SI APPENDIX

SUPPLEMENTAL FIGURES

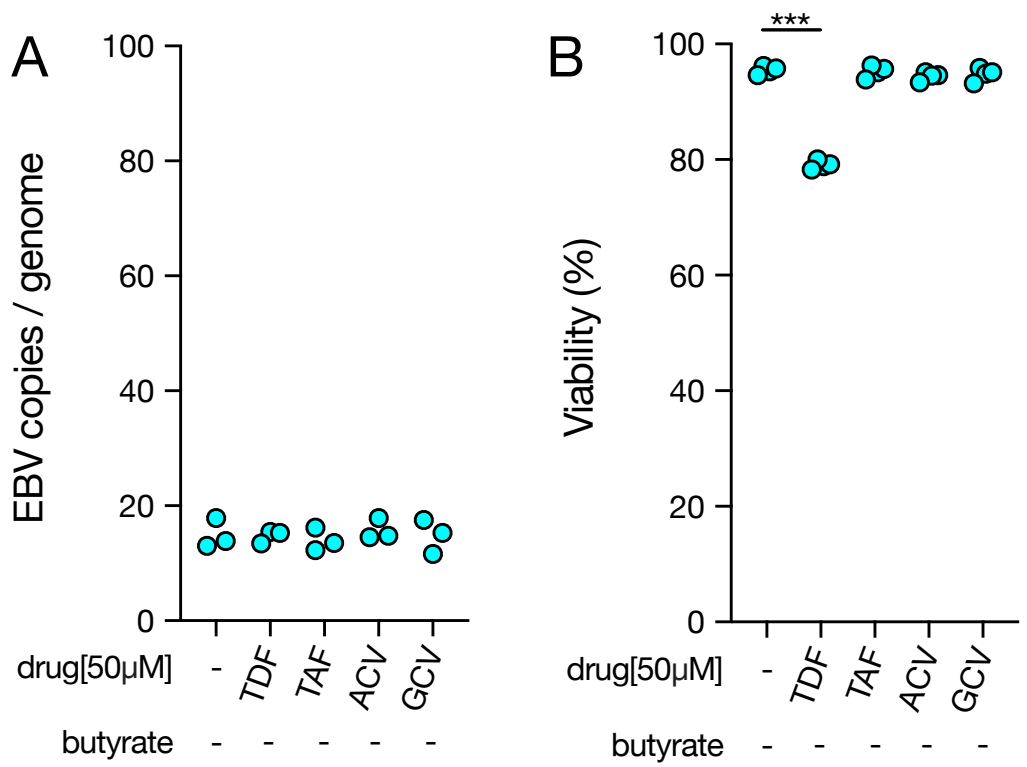

Figure S1: Effects of TDF and TAF on EBV DNA copies during latency

(A) HH514-16 cells were grown in the absence of butyrate for 96 hours and treated with the tenofovir prodrugs TDF and TAF, or the established anti-herpesviral drugs ACV and GCV. EBV copies per genome were measured by qPCR after 96 hours of treatment at a 50μM dose. Results were analyzed from 3 independent experiments, each value shown. One-way ANOVA was performed ($p>0.05$). (B) Viability was measured by trypan blue exclusion after 96 hours. Results were analyzed from 4 independent experiments, each value shown. One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between no drug and each condition. Statistical significance is highlighted by P values as follows: ***$P < 0.001$.

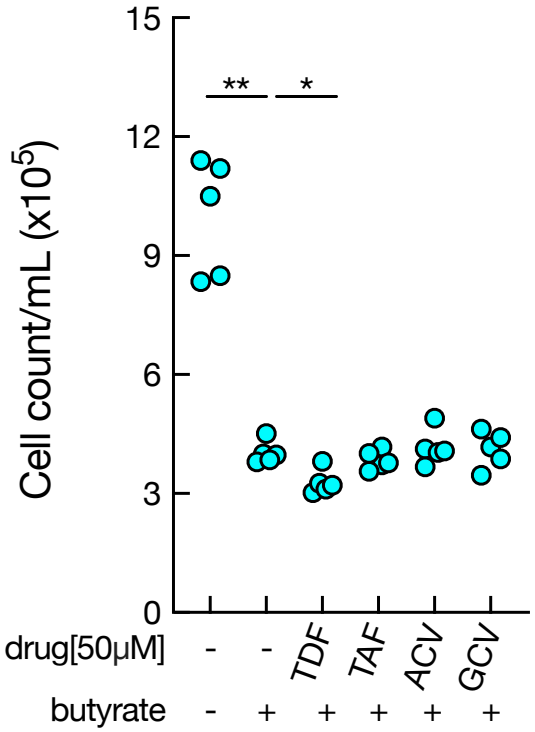

Figure S2: Effects of butyrate and drug treatment on the number of cells after 96 hours

HH514-16 cells were treated with the tenofovir prodrugs TDF and TAF, or the established anti-herpesviral drugs ACV and GCV. Cell count per mL was measured after 96 hours of treatment at a 50μM dose. Results were analyzed from 5 independent experiments, each value shown. (B) One-way ANOVA ($p<0.05$) was followed by multiple hypothesis testing between butyrate and each condition. Statistical significance is highlighted by P values as follows: *$P<0.05$; **$P < 0.01$

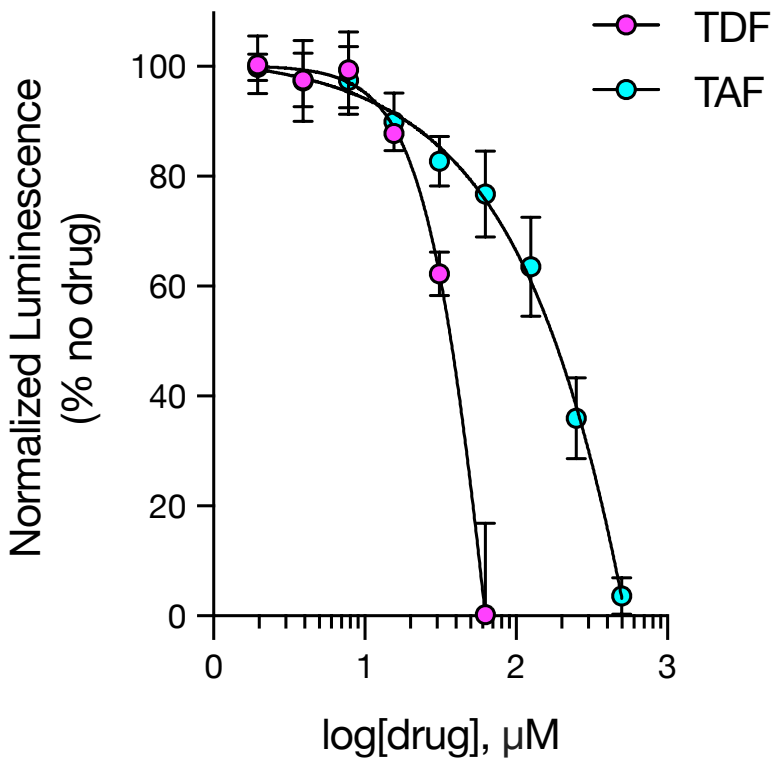

Figure S3: Effect of TDF and TAF dose on the number of viable cells in latency after 96 hours

HH514-16 cells were treated with TDF or TAF at indicated doses in the absence of butyrate. Total cell viability was measured by an ATP-dependent luminescence assay after 96 hours of treatment, which is dependent on both cell proliferation and percent viability. Luminescence was normalized using the signal from untreated samples (no cytotoxicity) and the signal from samples treated with 10μM podophyllotoxin (max. cytotoxicity). Results were analyzed from 3 independent experiments. Values shown represent mean and SD. A dose-response curve was fit by linear regression analysis.

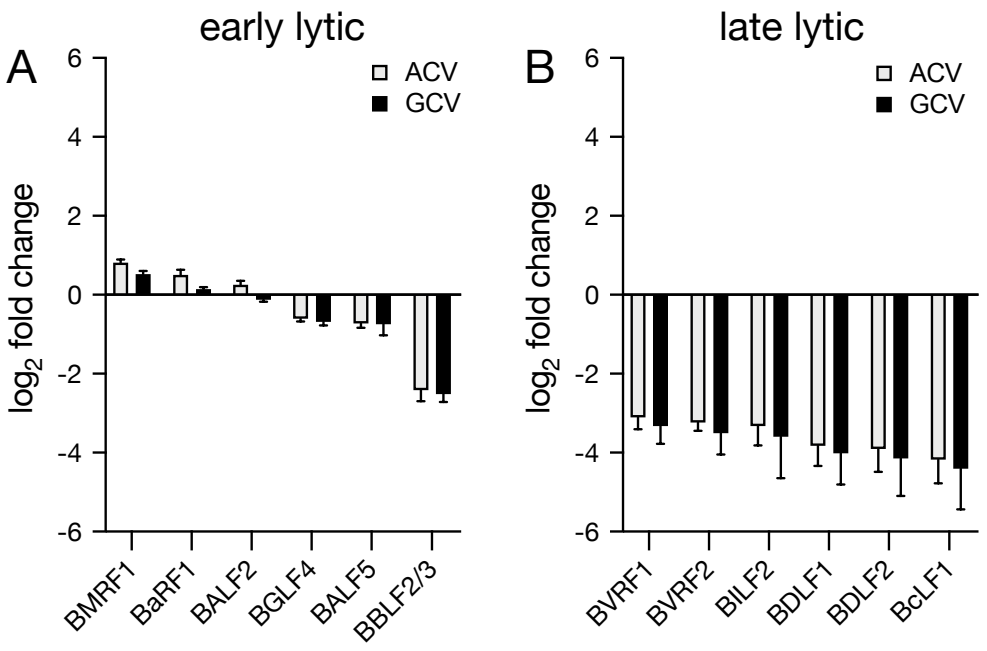

Figure S4: Effects of ACV and GCV on expression of early and late lytic viral genes

(A) HH514-16 cells were induced with 3mM butyrate and treated with ACV[62.5μM], GCV[5μM], or no drug. Total RNA was collected at 72 hours and used to measure the expression of six early and (B) six late lytic genes by RT-qPCR. Gene expression was normalized to the housekeeping gene HPRT1. Each column represents the mean and SD obtained from 3 independent experiments.

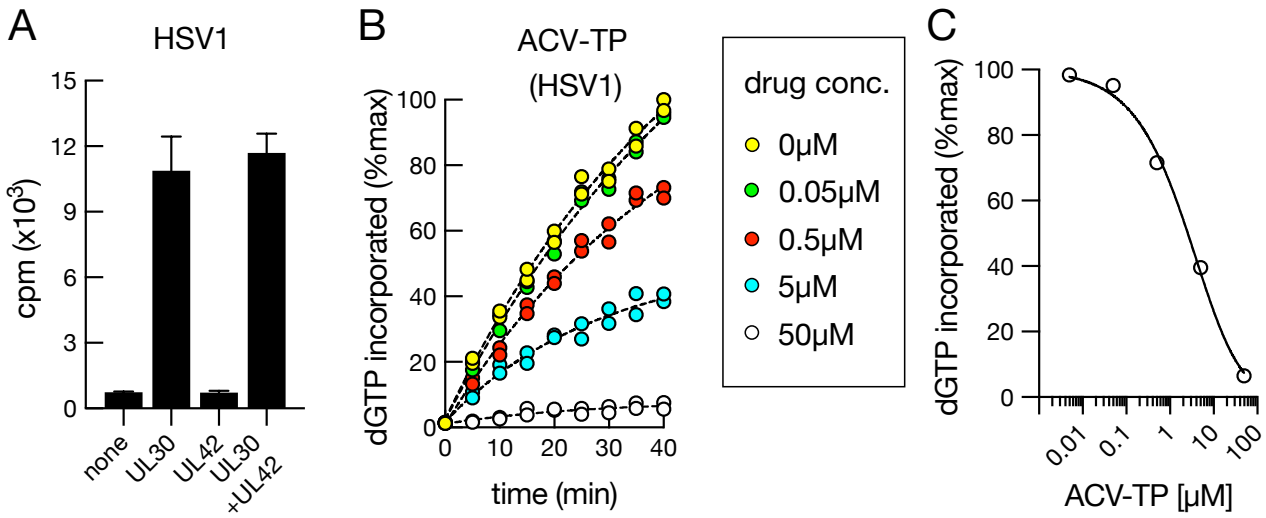

Figure S5: ACV-TP effects on the HSV-1 DNA polymerase

The HSV-1 polymerase subunits (UL30 and UL42) were cloned into pcDNA3.1+ vector and produced using a transcription/translation system in Rabbit reticulocyte lysates. (A) *In vitro* polymerase assays were performed by measuring the incorporation of [^{3}H]dGTP into activated calf thymus DNA during a 40 min reaction followed by DEAE filter-binding and scintillation counting. Each column represents the mean and SD of counts per minute (cpm) obtained from 3 independent experiments. (B) ACV-TP was added to the reaction in the presence of 1μM [^{3}H]dGTP from 0-50μM. Every 5 minutes, aliquots were removed and quenched with EDTA. Counts per minute were normalized to maximum counts obtained at 40 min. Results were analyzed from 2 independent experiments, each value shown. (C) Dose-response curves were generated from mean inhibition of dGTP incorporation at 40 min by five-parameter logistic regression analysis.

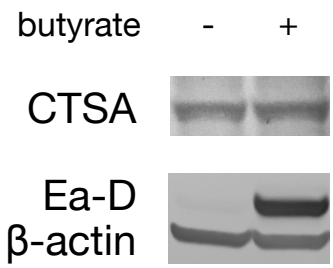

Figure S6: HH514-16 cells express cathepsin A

HH514-16 cells were induced with 3mM butyrate. Total protein was collected at 72 hours and stained for cathepsin A, Ea-D, or β-actin by western blot.

Table S1: Primers and probes for qPCR

Gene	Primer 1 (5'-3')	Primer 2 (5'-3')	Probe (FAM/ZEN/Iowa Black FQ)
BALF2	CTACCAGGAGGGAGAACTACAT	GTACAGGCTTGAGAGCTTAGTG	TCCTCAACACCTACCACAAGACCCTA
BALF5	CGGAAGCCCTCTGGACTTC	CCCTGTTTATCCGATGGAATG	TGTACACGCACGAGAAATGCGCC
BGLF4	GGCTGGTCCTGACTGATTATG	TCTGGCAATAGAGGCGATAGA	ATGCTGGATGTGCGGCTAAAGTCT
BaRF1	GATTCTTGTGTTCCTGCTGATTG	GAGCAGCTCATCCCTACTTATG	TGGCATCTGCCTGGCCAATAACTA
BBLF2/3	CAGTCCTCCTCAGATCTCAAAC	CTGCGGTCTGGGTAACATTAT	AATGATGGAAACACCCGCGGAGA
BMRF1	TTTACAGGTCTGGCATCATAGC	CTTCGGAGGCGTGGTTAAATA	AACACTAAGATCCAACGGCAGGTCC
BILF2	GGGAAGAAGACGACCAATACC	TTGTGGTGTGGGAGACTAATG	ACCCTCACTCAGCGTATGCATCTT
BcLF1	GTGGAGGCATGGATCGTAAA	TTGAGGCTGTTTAGGGTATGG	AATGCTCTCAAGGTTCTCTGTCACCC
BDLF1	AAATGGTGCCGGATGAGATAG	GATGCCAAAGACTGTAGGTAGG	TGACCTGTCAGTGGCGGATGATTT
BDLF2	GTGTCCGACCAATCCATTCT	ATGGCTGTCCGTGTTGTT	TATCCCGGCCGTGGTCATTAACAA
BVRF1	CCCAGTATGCAGTCTCAATCTC	GGTACCCACTGGTTGATGTT	ACGCTGCTTGGTAATCTGTACGGG
BVRF2	CTTCTTTGACCACGTGTCTATCT	GACGGTTCCAGGTCACTAAAG	ACGGCAGTCTACGGTACAGACCTT
HPRT1	TTGTTGTAGGATATGCCCTTGA	GCGATGTCAATAGGACTCCAG	AGCCTAAGATGAGAGTTCAAGTTGAGTTTGG

SUPPLEMENTAL METHODS

Quantification of EBV copy number by qPCR

Cells were centrifuged at 500xg for 3 min and washed once with PBS. Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen) and subjected to qPCR. A 20µL reaction was set up with 250ng genomic DNA. The PCR mix contained 1x PrimeTime qPCR Master Mix (Integrated DNA Technologies), 500 nM each primer, and 250 nM probe.

Primers and fluorescent probes (5'FAM/ZEN/3'IBFQ) for the BALF5 gene of EBV and GAPDH (Integrated DNA Technologies) are listed below:

BALF5 primers: 5'-CGGAAGCCCTCTGGACTTC-3', 5'-CCCTGTTTATCCGATGGAATG-3', BALF5 probe: 5'-TGTACACGCACGAGAAATGCGCC-3', GAPDH primers: 5'-ACATCACCCCTCTACCTCC-3', 5'-CTCCCACCTTTCTCATCCAAG-3', GAPDH probe: 5'-AAAGCCAGTCCCCAGAACCCC-3'.

The following cycling conditions were used: 95°C for 10 min followed by 40 cycles with 95°C for 15s and 60°C for 45s carried out on a LightCycler 480 system (Roche Diagnostics). GAPDH was used as an internal standard. The calculation of the relative amounts of BALF5 DNA compared to GAPDH DNA was performed using the $2^{-\Delta\Delta Cp}$ method using the Cp (second derivative maximum) calculated by the LightCycler software. Each experiment was performed with three biological replicates. To calculate the absolute number of EBV copies, the EBV BALF5 gene was cloned by PCR from HH514-16 genomic DNA into pcDNA3.1+ vector. Absolute EBV DNA copy number was calculated from a standard curve generated by serial dilutions of pcDNA3.1-BALF5 added to human genomic DNA extracted from the EBV-negative A549 cancer cell line (ATCC).

Measurement of cell viability by trypan blue

Cells (seeded at an initial concentration of $4x10^5$ cells/mL), were examined at timepoints and drug concentrations indicated in the text. Viability was measured by trypan blue exclusion using a 1:1 mixture of a cell suspension with 0.4% trypan blue in PBS, and quantified using an Auto T4 Cellometer Cell Counter (Nexcelom Biosciences).

Luminescent total cell viability assay

Cells were seeded at a density of $4x10^5$ cells/mL in a volume of 200µL in 96 well plates. After 96 hours, the cells were mixed with CellTiterGlo reagent (Promega) according to manufacturer's instructions, and luminescence was quantified using a Varioskan Flash plate reader (Thermo Fisher). Luminescence was normalized using the signal from untreated samples (no cytotoxicity) and the signal from samples treated with 10µM podophyllotoxin (Sigma) (max. cytotoxicity).

Dose-response curves for inhibition of EBV DNA replication and calculation of IC_{50}

Dose-response curves for inhibition of EBV lytic DNA replication were fit by four-parameter logistic regression analysis using the Prism 8 software (GraphPad), and IC_{50} measurements were calculated by the software using best-fit values.

Calculation of effective inhibitory concentration of TFV-DP in PBMCs from *in vitro* polymerase assays

Dose-response curves for inhibition of EBV lytic DNA replication were fit by five-parameter logistic regression analysis using the Prism 8 software (GraphPad) with top and bottom plateaus specified as 100 and 0, respectively. EC_{50} measurements and Hill slope were calculated by the

software using best-fit values. The following equation derived from the Hill equation was used to calculate EC_F, the concentration required to produce F, the percent response. Here, EC_{50} is the concentration that leads to 50% maximal response, and H is the Hill slope:

$$EC_F = \left(\frac{F}{100 - F}\right)^{\sqrt{H}} \times EC_{50}$$

Western blotting for early antigen and cathepsin A expression

Cells were centrifuged at 500xg for 3 min and washed once with ice-cold PBS. Cells were lysed in RIPA buffer supplemented with protease inhibitor tablets (Sigma) and phosphatase inhibitor cocktail (Thermo Fisher), on ice for 30 minutes. Cell lysates were centrifuged at 14,000xg for 30 min at 4ºC to remove debris, and supernatants were assayed for protein concentration by BCA assay to normalize for total protein. Samples were boiled in 1X NuPAGE LDS sample buffer (Thermo Fisher) with 2-mercaptoethanol at a final concentration of 2.5% for 10 minutes and loaded in a NuPAGE 10% Bis-Tris Protein Gel (Thermo Fisher). The gel was transferred to a nitrocellulose membrane using the iBlot gel transfer device (Thermo Fisher). Membranes were blocked for 30 min at room temperature in PBS + 0.1% Tween-20 (PBS-T) + 5% nonfat dry milk (Bio-Rad) and incubated with primary antibodies in with PBS-T with 10% StartingBlock blocking buffer (Thermo Fisher) overnight. Membranes were then washed with PBS-T for 15 min, incubated with HRP-linked secondary antibodies (Cell Signaling) for 2 hours at room temperature, washed in PBS-T for 30 minutes, and developed using 1 Step Ultra TMB Blotting Solution (Thermo Fisher). Blots were imaged with the Bio-Rad ChemiDoc XRS imaging system. Antibodies were obtained and used at the following dilutions: mouse anti-EBV EA-D (Thermo Fisher) at 1:100, rabbit anti-β-actin (Cell Signaling) at 1:1000, mouse anti-human CTSA (R&D

systems) at 1μg/mL, anti-rabbit IgG HRP (Cell Signaling) at 1:1000, and anti-mouse IgG HRP (Cell Signaling) at 1:1000.

Quantitative RT-PCR for measurement of gene expression

Total RNA was isolated from HH514-16 cells using the RNeasy Mini Kit (Qiagen). RNA (2.5μg) was used for synthesis of cDNA using the SuperScript IV VILO Master Mix with ezDNase kit (Thermo Fisher), and cDNA was then used at a 1:10 dilution for qPCR. Each reaction contained 1x PrimeTime qPCR Master Mix (Integrated DNA Technologies), 500 nM each primer, and 250nM fluorescent (5'FAM/ZEN/3'IBFQ) probe. The following cycling conditions were used: 95°C for 10 min followed by 45 cycles with 95°C for 15s and 60°C for 45s carried out on a LightCycler 480 system (Roche Diagnostics). HPRT1 was used as a housekeeping gene for normalization of gene expression. The calculation of relative gene expression was done by the $2^{-\Delta\Delta Ct}$ method using the Cp (second derivative maximum) calculated by the LightCycler software. Primers and fluorescent probes for gene expression (IDT) are listed in supplemental Table 1.

Recombinant HSV-1 DNA polymerase assays

Assays were performed as described for EBV DNA polymerase assays except that the UL30 and UL42 subunits were cloned by PCR from HSV-1 genomic DNA, MacIntyre strain (ATCC) into pcDNA3.1+ . For drug-triphosphate testing, only the UL30 protein was used for polymerase assays since the UL42 protein was dispensable for activity. After desalting, samples were diluted to normalize for polymerase activity to that obtained with the EBV polymerase.

Pre-treatment of cells in latency prior to induction of viral lytic DNA replication

Cells were seeded at an initial concentration of $4x10^5$ cells/mL and incubated for 48 hours in media without butyrate. After this time, drugs were added at the indicated concentrations, and cells were incubated for an additional 24 hours. Cells were then centrifuged at 500xg for 3 min and washed five times with fresh media without drug. After the final wash, cells were resuspended at a concentration of $4x10^5$ cells/mL in media containing 3mM butyrate. To remove supernatant for drug testing, half of the cell suspension from this step was re-centrifuged, and the butyrate-containing supernatant was filtered using a 0.2μM cellulose acetate syringe filter. Cells previously unexposed to drug were resuspended in this post-wash supernatant at a concentration of $4x10^5$ cells/mL. Genomic DNA was removed for measurements of EBV copy number every 24 hours.

Statistical Analysis

As indicated in text, a Brown-Forsythe and Welch ANOVA for unequal variances was initially used. Multiple hypothesis testing was then conducted, with correction for multiple testing using the Prism 8 software (GraphPad).