Activity and Mechanism of Action of HDVD, a Novel Pyrimidine Nucleoside Derivative with High Levels of Selectivity and Potency against Gammaherpesviruses


Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium; University of Georgia College of Pharmacy, Athens, Georgia, USA; Pathology Department, Universitair Ziekenhuis Leuven, Leuven, Belgium; Yale University School of Medicine, New Haven, Connecticut, USA

A novel nucleoside analogue, 1-(2,4S-2-(hydroxymethyl)-1,3-dioxolan-4-yl)-5-vinylpyrimidine-2,4(1H,3H)-dione, or HDVD, was evaluated against a wide variety of herpesviruses and was found to be a highly selective inhibitor of replication of the gammaherpesviruses Kaposi’s sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). HDVD had also a pronounced inhibitory activity against murine herpesvirus 68 (MHV-68) and herpes simplex virus 1 (HSV-1). In contrast, replication of herpesvirus saimiri (HVS), HSV-2, and varicella-zoster virus (VZV) was weakly inhibited by the compound, and no antiviral activity was determined against human cytomegalovirus (HCMV) and rhesus rhadinovirus (RRV). The HDVD-resistant virus phenotype contained point mutations in the viral thymidine kinase (TK) of HSV-1, MHV-68, and HVS isolates. These mutations conferred cross-resistance to other TK-dependent drugs, with the exception of an MHV-68 mutant (E358D) that exhibited resistance only to HDVD. HSV-1 and HVS TK-mutants isolated under selective pressure with bromovinyldeoxyuridine (BVDU) also showed reduced sensitivity to HDVD. Oral treatment with HDVD and BVDU was assessed in an intranasal model of MHV-68 infection in BALB/c mice. In contrast to BVDU treatment, HDVD-treated animals showed a reduction in viral DNA loads and diminished viral gene expression during acute viral replication in the lungs in comparison to levels in untreated controls. The valyl ester prodrug of HDVD (USS-02-71-44) suppressed the latent infection in the spleen to a greater extent than HDVD. In the present study, HDVD emerged as a highly potent antiviral with a unique spectrum of activity against herpesviruses, in particular, gammaherpesviruses, and may be of interest in the treatment of virus-associated diseases.

Herpesviruses are major human pathogens that have the ability to establish lifelong latent infections. Both primary infections and reactivations can cause life-threatening diseases, particularly in immunocompromised patients. Clinical manifestations of herpes simplex virus (HSV) infections range from asymptomatic infections or mild vesicular lesions up to serious diseases, such as encephalitis and disseminated neonatal infections (1). The human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV) have a tropism for lymphocytes and are capable of inducing a variety of lymphoproliferative disorders (2). EBV, the etiological agent of infectious mononucleosis, is associated with lymphomas, such as Burkitt’s lymphoma (BL), Hodgkin’s disease, and posttransplant lymphoproliferative diseases (PTLD), as well as other tumors of epithelial origin including nasopharyngeal carcinoma (NPC) and gastric carcinoma (1, 3). KSHV is the causative agent of Kaposi’s sarcoma (KS), a highly vascularized tumor of endothelial cell origin, and is involved in two lymphoproliferative disorders: primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD) (4).

The herpesvirus life cycle displays two distinct phases: latency and lytic replication. Most genes are expressed during the lytic phase, leading to production of infectious virions (5). Upon infection or reactivation, the virus follows a temporal and sequential gene expression pattern that can be divided into three phases: (i) immediate-early, (ii) early, and (iii) late. During the early phase of replication, herpesviruses synthesize several proteins involved in nucleoside and nucleotide metabolism, as well as DNA replication. Among these proteins, viral thymidine kinase (TK) and/or protein kinase (PK) enables the activation (phosphorylation) of several antiviral drugs, and the viral DNA polymerase is the target of almost all drugs currently available for the therapy of herpesvirus infections (6, 7).

FDA-approved antiviral drugs include nucleoside analogs such as acyclovir (ACV), penciclovir (PCV), ganciclovir (GCV), the acyclic nucleoside phosphonate derivative cidofovir (CDV), and the pyrophosphate analog foscarin (phosphonoformic acid [PFA]) (8). Except for PFA, acting as such directly on the viral DNA polymerase, the other drugs need to be converted into their active form by viral and/or cellular enzymes to eventually inhibit viral DNA synthesis (9). Currently, antiviral agents licensed for the treatment of herpes simplex virus 1 (HSV-1) and 2 (HSV-2), as well as varicella-zoster virus (VZV), include ACV, its valyl ester prodrug valacyclovir (VACV), and famciclovir (FCV), the oral prodrug of PCV (10, 11). CDV and PFA are mostly indicated for the treatment of ACV-resistant HSV infections mainly observed in immunocompromised patients (8, 11). CDV, GCV, and the oral prodrug valganciclovir (VGCV), have been licensed for the treatment of human cytomegalovirus (HCMV) infections. Unfortunately, the use of these drugs is limited by nephrotoxicity and poor oral bioavailability (CDV) as well as hematological toxicity (GCV) (8, 12).

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Address correspondence to G. Andrei, graciela.andrei@rega.kuleuven.be.
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Although antiviral agents inhibit gammaherpesvirus replication in vitro, no drug has been approved yet for the treatment of EBV and KSHV infections (13, 14). Their sensitivity to antiviral agents has been evaluated by chemical induction of lytic replication in latently infected cells (15). The lytic form of EBV can be effectively inhibited in vitro by guanine nucleoside analogs, such as ACV and GCV (16). Previous studies have demonstrated the requirement of the virus PK, and not the virus TK, for the phosphorylation of these drugs in EBV-infected cells (16–18). On the other hand, thymidine analogs such as bromovinyldeoxyuridine (BVDU), rather than guanine nucleoside analogs, are known substrates for EBV TK (16, 18). In contrast, the conversion of all nucleoside analogs to their monophosphate forms is performed by either the viral TK (UL23) in HSV-infected cells or by the viral PK (UL97) in HCMV-infected cells (16). These results indicate that the EBV TK has a narrower substrate specificity than the alphaherpesvirus TK. Therefore, thymidine analogs may be the most suitable antiviral agents to be activated by the EBV-encoded TK enzyme (18, 19). Similar to EBV TK, TKs of other gammaherpesviruses, such as KSHV and herpesvirus saimiri (HVS), have been found to possess comparable substrate specificities (17, 20–23). In addition, a closely related murine gammaherpesvirus (murine herpesvirus 68 [MHV-68]), encoding a TK with significant homology to the TKs of both EBV and KSHV, is used in a mouse model of infection for the evaluation of antiviral drugs against gammaherpesviruses (24–26).

1-Nucleoside analogs represent an important class of small molecules for the treatment of both viral infections and cancers (27, 28). Because of their interesting biological properties regarding reduced toxicity, increased antiviral potency, and metabolic stability, a number of 1-nucleosides have been synthesized and identified as potent antiviral agents, e.g., lamivudine (3TC), the first 1-nucleoside approved against human immunodeficiency virus and hepatitis B virus infections (29, 30). Previous studies have demonstrated that 1-dioxolane uracil nucleosides are a potent class of anti-EBV agents. So far, the 1-iodo derivative, 1-5-iododioxolane uracil [1-I-OddU], is the most active and selective compound in cell culture among all anti-EBV agents (50% effective concentration [EC50] of 0.03 μM; selectivity index [SI] of ≥6,250) (29, 31, 32).

In this study, we investigated the in vitro and in vivo antiviral effects of 1-dioxolane thymidine analog, 1-[(2S,4S-2-(hydroxymethyl)-1,3-dioxolan-4-yl)5-vinylpyrimidine-2,4(1H,3H)-dione, or HDVD, as well as its mechanism of action. We found that HDVD was active against human and animal herpesviruses, such as HSV-1, KSHV, EBV, HVS, and MHV-68. In addition, our data on drug resistance were consistent with the finding that nucleoside analogs of thymidine are preferentially phosphorylated by the gammaherpesvirus TK. Accordingly, we have mapped mutations associated with HDVD and BVDU resistance in the viral TK of HSV-1, HVS, and/or MHV-68 and further evaluated their cross-resistance profiles. Regarding the in vivo activity of HDVD and its produg, we were able to demonstrate their potent inhibitory effect on the acute replication of MHV-68 in mice.

**MATERIALS AND METHODS**

**Cell and virus culture.** Murine fibroblasts (NIH 3T3 cells; ATCC CRL-1685), owl monkey kidney cells (OMK; ATCC CRL-1536), and primary rhesus monkey fibroblasts (RF; kindly provided by S. Wong, Oregon Health and Science University, Beaverton, OR) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Merelbeke, Belgium) containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10 mM HEPES. Human embryonic lung (HEL) fibroblasts (ATCC CCL137) were cultured in modified Earle’s medium (MEM; Invitrogen) complemented with the supplements described above. Cells were grown at 37°C in a 5% CO2 humidified atmosphere. PEL-derived BCBL-1 (NIH AIDS Research and Reference Reagent Program) and BL-derived P3HR-1 cells (ATCC HTB-62) were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS, 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 1% HEPES.

Virally infected cells (MHV-68, clone G2.4; kindly provided by A. N. Nash, Edinburgh, United Kingdom), HELs (C-488; ATCC VR-1414), and rhesus rhadinovirus (RRV; strain 17577) (kindly provided by S. Wong) were grown in NIH 3T3, OMK, and RF cells, respectively. The following viruses were grown in HEL cells: HSV-1 (KOS strain; ATCC VR-1493), a thymidine kinase-deficient (TK-) HSV-1 KO strain resistant to ACV (ACVr), bearing a G insertion at nucleotides 430 to 436 of the viral TK gene (KOS/ACVr), two previously described KOS mutants selected under pressure with BVDU containing the A168T amino acid substitution (KOS/BVDU) (2000, dones 1 and 5) (33), HSV-2 (G strain; ATCC VR-734), VZV strain Oka, VZV TK- strain 07-1, and HCMV strains AD169 and Davis (ATCC VR-538 and ATCC VR-807, respectively). Two HSV-1 resistant clinical strains (70/5550 clone 4 and 70/6389 clone 2), harboring the R176Q amino acid substitution in TK, were also included in the phenotypic studies (34).

**Compounds.** HDVD, 1-[(2S,4S-2-(hydroxymethyl)-1,3-dioxolan-4-yl)5-vinylpyrimidine-2,4(1H,3H)-dione, and its valyl ester USS-02-71-44 were synthesized by C. K. Chu (University of Georgia, Athens, GA). The chemical structure of HDVD and USS-02-71-44 are shown in Fig. 1. The other compounds and their sources were as follows: ACV, 9-(2-hydroxyethoxymethyl)guanine (GlaxoSmithKline, Stevenage United Kingdom); brivudin (BVDU), (E)-3-(2-bromovinyl)-1-β-n-2-deoxyriboburanosyl-1-yl-uracil (Searle, United Kingdom); GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (Roche, Basel, Switzerland); CDV, (S)-1-(3-hydroxy-2-phosphonylethoxypyropyl)cytosine (Gilead Sciences, Foster City, CA); and the pyrophosphate analog PFA (phosphonoformate sodium salt; Sigma Chemicals, St. Louis, MO).

**Antiviral assays by real-time qPCR.** BCBL-1 and P3HR-1 cells were seeded in 48-well plates at a density of 3 × 104 cells/ml and 1 × 105 cells/ml, respectively. KSHV and EBV replication were induced by adding 20 ng/ml 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma-Aldrich, Bornem, Belgium) to the growing cells. The next day, cells were washed and resuspended in fresh medium in the presence or absence of various concentrations of antiviral drugs. At day 5 postinduction, total cellular DNA was extracted (QIAamp DNA kit; Qiagen Benelux B.V, Venlo, Netherlands), and viral DNA was quantified by quantitative PCR (qPCR) using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Thermocycling conditions for all qPCRs were done following the manufacturer’s instructions. Forward and reverse primers as well as TaqMan probe sequences allowing the detection of the target ORF73 of KSHV and BNRF1 of EBV have been described elsewhere (35). The concentrations required to effectively reduce KSHV and EBV DNA synthesis were able to demonstrate their potent inhibitory effect on the acute replication of MHV-68 in mice.

![FIG 1 Chemical structure of thymidine analogs HDVD, its valine ester produg (USS-02-71-44), and BVDU.](http://jvi.asm.org/Downloaded_from_http://jvi.asm.org)
in TPA-stimulated cells by 50% and 90% (EC_{50} and EC_{90}), respectively, were extrapolated from the standard curve using linear regression analysis, and results are shown as the means from at least three independent experiments.

**CPE and plaque reduction assays.** The drug susceptibility of other herpesviruses (HSV-1, HSV-2, VZV, HCMV, MHV-68, HVS, and RRV) were evaluated in 96-well microtiter plates on confluent cells and in their corresponding cell lines. The antiviral activity was calculated based on the inhibition of virus-induced cytopathic effect (CPE) or VZV plaque formation. Confluent cell cultures were infected with HSV-1, HSV-2, and HCMV at 100 times the tissue culture infectious dose that gives CPE in 50% of the cell cultures (TCID_{50}), with VZV at 20 PFU of cell-associated virus per well, or with MHV-68, HVS, and RRV at a multiplicity of infection of 0.02. After 2 h of adsorption at 37°C, residual virus was removed, and cultures were incubated in the presence of various concentrations of test compounds in duplicate. Viral CPE or plaque formation was recorded as soon as it reached completion in the untreated, virus-infected cells. The antiviral activity was expressed as the EC_{50}, the concentration required to reduce virus-induced CPE or viral plaque formation by 50% compared to the untreated control.

**Cytotoxicity assays.** The cytotoxic effect of the compounds was based on the inhibition of cell growth for each of the cell lines. HEL cells were seeded at a density of 5 × 10^4 cells/well into 96-well microtiter plates. NIH 3T3, OMK, and RF cells were seeded at a density of 3 × 10^4 cells/well, and uninduced BCBL-1 and P3HR-1 cells were seeded at a density of 3 × 10^5 cells/ml and 1 × 10^5 cells/ml, respectively. After 24 h of cell growth, medium containing different concentrations of the test compounds was added. After a period of 3 days of incubation at 37°C, cell counts were determined using a Coulter counter (Analis, Namur, Belgium). The cytotoxic concentration, or the concentration of the compound required to reduce cell growth by 50% (CC_{50}) relative to the number of cells in the untreated controls was calculated. Alternatively, cytotoxicity was expressed as the minimum cytotoxic concentration (MCC), that is, the compound concentration that causes a microscopically detectable alteration of cell morphology. The selectivity index (SI) was determined as the ratio of the CC_{50} to the EC_{50}.

**TK assay using [methyl-^3H]dThd as the natural substrate.** The activity of purified TK-1, TK-2, HSV-1 TK, and VZV TK and the 50% effective concentration of HDVD and BVDU were assayed in a 50-μl reaction mixture containing 50 mM Tris-Cl, pH 8.0, 2.5 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM 3’-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid, 3 mg/ml bovine serum albumin, 2.5 mM ATP, 1 μM [methyl-^3H]deoxothymidine ([methyl-^3H]dThd), and enzyme. The samples were incubated at 37°C for 30 min in the presence or absence of different concentrations (5-fold dilutions) of the test compounds. Aliquots of 45 μl of the reaction mixtures were spotted on Whatman DE-81 filter paper disks. The filters were washed three times for 5 min in 1 mM ammonium formate, once for 1 min in water, and once for 5 min in ethanol. The radioactivity was determined by scintillation counting.

**Selection and genotypic and phenotypic characterization of drug-resistant viruses.** Drug-resistant HSV-1, HVS, and MHV-68 were obtained by serial passages in their respective cell lines in the presence of an increasing concentration of HDVD or BVDU, starting at a concentration equivalent to the EC_{50}. After the drug reached a concentration of 50 or 200 μM, a last passage was performed in drug-free medium to obtain a virus stock. Independent procedures of selection were performed to isolate HDVD or BVDU. In both cases, the HVS mutant isolates were composed of a mixed population of variants. Hence, to identify the individual mutants, we characterized several HSV clones that were isolated by limiting dilution. DNA of all resistant viruses was extracted (Qiagen), and the entire virus TK, DNA polymerase, and/or PK gene was amplified by PCR using FastStart high-fidelity DNA polymerase (Roche Applied Science, Mannheim, Germany). PCR was performed following the manufacturer’s instructions. The PCR products were purified with a QIAquick Purification Kit (Qiagen), and gene amplicons were directly sequenced using the cycle sequencing BigDye Terminator kit, version 3.1, on an ABI 3730 sequencing system (Applied Biosystems) and a set of primers spanning the entire coding regions of the genes. The sequencing results were computer assembled and compared with the TK, PK, and DNA polymerase sequences from the wild-type (WT) strain using the software SeqScape, version 2.7 (Applied Biosystems). Drug susceptibility of the different mutants was evaluated by a CPE reduction assay. The level of drug resistance was expressed as the ratio of the EC_{50} of the resistant virus to the EC_{50} of the WT virus.

**Animal studies.** (i) Ethics statement. All animal work was approved by the Katholieke Universiteit Leuven Ethics Committee for Animal Care and Use (permit P097-2010). All animal experiments were conducted in accordance with the German and European guidelines for the protection of vertebrate animals used for experimental and other scientific purposes. (ii) Infection of mice. Female BALB/c mice were purchased from Elevage-Janvier (Le-Genest-St-Ise, France). All animal procedures were performed at biosafety level 2. Four-week-old mice were inoculated by intranasal route with 20 μl of MHV-68 (10,000 PFU) or mock inoculated with phosphate-buffered saline (PBS). Infections were performed under anesthesia using ketamine-xylazine in saline. HDVD and BVDU, diluted in PBS, were orally administered at a dose of 200 mg/kg of body weight once daily for five consecutive days, starting the day of infection. Mice were grouped as uninfected and infected untreated, HDVD treated, or BVDU treated, with five mice per group. An additional experiment was performed with UOS-02-71-44 and BVDU orally administered at a dose of 100 mg/kg of body weight two times per day for five consecutive days.

**Virus loads and RNA detection.** DNA was extracted from tissue sections stored in PBS using a QIAamp DNA Minikit (Qiagen) following manufacturer’s instructions. Viral DNA load was quantified by qPCR. The sequence of the forward primer of the glycoprotein B (gB) gene was 5’-GGCCAAAATCATATTAATGCT-3’, that of the reverse primer was 5’-CCTCTGGACACACTCTCAAGC-3’, and that of the probe was FAM-ACAAAGCTGACCACCGTCAACAC-TAMRA-3’ (where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine) (Applied Biosystems). RNA isolation from lungs and spleen was carried out with TRizol reagent (Invitrogen) and an RNeasy minikit (Qiagen). One-step reverse transcription qPCR (RT-qPCR) was performed using a TaqMan RNA-to-CT 1-Step Kit (Applied Biosystems) for relative quantitation (RQ) experiments on a real-time PCR system. Primers for the detection of the gB gene were identical to those described above; for the ORF73 gene, the forward primer was 5’-CCACCCTGCTCTGCGCCGTC-3’, and the reverse primer was 5’-GAGCCGCTTATCAAGGCCC-3’, and probe was FAM-CTATGCATTGGCGCCAGCT-TAMRA. gB and ORF73 mRNAs levels were normalized with a roden glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control (Applied Biosystems).

**Histopathology.** Sections of lungs and spleen from two mice per group were immediately fixed in 10% buffered formalin for 24 h. The following day, tissues were placed in PBS until further histopathological processing. Next, tissues were embedded in paraffin, and 5-μm sections were stained with hematoxylin-eosin.

**Statistical analyses.** All statistical analyses were done with GraphPad Prism, version 5, software (GraphPad Software Inc., La Jolla, CA). Viral DNA loads results were analyzed by a Mann-Whitney U test. Statistical significance was defined as follows: a P value of ≤0.01, very significant; a P value of ≤0.05, significant; and a P value of >0.05, not significant.
RESULTS

Antiherpetic activity of HDVD. The inhibitory effect of HDVD was evaluated against six human herpesviruses of three different subfamilies and against three animal gammaherpesviruses. ACV, BVDU, CDV, and/or GCV was included as a control in each assay. HDVD was found to be active against HSV-1 with an EC₅₀ of 0.1 μM, which is similar to that of ACV and BVDU (Table 1). However, HDVD completely lost its inhibitory activity against the mutant TK⁻ HSV-1 strain (EC₉₀ of >100 μM). A weak activity of HDVD was found against HSV-2 (EC₅₀ of 12 μM) and VZV Oka strain (EC₅₀ of 37 μM), whereas no inhibition of the mutant TK⁻ VZV 07-1 strain was observed. Nor was HDVD inhibitory to HCMV.

In the EBV and KSHV qPCR assays, intracellular viral DNA synthesis was found to be markedly inhibited by HDVD (Table 2), with EC₉₀ of 0.01 μM for EBV and 0.09 μM for KSHV. In addition, this compound kept its antiviral activity at very low concentrations since the EC₉₀ values for EBV and KSHV were 0.07 μM and 0.2 μM, respectively. In addition, HDVD appeared to be more potent than CDV (EC₅₀ of 2.5 μM and 1.0 μM against EBV and KSHV, respectively).

The replication of MHV-68 was also susceptible to the inhibitory effects of HDVD (EC₅₀ of 0.1 μM), which was in the range of that of BVDU (Table 3). HVS was moderately sensitive to the novel compound (EC₅₀ of 1.6 μM), but RRV, the other simian gammaherpesvirus, was not (Table 3).

For all five gammaherpesviruses, no differences in EC₉₀ were detected between HDVD and its valyl ester prodrug USS-02-71-44. No toxicity (measured by inhibition of cell proliferation or alteration of cell morphology) was observed for either HDVD or USS-02-71-44 in cell culture. In HEL, NIH 3T3, OMK, and RF cells, the CC₉₀ values were >100 μM. Hence, against HSV-1, EBV, KSHV, MHV-68, and HVS, high selectivity indices (ratio of CC₉₀/EC₅₀) were calculated for HDVD, ranging from >125 (HVS) to >7,700 (EBV), and for its prodru USS-02-71-44, ranging from >80 (HVS) to >10,000 (EBV).

Inhibitory activity on dThd phosphorylation by 2’-deoxy-5-fluorodeoxycytidine. HDVD was recognized as a good substrate by mitochondrial TK-2, HSV-1 TK, and VZV-TK (Table 4). The compound was inhibitory as an alternative substrate to dThd phosphorylation by these enzymes at 50% inhibitory concentrations (IC₅₀) of 0.3 μM, 2.9 μM, and 1.5 μM, respectively. In contrast, HDVD showed a 500-fold reduced inhibition of dThd phosphorylation catalyzed by TK-2. Thus, we demonstrated that HDVD had markedly lower affinity for this enzyme than BVDU, which may be of importance regarding its potential to cause mitochondrial toxicity. Cytosolic TK-1 was insensitive to the inhibitory activity of BVDU and HDVD against dThd phosphorylation. We observed a somewhat higher inhibitory effect of HDVD on dThd phosphorylation by HSV-1 TK than by VZV TK (IC₅₀ of 80 μM for HSV-1 TK versus 296 μM for VZV TK). These results correlate with the greater antiviral activity of HDVD against HSV-1 than against VZV, but it should also be realized that whereas the viral TK activity is required for the metabolic activation of the test compounds, the viral DNA polymerase represents the target that determines virus inhibition in cell culture.

Isolation and genotypic characterization of mutant HSV-1, MHV-68, and HVS strains resistant to HDVD and/or BVDU. To reveal the mechanism of action of HDVD, drug-resistant HSV-1, HVS, and MHV-68 strains were selected in cell culture. HDVD

### Table 1 Inhibitory activity of HDVD on the replication of alpha- and betaherpesviruses

<table>
<thead>
<tr>
<th>Compound</th>
<th>HSV-1</th>
<th>VZV</th>
<th>HCMV</th>
<th>Cytotoxicity (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (μM)</td>
<td>EC₉₀ (μM)</td>
<td>CC₉₀ (μM)</td>
<td>SI</td>
</tr>
<tr>
<td>HDVD</td>
<td>0.1 ± 0.06</td>
<td>&gt;100</td>
<td>12 ± 11</td>
<td>37</td>
</tr>
<tr>
<td>GCV</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ACV</td>
<td>0.4 ± 0.05</td>
<td>170 ± 41</td>
<td>0.5 ± 0.3</td>
<td>1.5</td>
</tr>
<tr>
<td>BVDU</td>
<td>0.06 ± 0.0</td>
<td>&gt;176</td>
<td>302 ± 0</td>
<td>0.003</td>
</tr>
<tr>
<td>CDV</td>
<td>1.2 ± 0.6</td>
<td>0.7 ± 0.9</td>
<td>1.0 ± 0.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

### Table 2 Inhibitory effects of HDVD on the replication of human gammaherpesviruses

<table>
<thead>
<tr>
<th>Compound</th>
<th>EBV (P3HR-1 cell) inhibition</th>
<th>KSHV (BCBL-1 cell) inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (μM)</td>
<td>EC₉₀ (μM)</td>
</tr>
<tr>
<td>HDVD</td>
<td>0.01 ± 0.003</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>USS-02-71-44</td>
<td>0.02 ± 0.02</td>
<td>≥ 0.4</td>
</tr>
<tr>
<td>GCV</td>
<td>4.3 ± 3.6</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>ACV</td>
<td>3.6 ± 0.4</td>
<td>16 ± 8</td>
</tr>
<tr>
<td>BVDU</td>
<td>6.9 ± 5.1</td>
<td>≥ 33</td>
</tr>
<tr>
<td>CDV</td>
<td>2.5 ± 1.3</td>
<td>25 ± 31</td>
</tr>
</tbody>
</table>

a Data are mean values of three independent experiments ± standard deviations. EC₅₀ and EC₉₀ effective concentrations required to reduce viral DNA synthesis by 50% and 90%, respectively; CC₉₀ cytostatic concentration required to reduce growth of uninduced cells by 50% after 3 days of incubation.

b SI, Selectivity Index (ratio of CC₉₀/EC₅₀).
None of the drug-resistant gammaherpesviruses had mutations in the viral DNA polymerase or in their PKs.

Phenotypic characterization of HDVD \( ^{\dagger} \) and BVDU \( ^{\ddagger} \) viruses. The inhibitory effects of HDVD, GCV, ACV, BVDU, and CDV against the different mutants, as well as the parental strains, were determined by CPE reduction assays in their respective cell lines. The results are presented in Table 5 and Table 6. All HSV-1 isolates selected under HDVD pressure and bearing mutations in the viral TK had an HDVD \( ^{\dagger} \) phenotype, with EC_{50} values of \( \approx 20 \) \( \mu \text{M} \). The four HSV-1 mutants were cross-resistant to ACV, BVDU, and GCV. Consistent with this, CDV remained equally active against the WT and drug-resistant viruses.

Two BVDU \( ^{\ddagger} \) HSV-1 clones bearing the A168T mutation resulting in a TK-altered phenotype were previously selected in our laboratory (33). These clones were resistant to BVDU (pyrimidine nucleoside derivative) but remained sensitive to ACV and GCV (purine derivatives). In this study, we confirmed these observations and demonstrated that the pyrimidine analog HDVD had reduced antiviral activity against these BVDU \( ^{\ddagger} \) HSV-1 mutants (A168T), with EC_{50} values of approximately 5 \( \mu \text{M} \) (Table 6). In contrast, the clinical strain harboring the R176Q mutation showed high levels of resistance to GCV and ACV (EC_{50} values of 7 \( \mu \text{M} \) and \( >20 \) \( \mu \text{M} \), respectively) but not to HDVD or very weak resistance to BVDU (14-fold), which is consistent with the amino acid change R176Q conferring resistance to purine but not to pyrimidine analogs (34).

A high level of resistance (\( \approx 1,900 \)-fold) against HDVD was determined for MHV-68 mutants bearing the T364P or Q401R mutation (Table 5). In addition, these isolates showed cross-resistance to BVDU but not to GCV and ACV. Interestingly, the E358D mutation conferred resistance only to HDVD.

HVS isolates resistant to HDVD presented a fold resistance of \( >25 \) (Table 5). Among the different compounds, only cross-resistance with BVDU (\( \approx 50 \)-fold) was observed. Similarly, the phenotypic analysis of BVDU \( ^{\ddagger} \) HVS demonstrated that the R45stop mutation conferred resistance to BVDU and had a drug susceptibility profile comparable to that of the HDVD \( ^{\dagger} \) strains (Table 6).

Inhibitory effects of HDVD and BVDU on viral replication and on establishment of latency in a mouse model of gammaherpesvirus infection. Following intranasal infection of mice, MHV-68 causes an acute productive infection in alveolar lung epithelial cells (36). Subsequently, the virus spreads to the mediastinal lymph nodes (MLNs) and establishes a latent infection in the spleen as well as in the lungs (37). We determined the

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### Table 3: Inhibitory effects of HDVD on the replication of animal gammaherpesviruses

<table>
<thead>
<tr>
<th>Compound</th>
<th>MHV-68 (NIH 3T3 cell) inhibition</th>
<th>HVS (OMK cell) inhibition</th>
<th>RRV (RF cell) inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC_{50} (( \mu \text{M} ))^a</td>
<td>CC_{50} (( \mu \text{M} ))^b</td>
<td>SI</td>
</tr>
<tr>
<td>HDVD</td>
<td>0.1 ± 0.03</td>
<td>&gt;200</td>
<td>&gt;2,000</td>
</tr>
<tr>
<td>USS-02-71-44</td>
<td>0.08 ± 0.04</td>
<td>&gt;200</td>
<td>&gt;2,500</td>
</tr>
<tr>
<td>GCV</td>
<td>18 ± 3.6</td>
<td>138 ± 39</td>
<td>8</td>
</tr>
<tr>
<td>ACV</td>
<td>8.9 ± 8.9</td>
<td>71 ± 31</td>
<td>8</td>
</tr>
<tr>
<td>BVDU</td>
<td>0.1 ± 0.06</td>
<td>12 ± 3</td>
<td>120</td>
</tr>
<tr>
<td>CDV</td>
<td>1.3 ± 0.3</td>
<td>105 ± 22</td>
<td>81</td>
</tr>
</tbody>
</table>

^a Data are mean values of three independent experiments ± standard deviations. EC_{50}, effective concentration required to reduce viral CPE by 50%; CC_{50}, cytotoxic concentration required to reduce cell growth by 50% after 3 days of incubation.

^b SI, selectivity index (ratio of CC_{50}/EC_{50}).

^c ND, not determined.

---

### Table 4: Inhibitory activity of HDVD and BVDU on dThd phosphorylation by 2'-deoxynucleoside kinases from different origins

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (( \mu \text{M} ))^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TK-1</td>
</tr>
<tr>
<td>HDVD</td>
<td>&gt;500</td>
</tr>
<tr>
<td>BVDU</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

^a Data are mean values of two independent experiments ± standard deviations. IC_{50}, 50% inhibitory concentration of the test compounds, which was calculated as the compound concentration required to inhibit 1\( \mu \text{M} \) [CH_{3}]^{3}HdTdT phosphorylation by 50%.
MHV-68 replication in the lungs at day 6 p.i., as well as establishment of latency in the spleen at early (6 days p.i.) and late (12 days p.i.) time points. MHV-68-infected BALB/c mice were treated orally with HDVD or BVDU once a day for 5 days, starting 2 h after infection. No signs of apparent toxicity related to repetitive administration of the drugs, i.e., HDVD and BVDU, were seen.

In a preliminary experiment, MHV-68-infected BALB/c mice were treated with 25 mg of HDVD per kg per day (for five consecutive days), administered intraperitoneally or per os. Independent of the route of administration, HDVD exerted a moderate inhibitory effect on the viral DNA loads in lungs at day 6 and resulted in a minor reduction of viral DNA copy numbers in the spleen at day 12 p.i., compared to the level in the untreated control (data not shown). However, these data were not significant ($P > 0.05$). As shown in this study, when dosage was increased to 200 mg per kg per day (oral administration), HDVD was able to significantly inhibit acute viral replication.

### Table 5: Phenotypic Characterization of HDVD-Resistant HSV-1, HVS, and MHV-68 Mutants

<table>
<thead>
<tr>
<th>Virus and/or Mutant</th>
<th>Amino Acid Change</th>
<th>EC$_{50}$ (µM)</th>
<th>HDVD</th>
<th>GCV</th>
<th>ACV</th>
<th>BVDU</th>
<th>CDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0</td>
<td>0.4 ± 0.04</td>
<td>0.06 ± 0.03</td>
<td>1.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>KOS A</td>
<td>R281Stop</td>
<td>&gt;20 (&gt;29)</td>
<td>11 ± 0.28</td>
<td>&gt;20 (&gt;50)</td>
<td>&gt;20 (&gt;333)</td>
<td>1.7 ± 0.7 (1)</td>
<td></td>
</tr>
<tr>
<td>KOS B</td>
<td>C deletion in a stretch of 4 cytosines (nt 1061–1064)</td>
<td>&gt;20 (&gt;29)</td>
<td>17 ± 0.5 (43)</td>
<td>&gt;20 (&gt;50)</td>
<td>&gt;20 (&gt;333)</td>
<td>1.6 ± 0.3 (1)</td>
<td></td>
</tr>
<tr>
<td>KOS C</td>
<td>C336Y</td>
<td>&gt;20 (&gt;29)</td>
<td>3.0 ± 0.8 (9)</td>
<td>&gt;20 (&gt;50)</td>
<td>&gt;20 (&gt;333)</td>
<td>1.7 ± 0.4 (1)</td>
<td></td>
</tr>
<tr>
<td>KOS D</td>
<td>C deletion in a stretch of 4 cytosines (nt 1061–1064)</td>
<td>&gt;20 (&gt;29)</td>
<td>3.5 ± 0.9 (9)</td>
<td>&gt;20 (&gt;50)</td>
<td>&gt;20 (&gt;333)</td>
<td>1.9 ± 0.8 (1)</td>
<td></td>
</tr>
<tr>
<td>MHV-68</td>
<td></td>
<td>0.08 ± 0.05</td>
<td>8.6 ± 6.3</td>
<td>4.9 ± 2.0</td>
<td>0.2 ± 0.03</td>
<td>2.4 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>&gt;20 (&gt;2,500)</td>
<td>11 ± 3.0 (1.3)</td>
<td>7.0 ± 0.1</td>
<td>24 ± 21 (120)</td>
<td>3.5 ± 2.2 (1)</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>T364P</td>
<td>13 ± 7 (163)</td>
<td>4.3 ± 2.4 (0.5)</td>
<td>4.4 ± 2.2 (1)</td>
<td>0.2 ± 0.09 (1)</td>
<td>1.4 ± 1.0 (1)</td>
<td></td>
</tr>
<tr>
<td>A7</td>
<td>E358D</td>
<td>152 ± 45 (1,900)</td>
<td>12 ± 5.5 (1.4)</td>
<td>6.2 ± 1.8 (1)</td>
<td>13 ± 11 (65)</td>
<td>3.5 ± 1.3 (1)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Q401R</td>
<td>8.0 ± 0</td>
<td>137 ± 43</td>
<td>160 ± 40</td>
<td>6.3 ± 4.5</td>
<td>2.1 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Clone 1</td>
<td>P326T</td>
<td>&gt;200 (&gt;25)</td>
<td>109 ± 67 (1)</td>
<td>49 ± 18.0 (3)</td>
<td>435 ± 285 (69)</td>
<td>1.9 ± 1.0 (1)</td>
<td></td>
</tr>
<tr>
<td>Clone 2</td>
<td>C deletion in a stretch of 3 cytosines (nt 213–215)</td>
<td>&gt;200 (&gt;25)</td>
<td>219 ± 114 (1)</td>
<td>271 ± 129 (2)</td>
<td>312 ± 21 (50)</td>
<td>2.2 ± 0.3 (1)</td>
<td></td>
</tr>
<tr>
<td>Clone 3</td>
<td>C deletion in a stretch of 3 cytosines (nt 213–215)</td>
<td>&gt;200 (&gt;25)</td>
<td>106 ± 51 (1)</td>
<td>178 ± 0 (1)</td>
<td>≥327 (≥52)</td>
<td>1.0 ± 0.3 (0.5)</td>
<td></td>
</tr>
<tr>
<td>Clone 4</td>
<td>C deletion in a stretch of 3 cytosines (nt 213–215)</td>
<td>&gt;200 (&gt;25)</td>
<td>141 ± 188 (1)</td>
<td>160 ± 27 (1)</td>
<td>381 ± 120 (61)</td>
<td>1.3 ± 1.0 (1)</td>
<td></td>
</tr>
</tbody>
</table>

a Relative to the WT strain. nt, nucleotide.
b EC$_{50}$, effective concentration required to reduce viral CPE formation by 50%. Values are the averages of at least three independent experiments ± standard deviations. Values in parentheses indicate the fold resistance ratios.
in the lungs (i.e., day 6 p.i.). The number of viral DNA copies showed a 1 log reduction ($P = 0.02$) in HDVD-treated mice compared to the untreated mice, while in BVDU-treated mice a 0.5 log reduction (not significant) was observed (Fig. 4A). At this time point, DNA copy numbers were as high as $10^6$ copies in the MLNs of untreated and HDVD- and BVDU treated mice (Fig. 4B). Low levels of MHV-68 DNA were detected in the spleen, and levels were significantly reduced in mice treated with HDVD ($P = 0.004$) or BVDU ($P = 0.03$) (Fig. 4C).

Next, we examined the viral DNA load at day 12 p.i., when latency is established in the spleen. Compared to loads at day 6 p.i., viral DNA loads were reduced in the lungs and MLNs of infected mice, yet viral DNA remained detectable (Fig. 4D and E). Meanwhile, DNA copy numbers were increased in the spleen of untreated mice and reached $10^4$ copies per mg of spleen tissue (Fig. 4F). However, oral treatment with HDVD and BVDU did not suppress the viral DNA load in the spleen at day 12 p.i.

Further, we analyzed the MHV-68 infection in BALB/c mice treated with the valyl ester prodrug, USS-02-71-44, compared to BVDU treatment. DNA loads were determined on day 6 in lungs, MLNs, and spleen (data not shown). Although the inhibition of viral DNA in the lungs was not greater than after treatment with HDVD, USS-02-71-44 was able to significantly ($P = 0.008$) reduce the viral DNA loads by 1 log in the spleens at 12 days p.i. (Fig. 5).

To characterize the pattern of MHV-68 lytic (gB) and latent (ORF73) gene expression in vivo following treatment with HDVD or BVDU, RNA was extracted from lung and spleen tissue. Both gB and ORF73 transcripts were detected in the lungs of infected mice early during infection, i.e., at day 6 p.i. Treatment with HDVD or BVDU resulted in a significant reduction of the relative gB expression in the lungs (Fig. 6A). HDVD was able to inhibit lytic gene expression more strongly than BVDU (65% versus 46% of inhibition, respectively, relative to the untreated control). In addition, ORF73 expression in the lungs was decreased by 94% and 65% in HDVD- and BVDU-treated mice, respectively.

At day 12 p.i., gB expression in the lungs of untreated mice was detected at very low levels; notably, the relative expression increased in mice treated with the antiviral drugs (Fig. 6B). When drug treatment was withdrawn at day 6 p.i., lytic gene expression appeared to increase in the lungs at day 12 p.i. At this time point, gB transcripts could not be detected in the lungs of one mouse in the control group and in one mouse of the HDVD-treated group. The difference in the levels of ORF73 in the lungs of HDVD- and BVDU-treated mice was not present at 12 days p.i., gB levels being significantly increased in mice treated with HDVD and BVDU (Fig. 6C). ORF73 expression in the spleen seemed to remain at relatively lower levels than in the untreated control, with a reduction of 72% after HDVD treatment and 12% after BVDU treatment, which was not significant ($P > 0.05$).

To further investigate the differences in pathology between control and treated mice during acute infection, lungs and spleens were harvested and examined histologically for inflammatory changes (Fig. 7). On day 6, when infectious virus peaks in the lungs of infected mice, we observed a diffusely increased interstitial cellularity, in both perivascular and peribronchial locations, compared to the level in uninfected mice. The same features were found in both drug-treated groups. At day 12, the inflammatory reaction in the lungs was slightly reduced but was dominated by mononuclear inflammatory cells. A similar course of inflammatory reaction in this tissue was observed in the HDVD- and BVDU-treated mice. One of the characteristics of MHV-68 infec-

**TABLE 6 Phenotypic characterization of in vitro-selected BVDU\(^{a}\) HSV-1 and HVS mutants and HSV-1 clinical strains**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amino acid change(^{a})</th>
<th>HDVD</th>
<th>GCV</th>
<th>ACV</th>
<th>BVDU</th>
<th>CDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KOS WT</td>
<td></td>
<td>0.7 ± 0.8</td>
<td>0.04 ± 0.03</td>
<td>0.2 ± 0.2</td>
<td>0.05 ± 0</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>BVDU(^{a}) clone 1</td>
<td>A168T</td>
<td>5.2 ± 3.4 (7)</td>
<td>0.04 ± 0.01 (1)</td>
<td>0.1 ± 0.01 (0.5)</td>
<td>&gt;20 (&gt;400)</td>
<td>0.9 ± 0.6 (1)</td>
</tr>
<tr>
<td>BVDU(^{a}) clone 5</td>
<td>A168T</td>
<td>4.8 ± 1.6 (7)</td>
<td>0.05 ± 0.03 (1)</td>
<td>0.1 ± 0.01 (0.5)</td>
<td>&gt;20 (&gt;400)</td>
<td>0.8 ± 0.3 (1)</td>
</tr>
<tr>
<td>Clinical strain 70/5550, clone 4</td>
<td>R176Q</td>
<td>0.3 ± 0.1 (4)</td>
<td>7.3 ± 2.0 (183)</td>
<td>&gt;20 (&gt;100)</td>
<td>0.7 ± 0.2 (14)</td>
<td>1.6 ± 1.3 (2)</td>
</tr>
<tr>
<td>Clinical strain 70/6389, clone 2</td>
<td>R176Q</td>
<td>0.2 ± 0.06 (0.3)</td>
<td>7.9 ± 1.3 (198)</td>
<td>&gt;20 (&gt;100)</td>
<td>0.7 ± 0.2 (14)</td>
<td>0.9 ± 0.5 (1)</td>
</tr>
<tr>
<td>HVS strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>8 ± 0</td>
<td>137 ± 43</td>
<td>160 ± 40</td>
<td>6.3 ± 4.5</td>
<td>2.1 ± 1.6</td>
</tr>
<tr>
<td>BVDU(^{a}) clone 2</td>
<td>R45stop</td>
<td>&gt;200 (&gt;25)</td>
<td>141 ± 24 (1)</td>
<td>204 ± 98 (1)</td>
<td>468 ± 165 (74)</td>
<td>1.7 ± 0.9 (1)</td>
</tr>
<tr>
<td>BVDU(^{a}) clone 3</td>
<td>R45stop, E363stop</td>
<td>&gt;200 (&gt;25)</td>
<td>121 ± 47 (1)</td>
<td>205 ± 93 (1)</td>
<td>534 ± 150 (85)</td>
<td>1.0 ± 0 (0.5)</td>
</tr>
</tbody>
</table>

\(^{a}\) Relative to the wild type.

\(^{b}\) EC\(_{50}\) effective concentration required to reduce viral CPE formation by 50%. Values represent the averages of at least three independent experiments ± standard deviations.

Values in parentheses indicate the fold resistance ratio.

**FIG 3** Kinetic analysis of MHV-68 gene expression in lungs and spleen of BALB/c mice. The graph demonstrates the relative quantification (RQ) of gB (lytic) and ORF73 (latent) expression in the lungs, as well as in the spleen of MHV-68-infected mice, over a time period of 18 days. The grey bars represent the days p.i. relevant to study the effect of antiviral agents. Gene expression levels were normalized to the endogenous control GAPDH. The error bars indicate the calculated maximum and minimum expression levels (RQ\(_{\text{Max}}\) and RQ\(_{\text{Min}}\) respectively) from the mean expression level (RQ). The RQ\(_{\text{Max}}\) was calculated as $\Delta \Delta C_T + s$, and the RQ\(_{\text{Min}}\) was calculated as $\Delta \Delta C_T - s$, where $C_T$ is threshold cycle and $s$ is the standard deviation of the $\Delta \Delta C_T$ value.
tion in BALB/c mice is the enlargement of MLNs and spleen as a result of a transient rise in leukocyte number (37). At both time points, we observed MLN enlargement in all infected mice, untreated as well as treated. Splenomegaly was observed at day 12 but not at day 6 in the untreated mice, as well as in the drug-treated mice. Compared to findings in the uninfected mice, the white pulp of the spleen of MHV-68-infected mice was rich in large, poorly delineated follicles with well-delineated follicle centers at this time point. The presence of prominent macrophages within follicles (Fig. 7, black arrows) was noted in infected mice and after treatment with the antiviral drugs.

DISCUSSION

In this study, we demonstrated the high selectivity of HDVD against EBV in vitro, as well as against other gammaherpesviruses. L-Nucleoside analogs, in particular, L-dioxolane uracil derivatives, were previously described to possess potent and selective anti-EBV activity (28, 29, 31). Therefore, new derivatives, including HDVD, were synthesized and evaluated for their inhibitory effect on EBV replication. Consistent with our data obtained in the P3HR-1 cell line, similar results were found in two different EBV-positive cell lines, i.e., B95-8 and H1 (unpublished data). Previously, Lin and colleagues described the structure-activity relationship of L-dioxolane uracil nucleosides against EBV, showing the importance of 5-position substituents in order to maintain high anti-EBV activity (31).

In the present study, HDVD, which contains a 5-vinyl on the uracil group, proved to possess potency and selectivity against EBV replication similar to those of the previously described derivative L-I-OddU. However, differences in antiviral spectrum between HDVD and L-I-OddU were noted. Despite the specific anti-EBV activity, L-I-OddU had weak antiviral activity against KSHV and poor inhibitory effects against HSV-1, HSV-2, VZV, and HCMV replication (29,32). In contrast, KSHV replication seemed to be highly sensitive to the effects of HDVD and the compound moderately inhibited HSV-1 replication. Similarly to L-I-OddU, HDVD has weak antiviral activity against HSV-2 and VZV and no inhibitory activity against HCMV. Also, it should be noted that the spectrum of activity of HDVD against herpesviruses differed from that of BVDU. Although HDVD and BVDU were equally active against HSV-1, HDVD was poorly active against VZV, as demonstrated by the plaque reduction assay and in agreement with its rather moderate interaction with VZV TK. Studies on structure-activity relationships of pyrimidine nucleosides revealed that the anti-VZV activity is dependent on the halovinyl group and that the antiviral activity increased with the increasing size of the halogen atom (which is absent in HDVD) (29). Among gammaherpesviruses, HDVD proved to be superior to BVDU in its capacity.

FIG 4 Analysis of MHV-68 viral DNA load in BALB/c mice treated with HDVD or BVDU. Mice were infected by intranasal route with 10^4 PFU of MHV-68 and treated with PBS (untreated), HDVD, or BVDU for five consecutive days, starting the day of infection. Viral DNA loads were determined by qPCR in lungs (A and D), MLNs (B and E), and spleen (C and F) at day 6 and day 12 p.i. In each group 5 mice were used, and the limit of detection was 100 copies per mg of tissue. Values are given as the mean viral copy number per mg of tissue ± standard deviation. Statistical significance was calculated using a Mann-Whitney U test: *, P < 0.05; **, P < 0.01.

FIG 5 MHV-68 DNA load in the spleen of USS-20-71-55-treated mice at day 12 p.i. MHV-68-infected BALB/c mice (10^4 PFU) were treated twice a day with PBS (untreated), or 100 mg/kg of USS-02-71-44, or BVDU for five consecutive days, starting the day of infection. Values are given as the mean viral copies per mg of tissue ± standard deviations. Statistical significance was calculated using a Mann-Whitney U test. **, P < 0.01.
to inhibit EBV and KSHV but completely lost its activity against RRV. Based on the alignment of the gammaherpesvirus TKs, the conserved regions in the RRV TK appeared to be highly similar to that of either KSHV or HVS. Therefore, it might be that other sites in the gammaherpesvirus TK play a major role in the recognition of HDVD since amino acid identity varies between 30% and 40% among gammaherpesvirus TKs. Also, BVDU and HDVD are differently recognized by the mitochondrial TK-2 since BVDU, and not HDVD, proved to be a good substrate of TK-2 (Table 4).

Since EBV TK was shown to be required for the activation of l-1-OdU to the l-1-OdUMP form, it was therefore reasonable to assume that the mechanism of antiherpesvirus activity of HDVD was dependent on the viral TK (32). The triphosphate form of HDVD is expected to act as a competitive inhibitor with the natural substrate dTTP for the viral DNA polymerase. HDVD-triphosphate is believed to inhibit the incorporation of dTTP in the viral DNA or, as an alternative substrate, could be incorporated into viral DNA via internucleotide linkages, as has been described for BVDU-triphosphate (40). Hence, we selected and characterized HDVD-resistant herpesviruses and demonstrated that all viruses with an HDVD-resistant phenotype exhibited point mutations or deletions in the viral TK. Since MHV-68 and HVS can be easily propagated at high titers and produce CPE in cell culture, these viruses were used for the selection and characterization of HDVD-resistant gammaherpesviruses. To our knowledge, this is the first time drug-resistant gammaherpesvirus mutants, i.e., HDVD-resistant MHV-68, and HVS, were selected in vitro. Mutations occurred in the viral TK and not in the viral PK, which is in agreement with previous studies reporting that thymidine analogs are preferentially phosphorylated by the gammaherpesvirus TK, rather than the viral PK (16, 18, 23). Comparative TK sequence analysis of 12 herpesviruses, including HSV-1, EBV, and HVS, allowed the identification of five conserved sites in this enzyme, i.e., the putative ATP-binding pocket (site I), the active site (site II), the nucleoside-binding domains (sites III and IV), and the arginine-rich domain which is believed to bind substrate phosphoryl groups (site V) (22, 41). The majority of the mutations in the HDVD-resistant HSV-1, HVS, and MHV-68 mutants were mapped in nonconserved regions of the TK, with the exception of one MHV-68 and one HVS mutant, which bore a mutation in the conserved site II (E358D) and site IV (P326T), respectively (Fig. 2).

We further examined the relationship between the drug susceptibilities of the different isolates and mutations in the viral TK. In HDVD-resistant MHV-68, amino acid substitutions were responsible for an altered (E358D) or diminished sensitivity (T364P and Q401R) to HDVD and BVDU. The glutamic acid at position 358 of MHV-68 TK is analogous to the glutamic acid at positions 83 (in HSV-1 TK) and 48 (in VZV TK) and acts as a potential proton acceptor in the ester formation during phosphorylation of substrates (42). The E83K (HSV-1) and E48K (VZV) amino acid changes, located in the conserved site II, were previously reported to be linked to drug resistance in site-directed mutagenesis studies or in ACV-resistant HSV-1 isolates (43–46). The positively
charged lysine in HSV-1 TK at this position is believed to induce a major change in the structure of the enzyme, leading to loss of its activity and resistance to other TK-dependent drugs (44). However, in the E358D MHV-68 TK, the aspartate closely resembles glutamic acid, and this amino acid change induced a TK-altered phenotype. Hence, this TK mutant virus remained sensitive to BVDU, in contrast to T364P and Q401R TK mutants, which had a TK-resistant phenotype displaying resistance to the pyrimidine analogues BVDU and HDVD.

Unlike MHV-68 mutants, HDVD<sup>r</sup> and BVDUr HVS mutants are expected to produce truncated proteins as a result of the introduction of a deletion of a cytosine at the N-terminal region of the viral TK or the appearance of a stop codon. One HDVD<sup>r</sup> isolate harbored a single nucleotide substitution that was located in the conserved nucleoside binding site IV (P326T). This region contains the amino acid motif Val-Phe-Pro, which is highly conserved in gammaherpesviruses, and the proline is conserved in all herpesviruses. Also, the crucial role of the phenylalanine in this motif for maintaining EBV TK activity due to an essential interaction with the pyrimidine nucleoside substrate has been demonstrated previously (22, 47). The P326T mutation maps into the Val-Phe-Pro motif. Proline is known to give conformational rigidity to chains and is commonly found in turns. It is reasonable to believe that replacement of the proline at this position by a threonine would alter the conformation of the nucleoside binding site, hence influencing its capacity to phosphorylate nucleoside analogs. In addition, substitutions of this conserved proline residue have been previously mapped in ACV-resistant HSV-1 isolates (48). Phenotypic characterization of all HVS resistant mutants confirmed the resistance to HDVD as well as cross-resistance to the pyrimidine nucleoside analog BVDU. Since PK of gammaherpesviruses, and not TK (18, 20), is believed to phosphorylate purine analogs, the antiviral activities of ACV and GCV were not altered against HVS TK mutants.

In contrast, each of the HDVD<sup>r</sup> HSV-1 strains showed cross-resistance to the nucleoside analogs evaluated, i.e., pyrimidine (BVDU) and purine (ACV and GCV) nucleoside analogs. This pattern of resistance was expected for the HSV-1 mutant bearing the C336Y mutation since this mutation is known to affect the stability and phosphorylation efficiency of HSV-1 TK (49). The replacement of the sulfhydryl group of a cysteine with a phenolic moiety of a tyrosine would induce a significant disruption of the ATP binding site, which modifies binding affinities, and the conformation of the active site. Furthermore, we selected HDVD<sup>r</sup> HSV-1 with a single nucleotide deletion in a stretch of five cytosine residues located at nucleotide positions 1061 to 1064 of the TK, which is considered a hot spot. Homopolymer nucleotide repeats of guanosine or cytidine have been shown to be particularly susceptible to nucleotide insertions or deletions, resulting in frame-shift mutations and responsible for the synthesis of truncated nonfunctional TKs (50). Mutational hot spots occur preferentially in herpesviruses with a high GC content, such as HSV-1 (68% GC content). In contrast, MHV-68 and HVS have lower GC contents (46% and 35%, respectively), possessing only a few (MHV-68) or
no (HVS) homopolymeric stretches within the TK. ACV HSV-1 isolates exhibiting a deletion of a cytosine between positions 1061 and 1064 have been previously described in patients. Such mutations resulted in a nonfunctional TK, indicating the importance of amino acids 355 to 376 (51). Accordingly, the C-terminal portion of the HSV-1 TK protein was found to be essential for the phosphorylation of ACV (51). The third mutation (R281stop) in the TK of HDVD HSV-1 is expected to result in a truncated protein lacking the TK C terminus. This amino acid substitution is located outside the active site and hot spot regions of the TK and was shown here to confer resistance to HDVD and other nucleoside analogs. Furthermore, the selection of R281stop has been previously reported in vitro in BVDU laboratory HSV-1 strain and in one ACV-resistant clinical isolate (33, 52). Characterization of resistant herpesviruses indicated that HDVD was recognized as a pyrimidine analog by the viral TK. These results were further strengthened by the data obtained with two HSV-1 mutants having a TK-altered phenotype, A168T or R176Q, that showed resistance only to pyrimidine analogs (BVDU and HDVD) or purine analogs (ACV and GCV), respectively. In this respect, it can also be noticed that site-directed mutagenesis studies on amino acid position 168 of HSV-1 TK revealed that the presence of the alanine at this position is important for efficient dThd phosphorylation. The intrinsic phosphorylation capacity of the A168T mutant TK for dThd indeed decreased by 14-fold but only by 2-fold for ganciclovir (53).

Furthermore, we evaluated the effect of HDVD and BVDU in an immunocompetent mouse model of MHV-68 infection. This in vivo model is known to reproduce similar features as those observed during EBV-induced infectious mononucleosis in humans, e.g., productive replication at mucosal sites, CD8+ T cell lymphocytosis, and establishment of a latent infection in B lymphocytes (54–56). Despite their similar anti-MHV-68 activities in vitro, HDVD proved to be more potent than BVDU when administered orally to mice. Since HDVD partially blocked the MHV-68 lytic phase in the lungs, smaller amounts of virus were able to further infect the lung tissue. BVDU, as well, had an inhibitory effect on acute viral replication in lungs, although less pronounced than that of HDVD. At day 6 p.i., both drugs were able to significantly reduce virus propagation to the spleen. These results suggest that the compounds delayed the establishment of latency in the spleen during treatment. However, when antiviral treatment was stopped (i.e., at 6 days p.i.), virus replication appeared to increase in the lungs, leading to the establishment of a latent MHV-68 infection in the spleen of HDVD- and BVDU-treated mice at day 12 p.i. Nevertheless, the slight reduction of ORF73 gene expression indicates that HDVD still exerted a minor antiviral effect on the establishment of a latent infection in the spleen. Of interest, and in contrast to HDVD, mice treated with the valyl ester prodrug USS-02-71-44 still had significantly reduced levels of viral DNA in the spleen at this time point. Despite the ability of HDVD and BVDU to slow down MHV-68 infection during treatment, these drugs no longer had a significant impact on disease development once antiviral treatment was ended. In line with previous results obtained by Neyts and De Clercq, BVDU treatment was ineffective in protecting immunocompromised SCID mice from MHV-68-induced mortality (25). In addition, another nucleoside analogue, 2’-deoxy-5-ethyl-beta-4’-thiouridine (4’-S-EtEdU), was reported to be a potent inhibitor of MHV-68 replication in the lungs of infected mice but was unable to prevent the establishment of latency despite delaying the onset of latent infection in the spleen (24). Furthermore, the effect of GCV on KSHV gene expression has been investigated in two humanized SCID mouse models, showing that GCV treatment affected lytic gene expression and was also able to delay, but not eliminate, latent gene expression (57, 58).

In summary, the data reported here showed the potency of HDVD against gammaherpesviruses in vitro, with a unique spectrum of antiviral activity against herpesviruses. When evaluated in vivo, HDVD and its prodrug proved to be inhibitors of acute MHV-68 replication and were able to delay the establishment of viral latency in the spleen during antiviral treatment. These studies showed the requirement of the gammaherpesvirus TK for the activation of pyrimidine nucleoside analogues and their potential use for the treatment of virus-associated diseases, as well as for the prevention or the delayed onset of virus-associated cancers in immunocompromised patients.

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