Oncolytic Reactivation of KSHV as a Therapeutic Approach for Primary Effusion Lymphoma

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Primary effusion lymphoma (PEL) is an aggressive subtype of non-Hodgkin lymphoma caused by Kaposi’s sarcoma-associated herpesvirus (KSHV) infection. Currently, treatment options for patients with PEL are limited. Oncolytic viruses have been engineered as anti-cancer agents and have recently shown increased therapeutic promise. Similarly, lytic activation of endogenous viruses from latently infected tumor cells can also be applied as a cancer therapy. In theory, such a therapeutic strategy would induce oncolysis by viral replication, while simultaneously stimulating an immune response to viral lytic cycle antigens. We examined the combination of the FDA-approved drug PEP005 (ingenol-3-angelate) with epigenetic drugs as a rational therapeutic approach for KSHV-mediated malignancies. JQ1, a bromodomain and extra terminal protein (BET) inhibitor, in combination with PEP005, not only robustly induced KSHV lytic replication, but also inhibited IL-6 production from PEL cells. Using the dosages of these agents that was found to be effective in reactivating HIV (as a means to clear latent virus with HAART therapy), we were able to inhibit PEL growth in vitro and delay tumor growth in a PEL xenograft tumor model. KSHV reactivation was mediated by activation of NF-κB pathway by PEP005, which led to increased occupancy of RNA polymerase II onto the KSHV genome. RNA-sequencing analysis further revealed cellular targets of PEP005, JQ1, and the synergistic effects of both. Thus, combination of PEP005 with a BET inhibitor may be considered as a rational therapeutic approach for the treatment of PEL.
Introduction

Gamma herpesviruses are responsible for a substantial proportion of virus-associated human cancers, particularly in immunocompromised individuals (1,2). Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8, is one of the tumorigenic viruses with a large double-stranded DNA genome. KSHV has been linked to Kaposi’s sarcoma (KS) as well as primary effusion lymphoma (PEL), or body-cavity B-lymphoma (BCBL), and a subset of multicentric Castleman’s disease.

The aggressive nature of PEL is evident by the poor median overall survival for this cancer, 10.2 months in one recent study in which patients received a multidrug cytotoxic regimen (3). Given the limited treatment options for this Non-Hodgkin’s lymphoma, novel, rationally designed therapeutic approaches are urgently needed. Although the introduction of antiretroviral therapy (i.e. HAART: highly active antiretroviral therapy) has reduced the incidence of KS lesions in HIV patients, KSHV-associated complications remains a significant problem. The absence of HAART before PEL diagnosis is also associated with poor outcome in a multivariate analysis (4), indicating a strong correlation of cancer progression with HIV replication. This is supported by the finding that complete remissions have been reported after treatment of PEL patients with HAART therapy alone (5-7). PEL may also be responsive to Zidovudine (AZT) alone or AZT in combination with interferon-alpha (IFNα) (8,9). Accordingly, using HAART to target HIV concomitantly with targeted therapy to inhibit PEL growth should be clinically beneficial, as has been suggested elsewhere (6,7,10).

PEP005 (ingenol-3-angelate), an FDA-approved drug for topical treatment of actinic keratosis, is natural extract from the plant, *Euphorbia peplus*, and has also been found to suppress the growth of some cancer cell types, including melanoma, acute myeloid leukemia, and non-Hodgkin B-cell lymphoma (NHL) (11-13). Mechanistically, PEP005 is an agonist of
protein kinase C and activates NF-κB. Treatment of cancer cell lines with PEP005 has been shown to induce apoptosis (13). Furthermore, we recently found that PEP005 robustly reactivated latently-infected HIV in cells purified from patients undergoing HAART (14), and that combined treatment of PEP005 with epigenetic drug, the bromodomain and extra-terminal (BET) family protein inhibitor, JQ1, synergistically increased reactivation of HIV (14). Accordingly, PEP005 represents a new group of lead compound for combating HIV latency (14).

JQ1 is a small molecule, which was developed as a mimetic to an acetylated histone tail (15). This then competes with, and antagonizes, bromo-domain containing proteins (mainly BRD2 and 4) for binding to acetylated histone tails, which consequently mutes the expression of pro-inflammatory genes in particular (16-18). BRD2/4 binds to the C-terminal positive transcription elongation factor b (pTEFb), which is a heterodimer composed of cyclin T and CDK9 (19,20). The pTEFb factor phosphorylates Ser-2 on the heptad repeats of the C-terminal domain (CTD) in the stalled RNA polymerase II, thereby stimulating transcription elongation and coupling the histone acetylation mark to the transcription activity (19-21). Accordingly, significant down-regulation of cellular gene expression was observed by treatment with JQ1, especially of those genes that are regulated transiently by strong and inducible transcriptional factors, like Myc and NF-κB (17,22). In addition, NF-κB (p65) undergoes protein acetylation once it becomes activated. The acetylated domain holds structural homology with the histone tail, thus the posttranslational modification allows BRD2/4 to directly bind to p65 (23). This mechanism makes JQ1 (BET inhibitor) particularly effective for inhibition of the NF-κB signaling pathway. BET inhibitors have also been proposed as a Myc pathway targeted therapeutic, with preclinical activity demonstrated in multiple lymphomas including primary
effusion lymphoma (PEL) as it down-regulates Myc expression and its downstream target genes (22,24-26).

Similar to the “shock and kill” strategy for elimination of the latent HIV reservoir, methods that employ lytic reactivation of viruses from tumors latently infected with an oncogenic herpesvirus represent a unique strategy of antineoplastic therapy, as it may increase the specificity of cytotoxic cancer therapeutics (27-29). This is conceptually similar to the mechanism of an oncolytic viruses-based therapy like the FDA-approved Talimogene Laherparepvec, which is designed to: (1) replicate specifically in cancer cells, but not in normal cells, (2) stimulate the host immune system to the tumor microenvironment, and (3) have their replication manageable by replication-suppressive drugs for patient safety. Accordingly, lytic reactivation of KSHV from latently infected cancer cells would theoretically be beneficial by providing additional highly specific direct tumor lytic effects as well as by evoking an increased cytotoxic immune response against cancer cells expressing lytic viral antigens.

In this study, we evaluated the effects of "shock and kill" strategy on latently KSHV-infected tumor cells, which was originally designed to target latently HIV-infected cells. We demonstrated that the same drug combination with the same dosage, which reactivates HIV from latently infected T-cells, could strongly reactivate KSHV in PEL cell lines and inhibit PEL cell growth in both in vitro tissue culture and in vivo xenograft tumor models.
Materials and Methods

Cell culture

HBL-6, JSC-1, and BC2 cell lines, obtained from Dr. Masahiro Fujimuro (Kyoto Pharmaceutical University, Japan) in 2015. BCBL-1 cell line was obtained from Dr. Ganem (University of California San Francisco) in 2001. These cell lines were cultured in RPMI 1640 medium supplemented with 15% FBS. The Flag-HA tagged-K-Rta-inducible, TREx-K-Rta BCBL-1 cell line was generated according to methods previously described (30). No testing for the cell authentication for HBL-6, JSC-1, BC2, and BCBL-1 cell lines was performed. BC3 cell line was obtained from ATCC, expanded and stored to obtain early passage stocks. The BC3 cell was used for mouse xenograft studies with early passages of cells within a month. Mycoplasma contamination was tested by PCR.

Antibodies

Anti-K-Rta and anti-K-bZIP antibodies were previously described (31). Anti-LANA antibody (Advanced Biotechnologies), anti-p-ΙκBα (Ser32/36), anti-ΙκBα, anti-p-NF-κB p65 (Ser536) and anti-NF-κB p65 antibody (Cell Signaling Technologies), anti-phospho-S2 RNA Polymerase II, anti-phospho-S5 RNA Polymerase II (Abcam), anti-RNA Polymerase II antibody (Active Motif), anti-BRD4 antibody (Bethyl Laboratories), and anti-Actin, anti-GAPDH, and normal mouse and rat IgG (Santa Cruz Biotechnologies) were commercially obtained.

Drugs
BET inhibitor (+)-JQ1 (ApexBio Technology), PEP005 (Tocris Bioscience), suberoylanilide hydroxamate (SAHA; also known as Vorinostat, Santa Cruz Biotechnologies), and GSK343 (32) (Sigma) were obtained from commercial sources. Drugs were added into culture media alone or in combination, and the effects on KSHV reactivation as well as PEL cell growth were monitored.

**Immunofluorescence Analysis (IFA)**

Cells were fixed with 4% formaldehyde and permeabilized with successive treatments of 1% SDS and 1% Triton-X 100 in PBS for 15 minutes each at room temperature. Primary antibody was incubated overnight in 2% BSA/PBS at 4°C. Secondary antibody (Alexa Fluor 488- or Alexa Fluor 555-conjugated antibodies; Invitrogen) was incubated for 1 hour at room temperature. Slides were mounted with Anti-fade Gold containing DAPI (Invitrogen).

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was isolated from cells using the RNeasy Kit (Qiagen) followed by digestion with DNase I (Invitrogen). First strand cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen). All cDNAs were analyzed by SYBR green-based quantitative PCR (qPCR) (Bio-Rad) with primers described previously (33). qPCR was performed in triplicate.

**RNA-seq library preparation and next-generation sequencing**
Whole transcriptome profiling was performed using a directional, strand-specific mRNA-Seq approach. Briefly, total RNA samples were submitted to the UC Davis Comprehensive Cancer Center’s Genomics Shared Resource (GSR), and indexed RNA-Seq libraries were prepared from total RNA (200 ng) using the KAPA Stranded mRNA-Seq Kit (Kapa Biosystems, Inc.) according to the manufacturer’s protocol. Briefly, poly-adenylated mRNA was purified by binding to oligo(dT) beads, which was followed by fragmentation by incubation at 94ºC in the presence of magnesium. Double-stranded cDNA was then generated by random-primed first-strand synthesis and second strand synthesis in the presence of dUTP for strand marking. Subsequently, the cDNA was 3’-A tailed and indexed, Illumina-compatible adapters were ligated. The libraries were then enriched by high-fidelity PCR amplification (13 cycles) with KAPA HiFi HotStart DNA Polymerase and adapter-specific primers. The libraries were combined for multiplex sequencing on an Illumina HiSeq 4000 System (50-bp, single read; ~30 million reads/sample).

Next Generation Sequence (NGS) data analysis

RNA-seq data was analyzed using a HISAT-Cufflinks workflow. Raw sequence reads (FASTQ format) were aligned to the reference human genome assembly (Dec. 2013, GRCh38) using HISAT2 v.2.0.3-beta (hierarchical indexing for spliced alignment of transcripts) software. Gene- and transcript-level expression were comprehensively quantified with Cufflinks v.2.2.1 software, which performed 1) transcript assembly, 2) identification of splice variants, 3) quantification of expression as FPKM (fragments per kilobase of exon per million mapped sequence reads) values, and 4) normalization. Normalized FPKM values (Cuffnorm output) were utilized for downstream analysis steps. Statistical analysis for treatment-specific DEGs (ANOVA, p<0.05),
comparison analyses (e.g., treatment vs. vehicle control), and hierarchical clustering of the data were performed with GeneSpring GX software (Agilent Technologies, Inc.). Gene Set Enrichment Analysis (GSEA) for each drug treatment was performed on GSEA web site (34) after conversion of gene ID to Affimetrix Human genome U133 Plus 2.0 Array ID. The raw sequence and processed expression data files are publicly available through NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/) with accession number GSE89478.

**Chromatin immunoprecipitation (ChIP) assays**

ChIP assays were performed as described previously (35). Briefly, BCBL-1 cells were pre-treated with JQ1 for 24 hrs, followed by treatment with PEP005 or vehicle control. Cells were fixed in 1% formaldehyde for 10 min at room temperature and quenched with glycine. DNA was sonicated to an average size of ~300 bp using a Diagenode Bioruptor. After pre-cleaning with BSA-blocked Dynabeads Protein A/G (Invitrogen), chromatin was incubated with antibodies at 4°C overnight. The chromatin immunocomplexes were collected with BSA-blocked Dynabeads. Immunoprecipitated chromatin was eluted from beads by heating for 30 min at 65°C in elution buffer (50 mM Tris-HCl, pH 8.0, 10 mm EDTA, 1% SDS). DNA was reverse crosslinked at 65°C overnight and purified with a PCR purification kit (Qiagen). All immunoprecipitated chromatin DNA samples were analyzed by SYBR green-based quantitative PCR (qPCR) (Bio-Rad) with specific primer pairs. The primer sequences are as follows: PAN promoter, 5’-GGT GGC TAA CCT GTC CAA AA-3’ (forward) and 5’-CAG CGA GCA CAA AAT CCA TA-3’ (reverse); K12 promoter, 5’-AGT GCT TTA ATG CGG GGA GG-3’ (forward) and 5’-GTT GCA CCA AGC ACA ACA TT-3’ (reverse).
ChIP-Seq analysis

Briefly, chromatin DNA from $1 \times 10^8$ BCBL-1 cells was used per each immunoprecipitation assay with 10 µg of BRD4 antibody. ChIP-enriched and input DNA samples (1 ng) were used to generate Illumina-compatible libraries with the KAPA LTP Library Preparation Kit (Kapa Biosystems, KR0453) according to the manufacturer’s recommendations. Libraries were submitted for sequencing (50-bp, single read) on an Illumina HiSeq 3000 sequencing system. The ChIP-Seq data (FASTQ sequence reads) was aligned to the human hg19 reference genome and reference KSHV genome sequence (Human herpesvirus 8 strain JSC-1 clone BAC16, GenBank: GQ994935.1) with Bowtie 2. Peak finding was performed with the MACS2 program utilizing the parameters and commands described following the developer’s manual. We used the default settings with a minimum FDR (q-value) cut-off of 0.05. The peaks and read alignments were visualized using the Integrative Genomics Viewer (IGV) browser from the Broad Institute.

Reporter assays

Reporter assays were performed as described previously (35). A dual luciferase reporter system (Promega) was used to quantify NF-κB activity. Briefly, 500 ng of pNF-κB-Luc reporter plasmid containing three direct repeats of the NF-κB responsive element was transfected into BCBL-1 cells in each well of 12-well plates. Two days after transfection, the cells were washed with PBS, and incubated with 250 µl passive lysis buffer provided by the manufacturer. A RenillaTK expression vector (10 ng/well) served as an internal control. At least three independent assays were carried out for each experiment.
Cell proliferation assays

WST-8 and MTT cell proliferation assays were performed using the Cell Counting Kit-8 (Sigma) or MTT reagents (Thermo Fisher) according to the manufacturer’s instructions. Assays were performed in triplicated wells and three independent assays were carried out.

Cytokine measurement by Luminex

Cytokines, IL-6, vascular endothelial growth factor (VEGF) and IL-10, in the culture supernatant were measured in triplicates with a combination of magnetic-beads set for each cytokine, an appropriate cytokine standard and a reagent kit from Bio-rad according to the company’s protocol. Each cytokine concentration was determined by a Bio-Plex Manager™ software (Bio-rad) based on the standard.

PEL xenografts

All animal studies were conducted according to an US Davis Institutional Animal Care and Use Committee (IACUC)-approved protocol. 5-week-old female NOD/SCID mice were injected intraperitoneally (i.p.) with $2 \times 10^7$ BC3 cells in 200 µL PBS. On day 3, mice were randomly assigned to Vehicle (5% DMSO in 2% Dextran PBS), PEP005 (10 µg/kg; daily), JQ1 (100 mg/kg; daily), and JQ1 in combination with PEP005 treatment groups. Treatments were administered by i.p. injection for 10 days. Mice were monitored daily and sacrificed when exhibiting signs of discomfort.
Statistics

SPSS version 18.0 was used for the statistical analysis of experimental data, with data presented as mean ± standard error of mean (SEM), calculated for all points from at least 3 independent experiments in triplicates. Statistical significance was determined using the ANOVA and Student’s t test. Survival curves were generated using Kaplan-Meier analysis, and statistical significance was determined using Gehan-Breslow-Wilcoxon test with Graphpad Prism software (San Diego, CA). $P$ values <0.05 were considered significant.
Results

**PEP005 induces KSHV reactivation in PEL cells *in vitro***.

KSHV-associated malignancies are most frequently seen in HIV infected patients. Ingenol-3-angelate (PEP005) has been identified as an effective drug to reactivate HIV within T cells of HIV-infected patients (14). Thus, we sought to characterize the ability of PEP005 to induce KSHV reactivation from latently infected PEL cells, because PEL is most frequently seen in HIV infected individuals. TREx K-Rta BCBL-1 cells were treated with different concentrations of PEP005 (10-1,000 nM) or doxycycline (Dox: 1 µg/ml). Dox was used for a positive control, as it induces K-Rta protein expression (under the control of a tetracycline/doxycycline-responsive promoter), which leads to KSHV lytic replication. Similar to its ability to reactivate HIV, PEP005 triggers KSHV lytic replication in a dose-dependent manner [Figure 1A (a)], at a concentration as low as 10 nM, which is the same concentration known to reactivate HIV in an *ex-vivo* model (14). In addition, PEP005 induced transient expression of both K-Rta and K-bZIP, with a peak expression occurring at 48 hours [Figure 1A (b); B, C]. High concentrations of PEP005 (500-1,000 nM) demonstrated apparent cell toxicity at 48 hours post-treatment [Figure 1D (a)].

PEP005-mediated KSHV reactivation was also seen in other PEL cell lines, HBL-6, JSC-1, BC2 and BC3 [Figure S1A (a)]. Similar to BCBL-1 cells, lower concentrations of PEP005 (*i.e.*, ≤100 nM) did not significantly induce cell death in other four PEL cell lines tested (Figure S1B). These results demonstrated that PEP005 can trigger KSHV reactivation under the same conditions in which it reacts HIV *in vitro* from latently infected T-cells isolated from patients. Although it is difficult to estimate clinical dosage for the systemic application of PEP005, we anticipate that greater than 100 nM would have significant side effects due to the molecular
action of this agent (PKC agonist). In preclinical studies, the maximum-tolerated dose in a rat model was determined as 30 µg/kg.

**PEP005-mediated KSHV reactivation from PEL cells is enhanced in combination with epigenetic drugs.**

Histone modification regulates the accessibility of transcriptional factors to the genome; thus, it has significant impacts on the gene regulation. PEP005-mediated KSHV reactivation with epigenetic drugs was next examined. We tested several agents in this group: the BET inhibitor, JQ1 (100 nM); the HDAC inhibitor, SAHA (100 nM); and the EZH2 inhibitor, GSK343 (2 µM). Cells were treated with each agent in both the presence and absence of PEP005, and KSHV reactivation was subsequently examined by immunoblotting for the expression of viral lytic antigens. Although JQ1, SAHA or GSK343 alone did not reactivate KSHV significantly at clinically-relevant dosages, all of three drugs clearly enhanced PEP005-mediated KSHV reactivation (Figure 2A). The combination of JQ1 with PEP005 has also been examined in detail for HIV reactivation (14). Thus, we decided to further study this combination. The kinetics of KSHV gene expression showed that PEP005 increased transcripts of immediate-early (a), early (b), and late (c) genes, and the combination with JQ1 increased viral gene expression of all kinetics class of the lytic genes (Figure 2B). The protein expression of K-Rta and K-bZIP confirmed the qRT-PCR results [Figure 2C, Figure S1A (b)], although timing of lytic protein expression was slightly different among cell lines.
PEP005 in combination with JQ1 induced PEL cell death and inhibited cell growth in vitro.

The effects of the drug combination on cell growth and killing were examined next. For this, the number of dead cells was counted by staining with trypan blue, and cell proliferation was examined by the MTT assay. The results showed that neither PEP005 nor JQ1 alone induced cell death at patient-relevant drug concentrations (i.e. PEP005: 10 nM, JQ1: 100 nM) (Figure 3A), although JQ1 alone could decrease cell growth in vitro (Figure 3B). Cell death rate was not increased in the drug combination treatment of PBMC B-cells from healthy donors. The combined treatment of PEP005 plus JQ1 exhibited cell killing effects on three PEL cell lines tested (i.e. BCBL-1, HBL-6, and BC3). Consistent with the cell killing effects, the growth of PEL cell lines (BCBL-1, HBL-6) was further decreased by the combination of PEP005 plus JQ1 treatment, while BJAB (KSHV negative) cell growth was not inhibited by either drug alone, or in combination (Figure 3B). PEP005 at 10 nM alone did not show significant growth-inhibitory effects in all cell lines except HBL-6 within a three-day culture period (Figure 1D). In contrast, the KSHV-negative BJAB cell was relatively resistant to treatment with the drug combination (Figure 3A, B).

Further stimulation of NF-κB signaling is a prominent component of the molecular mechanism of PEP005/JQ1-mediated KSHV reactivation.

The molecular mechanism underlying KSHV reactivation by the PEP005/JQ1 drug combination was examined next. In previous studies, PEP005 has been shown to activate the NF-κB pathway (14). NF-κB activation is tightly associated with the KSHV latency/reactivation
switch (35-37), and its activation is transiently regulated through the induction of own inhibitor, IκB. Accordingly, the phosphorylation levels of both IκB and NF-κB p65 were examined by immunoblotting. As shown in Figure 4A, PEP005 (12 nM) increased the levels of phosphorylated IκBα and NF-κB p65. The maximum level of IκBα phosphorylation was reached at 8 hours post-stimulation and decreased thereafter, while phosphorylation of NF-κB p65 exhibited a gradual and persistent increase during time course examined. Consistent with phosphorylation of IκBα, incubation with PEP005 promoted nuclear translocation of NF-κB p65 (Figure 4B, C) and lead to NF-κB p65 mediated transcriptional activity in BCBL-1 cells, which was measured with an NF-κB-specific luciferase reporter (Figure 4D).

Next, the effects of JQ1 on NF-κB recruitment to the KSHV genome were then examined by chromatin immunoprecipitation assays. First, the occupancies of BRD4, one of the major JQ1 targets, were determined in an unbiased manner by ChIP-seq analysis. The results showed that 24 hours after KSHV reactivation, BRD4 mainly bound to the KSHV IE and E gene cluster regions (Figure 4E). Based upon these results, we next designed specific primer pairs to regions that contained BRD4 recruitment sites that also overlapped with putative NF-κB target sequences on the KSHV genome (e.g., PAN promoter region), and examined the effects of the drug treatment on the recruitment of NF-κB. In addition, the K12 promoter region, where NF-κB directly binds was also included (35). Consistent with the observed nuclear translocation of NF-κB, the results demonstrated that PEP005 increased occupancies of NF-κB on the KSHV genome at the PAN RNA promoter region. We also found that PEP005 treatment alone induced dissociation of BRD4 weakly. As expected, implementation of JQ1 inhibited the binding of BRD4 to the KSHV promoter (Figure 4F). The dissociation of BRD4 enhanced recruitment of NF-κB and RNA polymerase II, and consequently increased PAN RNA
expression (Figure 2B). Similar effects were also seen in other KSHV promoters (Figure S2). Taken together, these results indicated that further activation of NF-κB by PEP005 may contribute to KSHV reactivation, and that JQ1 enhanced RNA polymerase II recruitment.

**JQ1 reduced IL-6 secretion from PEL cells.**

Inflammatory cytokine secretion from KSHV-infected cells plays an important role in the pathogenesis of KS and PEL (38). To explore the effects of the drug combination on cytokine secretion, Luminex assays were employed to measure the amount of cytokine secreted into tissue culture supernatants in response to drug treatments. Culture supernatants were harvested 24 hours after drug treatment, and the concentrations of IL-6, VEGF, and IL-10 were measured. Different drug combinations did not significantly affect cell viability at this time point (Figure 3B), thus bias in cytokine production caused by cell numbers should be minimal. The results demonstrated that overall a therapeutic dosage of JQ1 (100 nM) inhibited secretion of all three cytokines from KSHV-positive cell lines (Figure 5). Importantly, JQ1 and the combination with 10nM PEP005 reduced IL-6, an essential growth factor for PEL cells, in all three KSHV-positive cell lines tested. As expected, PEP005, an activator of NF-κB, showed a tendency of increasing the production of NF-κB-targets VEGF and IL-10 when used alone or in combination with JQ1, GSK343, or SAHA. Despite the latter, addition of PEP005 did not completely abolish the beneficial regulatory effect of JQ1 in terms of reducing inflammatory cytokine production when compared with the DMSO control, resulting in an overall favorable effect in the regulation of inflammatory cytokines. In contrast, treatment with JQ1 and PEP005, individually or as a combination, showed completely different modulatory effects on VEGF and
IL-10 production in KSHV-negative BJAB cells, which also express barely measurable amounts of IL-6.

Effects on cellular gene expression by treatment of PEL cell lines with PEP005, JQ1, or the combination.

Since the mechanisms of both PEP005 and JQ1 are known to impact gene expression via transcription factor activation (Figure 4) and/or epigenetic processes (e.g., NF-κB activation, BET inhibition, etc.), RNA-seq analysis was performed in order to better define their impact, alone and in combination, on the transcriptome in three PEL cell lines. First, unsupervised clustering of the 12 datasets (i.e., 3 cell lines × 4 treatments each) demonstrated that the data expectedly organized the samples into major clusters based on cell line, rather than treatment, thereby indicating the prominent heterogeneity of the PEL cell lines (Figure 6A). Next, comparison and statistical analyses were conducted for the identification of differentially-expressed genes (DEGs; treatment group relative to vehicle control). As shown in Figure 6B, numerous gene expression alterations were induced by the different treatments in each cell line; for instance, 2,650, 2,757, and 2,820 genes were altered in BC3 cells by PEP005, JQ1, and the combination, respectively. Notably, approximately 28.4-35.4% of the DEGs (389-435 genes) observed for each treatment were conserved among the three cell lines (Supplementary Figure S3). Of these, 226 genes exhibited statistically-significant expression changes (ANOVA, \( p<0.05 \)) in response to one or more treatments (i.e., relative to the control), and consistent with this result, hierarchical clustering of this DEG expression data organized the samples into major clusters based on treatment (Figure 6C). The significance of the PEP005+JQ1 combination treatment was then determined by evaluating its ability to further...
enhance or suppress the expression of genes that were differentially regulated by the individual treatments. The fold-change values for DEGs from each treatment (i.e., relative to control) of each cell line were visualized with heatmaps (Supplementary Figure S3B), and these showed that in addition to there being subsets of DEGs that were further up- or down-regulated by the addition of the second agent (i.e., combination treatment), there were also subsets that were counter-regulated by the combination.

Next, Gene Set Enrichment Analysis (GSEA) was performed on the DEGs that were induced by either treatment with PEP005 or JQ1 alone, and also by the PEP005+JQ1 combination. The results demonstrated that E2F target genes were significantly enriched in both JQ1 [Normalized enrichment score (NES) 1.640] and PEP005 treatment (NES 2.042) in BCBL-1, respectively. Importantly, enrichment score was further increased in the combined treatment (NES 2.362) (Figure 6D). Consistent with previous studies, treatment with JQ1 also displayed significant enrichment of MYC target genes in both JQ1 alone (NES 1.485) and in combination with PEP005 (NES 1.730) (Figure S3C) (22,39). Again, combination further increased NES score of MYC target genes (Figure S3C). The results indicated that the combination with PEP005 arguments the effects of JQ1 in PEL cells.

**PEP005 plus JQ1 delays the development of PEL in a xenograft model.**

Finally, the effects of the drug combination on *in vivo* tumor growth were evaluated in a PEL xenograft model. We inoculated female NOD/SCID mice with $2 \times 10^7$ BC-3 cells via intra-peritoneal injection followed by intra-peritoneal injections of JQ1 (100 mg/kg) alone or in combination with PEP005 (10 µg/kg), daily for 10 days starting from day 3 of tumor inoculation.
At the time point, tumors and ascites were not visible. Nonetheless, all mice that received injection of BC-3 cells developed PEL with ascites in the peritoneal cavity. Several mice also developed solid tumors subcutaneously near the site of injection. All mice in the control vehicle-treated groups died within 17 days; however, in mice that received JQ1 treatment, tumor growth was completely inhibited as evidenced by the absence of weight gain during the course of therapy on days 3-12 (Figure 7A), and extending the median survival from 12 to 23 days, compared with the vehicle-treated group (p=0.0017; Gehan-Breslow Wilcoxon test) (Figure 7B). Combination JQ1+PEP005 treatment further delayed tumor development (p=0.0049 compared to JQ-1 group) and extended the overall survival of the mice (median survival 23 and 30.5 for JQ1 and JQ1 + PEP005 group, respectively). Even though the both therapies effectively managed PEL tumor growth during the treatment period and extended mouse survival, they could not prevent tumor recurrence as evidenced by rapid weight gain as soon as the withdrawal of the therapies after day 13 (Figure 7A).

Discussion

KSHV-mediated malignancies are highly associated with HIV infection. Many drugs that target cancer cells induce stress signals (e.g. cell cycle arrest, ER stress), which in turn induce viral replication from latently infected cells. There is no clear answer if inducing latent viruses is clinically beneficial; however, having drugs (e.g. cART and ganciclovir) that only effective to the cells, in which viruses are actively replicating, oncolytic strategy to specifically target infected cells (e.g. malignant cells) could be an option for treatment. In this study, we demonstrated that PEP005 in combination with JQ1 induces KSHV reactivation in PEL cell lines. Our studies were leveraged on previous work that demonstrated effective reactivation of HIV with the same
drug combination and dosage. The main focus of this study was to determine if this “shock and kill” strategy for HIV would also be beneficial for managing KSHV associated malignancy.

KSHV reactivation was associated with transient PEP005-mediated further NF-κB activation. Interestingly, dissociation of BRD4 from the KSHV promoters appeared to enhance viral gene expression. This result was unexpected as BRD4 functions to recruit co-activators and elongation factors (17,40). However, our results are consistent with that of HIV study, which indicates BRD2/4 is a weaker activator and physically inhibits Tat recruitment into TAR/CyclinT1/CDK9 complex, therefore suppresses HIV transcription. When BRD2/4 binding to the viral genome is inhibited by JQ1, Tat binds to TAR/CyclinT1/CDK9 complex and phosphorylates CTD of RNA pol II, leading to possessive transcription of HIV. Another explanation would be that sequestration of BRD4 from cellular genomes may increase available (free) cellular RNA polymerase II and co-activators. Non-promoter bound RNA polymerase II could then be recruited to viral genomes (41,42). We think that viral transcriptional factors may have higher binding capability with RNA polymerase II (43), which then recruits RNA polymerase II to form viral transcriptional factories (41).

Our ChIP-seq study identified binding sites of BRD4, and that these sites largely overlapped with those for the latent KSHV protein LANA (44), supporting previous reports that LANA and BRD4 can physically interact with each other (45). In addition, BRD4 recruitment sites also overlapped with active chromatin marks, suggesting authenticity of the BRD4 binding sites identified in this study (46).

In our Luminex studies, we observed that JQ1 substantially reduced IL-6, VEGF and IL-10 production by PEL cell lines, a finding that was more prominent in KSHV-infected cells than KSHV-negative cells. This observation is supported by other studies demonstrating JQ1’s ability to
mute inflammation-associated gene expression. KSHV+ cells exhibit constitutively active NF-κB due to the presence of the latent viral proteins, and NF-κB activation is critical for both PEL cell growth and inhibition of cell apoptosis; this makes BET inhibitor an attractive option as a therapeutic approach for KSHV associated malignancies (36,37,47). In fact, PEP005 treatment alone, which further activates NF-κB and triggers KSHV reactivation, increased VEGF and IL-10 production in both KSHV+ (i.e. HBL-6, BC3 and BCBL-1) and KSHV- (i.e. BJAB) cell lines whereas PEP005 + JQ1 treatment reduced IL-6 in all KSHV-positive cell lines tested and controlled the levels of VEGF and IL-10 (Figure 5). Thus, PEP005 + JQ1 treatment had an overall favorable effect on the regulation of inflammatory cytokines.

RNA-sequencing was performed to comprehensively define each treatment’s impact on the transcriptome, and to gain insight into the downstream effects on cellular functions. In each of the cell lines, these treatments had marked effects upon gene expression accounting for up to 5.11% (3,098 genes) of the annotated transcriptome, such as observed for the combination treatment of HBL6 cells (Figure 6B). While we could identify treatment-specific DEGs that were conserved among the three cell lines (Figure 6C, panel b), the RNA-seq results also revealed that there was significant heterogeneity, or distinctiveness, in the responses of each cell line to these agents, since unsupervised clustering resulted in organization of the data according to cell line (Figure 6A). Consistent with previous work (22), Gene Set Enrichment Analysis demonstrated that JQ1 targeted E2F and Myc target gene expression, and the combination further potentiate the targeting effects.

In a NOD/SCID xenograft mouse model, we showed that the JQ1 successfully delayed and suppressed overall PEL tumor growth and prolonged host survival, which was further improved by combining with PEP005 to some extent (Figure 7). For treatment of skin cancer,
in which high local concentration achievable by topical application, PEP005 strongly induced cancer cell death (48). In our mouse model, however, we could not apply such higher PEP005 amount systemically due to its intrinsic toxicity. In addition, with our NOD/SCID PEL-xenograft mouse model, we could not expect immune stimulatory effects by the PKC agonist. Accordingly, we did not include PEP005-alone group in our xenograft studies after consideration of minimum effects on cell growth in vitro (Figure 1). Consistent with our in vitro observations that JQ1 did not possess strong cell killing capability (Figure 3A); the remaining viable PEL cells resumed growth as soon as the drug treatment was terminated. Similar results have been seen in other xenograft studies with JQ1 (22,39). Thus, the possible approach would be that administrating JQ1 to control tumor growth by targeting E2F and MYC (Figure 6D, Figure S3C), and providing specific cell killing effects by inducing KSHV reactivation and/or cytolytic drugs. While JQ1 provides other beneficial effects such as inhibition of IL-6, VEGF and IL-10 production in addition to cell growth inhibition (Figure 5), combining with other cancer drugs carrying cytolytic activities will be important to eradicate PEL cells. On the other hand, in vitro, we could readily increase the cell death rate by increasing PEP005 and/or JQ1 concentration (Figure 1D). This indicates that we can significantly improve prognosis if we could use higher concentration of drugs without evoking unfavorable side effects to patients. In this regard, new drug delivery methods such as nanotheranotics approaches, which allow increase in local drug concentration or promote drug uptake by tumors without increasing systemic drug concentration, should be the key for one of future interventions (49). Rationally selected drugs in conjunction with novel drug delivery methods, will improve prognosis of this devastating disease. Nonetheless, based on favorable effects on inflammatory cytokine production from PEL and enhancement of antigenic KSHV lytic gene expression in the cancer
cells, and mild side effects with convenient oral administration (50), BET inhibitor would be considered to be included in the current combinatorial regimens.

In summary, we have examined a drug combination, which is suggested to reactivate HIV from latently infected cells. We found same drug combination robustly activated KSHV from latently infected B-cells and has demonstrated some efficacy in preventing tumor growth in a pre-clinical PEL xenograft model.
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Figure Legends

Figure 1. Effects of PEP005 on KSHV latently infected cells. **(A)** KSHV reactivation by PEP005. Immunoblotting was performed 48 hours after incubation with PEP005. Indicated viral and cellular proteins were probed with specific antibody. *(a)* different doses of PEP005 and *(b)* time course was taken to examine KSHV reactivation. DOX: doxycycline. **(B)** Immunofluorescence analysis. KSHV reactivation was examined with IFA and **(C)** number of K-Rta positive cells was counted. **(D)** Effects on cell growth. TRExBCBL-1 cells were incubated with different dose of PEP005 and effects on cell death *(a)* and cell growth *(b)* were examined by MTT assays.

Figure 2. KSHV reactivation by PEP005 with epigenetic drugs. **(A)** Induction of KSHV lytic proteins. Viral protein expression was examined by immunoblotting. Indicated drug combination was used to treat four different PEL cell lines with 10 nM PEP005, 100 nM JQ1, 2 µM GSK343 or 100 nM SAHA, alone or in combination with 10 nM PEP005 for 48 hours. Forty-eight hours after treatment, indicated viral proteins were probed with specific antibody. Actin was used for a loading control. **(B)** qRT-PCR. Total RNA was harvested from BCBL-1 after treating cells with 10 nM PEP005, 100 nM JQ1 or in combination at indicated time points. Viral gene expression was measured by qRT-PCR. Viral gene names were shown and actin was used for internal control. **(C)** Dynamics of lytic protein induction. KSHV lytic protein expression at indicated time point after the drug treatment was examined.

Figure 3. Effects of drug combinations on PEL cell growth. **(A)** JQ1 alone does not induce cell death. Indicated drugs (PEP005, 10 nM; JQ1, 100 nM) were incubated with different B-cell lymphoma lines, and cell killing effects were measured by trypan blue staining after 48 hours of incubation. **(B)** Inhibition of cell growth by the combination PEP005 with JQ1. B-cell lymphoma lines were incubated with indicated drugs (PEP005 10 nM, JQ1 100 nM) and cell growth were measured with MTT analyses in triplicate. At day 3 (72 hrs), all wells were reseeded once again with same numbers of cells with appropriate dilution to prevent overgrowth. Each drugs
were also added, when we reseed the cells. Dilution factors were multiplied (where necessary, e.g. control) to the value of O.D. 570 at 96 and 144 hrs time points. Values are represented as means ± SEM. ns; not significant, * $p<0.05$, **$p<0.01$

**Figure 4. A molecular mechanism of KSHV reactivation by the drug combination. (A) Activation of NF-κB pathway by PEP005 in PEL cells.** BCBL-1 cells were treated with PEP005 and harvested at indicated time points. NF-κB activation was examined with phosphor-specific antibodies, nuclear translocation of p65 with IFA (B), immunoblotting after fractionation of nuclear protein (C), and reporter analysis (D). (E) BRD4 recruitment sites on KSHV genome. ChIP-sequence analysis was performed with anti-BRD4 specific antibody in TREx BCBL-1 during KSHV reactivation. Enrichments over input DNA is shown. (F) Effects of PEP005 and PEP005/JQ1 combination. qPCR was used to determined recruitment of NF-κB, BRD4, RNA polymerase II, and phosphorylated form of RNA polymerase II. Mouse IgG was used as background control.

**Figure 5. Inhibition of cytokine production by JQ1.** Amount of cytokines produced in supernatant was measured by Luminex assay. Indicated drugs were added in culture media and supernatants were harvested at 24 hours post treatment. Three cytokines were measured for four different B cell lymphoma lines, including KSHV negative BJAB cells. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, *$p<0.05$.

**Figure 6. Transcriptome profiling of PEL cell lines treated with KSHV-reactivating agents.** Three PEL cell lines were treated for 24 hours with vehicle control (DMSO), JQ1, PEP005, or the combination (JQ1 + PEP005). RNA-seq analysis was performed on total RNA samples (200 ng) followed by data analysis with a HISAT-Cufflinks pipeline to yield normalized FPKM expression values. (A) Hierarchical clustering of the 12 transcriptomes (i.e., all values passing filter) was performed using the CummeRbund package in R. (B) The relatedness of differentially-regulated genes (by ≥2-fold relative to DMSO) associated with different KSHV-reactivating treatments in each
cell line were identified by intersection analysis and depicted with Venn diagrams. The number of DEGs that were either up- or down regulated are indicated in each section of the diagram. (C) Hierarchical clustering performed on treatment-induced DEGs (ANOVA, \( p < 0.05 \), \( n = 226 \)) demonstrates clustering of the samples according to treatment, as shown by the (a) dendrogram and (b) heatmap of expression values. (D) Gene Set Enrichment Analysis. Gene enrichment score plot shows enrichment of gene sets containing target genes of E2F among affected by JQ1, PEP or in combination in BCBL-1 cells. NES, normalized enrichment score; FDR, false discovery rate.

**Figure 7. Inhibition of PEL growth in xenograft PEL model.** NOD/SCID mice were injected i.p. with \( 2 \times 10^7 \) BC3 cells. On day 3, JQ1 (100 mg/kg; daily)(n=5), JQ1 (100 mg/kg; daily) + PEPO05 (10 \( \mu \)g/kg; daily)(n=10) or vehicle (n = 10) were administered i.p., once daily, for 10 days. Mice were monitored daily and PEL tumor-related deaths were recorded. (A) Average weight gain of surviving mice in each group at indicated time points are shown. Control vs JQ1, and Control vs JQ1/PEP are statistically different on day 4 \( (p<0.05) \) and day 5-12 \( (p<0.001) \) after tumor inoculation. (B) Kaplan-Meier plot of PEL tumor-related deaths in control (median survival 12 days), JQ1 (median survival 23 days) and JQ1/PEP (median survival 30.5 days) group are shown. Survival curves are statistically different between control and JQ1 \( (p=0.0017) \) and control and JQ1/PEP \( (p<0.0001) \), and JQ-1 and JQ-1/PEP \( (p=0.0049) \).
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