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Supplementary Information

Assessment of combined antiviral drug treatment against selected α-, β-, and γ-herpesviruses

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Supplementary Methods

Cell culture and viruses

Primary human foreskin fibroblasts (HFFs) were cultivated in minimal essential medium (MEM, 21090022, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS, F7524, Sigma Aldrich, St. Louis, MO, USA), 1% GlutaMAX^{TM} (35050038, Thermo Fisher Scientific), and 10 µg/ml gentamicin and incubated at 37 °C, 5% CO_2, and 80% humidity. African green monkey kidney cells (Vero76) were specifically cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) as well supplemented with 10% fetal bovine serum (FBS, F7524, Sigma Aldrich), 1% GlutaMAX^{TM} (35050038, Thermo Fisher Scientific), and 10 µg/ml gentamicin and incubated at 37 °C, 5% CO_2, 80% humidity. HFF cells were infected with HSV-1 VP22-GFP and HCMV AD169-GFP, while Vero76 cells were infected with MHV-68-Luc as a murine EBV homolog.

Antiviral compounds

Antiviral compounds were obtained from the following sources: abemaciclib (ABE; MedChemExpress, Monmouth Junction, NJ, USA), brincidofovir (BCV; Biomol GmbH, Hamburg, Germany), LDC4297 (Lead Discovery Center GmbH, Dortmund, Germany) and the NEC-binding investigational drug P-020 (AiCuris AG, Wuppertal, Germany). The compounds were dissolved in sterile DMSO (Sigma Aldrich) and stored at -20 °C.

Cytotoxicity assessment via Neutral Red (NR) uptake assay

To determine the cytotoxicity of the compounds (1) used, a cell viability assay based on NR uptake was performed. For this purpose, 1.35×10^4 cells/well were seeded in a 96-well plate. The following day, they were treated with either the compound, DMSO as a solvent control, or 1 µM staurosporine as a cytotoxic positive control. At 7d post-infection (p.i.), 40 µg/mL of Neutral Red (NR) was added and the plates were incubated. After removing of the NR solution and destaining of the cells, cellular neutral red uptake was quantified using a Victor X4 microplate reader (PerkinElmer, Waltham, MA, USA) (560/630 nm).

Assessment of viral replication via fluorescence- and luminescence-based reporter readouts

To assess the antiviral efficacy of compounds, we used green fluorescence protein (GFP) (2) and luciferase (Luc) (3, 4) based reporter systems, as already described previously. In detail for HSV-1 and HCMV, 1.35×10^4 HFF cells/well were seeded in a 96-well plate. Infection was performed with HSV-1 VP22-GFP (5) or HCMV AD169-GFP (2) MOI 0.25-0.5 GFP-FU/7d, respectively. 32h p.i for HSV-1 and 7d p.i. for HCMV following infection, the cells were fixed with 10% formalin and viral reporter fluorescence was quantified using a Victor X4 microplate reader, alternatively, after fixation, cells were permeabilized using 0.2% Triton-100 and the nuclei were stained using Höchst dye before measurement using the ImageXpress Pico (Molecular Devices, LLC, San Jose, CA, USA). Regarding MHV-68, we employed a luciferase-based

assay system. For this purpose, 3×10^4 Vero cells/well were seeded in 96 well format and infected with MHV-68 (MOI 0.25-0.5 Luc-FU/7d). 4 days p.i cells were lysed using a lysis buffer consisting of a 100 mM phosphate buffer and 15 mM $MgSO_4$, to which 1% Triton X-100 was added. Then 50 µl of measurement buffer, consisting of base buffer with 10% ATP and 1 mM D-luciferin, was added per well. The resulting emitted luminescence was immediately detected using a Victor X4 microplate reader (PerkinElmer).

Compound interaction analysis via Loewe additivity fixed-dose assay

To evaluate the potential for dose reduction while maintaining efficacy, we utilized the Loewe additivity fixed-dose assay with defined dosages of each compound. This assay examines the antiviral interactions of two inhibitors. For this purpose, we selected a fixed ratio of the antiviral agents and then performed eight-fold serial dilutions chosen to cover the biologically relevant range of activity. The antiviral efficacy was determined using CompuSyn software (6) and quantified using the combination index (CI) (7-9). The subsequently calculated weighted CI (CI_{wt}) allows to interpret the interaction pattern. Only results with an r-value greater than 0.9 were included in the final evaluation.

Compound interaction analysis via Bliss independence checkerboard assay

The Bliss independence checkerboard assay was used to analyze substance interactions (10) across a broad matrix of concentration pairs, as already described previously (11). For this purpose, 1.35×10^4 cells/well were seeded in a 96-well plate for HSV-1, and 90 minutes after infection, the inhibitors, according to an 8x8 matrix, DMSO as a solvent control, or MEM were added. Technical triplicates were used, and the cells were fixed, using a slightly modified form of the GFP assay, 7 days post-infection. The cells were then measured using a Victor X4 Microplate Reader (PerkinElmer).

Time-of-addition (ToA) antiviral drug analysis

To determine whether the timing of the compound addition affects its effectiveness against an infection with HCMV; ABE, BCV, LDC4297 or P-020 was added at different time points: (i) three days before infection with repeated application on the day of infection; (ii) on the day of infection; (iii) two days after infection; and (iv) as a single dose two days before infection. 1.35×10^4 HFF cells/well were seeded in a 96-well plate and infected with HCMV AD169-GFP (MOI 0.25-0.5 GFP-FU/7d). 7d p.i., the cells were fixed and measured using a Victor X4 microplate reader.

Supplementary Figure

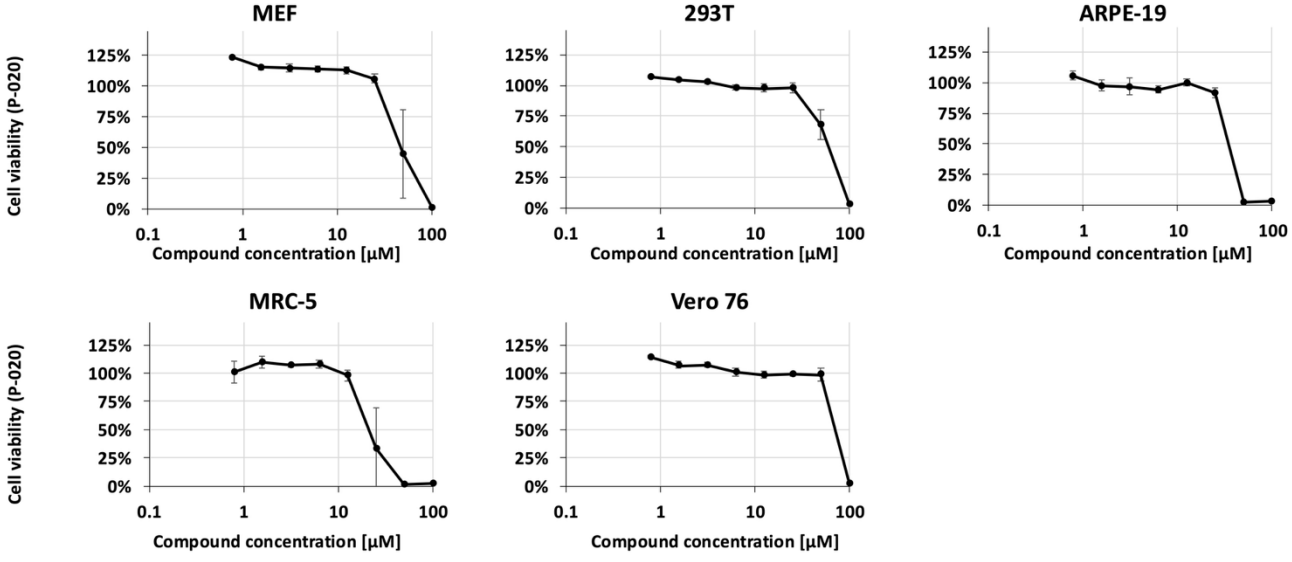

Fig. S1. Levels of cytotoxicity determined for the recently identified pharmacophore compound P-020. Selected cell types were treated with P-020 at a series of concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µM. At 5 d post-treatment, cell viability was measured by Neutral Red uptake assay (triplicate determination), and values were expressed as a percentage relative to DMSO.

Supplementary References

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