have acquired in the art. Such definitions can be found in any technical dictionary or reference known to the skilled artisan, such as the *McGraw-Hill Dictionary of Scientific and Technical Terms* (McGraw-Hill, Inc.), *Molecular Cloning: A Laboratory Manual* (Cold Springs Harbor, New York), and *Remington's Pharmaceutical Sciences* (Mack Publishing, PA). These references, along with those references and patents cited herein are hereby incorporated by reference in their entirety.

At least one aspect of the present invention is directed to a composition for treating human herpesvirus 8 (HHV8). The composition comprises an antisense antiviral oligomer. As used herein, "antisense" refers to an oligonucleotide strand having a sequence complementary to the messenger ribonucleic acid (mRNA) of a targeted sequence. The antisense antiviral oligomer acts by binding to viral mRNA, which blocks or inhibits the translation of the viral mRNA. As such, protein synthesis, viral replication, and viral-induced transformation may be inhibited or blocked.

The antisense antiviral oligomer comprises a sufficient number of nucleotides to bind to a portion of the mRNA of a HHV8 targeted gene and suppress HHV8 replication or HHV8-induced transformation. The oligomer preferably comprises at least 10, at least 15, at least 18, at least 20, at least 22, at least 23, or at least 25 nucleotides or a range of nucleotides effective to reduce expression of the targeted gene. The oligomer can be composed of any types of nucleotides, such as deoxyribonucleotides or ribonucleotides or analogs thereof. In another aspect of the invention, one or more of the nucleotides may be modified in the antisense antiviral oligomer. For example, the base of the nucleotides may be any of the known naturally occurring bases including purines, such as adenine, guanine, deazaadenine, and deazaguanine, and pyrimidines, such as thymine, uracil, and cytosine. Alternatively,

analogs or derivatives of the bases may be utilized in the antisense antiviral oligomer, as are known in the art.

In another aspect of the invention, at least one of the sugars or phosphate groups of the nucleotides in the antisense antiviral oligomer is modified. For example, the sugar may be a ribose or deoxyribose, morpholino analog, or peptide nucleic acid (PNA) analogs. The phosphate group may also be modified in at least one nucleotide of the antisense antiviral oligomer. The phosphate group may be substituted with a moiety such as C_1-C_5 alkyl, amino, -NR_2, wherein R is a C_1-C_5 alkyl group, or the like. Suitable antisense DNA or RNA analogs include various combinations of the above-mentioned modifications involving linkage groups and/or structural modifications of the sugar or base for the purpose of improving antisense oligomer stability and specificity in inhibition of target RNA translation.

In an aspect of the present invention, the antisense antiviral oligomers are phosphorodiamidate morpholino oligonucleotides (PMOs), which have a morpholine ring and phosphorodiamidate linkages instead of a deoxyribose sugar and phosphodiester linkages as found in DNA, as depicted in Figure 1. PMOs are uncharged, water soluble molecules. They are resistant to nucleases, stable to extremes of temperature and chemically inert. PMOs dissolve in water to give a solution with a neutral pH and decompose in low acid (pH<4) and high alkali (pH>11) conditions. In addition, these antisense antiviral oligomers bind to mRNA by Watson-Crick base pairing and prevent translation by steric blockade of translation initiation, an RNase H-independent mechanism. Morpholino antisense oligomers have been found to provide good target predictability, high in-cell efficacy and sequence specificity. The oligomers of the present invention are effectively delivered into the cytosol and nucleus.

The antisense antiviral oligomers of the present invention act to inhibit viral replication, inhibit viral protein synthesis, or inhibit viral transformation by "binding" to the targeted mRNA sequence. "Binding" refers to the antisense antiviral oligomers' ability to bind to viral mRNA through base pairing. "Base pairing" means hybridizing or the hydrogen bonding of complementary purine and pyrimidine bases – adenine with thymine (adenine with uracil), guanine with cytosine – in double-stranded DNA or RNA or in DNA/RNA hybrid molecules. The antisense oligomer must be sufficiently complementary to the target mRNA sequence to hybridize therewith to be effective in inhibiting translation of the target mRNA sequence or inhibition of HHV8 viral replication or transformation. In one aspect of the invention, the antisense oligomer may be 100% complementary to the targeted mRNA sequence. In another aspect of the invention, the antisense oligomer may be at least 90% complementary to the targeted mRNA sequence. In another aspect of the invention, the antisense oligomer may be at least 80% complementary to the targeted mRNA sequence. In another aspect of the invention, the antisense oligomer may be at least 70% complementary to the targeted mRNA sequence.

The antisense antiviral oligomers are oligonucleotides designed to target HHV8 RNA sequences, the binding to which would inhibit or alter the expression of the target sequence. The target sequence may be RNA, such as a pre-mRNA or an mRNA. mRNA target sequences include an initiation codon region, a coding region, a polyadenylation region, an mRNA cap site or a splice junction. Exemplary genes with gene products that either regulate virus replication or cause transformation include, but are not limited to, LANA, RTA, vIL-6, vIRF-1, v-Cyclin, vGCR, ORF-K1, ORF-K4/vMIP-I, ORF-K6/vMIP-II, ORF-K3, ORF-K5, ORF16/vBCL-2, ORF-K12/Kaposin, ORF-K10.5/LANA-2, ORF71/vFLIP, ORF-K15 and ORF59/DNA replication processivity factor. The genes for each of the HHV8 proteins are

known. For example, the nucleotide sequences for each of the HHV8 proteins can be found in GenBank Accession number U75698.

In one embodiment, the antisense oligomer targets the HHV8 LANA gene. In HHV8 latent infection, 90% of the tumor cells are latently infected with HHV8 and express only a limited number of viral genes, including LANA, the most immunodominant major latent antigen (14, 15). LANA maintains HHV8 latent infection (16-18), interacts with p53 to repress its transcriptional activity (19), targets retinoblastoma-E2F transcriptional regulatory pathway, and transforms primary rat cells in cooperation with oncogene *Hras* (20). LANA also binds RING3 (21) and relocates it to nuclear heterochromatin regions (22). LANA can interact with activating transcription factor (ATF) 4/cAMP response element binding protein (CREB) 2 (23), and the proteins of the mSin3 corepressor complex (24). It has been reported that LANA modulates cellular gene expression by upregulating six known genes and nine expressed sequence tags in a stably transfected B cell line, and that LANA upregulates its own expression (25). Thus, like EBV latency proteins, HHV8 LANA has important roles in maintaining viral latent infection, modulating cellular gene expression, and causing cellular transformation.

In another embodiment, the antisense oligomer targets the HHV8 RTA gene. The activation from HHV8 latency to the lytic productive cycle requires an immediate early protein, RTA encoded by HHV8 ORF50 (12). RTA activates downstream viral genes of both early and late kinetic class genes, including many of the cellular homologues and nonstructural genes, such as vIRF-1, homologues of macrophage inflammatory protein I and II, vIL-6, v-Cyclin, vGCR, and a homologue of *Bcl*-2 (reviewed by (26)). The proinflammatory cytokines have long been considered to be essential for the development of HHV8-associated malignancies (26,27). Down-regulating RTA expression with

methotrexate, a potent anti-inflammatory agent, inhibits the switch of HHV8 to lytic replication and downstream gene expression (28). In HHV8-infected PEL cells and in KS lesions, there is a low percentage (<5%) of cells supporting HHV8 lytic infection (29). Lytic cycle activation is a pivotal event of the cascade leading to the expression of the proinflammatory cytokines. Therefore, RTA is essential to control viral lytic replication and contribute to the pathogenesis of HHV8-associated diseases.

In another embodiment, the antisense oligomer targets the HHV8 vIRF-1 gene. HHV8 ORFK9 encodes vIRF-1 that 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 causes transformation of NIH3T3 cells (30-33). 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 (34). The vIRF-1 protein directly interacts with these DNA binding proteins to interfere with their functions (35,36).

In another embodiment, the antisense oligomer targets the HHV8 vGCR gene. HHV8 ORF74 encodes a viral G-protein coupled receptor (vGCR) homologous to the human receptor for the angiogenic chemokine interleukin-8 (IL-8R) (37,38). The vGCR can bind a broad spectrum of chemokines and has been shown to be agonist-independent, constitutively active and capable of modulating host gene expression (39-41). Ectopic expression of vGCR in rodent kidney and fibroblast cells has been shown to elicit a constitutively active signal that strongly stimulates both proliferation and angiogenesis (39,42). The angiogenic response induced by vGCR is mediated by upregulation of vascular endothelial growth factor (VEGF). Because vGCR is expressed during the early phase of viral lytic replication, it is postulated

that vGCR acts through a paracrine mechanism to induce upregulation of angiogenic growth factor VEGF and inflammatory cytokines, resulting in angiogenesis and infiltration of lymphocytes into the KS lesion (43). Transgenic expression of vGCR has induced angioproliferative disease resembling KS lesions (44), suggesting vGCR's contribution to dysregulated angiogenesis and increased tumorigenicity of HHV8-infected cells.

In another embodiment, the antisense oligomer targets the HHV8 v-Cyclin gene. HHV8 ORF72 encodes v-Cyclin, which is similar to cellular cyclin D2, and is expressed mostly in the latent stage of viral replication (45). The v-Cyclin predominantly associates with CDK6, a cellular cyclin-dependent kinase (CDK) known to interact with type D cyclin. Unlike CDK6 activated by cellular cyclin, CDK6 activated by v-Cyclin can proceed in the absence of phosphorylation by CDK-activating kinase (46), and is capable of phosphorylating not only the retinoblastoma protein but also histone H1(47). Complexes of v-Cyclin with CDK6 phosphorylate p27 (Kip) and abolish p27's inhibitory effect (48). In a KS biopsy study, p27 (Kip) levels in late stages of KS were lower than in early stages of KS (49). The v-Cyclin-CDK6 complex can phosphorylate and inactivate *Bcl-2*, interfering with host apoptotic signaling pathway (50). These findings suggest that v-Cyclin can deregulate cell growth control in HHV8-infected cells and play a role in KS progression.

In another embodiment, the antisense oligomer targets the HHV8 vIL-6 gene. HHV8 ORFK2 encodes vIL-6 (26). Cellular IL-6 is expressed in lymphomocytes, macrophages and endothelial cells. It acts on most cells, and is considered to be an important growth factor for lymphoma and leukemia. The vIL-6 is expressed in a few cells of KS lesions but in most cells of primary effusion lymphomas and MCD lesions, indicating a role of vIL-6 in HHV8-associated lymphoproliferative disorders (51-53). Cellular IL-6 and vIL-6 display differences in receptor usage for IL-6 has an absolute requirement for both of the IL-6R alpha and the

gp130 subunits, but vIL-6 appears to require only gp130 (54), however, vIL-6 can stimulate all of the known IL-6-induced signaling pathways (54, 55). Studies *in vitro* and *in vivo* have shown that vIL-6 can stimulate the growth of HHV8-infected lymphoma cells, promote hematopoiesis, and act as an angiogenic factor through the induction of VEGF (56, 57). Thus, vIL-6 is a multifunctional cytokine that potentially contributes to the HHV8-associated lymphoproliferative disorders.

In another embodiment, the antisense oligomer targets the HHV8 v-FLIP gene. Apoptosis is related to many diseases and induced by a family of cell death receptors and their ligands. Cell death signals are transduced by death domain (DD)-containing adapter molecules and members of the ICE/CED-3 protease family. Caspases-8 (FLICE) and -10 (FLICE2) are two pivotal members in the ICE/CED-3 protease family. FLICE-inhibitory proteins were identified in virus and human and designated v-FLIPs and FLIP, respectively (81,82). KSHV ORF71/K13 encodes v-FLIP, which is expressed during latent phase.

An aspect of the invention is the design of an antisense PMO effective to inhibit HHV8 viral replication, HHV8 viral protein synthesis, or HHV8-induced transformation. It has been found that PMOs are generally effective against most HHV8 target mRNA sequences from the 5' terminus to about 20 bases past the AUG translational start site of the targeted mRNA. *See, e.g.,* (58). While not wanting to be limited by any theory, it is postulated that the effective targeting predictability is a consequence of PMOs' high-affinity, making them able to invade RNA secondary structures. In designing PMOs that target HHV8 mRNAs, it is recommended, but not critical, to identify a target sequence selected from the post-spliced mRNA in the region from the 5' cap to about 20 bases 3' to the AUG translational start site. In addition, it is recommended, but not required, to have a PMO that has little or no self-complementary regions, has no more than 4 contiguous intra-strand base

pairs, does not contain over about 36% guanines or more than 3 contiguous guanines because of reduced water solubility, or does not have more than 16 contiguous bases identical to the host chromosome sequences or non-target HHV8 sequences to ensure PMO specificity. The PMO may be any size, such as between 10-35 nucleotides, 15-30 nucleotides, or 18-25 nucleotides.

Once the targeted mRNA sequence is selected and the antisense antiviral oligomer is designed, such as in the form of a PMO, the oligomers are synthesized according to methods known in the art. In one aspect of the invention, the PMOs targeting HHV8 genes include those having the nucleic acid sequences shown by SEQ ID NOS: 2-4, 6-8, 10-12, 14-16, 18, 20-21, 23-26, 29, 30, 34, 36-41, targeted to the HHV8 gene sequences having the nucleic acid sequences shown by SEQ ID NOS:1, 5, 9, 13, 17, 19, and 22, as depicted in Table 1.

The antisense antiviral oligomers of the present invention can be assayed for effectiveness in accordance with the present invention. In one embodiment, an *in vitro* transcription/translation assay may be used to select the antisense antiviral oligomers, such as PMOs, that can bind target sequences and inhibit the expression of a downstream reporter gene. The target sequence is synthesized and cloned into a reporter plasmid upstream of a reporter gene, such as luciferase. Any reporter plasmid available to the skilled artisan is suitable for cloning the target sequence into. A nonlimiting example of a suitable reporter plasmid is pCiNeolucrΔA* (modified from pCI-neo, a vector from Promega Corporation, Madison, WI). An *in vitro* transcription/translation assay, such as the TNT® Coupled Reticulocyte Lysate Systems (Promega), is conducted to determine the effect of the antisense antiviral oligomer. If the antisense antiviral oligomer binds its target sequence in the reporter plasmid, the expression of the reporter gene will be blocked. There will be low or no reporter gene activity in the assay.

In an alternative assay to determine the effectiveness of the antisense antiviral oligomer, the whole target gene sequence can be cloned and fused to a reporter gene, such as green fluorescent protein (GFP) to examine the oligomer's effect on target gene expression in transiently transfected cells. Reporter gene expression will reflect the target gene expression. The number of reporter gene-positive cells and intensity of the reporter gene will be reduced if the antisense antiviral oligomer is effective.

Another method of determining the effectiveness of the antisense antiviral oligomers is to deliver the oligomer into an HHV8-infected cell. Any cell line suitable for infection by HHV8 may be used to test the inhibition of target gene expression. For example, BCBL-1, BC-1, BC-2, BC-3, BCP-1, and JSC-1 cells may be used. The delivery of the antisense antiviral oligomers to HHV8-infected cells may be accomplished by any method known to the skilled artisan, including, but not limited to, commercially available delivery systems. In one embodiment, the antisense oligomer is conjugated to a peptide to facilitate delivery into cells. In another embodiment, a delivery system such as one provided by Gene Tools, LLC, is used. In this system, a partially complementary DNA oligomer pairs with the antisense antiviral oligomer oligomer and also interacts ionically with Ethoxylated Polyethylenimine (EPEI). The complex is efficiently endocytosed by cells due to the positive charges on EPEI. Once inside the endosome/lysozome, the pH drop further ionizes the EPEI, allowing the complex to pass through the endosome/lysozome membrane, thus delivering the antisense antiviral oligomer/DNA complex into the cytosol compartment of the cell. This method is extremely efficient in achieving maximum delivery at only 1 μM final morpholino oligomer concentration.

Another method of delivering the antiviral antisense oligomer includes a scrape-loading method is used to deliver the oligomer into the adherent cells. *See, e.g.,* (59). In this

method, when the cells are scraped off of the plate, desmosomes connecting the cell to the plate are pulled out of the cell membrane, leaving transient holes in the cytoplasmic membrane. This allows oligomers to freely enter the cytoplasm for as much as a minute before the holes in the membrane reseal. This method works well both in the presence and absence of serum and causes minimal damage to cells.

If the selected HHV8 target gene is expressed during lytic replication, the HHV8-infected cells are then treated with a chemical inducer, such as tetradecanoyl phorbol acetate (TPA), to induce viral lytic replication after delivery of the antiviral antisense oligomer to the cells. Northern-blotting and Western-blotting analysis are conducted to determine the levels of target gene mRNA and protein, respectively. Immunofluorescence and flow cytometry assays are performed to visualize PMO delivery to cells. Antibodies against selected HSHV genes are commercially available or obtained from other research laboratories.

Alternatively, the effectiveness of the antisense antiviral oligomer to suppress HHV8 latent or lytic replication can be accomplished by determining the HHV8 genome copy number by dot-blot hybridization, as is known in the art. In this manner, DNA is extracted from the antisense antiviral oligomer-treated cells and the genome copy number compared to negative controls. It is expected that the copy number of PMO-treated cells will be less than that of untreated cells.

The above-described methods are well known to the skilled artisan and may be performed as described in, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, 2nd ed., Cold Springs Harbor, New York (1989). Other references describing molecular biology and recombinant DNA techniques include, for example, *DNA Cloning 1: Core Techniques*, (D. N. Glover, *et al.*, eds., IRL Press, 1995);

DNA Cloning 2: Expression Systems, (B. D. Hames, *et al.*, eds., IRL Press, 1995); *DNA Cloning 3: A Practical Approach*, (D. N. Glover, *et al.*, eds., IRL Press, 1995); *DNA Cloning 4: Mammalian Systems*, (D. N. Glover, *et al.*, eds., IRL Press, 1995); *Oligonucleotide Synthesis* (M. J. Gait, ed., IRL Press, 1992); *Nucleic Acid Hybridization: A Practical Approach,* (S. J. Higgins and B. D. Hames, eds., IRL Press, 1991); *Transcription and Translation: A Practical Approach,* (S. J. Higgins & B. D. Hames, eds., IRL Press, 1996); R. I. Freshney, *Culture of Animal Cells: A Manual of Basic Technique*, 4th Edition (Wiley-Liss, 1986); and B. Perbal, *A Practical Guide To Molecular Cloning, 2nd Edition,* (John Wiley & Sons, 1988); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., John Wiley & Sons), which is regularly and periodically updated.

In a further embodiment of the invention, PMOs that may overcome HHV8-induced transformation by inhibiting the expression of target genes are identified. The selected target genes are able to transform cells or control HHV8 replication. The PMOs selected from the above-described methods for the ability to suppress HHV8 latent or lytic replication are tested for their effects on suppression of HHV8-induced transformation. If HHV8 latent and lytic replication is suppressed and expression of the genes causing transformation is inhibited, cell growth of the HHV8-infected cells is likely to slow down and their transformed phenotypes would be reversed.

Another aspect of the invention is directed to compositions comprising the antisense antiviral oligomer with a pharmaceutically acceptable carrier, such as a diluent or excipient which may include fillers, extenders, binding, wetting agents, disintegrants, surface-active agents, erodible polymers or lubricants. Other suitable pharmaceutical carriers includes a variety of cationic lipids, including, but not limited to N-(1-2,3-dioleyloxy)propyl)-n,n,n-trimethylammonium chloride (DOTMA) and dioleoylphophotidylethanolamine (DOPE).

Liposomes are also suitable carriers for the antisense oligomers of the invention. Another suitable carrier is a slow-release gel or polymer comprising the claimed antisense molecules.

Antisense antiviral oligomers of the present invention may be present in mixtures of any of the antisense oligomers alone or in combination with native DNA or RNA. Alternatively, the oligomers may be in combination with other agents that do not affect that ability of the antisense oligomer to bind to the targeted gene. The antisense oligomers or analogs thereof may also be administered either singly or in combination with one or more additional oligonucleotides or analogs thereof or in combination with other agents, which do not affect the ability of the antisense oligomer to bind to the targeted gene.

The antisense oligomers may be administered to patients by any effective route, including intravenous, intramuscular, intrathecal, intranasal, intraperitoneal, intratumoral, subcutaneous injection, in situ injection, topical, and oral administration. It may be beneficial to have pharmaceutical formulations containing acid resistant antisense oligomers that may come in contact with acid conditions during their manufacture or when such formulations may themselves be made acidic, to some extent, in order to be more compatible with the conditions prevailing at the site of application. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., latest edition. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, and capsules.

Oral administration may require enteric coatings to protect the claimed antisense molecules and analogs thereof from degradation along the gastrointestinal tract. The antisense oligomers may be mixed with an amount of a physiologically acceptable carrier or diluent, such as a saline solution or other suitable liquid. The antisense oligomers may also be combined with other carrier means to protect the antisense molecules or analogs thereof

from degradation until they reach their targets and/or facilitate movement of the antisense molecules or analogs thereof across tissue barriers.

Systemic administration of the claimed oligomers can be achieved by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use of nasal sprays, for example, as well as formulations suitable for administration by inhalation, or suppositories.

The antisense oligomers are administered in amounts effective to inhibit translation of the targeted gene, HHV8 viral replication, or HHV8-induced transformation. The actual amount of any particular antisense oligomer administered will depend on factors such as the toxicity of the antisense oligomer to other cells of the body, its rate of uptake by HHV-infected cells, and the weight and age of the individual to whom the antisense oligomer is administered. An effective dosage for the patient can be ascertained by conventional methods such as incrementally increasing the dosage of the antisense oligomer from an amount ineffective to inhibit protein expression to an effective amount.

The present invention further comprises vectors for transfecting HHV8-infected cells. The vector comprises a nucleotide sequence that encodes an antisense RNA which reduces the expression of the targeted HHV8 gene. The antisense RNA expressed from the vector-delivered nucleotide sequence binds with a sequence portion of RNA expressed from the targeted HHV8 gene. The antisense RNA reduces the expression of at least one targeted HHV8 gene product, thereby inhibiting viral replication or transformation.

The present invention further involves a method using the claimed vector for

suppressing the expression of the targeted HHV8 gene by introducing into cells the claimed antisense oligonucleotide as an RNA. The method comprises the step of transfecting the cells with the claimed vector which comprises a sequence that encodes an antisense RNA which is substantially complementary to and binds the targeted HHV8 gene. A further step involves the expression of the sequence encoding the antisense RNA, which thereby results in suppression of the targeted HHV8 gene in the HHV8-infected cells.

Vectors for transfecting/transforming mammalian cells, which vectors comprise nucleotide sequences coding for antisense RNA that inhibit the expression of target genes are well known in the art.

The antisense antiviral oligomers of the present invention may be used to inactivate, inhibit, or alter expression of the targeted HHV8 gene in a living cell, allowing selective inactivation, inhibition, or alteration of expression. In this manner the antisense antiviral oligomers of the present invention may be used to treat HHV8-infected animals, including humans, or prevent HHV8 infections.

Another aspect of the invention is a method for inhibiting target gene expression of an HHV8 gene using an antisense antiviral oligomer. The method involves introducing the antisense antiviral oligomer to the HHV8-infected cells. The oligomer binds to the targeted gene under conditions sufficient to reduce expression of the target HHV8 gene in the infected cells.

Another aspect of the invention is a method for inhibiting or blocking viral replication. The antisense antiviral oligomer is introduced into the HHV8-infected cells, which bind to the targeted gene under sufficient conditions. The binding blocks the

expression of the protein needed for viral replication thereby inhibiting or blocking viral replication.

Another aspect of the invention is a method for inhibiting or suppressing transformation of HHV8 into a host cell. In this method, the antisense antiviral oligomer is introduced to an HHV8-infected cell under conditions sufficient to allow the oligomer to bind to the targeted HHV8 gene. The binding blocks the expression of the gene product, which is necessary for transformation thereby inhibiting or suppressing transformation.

Preferably, cell proliferation and transformation assays are carried out to determine the effects of the antisense antiviral oligomers, such as PMOs, on HHV8-infected cells. The cell proliferation assay determines changes of cell growth rate of HHV8-infected cells in the presence or absence of these oligomers. In the transformation assay, contact inhibition is measured by colony formation in soft agar. The transformation assay determines if the HHV8-infected cells lose contact inhibition as tumor cells. If the PMOs are effective in overcoming HHV8-induced transformation, the treated cells will form much fewer colonies in soft agar than non-treated control. DNA damage assay are also conducted to determine apoptosis of the PMO-treated cells. DNA nicking in PMO-treated cells are assayed by agarose gel electrophoresis and terminal deoxytransferase-mediated dUTP nick end-labeling (60).

Reference will now be made to specific examples illustrating the constructs and methods above. It is to be understood that the examples are provided to illustrate preferred embodiments and that no limitation to the scope of the invention is intended thereby.

Examples

Example 1: PMO Design and Synthesis

HHV8 ORFs K2, K9, 50, 72, 73 and 74 genes were selected as antisense PMO targets. Sequences of these target genes, including 5'-noncoding regions, were downloaded from GenBank and aligned. Alignment of available sequences showed that these regions are conserved. Three 25-mer PMOs were designed for each target gene (Table 1), as studies on calicivirus showed that there is variable effect among PMOs against the same target gene. For ORFs 50, K2, K9, and 72, target sequences were selected from around or near AUG translation initiation sites. For ORF73, two PMOs were designed around the AUG region and one was chosen from the 5' noncoding region. For ORF74, the PMO target sequence was selected from the 5' noncoding region as the AUG translational initiation region contains more than 16 contiguous bases identical to host chromosome sequences. The PMOs are synthesized at 300 nanomole scale by GeneTools, LLC (Philomath, OR) or by AVI Biopharma (Corvallis, OR). The reconstitution and storage of the PMOs follows the manufacturer's instructions.

Example 2: *In vitro* reporter assay

A 40-nt oligomer of a target sequence is synthesized and cloned into a reporter plasmid, pCiNeolucrΔA* for each target gene. This plasmid is a modification of pCiNeo (Promega Corporation, Madison, WI), designed to express a fusion protein from a customized 'leader' sequence and a following reporter gene, luciferase. The 40-nt oligomer is inserted as a leader sequence upstream of luciferase sequence. Because PMOs are designed to target the translation initiation site in the 40-mer, the AUG of the luciferase gene is removed and the translation of luciferase starts from an AUG in the 40-mer. To ensure that the insert is in

frame, three variants of pCiNeolucrΔA with single nucleotide differences at multiple cloning site have been developed to accommodate different frames of ORF encoding in an insert. DNA sequencing is performed to verify the insert in recombinant plasmid. For those PMOs targeting upstream of the AUG initiation regions of ORFs73 and 74, fragments including target sequence and AUG initiation region are engineered and cloned upstream of luciferase gene in the reporter plasmid.

Example 3: Inhibition of HHV8 RTA and LANA by PMOs

Cells and viruses. KSHV-infected cells BC-1 (EBV-positive) and BCBL-1 (EBV-negative) were derived from body cavity-based lymphomas (11, 61). BJAB is a KSHV- and EBV-negative lymphoma cell line (69). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cell numbers were counted on a hemacytometer and cell viability was assessed by trypan blue exclusion. For induction of KSHV lytic replication, TPA (Sigma, St Louis, MO) was added to the cell growth medium to a final concentration of 20 ng/mL.

PMO design and synthesis. PMOs were synthesized and purified at AVI BioPharma (Corvallis, OR) as previously described (75) and were designed to be complementary to specific KSHV RNA sequences. PMOs used in this study and their target sites are summarized in Table 2 and depicted in Fig. 1. PMOs were covalently conjugated at the 5' end to a peptide, NH2-RRRRRFFRRRRC-CONH2 designated $R_5F_2R_4$ (Fig. 1). The conjugation, purification, and analysis of $R_5F_2R_4$-PMO (P4-PMO) compounds were similar to the methods described elsewhere (72). Additionally, P4-PMO-F1 and PMO-F1 were conjugated at the 3' end to fluorescein and used to assess PMO uptake by BCBL-1 cells.

Plasmid construction and cell-free luciferase reporter assay. Oligonucleotides of ORF50 sequence from bases -22 to +14 relative to the 'A' of the AUG translation start-site codon, which includes the RP1 target region, were cloned upstream of AUG-ablated luciferase sequence in a reporter vector as described previously (73). DNA sequencing was conducted to confirm that the insert sequence was in-frame with the luciferase open reading frame. The plasmid DNA was linearized downstream of the target-luciferase fusion gene and *in vitro* transcription was conducted with MegaScript Kit following manufacturer's instructions (Ambion, Austin, TX). Cell-free translation was performed with Rabbit Reticulocyte Lysate System (Promega, Madison, WI). Different concentrations of RP1 were added to the reactions in order to determine its ability to bind to target RNA and inhibit luciferase translation. A PMO targeting a different KSHV gene was included in the assay for comparison. Luciferase activity in the cell-free translation system was measured according to the supplier's instructions (Promega).

PMO uptake analysis. BCBL-1 cells were counted, pelleted and resuspended in RPMI 1640 medium supplemented with 100 μg/mL bovine serum albumin. The cells were transferred to 12- well cell culture plates at 10^6 cells per well immediately before PMO application. To assess uptake by BCBL-1 cells, P4-PMO-F1 was added to a final concentration of 2, 4, or 8 μM. PMOF1 was included for comparison. The plate was placed at 37°C incubator after being shaken gently. After 4 h incubation, the cells were then pelleted and washed with phosphate-buffered saline (PBS), pH 7.2, to remove free PMO, and then for observed under a fluorescence microscope.

For flow cytometry analysis, the PMO-treated BCBL-1 cells were pelleted, fixed with 1% paraformaldehyde in PBS for 15 minutes at room temperature, and analyzed in the

FACSCalibur system with CellQuest program version 3.3 (BD Biosciences, San Jose, CA). Cells exhibiting green fluorescence were detected with a 525 nm band pass filter.

To test whether PMO stably persists in BCBL-1 cells, the cells were cultured in growth medium (RPMI 1640 supplemented with 10% fetal bovine serum), treated with P4-PMO-F1 as described above and harvested at 24, 48 or 72 h for analysis by fluorescence microscopy and flow cytometry, as described above.

PMO treatment of KSHV-infected cells. KSHV-infected BC-1 and BCBL-1 cells were counted, pelleted and resuspended in RPMI 1640 medium supplemented with 100 μg/mL bovine serum albumin. The cells were transferred to a 12-well cell culture plate at 10^6 cells per well immediately before PMO treatment. PMOs were diluted and added to the cells. Control PMO CP1 was included as a negative control. Cells treated with PBS were included as a mock-treatment control. After mixing, the cells were incubated for 4 h at 37°C. Growth medium supplemented with TPA at 20 ng/mL was then added. The cells were incubated for 48 h at 37°C and harvested for further analysis. For time-kinetic analysis, TPA was added to the cells at 0, 0.5, 1, 2, 3, or 4 h after PMO application. Growth medium was added to the cells 4 h after PMO treatment.

For LANA PMO treatment, the same protocol was followed but TPA was omitted in the growth medium. Recurrent applications of the LANA PMOs at an interval of two days were tested on the same BCBL-1 cells, as described above.

Immunofluorescence assay (IFA). IFA was utilized to detect proteins in cells as described previously (63). Briefly, cells were pelleted, rinsed in PBS three times and spotted onto slides. The slides were heat-fixed for 2 h at 56°C. Polyclonal rabbit anti-RTA antibody was used to detect RTA expression (77). Mouse monoclonal antibody against vIRF-1-1 (a gift

from Dr. Keiji Ueda of Osaka University School of Medicine, Japan), mouse monoclonal antibody against a glycoprotein encoded by KSHV ORF-K8.1A, rat anti-LANA monoclonal antibody and polyclonal rabbit anti-vIL-6 antibody (Advanced Biotechnologies Inc., Columbia, MD) were used to detect vIRF-1-1, K8.1A product, LANA and vIL-6 proteins in the cells, respectively. Goat anti-mouse IgG-fluorescein isothiocynate (FITC), anti-rat IgG-FITC or anti-rabbit IgG-FITC (Sigma) conjugates were used to identify specific reactions. Stained cells were observed under fluorescence microscopy.

For flow cytometry analysis, cells were fixed in 1% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. The rabbit anti-RTA antibody and goat anti-rabbit IgG-FITC conjugate were used to detect RTA protein. The stained cells were resuspended in PBS and analyzed in the FACSCalibur system (BD Biosciences) as described above. Cells were mock-stained or stained with secondary antibody only when included as negative controls.

Western-blot analysis. RTA protein expression in KSHV-infected cells was identified by Western-blot analysis. BCBL-1 cells were lysed and resolved in 12% polyacrylamide gels with SDS-PAGE. The separated proteins were transferred to nitrocellulose membrane and reacted with rabbit anti-RTA antiserum. Any specific reaction was detected with goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) and addition of chemiluminescence substrate. Chemiluminiscence signals were collected in VersaDoc 3000 digital imaging system (Bio-Rad Laboratories, Hercules, CA). Tubulin was detected on the same blot membrane to normalize the protein loading in the blot analysis. Similarly, protein expression of vIRF-1-1, vIL-6, K8.1A gene product and LANA were detected with respective antibodies described above. Digital image analyses were conducted with Quantity One (Version 4.4) following the instructions of the software producer (Bio-Rad).

Cell viability assay. The viability of BCBL-1 cells after PMO treatment was determined with CellTiter-Blue (Promega) following the manufacturer's instructions. Briefly, BCBL-1 cells were treated with RP1 or CP1 under identical conditions as described above. Mock-treated cells were included for comparison. The same number of BCBL-1 cells without TPA induction was also included as a control. CellTiter-Blue reagent was added and incubated for 1 h at 37°C. The fluorescence signal was measured with a Synergy HT Multi-Detection Microplate Reader (Bio Tek Instruments, Inc., Winooski, VT). The fluorescence intensity reflecting the number of viable cells was analyzed and compared among different treatments.

RESULTS

Design of PMOs against KSHV RTA and LANA transcripts. For ORF50/RTA, a PMO was designed to be complementary to an immediate early transcript that includes the RTA translational initiation codon region (Fig 1). A control PMO, mRP1, was designed with five-base changes in RP1 sequences (Table 2) to verify RP1-mediated sequence-specific inhibition of RTA expression. For ORF73/LANA, three PMOs were designed. LP1 was designed to target the LANA translation initiation region around the AUG (Fig. 1). LP2 and LP3 were designed against target regions located in the 5' untranslated region (UTR) of a latent transcript encoding LANA (Fig. 1). The LP3 targets a splice donor site in the 5'UTR of the latent transcript (62, 76).

Cellular uptake of PMO by BCBL-1 cells. PMOs are non-charged oligomers and their conjugation to arginine-rich peptides assists delivery into a variety of cells (71, 73); however, there was no information available about PMO delivery to B lymphocytes. To test whether BCBL-1 cells can take up PMOs efficiently, P4-PMO-F1 was added to the cells. PMO-F1 was included for comparison. After 4 h incubation, almost all of the cells treated with P4-

PMO-F1 exhibited green fluorescence when observed under fluorescence microscope (Fig. 2A), while no fluorescence was observed in the cells treated with PMO-F1. The results suggested high efficiency uptake of P4-PMO-F1 by the BCBL-1 cells and failure in uptake of PMO-F1.

Flow cytometry analysis was conducted to quantify the cellular uptake efficiency. Almost all BCBL-1 cells treated with P4-PMO-F1 were fluorescence-positive (Fig. 2C-E), while few cells were found to be fluorescence-positive of those after mock-treatment or treatment with PMO-F1.

To determine whether the P4-PMO-F1 stably persisted in BCBL-1 cells after uptake, the cells were treated with P4-PMO-F1 for 4 h, rinsed, replenished with media, and harvested 24, 48 and 72 h later for analysis by fluorescence microscopy and flow cytometry. Green fluorescence was still observed in almost all cells at all three time points. Flow cytometry analysis showed that over 95% of the cells had green fluorescence (Fig. 2F), indicating the stable presence and partition of the PMO after cell division. BCBL-1 cells treated with PBS were included as a mock-treatment control and no fluorescence was observed in these cells. Green fluorescence in the P4-PMO-F1 treated cells was observed at day 7 after treatment but at reduced intensity. The fluorescence intensity reduction was possibly due to cell division.

RP1 inhibition of target RNA translation in a cell-free luciferase reporter assay. The effect of RP1 on translation of its intended target RNA was evaluated in a rabbit reticulocyte cell-free translation assay. Binding of the PMO to target RNA was expected to block the translation of downstream luciferase reporter transcript. In comparison with a mock-treatment control, the luciferase activity was significantly reduced by RP1 treatment in a dose-responsive manner, up to 98% when 300 nM RP1 was added; while treatment with CP1

did not have any effect (Fig. 3). The results indicated that the RP1 blocked target RNA translation in a dose-responsive manner.

PMO dose-dependent inhibitory effect on RTA protein expression. Having demonstrated the high efficiency of PMO uptake by BCBL-1 cells and effective inhibition of target RNA translation in the cell-free reporter assay, we applied RP1 at 16 μM to BCBL-1 cells to assess its effect on RTA protein expression. In non-induced BCBL-1 cells, less than 1% of cells were RTA-positive, indicating that a small number of cells have spontaneous KSHV lytic replication, as expected. After TPA induction, approximately 20% of BCBL-1 cells expressed RTA, as detected by IFA (Fig. 4). Compared with cells treated with CP1, the cells treated with RP1 and induced with TPA had a much lower number of RTA-positive cells; around 5% of cells positive for RTA (Fig. 4). CP1-treated cells had a similar number of RTA-positive cells after TPA induction to those mock-treated; all were approximately 20%.

The inhibitory effect of RP1 on RTA expression was further examined in KSHV-infected BC-1 cells that had EBV co-infection. BC-1 cells were treated in the same manner as described above for BCBL-1 cells. IFA was conducted and it was found that in TPA-induced BC-1 cells RP1 treatment reduced the number of RTA-positive cells compared to mock-treated cells, while CP1 did not have observable effect.

As a confirmation of the IFA observation of RP1 effect, Western-blot analysis was performed to determine RTA protein level in the cells after PMO treatment and TPA induction. BCBL-1 cells were treated with RP1 or CP1 at a series of two-fold concentration dilutions ranging from 0.125 to 16 μM. Cells treated with PBS were included as a mock treatment control. Total cell lysates were subjected to SDS-PAGE and Western-blot analysis. The analysis demonstrated that RTA protein expression in TPA-induced BCBL-1 cells was

reduced along with the increase of RP1 concentration in the treatment of BCBL-1 cells (Fig. 5A), confirming the IFA observation, while identical treatment with CP1 did not yield observable change in RTA expression in TPA-induced BCBL-1 cells (Fig. 5B). Quantitative digital image analysis revealed that RP1 treatment of TPA-induced BCBL-1 cells inhibited RTA expression up to 91% in comparison with mock-treatment (Fig. 5C). These results demonstrated that RP1 inhibited RTA protein expression in BCBL-1 cells in a dose-dependent, sequence-specific manner.

It was shown that ORF50/RTA transcription is induced within 4 hours of the addition of TPA, and KSHV early lytic gene transcripts appear 8 to 13 h after chemical induction (53). The above experiments showed that RP1 inhibited RTA protein expression when TPA was added 4 h after PMO treatment. Because RP1 was expected to bind IE transcript and cause blockage of its translation, TPA was added at different time points in cells treated with RP1 to determine kinetics of RP1 effect. For BCBL-1 cells, TPA was added at 0, 0.5, 1, 2, 3 or 4 h after PMO application. IFA analysis showed that RP1 treatment reduced RTA protein expression at all of the above time points.

A mismatch PMO (mRP1) with five-base changes in RP1 sequence was also tested in BCBL-1 cells under identical conditions as RP1, and no observable effect on RTA protein expression was detected (Fig. 5D), confirming the sequence-specific inhibition of RTA expression by RP1 in BCBL-1 cells.

Effect of RP1 on KSHV lytic replication. Because RTA is an immediate-early gene that plays a key role in activating KSHV lytic replication, inhibition of RTA protein expression may block KSHV lytic replication. To investigate this possibility, expressions of KSHV lytic genes vIL-6, vIRF-1-1 and a glycoprotein encoded by ORF-K8.1A, were analyzed in RP1-

treated BCBL-1 cells by Western-blot analysis. In comparison with mock-treated BCBL-1 cells after TPA induction, expression of these lytic genes in cells treated with RP1 was decreased in a dose-dependent manner (Fig. 6A), up to 98% for vIL-6 (Fig. 6C) and 93% for vIRF-1-1 (Fig. 6D). In contrast, the expression level of these lytic genes did not have observable change in the cells treated with CP1 (Fig. 6B). These results indicated that inhibition of RTA expression by RP1 resulted in reduction of KSHV lytic replication.

LANA is a latent gene product that is expressed constitutively in KSHV-infected cells. To determine if RP1 treatment has any effect on KSHV latency, Western-blot analysis of LANA in BCBL-1 cells treated with RP1 was conducted. No detectable change in signal generated with a LANA antibody was found in RP1-treated cells in comparison with mock-treated or CP1-treated cells.

Flow cytometry analysis. A second confirmation of the IFA observation of RP1 effect was obtained by flow cytometry. BCBL-1 cells were treated with the RP1 or CP1 at 8 μM. RTA was detected with rabbit anti-RTA antibody. As expected, around 25% BCBL-1 cells expressed RTA after TPA induction. In non-induced BCBL-1 cells, less than 1% of cells were RTA-positive. Approximately 7% of cells treated with RP1 were RTA-positive and the number was significantly lower than that in CP1-treated or mock-treated cells ($P<0.001$) (Fig. 7). Cells treated with CP1 had the same profile as mock treatment control. These results confirmed the IFA and Western-blot data that showed RP1 specifically inhibited RTA protein expression in BCBL-1 cells.

Cell viability assay of BCBL-1 cells after RP1 treatment. Cell viability assays were conducted with RP1 in order to assess cytotoxicity of the PMO and exclude the possibility that reduction of target gene expression in the test cells was mis-interpreted. BCBL-1 cells

were treated with either RP1 or CP1 at 8 and 16 μM under identical conditions as the above experiments. BCBL-1 cells without PMO treatment were included as mock treatment control. Relative percentages of fluorescence intensity were calculated in comparison with mock-treatment control. The assay was repeated three times and the averages of the percentages were 103% and 96% for cells treated with 8 and 16 μM RP1, 106% and 107% for cells treated with 8 and 16 μM CP1, and 103% for no-induction control. These results demonstrated that the numbers of viable cells were similar between RP1 treatment, CP1 treatment, mock-treatment, and no-TPA control, indicating that RP1 and CP1 were not cytotoxic.

The suppression of LANA by PMO treatment. Three PMOs against LANA were designed sequentially and evaluated in BCBL-1 cells. Application of LP1 at 8 μM in BCBL-1 cells did not cause an observable change in LANA expression detected by IFA and Western-blot analysis. Because LANA is constitutively expressed in BCBL-1 cells at high level, recurrent applications of LP1 every two days were tested, but no observable change in LANA level was detected. To determine if the PMO can bind to its target RNA, a cell-free reporter assay of LP1 was conducted and LP1 was found to be effective in blocking the translation of target RNA. These results indicated that LP1 might be inaccessible to target RNA in the cells. Then two other PMOs were designed and evaluated in BCBL-1 cells.

Application of LP2 and LP3 at 1 and 8 μM in BCBL-1 cells resulted in reduction of LANA protein level detected by IFA and Western-blot (Fig. 8). IFA showed that three repeated treatments of the same BCBL-1 cells with LP2 or LP3 at an interval of two days had reduced LANA protein expression, while the same recurrent applications of CP1 or mock treatment had no observable effect (Fig. 8A). LANA protein level in cells after treatment

with the above three compounds was also evaluated by Western-blot analysis. LANA protein expression after three- time treatment with LP2 or LP3 at 8 μM in BCBL-1 cells was significantly reduced (Fig. 8B), but it remained stable in the mock-treatment control. In comparison with the mock-treatment control, cells treated with LP2 or LP3 had over 60% reduction in LANA protein level after the second application and up to 92% reduction after the third application (Fig. 8C). LP2 appeared to be somewhat more potent than LP3 in the suppression of LANA protein expression in this experiment.

Because the latent transcript encoding ORF72/vCyclin and ORF71/vFLIP is spliced from the same latent transcript as LANA (62, 76), the LP2 and LP3 should be effective in blocking the translation of the latent transcript encoding vCyclin and vFLIP. IFA with anti-vCyclin antibodies was conducted in BCBL-1 cells treated with the two PMOs and vCyclin was moderately reduced in comparison with CP1 or mock-treated cells.

DISCUSSION

This is the first report of application of antisense morpholino oligomers against a DNA virus. Our study demonstrated that PMOs specifically suppressed protein expression of KSHV RTA and LANA. RTA is a switch protein for KSHV lytic replication and, after its expression is initiated, viral proinflammatory cytokines and transforming genes are expressed. LANA is crucial in maintaining KSHV latency. Thus, control of RTA and LANA protein expression may be an effective strategy to block KSHV replication.

Delivery into relevant cells *in vivo* has been a challenge for nucleic acid-based compound strategies to impact target gene expression. KSHV-infected BCBL-1 cells are difficult to transfect to high efficiency with common transfection reagents (66, 67, 78). A recent report described that the transfection efficiency of BCBL-1 cells with Lipofectamine

2000 liposome (Invitrogen) was approximately 10% (78). PMOs are non-charged compounds and, therefore, cationic lipids for delivery into cultured cells don't work well for them (64, 70). Conjugation of arginine-rich peptides to PMOs has been proven to be an efficient means to deliver PMOs into cultured cells (71, 73). In this study, $R_5F_2R_4$ PMOs were tested for cellular uptake into and persistence in BCBL-1 cells. The P4-PMO-F1 had high uptake efficiency and persisted for one week after a single application of 8 μM. The presence of P4-PMO-F1 in treated BCBL-1 cells indicated that $R_5F_2R_4$ assisted the PMO uptake, as the corresponding PMO-F1 without peptide failed in the uptake assay. Flow cytometry analysis confirmed that over 95% of the cells had P4- PMO-F1 uptake.

To test the efficacy and specificity of PMOs, we applied them to KSHV-infected BCBL- 1 and BC-1 cells in culture. Because these cells were derived from patients with PEL, testing the PMO's effect on target protein expression in these cells has relevance to a natural KSHV infection. The IFA and Western-blotting analyses results demonstrate that the RP1 is effective in blocking RTA protein expression in BCBL-1 cells in a dose-responsive manner, whereas CP1 and mRP1 do not have any effect. The RP1-mediated inhibition is sequence-specific because mRP1 containing five-base changes in RP1 sequence had no detectable inhibitory effect on RTA protein expression in BCBL-1 cells. The result indicates that the sequence change abolishes binding to target RNA. There was slight RTA protein expression in BCBL-1 cells treated with 16 μM RP1, which may indicate that there remained some cells with little or no uptake of PMO. Another possible reason for this detectable activity may be that an insufficient quantity of PMO made its way into the subcellular locations where RTA mRNA is translated. The above speculation also provide potential explanations as to why higher PMO concentration was required in cultured cells than that

needed in the cell-free reporter assay to achieve an equivalent degree of inhibition on target RNA translation.

Our study showed that RP1 suppressed RTA protein expression even when TPA induction was conducted at the same time as PMO treatment. The inhibition of RTA protein expression was less effective when RP1 was applied after TPA induction. This observation is consistent with the predicted mechanism of RTA PMO action, that is, blocking the initiation of RNA translation into protein (75).

A cell viability assay indicated that RP1 was not cytotoxic to the cells and that suppression of RTA or other lytic gene expression was due to PMO's specific binding and inhibition of RTA mRNA translation. Application of high concentration of the PMO, however, may have some adverse effect to cells and we have observed that application of RP1 at 16 μM led to about 5% cell death.

RP1 application to BCBL-1 cells led to ablation of expression of a series of viral genes, vIL-6, vIRF-1-1, and ORF-K8.1A, which are typically expressed temporally downstream of RTA. These results suggest that KSHV lytic replication was abolished in BCBL-1 cells after RTA PMO application. This pattern of RTA pre-eminence is consistent with previous publications (28, 78). Curreli et al. (28) showed that methotrexate, a potent anti-inflammatory agent and an inhibitor of cellular dihydrofolate reductase, reduces the expression of ORF50/RTA and prevents expression of other lytic genes. The mechanism of this relationship is not known. RNase P-based cleavage has also been explored to knock-down RTA expression and KSHV lytic replication (78). In this approach, a DNA-based external guidance sequence (EGS) targeting RTA mRNA was used and found to be effective

in BCBL-1 cells, despite limited transfection efficiency (78). These data strongly suggest that RTA is an appropriate target for antiviral therapy.

We also found two effective PMOs against LANA among the three antisense compounds evaluated. The two PMOs, LP2 and LP3, targeting the 5'UTR of a latent transcript encoding LANA, were effective in binding to and blocking target mRNA translation, indicating that the target regions are essential for translation of the latent transcript. The inhibitory effect of LP3 indicates that it inhibits the 5'UTR splicing of the 5.7 Kb latent transcript (Fig.1), leading to reduction of 5.4 Kb transcript encoding LANA. Translation of ORF72/vCyclin and ORF71/vFLIP should be reduced in cells treated with both the LP2 and LP3 because the mRNA encoding these two proteins is spliced from the same latent transcript as the mRNA encoding LANA. Our IFA result with vCyclin antibodies supports this speculation. LP1 was designed against the LANA translational initiation region, but had no observable suppressive effect on LANA protein expression. We speculate that the target site on the latent transcript encoding LANA may not be particularly accessible, because the same PMO was effective in blocking translation of its target RNA in a cell-free reporter assay. RNA secondary structural analysis of the latent transcript showed there are potential complex secondary structures in the translational initiation region.

Current anti-herpesvirus drugs target the lytic phase rather than latent cycle, and it has been reported that cidofovir and foscarnet are reported to be the most efficient commercially available compounds against KSHV (65, 68, 74). Cidofovir and foscarnet have no effect on expression of latent or early lytic genes (28), as these drugs are inhibitors of the viral DNA polymerase, and transcription of early lytic genes occurs before KSHV DNA replication. Considering the significance of LANA, RTA, and early lytic genes such as vIL-6 (52, 56, 57) and vIRF-1-1 (30, 31) in KSHV replication and pathogenesis, blocking their expression and

that of down-stream genes could contribute to the treatment of KSHV-associated malignancies.

Example 4: Blockade of expression of a growth factor homologue vIL-6 of HHV8

KSHV ORF-K2 encodes viral interleukin 6 (vIL-6), an early lytic gene product homologous to human interleukin 6 (hIL-6) that is highly expressed in infected hematopoietic cells. The hIL-6 is a pro-inflammatory cytokine, as well as an autocrine/paracrine growth factor that stimulates growth and proliferation of B cells, induces growth arrest and differentiation of macrophages, and also has anti-apoptotic activity. The vIL-6 is capable of substituting for hIL-6 in its growth factor and anti-apoptotic activity, and can induce hIL-6 expression. Thus, vIL-6 is believed to be a major contributor to PEL and MCD pathogenesis.

Two KSHV-infected PEL cell lines, BCBL-1 and BC-1, were treated with varying concentrations of vIL-6 PMO or control (scramble) PMO. Immunofluorescence assay (IFA) was used to detect vIL-6 in the PEL cells. IFA showed that the number of vIL-6 positive cells in cells treated with the vIL-6 PMO was reduced in comparison with cells treated with the control PMO. Subsequently, Western-Blot confirmed the reduction of vIL-6 expression and found the suppression was in a dose-dependent manner. To detect the changes of hIL-6 after PMO treatment, enzyme immunoassay was performed and it was found that hIL-6 expression was reduced in cells treated with vIL-6 PMO in comparison with cells treated with the control PMO. The result indicates that vIL-6 PMO is effective in blocking the expression of both vIL-6 and that the reduction of vIL-6 leads to the decrease of hIL-6.

PMOs target the vIL-6 of KSHV. PMOs were designed against the translation initiation region of the vIL-6 mRNA, and were synthesized by AVI Biopharma, Inc. as peptide-conjugated compounds (Table 3). In addition, three PMOs were designed against vIL-6 mRNA, which complement the 5' untranslated region (UTR) of the transcript (Table 4).

Exploring the efficacy of ILP1 PMO against vIL-6 in blocking expression of vIL-6 in KSHV-infected cells. BCBL-1 cells were treated with varying concentrations of ILP1 or Control PMO (8 and 16μM) for 4 h at 37°C. The control PMO used was a scrambled sequence not specific for any KSHV gene (Table 3). After 4h, growth medium containing TPA (12-O-tetratdecanoylphorbol 13-acetate) at 20 ng/mL was added to the cells to induce KHSV lytic replication. The BCBL-1 cells were harvested 48h after PMO treatment. Upon harvest, a portion of cells were washed three times in 1X PBS, then added to 12-well glass slides, dried and fixed by heating at 56°C for 2 hours. Immunofluorescence assay (IFA) was then performed using vIL-6 antibodies to determine the effect of PMO treatment upon suppression of vIL-6 protein expression. A second portion of harvested cells was resuspended in 1X PBS and lysed in 2X SDS sample buffer. SDS-PAGE was performed on this portion and Western-blot was conducted with rabbit anti-vIL-6 antibodies (ABI).

a. ILP1 diminished expression of vIL-6 protein

Observation by fluorescence microscopy revealed a dose-dependent decrease in vIL-6 expression as a result of vIL-6-specific PMO treatment. Compared with controls, ILP1 treatment of BCBL-1 cells lead to a significant decrease in the amount of fluorescence observed. Cells treated with 8μM ILP1 exhibited approximately 50% less fluorescence than those treated with 8μM control PMO, while greater than 90% reduction was observed in cells treated with the 16μM concentration vs. the 16μM control-treated cells (Fig. 9).

b. Western-blot analysis confirmed the ablation of vIL-6 expression by ILP1 in both BCBL-1 and BC-1 cells

In BCBL-1 cells, ILP1 was shown to decrease vIL-6 protein expression by 60% at the 8μM concentration and over 90% when 16μM was used (Fig. 10). When another KSHV-infected cell line, BC-1, was treated as described in (a.), vIL-6 expression was knocked down

to similar levels (Fig. 11). This demonstrates that vIL-6 PMO is effective in diminishing vIL-6 protein expression, and the effectiveness of this PMO is not cell-line specific.

Determining the effect of vIL-6 PMO treatment on the expression of hIL-6. Since vIL-6 is both a structural and functional homolog of hIL-6, and vIL-6 has been shown to up-regulate the expression of hIL-6 in KSHV-infected cells, we sought to determine whether ablation of vIL-6 expression also caused down-regulation of the expression of hIL-6. As hIL-6 is a secreted cytokine, supernatants from each well of PMO-treated cells were analyzed by ELISA (hIL-6 kit by Assay Designs) to assess hIL-6 expression under the differing treatment conditions.

As shown in Fig. 12, the expression of hIL-6 was decreased nearly 2 fold after vIL-6 PMO treatment of BCBL-1 and BC-1 cells. Thus, there may be a negative effect on hIL-6 expression as a result of vIL-6 PMO treatment.

As a result of this study, we have shown that vIL-6 PMO treatment ablated protein expression of vIL-6 in KSHV-infected cells in a dose-dependent manner. This effect was observed in both KSHV cell lines tested, thus indicating that the vIL-6 PMO inhibition was target specific. Inhibition of vIL-6 expression via PMO was sequence-specific, as no reduction in expression level was observed in cells treated with the control PMO. In addition, hIL-6 protein expression was decreased as a result of vIL-6 PMO-treatment.

Example 5: PMOs targeted to vFLIP

PMOs as identified in Table 4 targeting the KSHV ORF71/K13, which encodes vFLIP, were designed. These PMOs are contacted to the HHV8-infected cells. The mRNA translation of these targeted HHV8 genes can be assayed in accordance with the methods described in Example 3.

Example 6: Identification of PMOs that can overcome the transformed phenotype of HHV8-infected cells *in vitro*

Cell proliferation and growth in soft agar. To determine if suppression of target gene expression and inhibition of HHV8 latent and lytic replication can cause growth regression of HHV8-infected BCBL-1 cells, a cell proliferation assay is run to detect the number of viable BCBL-1 cells in the presence and absence of PMOs (Cell Proliferation Assay, Promega). A transformation assay is conducted to detect the contact inhibition of cell growth in soft agar (31). BCBL-1 cells treated with scramble PMO and untreated BCBL-1 cells are included as controls for the assay. If the PMOs works, the PMO-treated cells may not form colonies or form much smaller and fewer colonies, while control cells will form many colonies (31).

DNA damage assay. To determine if the PMO treatment induces apoptosis in HHV8-infected cells, DNA nicking in PMO-treated BCBL-1 cells is assayed by agarose gel electrophoresis and terminal deoxytransferase-mediated dUTP nick end-labeling (60).

HHV8-infected endothelial cells. Moses et al. (84) established an *in vitro* model utilizing dermal microvascular endothelial cells that support significant HHV8 infection. The HHV8 infection induced phenotypic changes in infected endothelial cells resembling characteristics of KS spindle cells *in vivo*. Thus, this model is used to determine if the PMOs can suppress the transformed phenotypes of the HHV8-infected endothelial cells. PMO-mediated changes of cell phenotypes and contact inhibition of the HHV8-infected endothelial are determined by cell proliferation, colony formation in soft agar, and apoptosis detection as described above.

Example 7: PMOs targeted to v-Cyclin, vIRF-1, and vGCR

An antisense antiviral oligomer as identified in Table 1 targeting the HHV8 v-Cyclin, vIRF-1, or vGCR can be made by known methods. These PMOs are contacted to the HHV8-

infected cells. The mRNA translation of these targeted HHV8 genes can be assayed in accordance with the methods described in Example 3.

While the invention has been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected. In addition, all references cited herein are indicative of the level of skill in the art and are hereby incorporated by reference in their entirety.

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CLAIMS

We claim:

1. An antisense antiviral oligomer having at least 10 nucleotides, which specifically binds to a portion of targeted RNA expressed from the human herpesvirus 8 (HHV8), wherein said oligomer is effective for reducing the expression of said RNA.

2. The antisense antiviral oligomer of claim 1, wherein said oligomer has at least 15 nucleotides.

3. The antisense antiviral oligomer of claim 1, wherein said oligomer has at least 20 nucleotides.

4. The antisense antiviral oligomer of claim 1, wherein said oligomer has at least 25 nucleotides.

5. The antisense oligomer of claim 1, wherein said HHV8 RNA is selected from genes whose gene products affect the replication or transformation of HHV8.

6. The antisense oligomer of claim 1, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 selected from the group consisting of the RTA, vIL-6, vIRF-1, v-Cyclin, LANA, vGCR, and vFLIP gene.

7. The antisense oligomer of claim 6, wherein said antisense oligomer has a nucleic acid sequence effective to reduce expression of the RTA gene.

8. The antisense oligomer of claim 7, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4, and 26.

9. The antisense oligomer of claim 6, wherein said antisense oligomer has a nucleic acid sequence effective to reduce expression of the vIL-6 gene.

10. The antisense oligomer of claim 9, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 6-8, 34, and 36-38.

11. The antisense oligomer of claim 6, wherein said antisense oligomer has a nucleic acid sequence effective to reduce expression of the vIRF-1 gene.

12. The antisense oligomer of claim 11, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 10-12.

13. The antisense oligomer of claim 6, wherein said antisense oligomer has a nucleic acid sequence effective to reduce expression of the vCyclin gene.

14. The antisense oligomer of claim 13, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 14-16.

15. The antisense oligomer of claim 6, wherein said antisense oligomer has a nucleic acid sequence effective to reduce expression of the LANA gene.

16. The antisense oligomer of claim 15, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 18, 20, 21, 29, and 30.

17. The antisense oligomer of claim 6, wherein said antisense oligomer has a nucleic acid sequence effective to reduce expression of the vGCR gene.

18. The antisense oligomer of claim 17, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 23-25.

19. The antisense oligomer of claim 6, wherein said antisense oligomer has a nucleic acid sequence effective to reduce expression of the vFLIP gene.

20. The antisense oligomer of claim 19, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 39-41.

21. The antisense oligomer of claim 1, wherein said oligomer comprises a morpholine ring and a phosphorodiamidate linkage.

22. The antisense oligomer of claim 1, wherein said oligomer does not contain over about 36% guanines or more than 3 contiguous guanines.

23. The antisense oligomer of claim 1, wherein said oligomer does not comprise more than 16 contiguous bases identical to host chromosome sequences or non-target HHV8 sequences.

24. A composition comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 RTA gene, wherein said oligomer is effective for reducing the expression of said RTA gene, and a pharmaceutically acceptable carrier.

25. The composition of claim 24, wherein said oligomer is at least 15 nucleotides in length.

26. The composition of claim 24, wherein said oligomer is at least 20 nucleotides in length.

27. The composition of claim 24, wherein said oligomer is at least 25 nucleotides in length.

28. The composition of claim 24, wherein the oligomer is selected from the group consisting of SEQ ID NOS: 2, 3, 4, and 26.

29. A composition comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 vIL-6 gene, wherein said oligomer is effective for reducing the expression of said vIL-6 gene, and a pharmaceutically acceptable carrier.

30. The composition of claim 29, wherein said oligomer is at least 15 nucleotides in length.

31. The composition of claim 29, wherein said oligomer is at least 20 nucleotides in length.

32. The composition of claim 29, wherein said oligomer is at least 25 nucleotides in length.

33. The composition of claim 29, wherein the oligomer is selected from the group consisting of SEQ ID NOS: 6-8, 34, and 36-38.

34. A composition comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 vIRF-1 gene, wherein said oligomer is effective for reducing the expression of said vIRF-1 gene, and a pharmaceutically acceptable carrier.

35. The composition of claim 34, wherein said oligomer is at least 15 nucleotides in length.

36. The composition of claim 34, wherein said oligomer is at least 20 nucleotides in length.

37. The composition of claim 34, wherein said oligomer is at least 25 nucleotides in length.

38. The composition of claim 34, wherein the oligomer is selected from the group consisting of SEQ ID NOS: 10-12.

39. A composition comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 v-Cyclin gene, wherein said oligomer is effective for reducing the expression of said v-Cyclin gene, and a pharmaceutically acceptable carrier.

40. The composition of claim 39, wherein said oligomer is at least 15 nucleotides in length.

41. The composition of claim 39, wherein said oligomer is at least 20 nucleotides in length.

42. The composition of claim 39, wherein said oligomer is at least 25 nucleotides in length.

43. The composition of claim 39, wherein the oligomer is selected from the group consisting of SEQ ID NOS: 14-16.

44. A composition comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8

LANA gene, wherein said oligomer is effective for reducing the expression of said LANA gene, and a pharmaceutically acceptable carrier.

45. The composition of claim 44, wherein said oligomer is at least 15 nucleotides in length.

46. The composition of claim 44, wherein said oligomer is at least 20 nucleotides in length.

47. The composition of claim 44, wherein said oligomer is at least 25 nucleotides in length.

48. The composition of claim 44, wherein the oligomer is selected from the group consisting of SEQ ID NOS: 18, 20, 21, 29, and 30.

49. A composition comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 vGCR gene, wherein said oligomer is effective for reducing the expression of said vGCR gene, and a pharmaceutically acceptable carrier.

50. The composition of claim 49, wherein said oligomer is at least 15 nucleotides in length.

51. The composition of claim 49, wherein said oligomer is at least 20 nucleotides in length.

52. The composition of claim 49, wherein said oligomer is at least 25 nucleotides in length.

53. The composition of claim 49, wherein the oligomer is selected from the group consisting of SEQ ID NOS: 23-25.

54. A composition comprising an antisense oligomer of at least 10 nucleotides in length, which specifically binds to a portion of RNA expressed from the human herpesvirus 8 vFLIP gene, wherein said oligomer is effective for reducing the expression of said vFLIP gene, and a pharmaceutically acceptable carrier.

55. The composition of claim 54, wherein said oligomer is at least 15 nucleotides in length.

56. The composition of claim 54, wherein said oligomer is at least 20 nucleotides in length.

57. The composition of claim 54, wherein said oligomer is at least 25 nucleotides in length.

58. The composition of claim 54, wherein the oligomer is selected from the group consisting of SEQ ID NOS: 39-41.

59. A method for inhibiting the expression of a human herpesvirus 8 (HHV8) gene comprising introducing an antisense antiviral oligomer having at least 10 nucleotides, which specifically binds to a portion of RNA of the HHV8 gene, wherein said oligomer is effective for reducing the expression of said gene, under conditions sufficient for the antisense antiviral oligomer to bind to the HHV8 gene.

60. The method of claim 59, wherein said oligomer is at least 15 nucleotides in length.

61. The method of claim 59, wherein said oligomer is at least 20 nucleotides in length.

62. The method of claim 59, wherein said oligomer is at least 25 nucleotides in length.

63. The method of claim 59, wherein said HHV8 gene is selected from the group consisting of RTA, vIL-6, vIRF-1_3 v-Cyclin, LANA, vGCR, and vFLIP.

64. The method of claim 59, wherein said antisense oligomer has a nucleic acid sequence effective to reduce expression of the RTA gene.

65. The method of claim 64, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4, or 26.

66. The method of claim 59, wherein said antisense oligomer has a nucleic acid sequence effective to reduce expression of the vIL-6 gene.

67. The method of claim 66, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 6-8, 34, and 36-38.

68. The method of claim 59, wherein said antisense oligomer has the nucleic acid sequence effective to reduce expression of the vIRF-1 gene.

69. The method of claim 68, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 10-12.

70. The method of claim 59, wherein said antisense oligomer has the nucleic acid sequence effective to reduce expression of the vCyclin gene.

71. The method of claim 70, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 14-16.

72. The method of claim 59, wherein said antisense oligomer has the nucleic acid sequence effective to reduce expression of the LANA gene.

73. The method of claim 72, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 18, 20, 21, 29, and 30.

74. The method of claim 59, wherein said antisense oligomer has the nucleic acid sequence effective to reduce expression of the vGCR gene.

75. The method of claim 74, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 23-25.

76. The method of claim 59, wherein said antisense oligomer has the nucleic acid sequence effective to reduce expression of the vFLIP gene.

77. The method of claim 76, wherein said antisense oligomer has a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 39-41.

78. The method of claim 59, wherein said antisense oligomer comprises a morpholine ring and a phosphorodiamidate linkage.

79. The method of claim 59, wherein said antisense oligomer does not contain over about 36% guanines or more than 3 contiguous guanines.

80. The method of claim 59, wherein said antisense oligomer does not comprise more than 16 contiguous bases identical to host chromosome sequences or non-target HHV8 sequences.

Figure 1.

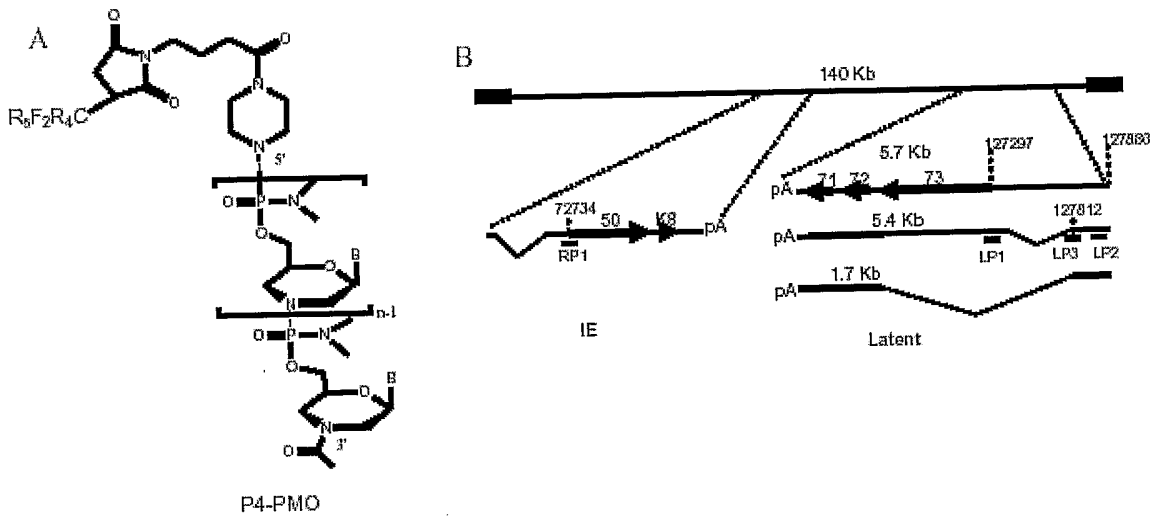

A
$R_5F_2R_4C$
5'
3'
n-1
P4-PMO
B
140 Kb
5.7 Kb
127297
127883
pA
71
72
73
72734
50
K8
pA
RP1
IE
5.4 Kb
127812
pA
LP1
LP3
LP2
1.7 Kb
pA
Latent

Figure 2.

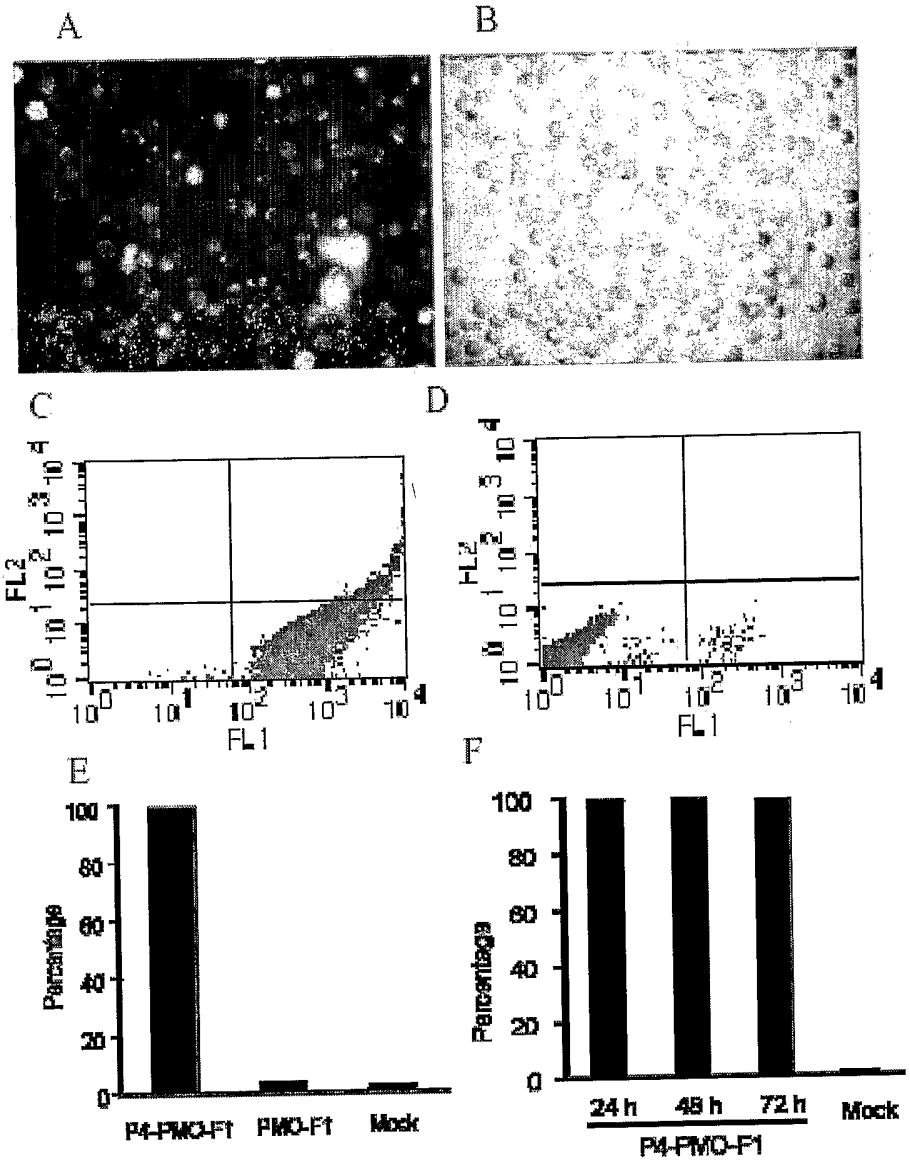

A
B
C
D
FL2
FL1
E
F
Percentage
P4-PMO-F1
PMO-F1
Mock
24 h
48 h
72 h
P4-PMO-F1

Figure 3.

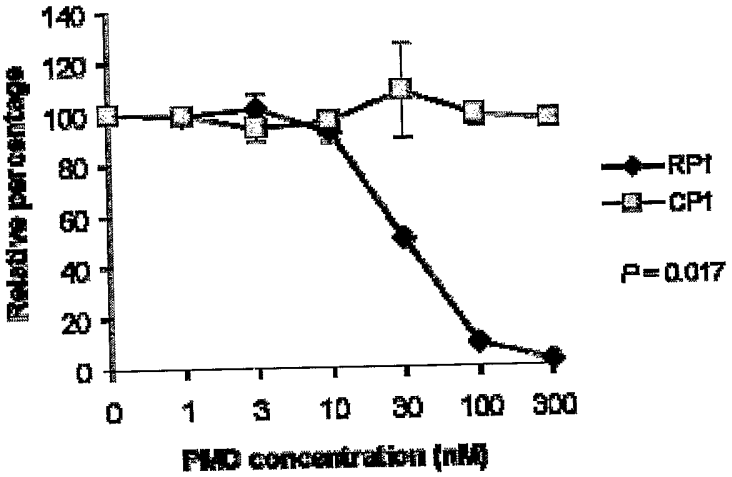

140
120
100
80
60
40
20
0
Relative percentage
0
1
3
10
30
100
300
PMD concentration (nM)
RP1
CP1
P = 0.017

Figure 4.

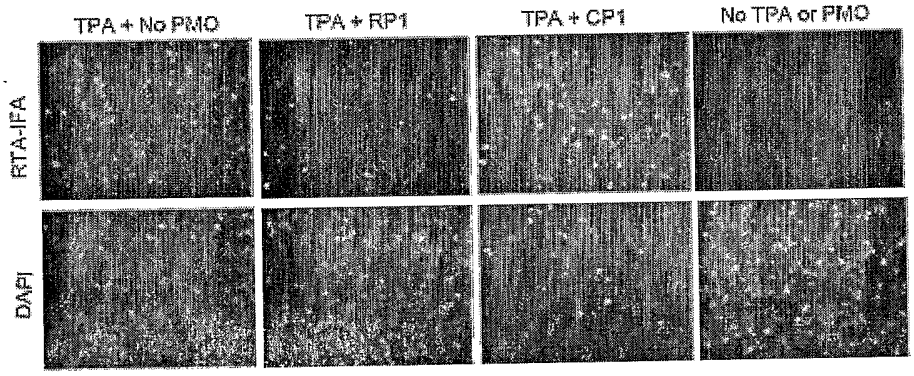


Figure 5.

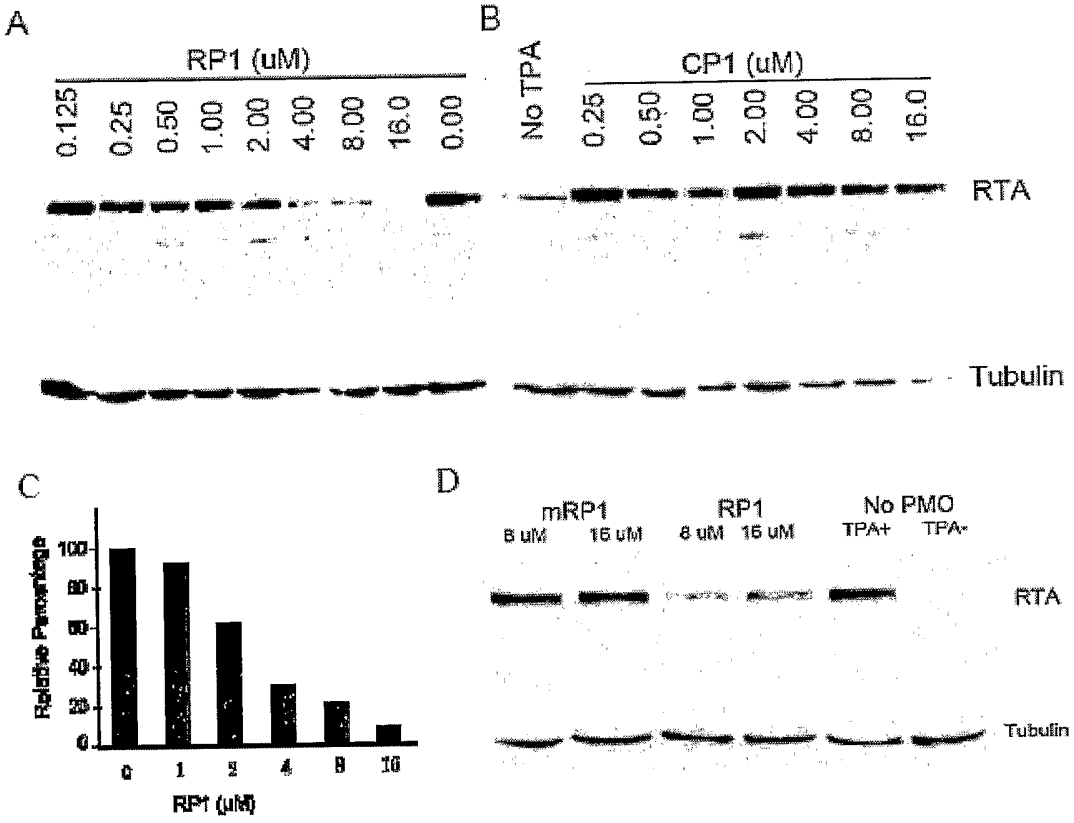

A
RP1 (uM)
0.125
0.25
0.50
1.00
2.00
4.00
8.00
16.0
0.00
B
No TPA
CP1 (uM)
0.25
0.50
1.00
2.00
4.00
8.00
16.0
RTA
Tubulin
C
Relative Percentage
100
80
60
40
20
0
0
1
2
4
8
16
RP1 (uM)
D
mRP1
8 uM
16 uM
RP1
8 uM
16 uM
No PMO
TPA+
TPA-
RTA
Tubulin

Figure 6.

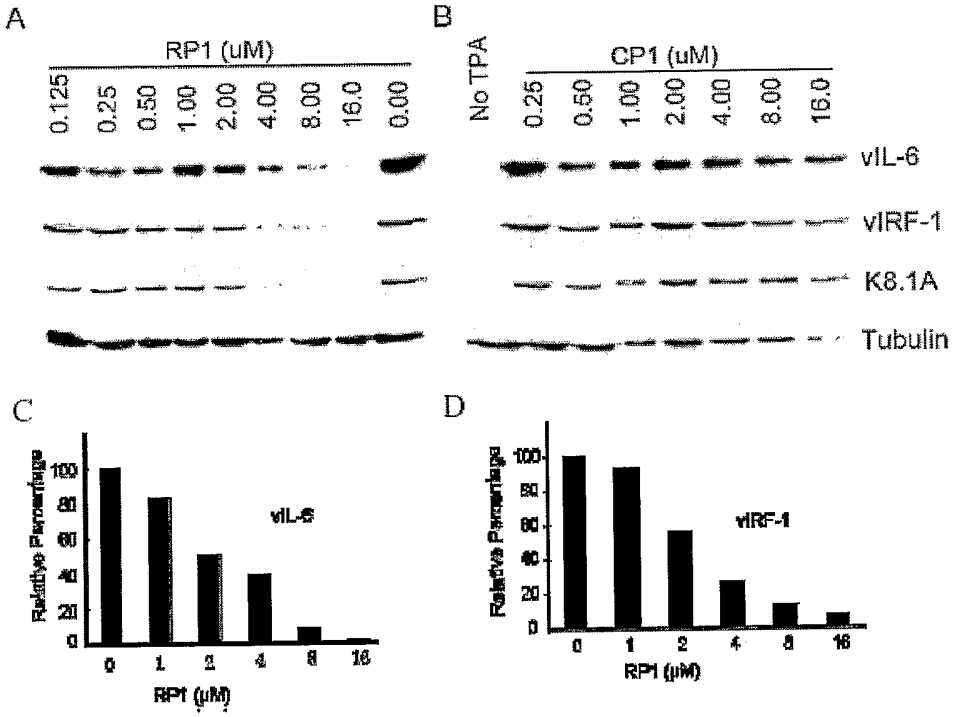

A
RP1 (uM)
0.125
0.25
0.50
1.00
2.00
4.00
8.00
16.0
0.00
B
No TPA
CP1 (uM)
0.25
0.50
1.00
2.00
4.00
8.00
16.0
vIL-6
vIRF-1
K8.1A
Tubulin
C
vIL-6
RP1 (μM)
D
vIRF-1
RP1 (μM)

Figure 7.

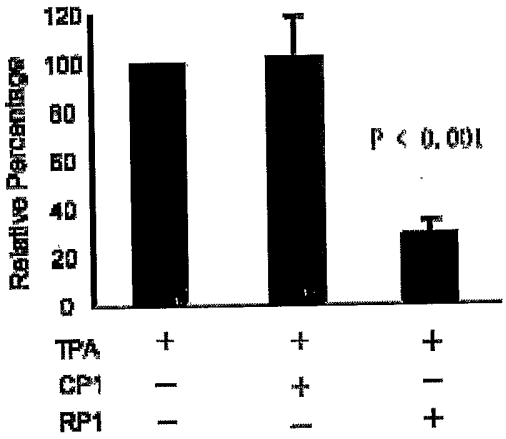


Figure 8.

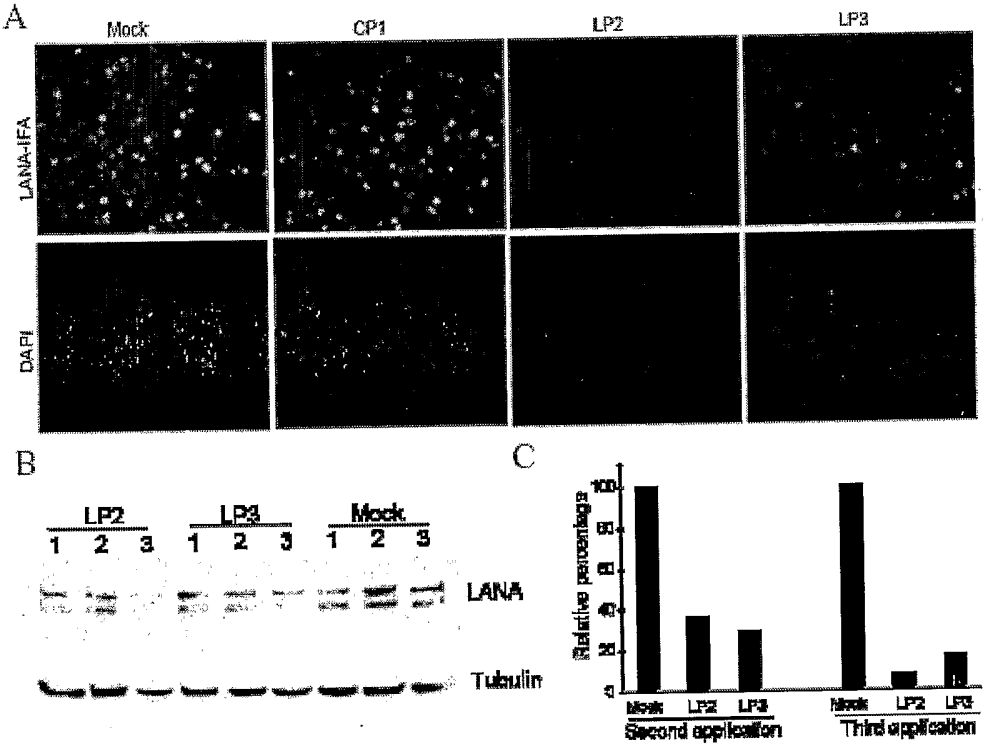

A
Mock
CP1
LP2
LP3
LANA-IFA
DAPI
B
LP2
LP3
Mock
1 2 3 1 2 3 1 2 3
LANA
Tubulin
C
Relative percentage
Mock
LP2
LP3
Second application
Mock
LP2
LP3
Third application

Figure 9.

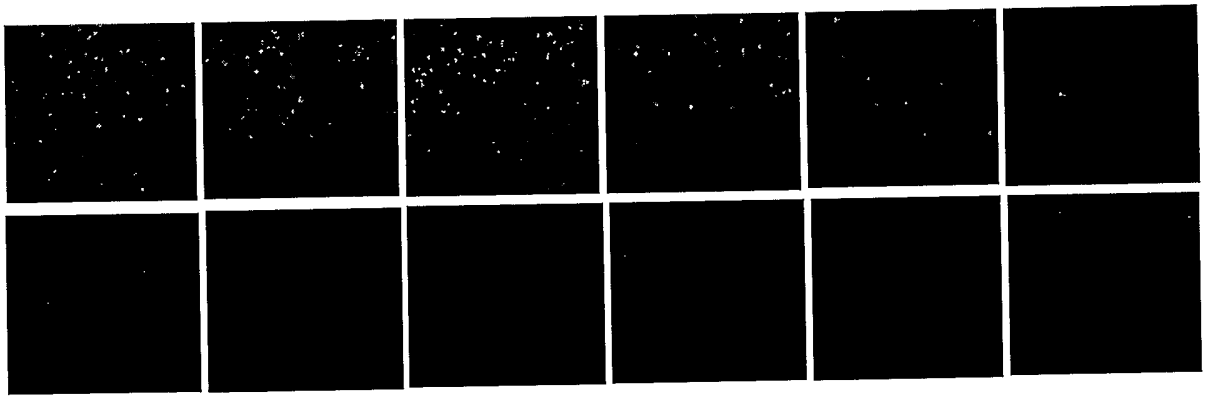


Figure 10.

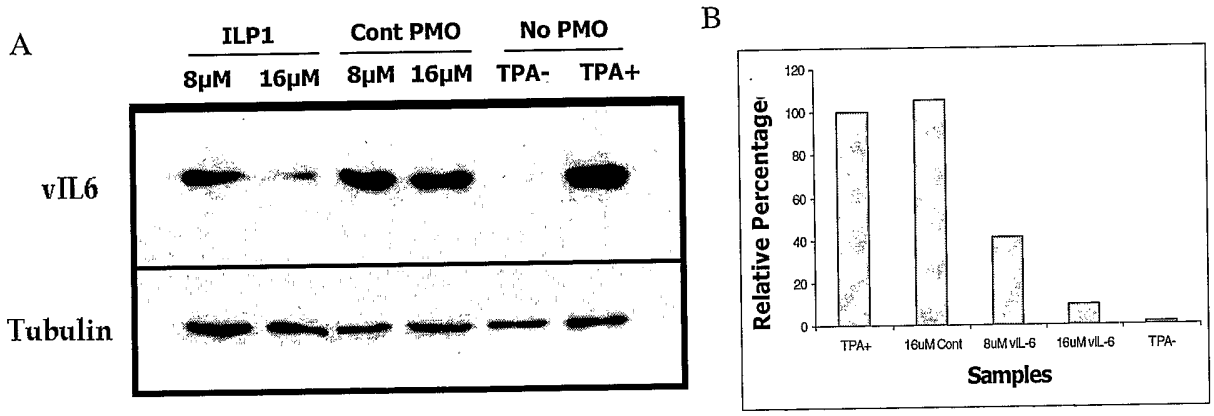
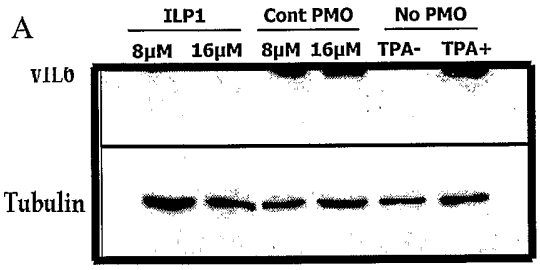

A
ILP1
Cont PMO
No PMO
8μM
16μM
8μM
16μM
TPA-
TPA+
vIL6
Tubulin
B
Relative Percentage
120
100
80
60
40
20
0
TPA+
16uM Cont
8uM vIL-6
16uM vIL-6
TPA-
Samples

Figure 11.

A


ILP1
Cont PMO
No PMO
8μM 16μM 8μM 16μM TPA- TPA+
vIL6
Tubulin

B

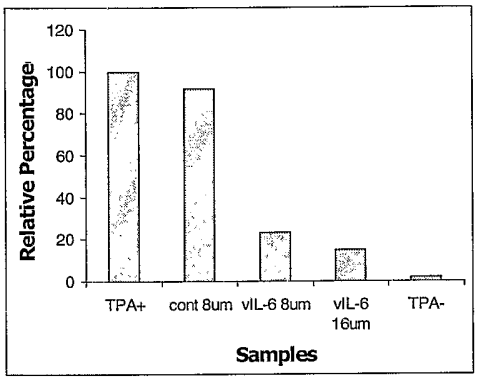
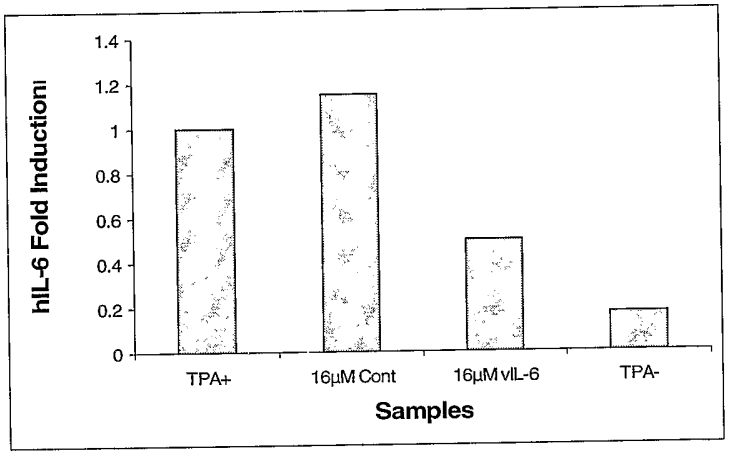

Relative Percentage
120
100
80
60
40
20
0
TPA+
cont 8um
vIL-6 8um
vIL-6 16um
TPA-
Samples

Figure 12.


hIL-6 Fold Induction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TPA+
16μM Cont
16μM vIL-6
TPA-
Samples

ORF PMO	Target HHV8 sequence and antisense PMOs
	73 108
ORF50	5′-CAGCCACAAAAAUGGCGCAAG**AUG**ACAAGGGUAAGA (SEQ ID NO:1)
pRTA-1	GGTGTTTTTACCGCGTTC**TAC**TGTT-5′(2)
pRTA-2	TCGGTGTTTTTACCGCGTTC**TAC**TG-5′(3)
pRTA-3	TTACCGCGTTC**TAC**TGTTCCCATTC-5′ (4)
	17911 17877
ORFK2	5′-GGGCACCACCGGCUAGUGU**AUG**CCGCGUUAGCAGC (5)
pvIL6-1	GTGGCCGATCACA**TAC**GGCGCAATC-5′ (6)
pvIL6-2	CCGATCACA**TAC**GGCGCAATCGTCG-5′ (7)
pvIL6-3	CGTGGTGGCCGATCACA**TAC**GGCGC-5′ (8)
	136 173
ORFK9	5′-GCGAGCUAGUCUGGUUGCGGGACA**AUG**GACCCAGGCCA (9)
pvIRF-1	ACCAACGCCCTGT**TAC**CTGGGTCCG-5′ (10)
pvIRF-2	ATCAGACCAACGCCCTGT**TAC**CTGG-5′ (11)
pvIRF-3	CTCGATCAGACCAACGCCCTGTT**AC**-5′ (12)
	123601 123561
ORF72	5′-UCUACAGGUAGGCCACAUACGCUCGCCACUCUAU**AUG**GCAA (13)
pvCyclin-1	TGTATGCGAGCGGTGAGATA**TAC**CG-5′ (14)
pvCyclin-2	TCCATCCGGTGTATGCGAGCGGTGA-5′ (15)
pvCyclin-3	ATGTCCATCCGGGTGTATGCGAGCGG-5′ (16)
	127387 127352
ORF73	5′-CCUUGUGGUCACUACGGGUAUUGCAUAAUGUGAAUA (17)
pLANA-1	ACACCAGTGATGCCCATAACGTATT-5′ (18)
	127325 127288
ORF73	5′-UUUGGUUGUCAGACCAGAUUUCCCGAGG**AUG**GCGCCCC (19)
pLANA-2	TCTGGTCTAAAGGGCTCC**TAC**CGCG-5′ (20)
pLANA-3	AGTCTGGTCTAAAGGGCTCC**TAC**CG-5′ (21)
	1236 1273
ORF74	5′-UCCCCAUUUUGGUGUCGCGUUUUGGGUUUUGCCCUGCC (22)
pvGCR-1	AACCACAGCGCAAAACCCAAAACGG-5′ (23)
pvGCR-2	GGTAAAACCACAGCGCAAAACCCAA-5′ (24)
pvGCR-3	CACAGCGCAAAACCCAAAACGGGAC-5′ (25)

Table 1. Alignment of PMOs targeting HHV8 ORFs 50, K2, K9, 73 and 74 and their target RNA sequences. AUG translation initiation codons are shown in bold case. GenBank numbers are: AF091350 for ORF50 mRNA, U75698 for ORFs K2, 72 and 73 genes, AF145701 for ORFK9 mRNA, and AF367765 for ORF74 mRNA.
The number in parentheses at the end of each sequence indicates the SEQ ID NO.

Table 2. PMOs[a] against RTA and LANA and their target sites.

PMO	Sequence (5' to 3')		Target site
RP1[b]	GTCATCTTGCGCCATTTTTGTGG	(SEQ ID NO:26)	ORF50 initiation region (76-99)[c]
mRP1[d]	GTCAACTTCCGCGATTATTGTCG	(27)	ORF50 initiation region (76-99)
LP1	GCCATCCTCGGGAAATCTGG	(28)	ORF73 initiation region (127293-127312)[e]
LP2	GACGTGACTGCTTCGTGGCGCA	(29)	5'UTR of latent transcript (127852-127873)
LP3	GCGGGCGCTACTCACTGTTTAT	(30)	Splicing donor site on 5'UTR of latent transcript (127799-127820)
P4-PMO-F1[f]	AGTCTCGACTTGCTACCTCA	(31)	Scramble control
PMO-F1[f]	AGTCTCGACTTGCTACCTCA	(32)	Scramble control
CP1	GATATACACAACACCCAATT	(33)	Scramble control

[a] The peptide $R_5F_2R_4$ (designated 'P4') is conjugated to the 5'end of all the PMOs except PMO-F1.

[b] The underlined nucleotides CAT correspond to the AUG translation initiation codon of RTA mRNA.

[c] GenBank accession number for the cDNA of RTA mRNA is AF091350.

[d] The underlined nucleotides indicate those changed in comparison to the RP1 sequence.

[e] GenBank accession number for the KSHV genome sequence is U75698. The numbers in parentheses indicate nucleotide positions in the genome.

[f] P4-PMO-F1 and PMO-F1 were fluorescein-labeled at 3'end.

The number in parentheses at the end of each sequence indicates the SEQ ID NO.

Table 3. PMOs against vIL-6 and their target sites

PMO	Sequence (5' to 3')		Target Site
ILP1 (vIL-6 PMO)	GCGGCATACACTAGCCGGTGG (SEQ ID NO: 34)		ORF-K2 translation initiation region
CONT-PMO	GATATACACAACACCCAATT	(35)	Scramble control
ILP2	GAAACACGTCACACGCAAAGTCA	(36)	5'UTR of vIL-6 mRNA
ILP3	ACGCCGTATCCGAACTCTGAGT	(37)	5'UTR of vIL-6 mRNA
ILP4	AGCGGGAGTTACGAAGTCTCAC	(38)	5'UTR of vIL-6 mRNA

The number in parentheses at the end of each sequence indicates the SEQ ID NO.

Table 4. PMOs for KSHV ORF71/K13/vFLIP

PMO	Sequence (5' to 3')		Target site
VFP1[a]	CATGGTGCCGGCTTGTATATGTG	(39)	ORF71 initiation region (122709-122731)[b]
VFP2	ACCTCGTAAGTGGCCATGGTGCC	(40)	ORF71 initiation region (122695-122717)
VFP3	CCAGATTTCCCGAGGATGGC	(41)	ORF71 initiation region (122726-122748)

[a] The underlined nucleotides CAT correspond to the AUG translation initiation codon of vFLIP mRNA.

[b] GenBank accession number for the KSHV genome sequence is U75698. The numbers in parentheses indicate nucleotide positions in the genome.

The number in parentheses at the end of each sequence indicates the SEQ ID NO.