Pharmacological Study of ASP2151 (Amenamevir), Helicase-primase Inhibitor Possessing Antiviral Activity against Varicella-zoster Virus and Herpes Simplex Virus Type 1 and 2

Koji Chono

Department of Virology,

Graduate School of Medicine and Pharmaceutical Sciences,

University of Toyama

Contents

Executive Summary

ASP2151 (amenamevir) is a helicase–primase complex inhibitor that shows antiviral activity against herpes simplex virus (HSV)-1, HSV-2, and varicella-zoster virus (VZV). We conducted pharmacological studies to evaluate and characterize the efficacy, mechanism of action, ASP2151-resistant mutants emerging risk *in vitro* and *in vivo*.

At the first, an inhibitory effect of ASP2151 on enzymatic activities associated with a recombinant HSV-1 helicase-primase complex was assessed. To investigate the effect on viral DNA replication, we analyzed viral DNA in cells infected with herpes viruses (HSV, VZV, and human cytomegalovirus). *In vitro* and *in vivo* antiviral activities were evaluated using a plaque reduction assay and an HSV-1–infected zosteriform-spread model in mice. ASP2151 inhibited the single-stranded DNA-dependent ATPase, helicase and primase activities associated with the HSV-1 helicase-primase complex. Antiviral assays revealed that ASP2151, unlike other known HSV helicase-primase inhibitors, exerts equipotent activity against HSV-1, HSV-2, and VZV through prevention of viral DNA replication. Further, the anti-VZV activity of ASP2151 (EC_{50}: 0.038–0.10 μmol/L) was more potent against all strains tested than that of acyclovir (ACV) (EC_{50}: 1.3–27 μmol/L). ASP2151 was also active against ACV-resistant VZV mutants. In a mouse zosteriform-spread model, ASP2151 was orally active and inhibited disease progression more potently than valacyclovir (VACV).

In the next step, we characterized the ASP2151-resistant HSV-1, HSV-2, and VZV variants or

mutants based on findings from sequencing analysis, growth, pathogenicity, and susceptibility testing, identifying several single base-pair substitutions resulting in amino acid changes in the helicase and primase subunit of ASP2151-resistant mutants. Amino acid alterations in the helicase subunit were clustered near or within helicase motif IV, which is one of the six helicase motifs forming the functional active site, in the helicase gene of HSV-1, HSV-2, and VZV, while the primase subunit substitution associated with reduced susceptibility was found in ASP2151-resistant HSV-1 mutants. However, while susceptibility in the ASP2151-resistant HSV mutants to existing antiherpes agents was equivalent to that in wild-type HSV strains, ASP2151-resistant HSV and VZV mutants showed attenuated *in vitro* growth capability. Moreover, ASP2151-resistant HSV showed *in vivo* pathogenicity compared with the parent strains.

Finally, to assess combination therapy of ASP2151 with existing antiherpes agents against HSV-1, HSV-2, and VZV, we conducted two-drug combination studies *in vitro* and *in vivo*. Combination activity effect of ASP2151 with ACV was tested by a plaque reduction assay and the data was analyzed using isobologram and response surface model. *In vivo* combination therapy of ASP2151 with VACV was studied in HSV-1 infected zosteriform-spread mice model. The antiviral activity of ASP2151 combined with ACV against ACV-susceptible HSV-1, HSV-2, and VZV showed statistically significant synergistic effect ($P<0.05$). In mouse zosteriform-spread model, the inhibition of disease progression by the combination therapy was more potent than that of each monotherapy ($P<0.05$).

Here, we showed that ASP2151 possesses potent antiviral activity against not only HSV-1

and HSV-2 but also against VZV including ACV-resistant mutants. ASP2151 inhibited the multiple enzymatic activities associated with ssDNA-dependent ATPase, DNA helicase and primase assays. These data suggest that the antiviral activity of ASP2151 results from inhibition of the helicase-primase complex. Our second study findings demonstrated that important amino acid substitutions associated with reduced susceptibilities of HSV-1, HSV-2, and VZV to ASP2151 exist in both the helicase and primase subunits of the helicase-primase complex, and that mutations in the enzyme complex against ASP2151 might confer defects in viral replication and pathogenicity. The results of combination therapy study indicate that an antiherpes efficacy of the combination therapy of ASP2151 with ACV or VACV could show the synergistic effect against HSV and VZV infections and the strong anti-herpetic therapy would be feasible in severe diseases, such as encephalitis or in patients with immunosuppression.

In conclusion, ASP2151 is a novel viral helicase-primase inhibitor with potent activity against not only HSV-1, HSV-2 but also VZV. Based on our results, ASP2151 warrants further investigation for the treatment of VZV, HSV-1, and HSV-2 infections.

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(1) Chono K, Katsumata K, Kontani T, Kobayashi M, Sudo K, Yokota T, et al. ASP2151, a novel helicase-primase inhibitor, possesses antiviral activity against varicella-zoster virus and herpes simplex virus types 1 and 2. J Antimicrob Chemother 2010;65:1733-41.

(2) Chono K, Katsumata K, Kontani T, Shiraki K, Suzuki H. Characterization of virus strains

resistant to the herpes virus helicase-primase inhibitor ASP2151 (Amenamevir). Biochem Pharmacol 2012;84:459-67.

(3) Chono K, Katsumata K, Suzukia H, Shiraki K. Synergistic Activity of Amenamevir (ASP2151) with Acyclovir against Herpes Simplex Virus Type 1, 2 and Varicella-zoster Virus. Antiviral Res 2013;97:154-60.

Chapter 1 General Introduction

1.1. Pathogenicity and Treatment of Herpes Simplex Virus Type 1, Type 2, and Varicella-zoster Virus

Herpes simplex virus type 1 (HSV-1), HSV-2, and varicella-zoster virus (VZV) are widely prevalent pathogens belonging to the human herpesvirus subfamily *Alphaherpesvirinae* of family *Herpesviridae* [Pellet and Roizman, 2007]. Both HSV and VZV establish life-long latent infections in sensory ganglia after the primary infection and eventually reactivate, leading to recurrent episodes. HSV infections are the most widespread infectious disease in the world, with no seasonal variation, and naturally occurring only in human beings [Whitley and Roizman, 2001], affecting nearly 60% to 95% of the adult human population globally [Fatahzadeh and Schwartz, 2007]. HSV-1 and HSV-2 cause genital herpes, herpes labialis or herpetic keratitis, and frequent disease recurrence dramatically affects the quality of life of afflicted individuals [Roizman et al., 2007]. VZV are also one of the most worldwide spread virus pathogens. Infection with VZV leads to the development of two distinct disease episodes: varicella as the primary episode and herpes zoster as the recurrent episode [Levin and Schmader, 2007].

Since the late 1970s, synthetic nucleoside analogs targeting viral DNA polymerase, such as acyclovir (ACV), penciclovir (PCV), valaciclovir (VACV), and famciclovir, have been developed for the treatment of HSV and VZV infections [Brady and Bernstein, 2004]. These nucleoside analogs represent safe and effective therapies for HSV and VZV infections. Nucleoside analogs

share the same mechanism of action, requiring phosphorylation by viral thymidine kinase (TK) and host kinases; after their phosphorylation, the analogs interfere with viral DNA polymerization through competitive inhibition with guanosine triphosphate and obligatory chain termination [Biron and Elion, 1980; Elion et al., 1977; Miller and Miller, 1980; Miller and Miller, 1982; Morfin and Thouvenot, 2003]. As viral TK is not essential for viral replication, HSV and VZV lacking a functional TK (TK-negative or TK-partial mutants) are still viable and result in cross-resistance to the nucleoside analog drug class [Coen and Schaffer, 1980; Larder and Darby, 1986; Piret and Boivin, 2011].

The pyrophosphate analog foscarnet (FOS) is a viral DNA polymerase inhibitor that interferes with the binding of diphosphate to DNA polymerase. Given that FOS does not require activation by TK, its antiviral effect can be exerted against ACV-resistant mutants lacking a functional TK [Oberg, 1989]. However, a number of nucleoside analog-resistant mutants with modifications to their DNA polymerase have been found to be resistant to FOS as well as other nucleoside analog antivirals [Piret and Boivin, 2011]. The emergence of such potentially multi-resistant mutants represents a growing concern, particularly among immunocompromised patients [Morfin and Thouvenot, 2003], highlighting the need for novel antiherpetic drugs with alternative mechanisms of action [Stránská et al., 2004]. These limitations of the current standard of care highlight the need for developing novel anti-herpes drugs with potent antiviral activity based on alternative mechanisms of action.

1.2. Helicase-primase Inhibitors as an Anti-herpes Agents

Viral gene products essential for virus replication, such as the herpesvirus helicase-primase complex, are potential targets for novel antiviral agents. The helicase-primase complex performs essential functions in viral genome DNA replication and hence viral replication, responsible for both unwinding viral DNA at the replication fork and priming DNA synthesis [Crute et al., 2002; Crute et al., 1988; Crute et al., 1989; Dodson et al., 1989]. This complex consists of three proteins—the helicase, primase, and cofactor subunits. The helicase-primase complex possesses multi-enzymatic activities, including DNA-dependent ATPase and helicase localized in the helicase subunit and primase in the primase subunit; all of these enzymatic activities are needed for the helicase-primase complex to function in viral DNA replication and hence virus growth.

The helicase-primase complex is well conserved among members of family *Herpesviridae* viruses. For instance, the genes encoding the HSV-1 and HSV-2 helicase subunit (U_L5), primase subunit (U_L52) and cofactor subunit (U_L8) share homology with the ORF55, ORF6 and ORF52 genes of VZV [Davison and Scott, 1986] and the U_L105, U_L70 and U_L102 genes of the cytomegalovirus [Chee et al., 1990]. Therefore, agents that target the helicase-primase complex have the potential to represent novel, broad-spectrum, anti-herpes agents.

Helicase-primase inhibitors (HPIs) could supplement the currently available therapies. Indeed, the amino-thiazolylphenyl-containing compound, BILS 179 BS, and the thiazole urea derivative, AIC316 (formerly known as BAY 57-1293), have been reported as helicase-primase inhibitors (HPIs) with anti-HSV activity [Crute et al., 2002; Kleymann et al., 2002]. BILS 179 BS has been

shown to have 10-fold more potent activity against HSV than ACV *in vitro*, while its *in vivo* efficacy was comparable to ACV in animal studies [Crute et al., 2002]. AIC316 showed two orders of magnitude greater potency against HSV than ACV *in vitro*, and superior *in vivo* activities compared with valaciclovir in mice and guinea pigs models [Kleymann et al., 2002]. Moreover, one phase 2 clinical study result of AIC316 were reported (ClinicalTrials.gov Identifier: NCT01047540) and another phase 2 study have been conducted for genital herpes patients [Tyring et al., 2012]. However, despite the potential shown by these compounds, their antiviral spectrum is limited, as both compounds inhibit HSV-1 and HSV-2 but not other human herpesviruses. As HPIs seem to be a promising class of anti-herpes drugs, further investigation is warranted to optimize the antiviral spectrum, potency, and *in vivo* efficacy of this class of drugs.

1.3. Structurally Novel Class of Helicase-primase Inhibitor, ASP2151

Here, we study that ASP2151 (international nonproprietary name: amenamevir), an oxadiazolephenyl derivative, is a structurally novel class of HPI that possesses potent antiviral activity against not only HSV-1 and HSV-2 but also VZV (Chapter 2). Due to promising preclinical profiles on antiviral activity, safety, tolerability and pharmacokinetics, ASP2151 was selected as a development candidate and the clinical efficacy has been evaluated in two phase 2 clinical studies for the patients with herpes zoster (ClinicalTrials.gov Identifier: NCT00487682) and genital herpes [Tyring et al., 2012].

Mutants resistant to HSV-specific HPIs AIC316 and BILS 179 BS were previously

characterized in terms of prevalence of pre-existing resistance, ability of replication *in vitro*, and pathogenicity in mice. However, few studies have characterized ASP2151-resistant HSV and VZV variants or mutants. To analyze the effect of amino acid mutations in helicase and primase on *in vitro* and *in vivo* growth characteristics, we characterized the ASP2151-resistant HSV-1, HSV-2, and VZV variants or mutants based on findings for sequencing analysis, growth, pathogenicity, and susceptibility testing (Chapter 3).

Given its different mechanism of action compared with nucleoside analog drugs, ASP2151 is expected to exhibit a combination effect with existing nucleoside analog antiherpes drugs against HSVs and VZV strains as well as nucleoside analog drug-resistant mutants. However, while combination therapy of ASP2151 with nucleoside analog antiherpes drugs may be a promising option in cases of severe disease, such as herpes encephalitis or in patients with immunosuppression, whether or not such combination therapy will have synergistic, additive, or antagonistic effects compared to either drug in monotherapy remains unclear. To assess the combination therapy of ASP2151 and existing nucleoside analog antiherpes drugs, we tested the antiviral activity of ASP2151 combined with ACV and other nucleoside analogs (PCV and vidarabine) *in vitro* and a combination therapy with ASP2151 and VACV in a mouse model of zosteriform spread (Chapter 4).

Chapter 2 ASP2151, a Structurally Novel Helicase-primase Inhibitor, Possesses Antiviral Activity against Varicella-zoster Virus, Herpes Simplex Virus Types 1, and 2

2.1. Introduction

ASP2151, an oxadiazolephenyl derivative, is a structurally novel class of helicase-primase inhibitor that possesses potent antiviral activity against not only herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) but also varicella-zoster virus (VZV). In this chapter, we report that an inhibitory effect of ASP2151 on enzymatic activities associated with a recombinant HSV-1 helicase-primase complex was assessed and viral DNA in cells infected with herpes viruses (HSV, VZV, and human cytomegalovirus) was analyzed to investigate the effect on viral DNA replication. Moreover, *in vitro* and *in vivo* antiviral activities were evaluated using a plaque reduction assay and an HSV-1–infected zosteriform-spread model in mice.

2.2. Materials and Methods

2.2.1. Ethics Statement

All animals were housed and handled according to the Animal Ethical Committee guidelines of Yamanouchi Pharmaceutical Co., Ltd., which is now known as Astellas Pharma Inc. and the Astellas Pharma's Institutional Animal Care and Use Committee guidelines.

2.2.2. Compounds

ASP2151 (molecular weight, 482.55; international non-proprietary name, amenamevir) was

synthesized by Astellas Pharma Inc. (Tokyo, Japan). Acyclovir (ACV; Sigma-Aldrich, St. Louis, MO, USA), penciclovir (PCV; LKT Laboratories, St. Paul, MN, USA), vidarabine (VDB; Sigma-Aldrich, St. Louis, MO, USA), and valaciclovir (VACV) as Valtrex® film tablets (GlaxoSmithKline, Middlesex, UK) were purchased from commercial suppliers.

2.2.3. Viruses and Cell Lines

Four VZV strains clinically isolated in the United States were kindly provided by Dr. Ann M. Arvin (Stanford University School of Medicine, Palo Alto, CA, USA). All other viruses and cell lines were provided by Rational Drug Design Laboratories (Fukushima, Japan). Human embryonic fibroblast (HEF) cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). VZV, HSV-1 and HSV-2 were propagated using HEF cells in maintenance medium containing 2% FBS. Human cytomegalovirus (HCMV) was cultured using MRC-5 cells.

2.2.4. Preparation of Helicase-primase Complex

Recombinant baculoviruses expressing HSV-1 U_L5 (helicase), U_L52 (primase) and N-terminally histidine-tagged U_L8 (cofactor) of wild-type HSV-1 KOS strain were prepared using the Bac-to-Bac® Baculovirus Expression System (Invitrogen). The recombinant HSV-1 strain KOS helicase-primase complex was expressed in Sf9 cells triply infected with the baculoviruses, and then purified using Ni-NTA agarose resin (Invitrogen) in accordance with a previously described

method [Calder and Stow, 1990; Crute et al., 1991; Ramirez-Aguilar et al., 2002].

2.2.5. ATPase Assay

Single stranded DNA-dependent ATPase activity of the HSV-1 helicase-primase complex was determined using an assay modified from a previously described method [Crute and Lehman, 1991]. The reaction buffer contained 20 mmol/L HEPES (pH 7.6), 2 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol (DTT), 9 μg/mL ssDNA prepared using calf thymus DNA (Sigma-Aldrich), 90 μmol/L ATP (Roche Diagnostics K.K., Tokyo, Japan) and 25 ng of the enzyme complex in a reaction volume of 10 μL. The mixture containing ASP2151 at a concentration from 0.0001 μmol/L to 3 μmol/L was incubated for 75 min at 37 °C. ATP hydrolysis was determined by adding 10 μL of Biomol® green according to the manufacturer's instruction (Enzo Life Science, Farmingdale, NY, USA).

2.2.6. DNA Helicase Assay

Forked DNA helicase substrate was prepared using the oligonucleotides: 5′-CAGTCACGACGTTGTAAAACGACGGCCAGTGTTATTGCATGAAAGCCCGGCTG-3′ labeled at the 5' end with Alexa Fluor® 488 (Invitrogen), and unlabeled 5′-GTCGGCCCACCTTCCTGTTATTGACTGGCCGTCGTTTTACAACGTCGTGACTG-3′ as previously reported [Graves-Woodward et al., 1997]. The reaction mixture (10 μL) contained 20 mmol/L HEPES (pH 7.6), 1 mmol/L DTT, 5 mmol/L $MgCl_2$, 2 mmol/L ATP, 1 μg

helicase-primase complex, 20 nmol/L forked DNA helicase substrate and a 5-μmol/L concentration of a capture strand (*5′*-CAGTCACGACGTTGTAAAACGACGGCCAGT-3′). Reactions containing ASP2151 were allowed to proceed for 60 min at 30 °C, and then the products were electrophoresed through a 20% nondenaturing polyacrylamide gel. Fluorescence was detected using the ProXPRESS® 2D Proteomic Imaging System (PerkinElmer, Waltham, MA, USA).

2.2.7. Primase Assay

Primase activity was measured by detecting synthesized RNA primers in the presence of fluorescence-labeled CTP using the 51-mer DNA oligonucleotide: 5′-CTTCTTCGGTTCCGACTACCCCTCCCGACTGCCTATGATGTTTATCCTTTG-3′ as a template [Crute et al., 2002; Ramirez-Aguilar et al., 2002]. Reaction mixtures (10 μL) containing 50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L ATP, 1 mmol/L GTP, 1 mmol/L UTP, 2 μmol/L fluorescence-labeled CTP, 1 μmol/L of the 51-mer template and 2 μg of the helicase-primase complex were incubated at 30 °C for 90 min in the presence of vehicle (0.1% DMSO) or ASP2151. Reactions were then quenched by 10 μL of stop buffer containing 50 mmol/L EDTA (pH 8.0) and 90% (v/v) formamide. The products were heat denatured at 95 °C for 5 min and separated via denaturing polyacrylamide gel electrophoresis (15% polyacrylamide, 7 mol/L urea), and then fluorescence was detected using the ProXPRESS® 2D Proteomic Imaging System (PerkinElmer).

2.2.8. Polyacrylamide Gel Electrophoresis of Virus-specific PCR Fragments

HEF cells infected with HSV-1, HSV-2, VZV, and HCMV were exposed to ASP2151 and incubated until plaques clearly appeared in virus control wells. Cells were then collected to extract whole DNA using the Gentra® Puregene® Cell Kit (Qiagen, Valencia, CA, USA). PCR was performed using a specific primer set targeting U_S4, ORF31, or U_L83 for HSV-1 and HSV-2, VZV or HCMV, respectively (Table 1). Each PCR reaction was electrophoresed, stained, and visualized. Human β-actin gene was used as an internal control.

2.2.9. Real-time PCR

Real-time PCR was performed to quantify the VZV DNA in virus-infected HEF cells using ABI Prism 7900 HT (Applied Biosystems) with the primers and probe for VZV glycoprotein B gene [Pevenstein et al., 1999]. To normalize each of the DNA extracts, human β-actin gene was used as an internal control (TaqMan β-actin Control Reagents; Applied Biosystems, Carlsbad, CA, USA).

2.2.10. Plaque Reduction Assay (PRA) and Cytotoxicity Assay

HEF cells were seeded into multi-well plates and incubated until the cells formed a monolayer. After the medium was removed, the cells were infected with HSV-1, HSV-2, or VZV at a titer of 40 plaque-forming units (pfu)/well. The plates were then incubated for 1 h at 37 °C. After being washed twice with maintenance medium, cells were treated with the test compound until

clear plaques appeared. The cells were then fixed with 10% formalin in phosphate-buffered saline, and stained with 0.02% crystal violet solution. The number of plaques present was counted under a microscope. MTT assay or neutral red assay were conducted using HEF cells to determine the cytotoxic concentration causing 50% reduction in the number of viable cells (CC_{50}).

2.2.11. *In Vivo* Antiviral Activity

Hairless mice (HOS:HR-1, female, aged 7 weeks at virus infection) were infected (designated as day 0 post-infection) with HSV-1 strain WT51 (15 μL/mouse of suspension at a titre of 8.0×10^5 pfu/mL) at dorsolateral skin that had been scratched in a grid-like pattern with a 23-gauge needle under anesthesia. ASP2151 at doses of 0.3, 1, 3, 10, and 30 mg/kg, or VACV at doses of 3, 10, 30 and 100 mg/kg (suspension in 0.5% methylcellulose solution) was orally administered twice daily for 5 days starting 3 h after viral inoculation. Ten mice per test group were used. Disease course was monitored daily for 17 days and scored on a composite scale from 0 to 7 based on the severity of zosteriform lesions and general symptoms according to the following criteria: Score 0, no sign of infection; Score 1, localized, barely perceptible small vesicles; Score 2, slight vesicle spread; Score 3, large patches of vesicles formed; Score 4, zosteriform vesicles; Score 5, large patches of ulcers formed; Score 6, large zosteriform ulcers (severe); Score 7, hind limb paralysis or death.

2.2.12. Statistical Analyses

Statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA). The 50% inhibition concentration (IC_{50}) for the ssDNA-dependent ATPase assay, 50% effective concentration (EC_{50}) for real-time PCR, and EC_{50} and 90% (EC_{90}) effective concentration values for PRA, were calculated using nonlinear regression analysis with a sigmoid-E_{max} model. The 50% effective dose (ED_{50}) values for ASP2151 and valaciclovir were calculated using linear regression analysis.

2.3. Results

2.3.1. Antiviral activity of ASP2151 against HSV-1, HSV-2, and VZV

We focused on inhibitors of the herpesvirus helicase-primase complex as a new class of anti-herpesvirus agents. Our medicinal chemistry process was initiated based upon a 2-amino thiazole-containing HSV helicase-primase inhibitor (HPI) [Spector et al., 1998] to create more potent viral-specific agents with broader spectra and we consequently selected ASP2151 as a candidate for a novel anti-herpes agent. ASP2151 is a structurally new type of HPI containing an oxadiazolyl-phenyl as an essential moiety (Figure 1A). Full details of its synthesis, as well as the structure-activity relationship among ASP2151 and its related derivatives, will be published elsewhere.

The inhibitory activity of ASP2151 against helicase-primase complex was assayed using the recombinant U_L5-U_L52-U_L8 complex of HSV-1 strain KOS. HSV helicase-primase complex has

multiple enzymatic activities, namely DNA helicase and ssDNA-dependent ATPase activity catalyzed by the U_L5 helicase subunit [Dodson and Lehman, 1991; Zhu and Weller, 1992] and primase activity catalyzed by the U_L52 primase subunit [Klinedinst and Challberg, 1994]. Results showed that ASP2151 inhibited the DNA helicase activity of the complex at concentrations of 0.1 μmol/L or higher (Figure 1B). Similarly, the ssDNA-dependent ATPase activity was inhibited in a concentration-dependent manner with a mean IC_{50} value of 0.078 μmol/L (n=3, standard error: 0.016 μmol/L). Interestingly, ASP2151 also inhibited the primase activity at concentrations of 0.03 μmol/L or higher (Figure 1C).

We then conducted a PRA to compare the potential antiviral activity and specificity of ASP2151 against herpes family viruses *in vitro* with that of two known HSV HPIs; BILS 179 BS and BAY 57-1293 [Crute et al., 2002; Kleymann et al., 2002]. The EC_{50} values of BILS 179 BS against HSV-1 and HSV-2 were 0.060 μmol/L and 0.046 μmol/L, respectively (Table 2). BAY 57-1293 also inhibited HSV-1 and HSV-2 replication at similar EC_{50} values of 0.014 μmol/L and 0.023 μmol/L, respectively (Table 2). In contrast, the EC_{50} values of BILS 179 BS and BAY 57-1293 against VZV were 4.1 μmol/L and 11 μmol/L, respectively. The EC_{50} value ratio for the anti-VZV activities of BILS 179 BS and BAY 57-1293 were approximately 1/70 and 1/790 of that against HSV-1.

The oxadiazolyl-phenyl derivative ASP2151 constitutes a novel class of HPI distinguishable from the currently known HPIs. This distinction can be attributed to the equipotent antiviral activity of ASP2151 against VZV, HSV-1, and HSV-2. In the present study, ASP2151 inhibited VZV,

HSV-1 and HSV-2 replication with EC_{50} values of 0.047, 0.036 and 0.028 μmol/L, respectively (Table 2). In addition, EC_{90} values of ASP2151 against VZV, HSV-1, and HSV-2 also indicated similar antiviral potency against these viruses (Table 2). Importantly, ASP2151 showed no obvious cytotoxic effects at higher concentrations (CC_{50} value: >30 μmol/L), and the selectivity index (SI) was calculated to be at least 638 (Table 2).

The anti-VZV activity of ASP2151 compared with ACV was further evaluated using several strains of VZV that included clinical isolates and an ACV-resistant mutant. ASP2151 inhibited the replication of all the VZV strains tested. The EC_{50} values of ASP2151 and ACV for ACV-susceptible VZV strains ranged from 0.038 to 0.10 μmol/L and 1.3 to 5.9 μmol/L, respectively (Table 3). ASP2151 was also active against the ACV-resistant mutant Kanno-Br, which showed reduced susceptibility to ACV (EC_{50} value: 27 μmol/L), with an EC_{50} value of 0.082 μmol/L. The CC_{50} of ASP2151 was determined to be greater than 200 μmol/L (same as ACV) in HEF cells in a neutral red re-uptake assay, which provided an SI higher than 2000 (Table 3). No antiviral activity for ASP2151 was observed against HCMV, respiratory syncytial virus, influenza virus and human immunodeficiency virus-1 for concentrations up to 25 μmol/L.

2.3.2. Inhibitory Effect on Virus DNA Replication

Enzymatic activity of the helicase-primase complex is essential for virus replication, and inhibition of the initiation of DNA replication is believed to be the mechanism behind the antiviral activity of the HPIs. In order to study the effect of ASP2151 on viral DNA replication, we

measured the quantity of viral DNA in virus-infected cells exposed to ASP2151. At concentrations of 0.03 μmol/L or more, ASP2151 inhibited the DNA synthesis of VZV, HSV-1 and HSV-2 (Figure 2A). However, no effect on viral DNA replication was observed in HCMV-infected cells at ASP2151 concentrations of up to 1 μmol/L. This observation was consistent with the PRA data.

The IC_{50} value for VZV DNA replication was determined using real-time PCR. Both ASP2151 and ACV reduced the quantity of VZV DNA in a concentration-dependent manner, with IC_{50} values of 0.057 μmol/L and 0.44 μmol/L, respectively (Figure 2B). The IC_{50} value of ASP2151 assessed via real-time PCR corresponded with the EC_{50} value for anti-VZV effect assessed using PRA.

2.3.3. *In Vivo* Antiviral Activity

The *in vivo* activity of ASP2151 was evaluated in mice cutaneously infected with HSV-1. In mice, cutaneous infection with HSV-1 leads to a progressive disease course due to virus zosteriform-spread [De Clercq, 1984]. When compared with vehicle, oral administration of ASP2151 and valaciclovir significantly reduced mortality on days 17, the cumulative disease score, and area under the disease score-time curve for the period from days 0 to 17 post-infection ($AUC_{day0-17}$) at doses of ≥1 mg/kg twice daily and ≥10 mg/kg twice daily, respectively ($P<0.05$) (Table 5 and Figure 3). Based on the $AUC_{day0-17}$, ED_{50} values (95% confidence interval) of ASP2151 and valaciclovir were calculated as 1.9 (0.9-3.4) mg/kg twice daily and 27 (14-74) mg/kg twice daily, respectively. ASP2151 was statistically significantly 14-fold potent to VACV in the

model.

2.4. Discussion

Here, we showed that the novel oxadiazolyl-phenyl type herpesvirus HPI ASP2151 (amenamevir) possesses potent antiviral activity against not only HSV-1 and HSV-2 but also against VZV. ASP2151 is selective with low cytotoxicity *in vitro,* and is orally available and well tolerated in mice. So far, two classes of HPIs (thiazole urea [Kleymann et al., 2002] and 2 amino-thiazolylphenyl [Crute et al., 2002; Spector et al., 1998] derivatives) have found to exert potent antiviral activity against HSV-1 and HSV-2 *in vitro* and *in vivo*, but both classes of agent were reported to be inactive against VZV. Thus, agents such as ASP2151 that target the helicase-primase complex also represent potential anti-herpesvirus agents with activity against VZV.

The herpes helicase-primase complex is a heterotrimeric viral protein complex comprising helicase, primase, and cofactor subunits [Crute et al., 1991], which has essential functions involved in viral DNA replication. In the present study, ASP2151 inhibited the multiple enzymatic activities associated with the recombinant helicase-primase complex of HSV-1 strain KOS with similar potency, as assessed by ssDNA-dependent ATPase, DNA helicase and primase assays. The IC_{50} value of ASP2151 against ssDNA-dependent ATPase (0.078 μmol/L) was found to be consistent with that of the antiviral activity against HSV-1 strain KOS as assessed in PRA (EC_{50}, 0.036 μmol/L, Table 2). In addition, the minimum concentration of ASP2151 at which DNA

replication of HSV-1 was almost completely inhibited was 0.1 μmol/L (Figure 2A), indicating a striking agreement between the inhibitory effect of ASP2151 on viral DNA replication and its antiviral effect as shown by the EC_{90} values of ASP2151 for HSV-1 using PRA (Table 2). These data suggest that the anti-HSV-1 activity of ASP2151 results from inhibition of the helicase-primase complex.

Thiazole urea- and 2 amino-thiazolylphenyl-type HPIs have been reported to possess comparable antiviral potency against HSV-1 and HSV-2 [Crute et al., 2002; Kleymann et al., 2002; Spector et al., 1998]. Indeed, PRA results demonstrated that BILS 179 BS and BAY 57-1293 had similar potency against HSV-1 and HSV-2 (Table 2). Of particular interest, in addition to its activity with regard to HSV-1 and HSV-2, ASP2151 demonstrated potent antiviral activity against VZV as well. The anti-VZV activity of ASP2151 was evaluated in VZV DNA quantification and PRA using not only laboratory-stocked strains but also several clinical isolates. In the PRA, the anti-VZV EC_{50} values for strains tested ranged from 0.038 to 0.10 μmol/L for ASP2151 compared with 1.3–5.9 μmol/L for ACV (Table 2). These PRA findings indicate that ASP2151 exerted more potent anti-VZV activity than did acyclovir in PRA, and this activity was also demonstrated in VZV-DNA quantification using real-time PCR. It is known that particular series of thymidine analogs like sorivudine possesses extremely potent *in vitro* anti-VZV activity to ACV and showed clinical efficacy more potent to ACV in herpes zoster patients [Gnann et al., 1998]. No helicase-primase inhibitor, however, has been reported to show anti-VZV activity so far. To our knowledge, ASP2151 is the first helicase-primase inhibitor exerting more potent anti-VZV activity than ACV.

As expected, ASP2151 was also active against an ACV-resistant VZV mutant (Table 3). Given that current nucleoside analog drugs, such as ACV and penciclovir, depend on viral TK for phosphorylation to an active form, drug-resistant mutants can be developed either through TK-negative mutations or reduced TK activity [Field, 2001; Morfin and Thouvenot, 2003]. Although the prevalence of ACV-resistant viruses is thought to be limited in immunocompetent patients (<1%), it cannot be ruled out that cross-resistant mutants may emerge, especially in immunocompromised patients [Field, 2001]. ASP2151 may therefore offer a therapeutic option for treating ACV- or penciclovir-resistant virus infections.

Our *in vivo* studies using acute oral administration of ASP2151 showed that the course of disease progression for HSV-1 was ameliorated by ASP2151 in a dose-dependent manner and that cutaneous lesions and the mortality on day 17 post-infection due to HSV-1 infection were significantly improved. Since VZV is hard to infect to and replicate in animals, no conventional animal model has yet been developed to evaluate anti-VZV efficacy *in vivo*. In the present study, we assessed *in vivo* antiviral activity of ASP2151 using an HSV-1 zosteriform-spread model in mice to mimic zoster infections. In the present study, ASP2151 demonstrated 14-fold potent anti-HSV activity to valaciclovir in the model (Figure 3). Because ASP2151 showed equipotent antiviral activity against HSV-1 and VZV *in vitro*, the present *in vivo* data suggest the therapeutic potential of ASP2151 against VZV infections. Importantly, ASP2151 was well tolerated and revealed no obvious safety concerns in the 5-day experiment of dosing up to 30 mg/kg twice daily in mice. Furthermore, no safety issues were apparent in toxicology assessments in mice that

received ASP2151 for 4 weeks up to 500 mg/kg. The safety profile of ASP2151 may be explained at least partly by the high selectivity index (Table 2 and Table 3). Nevertheless, it is necessary to conduct further evaluations in terms of the tolerability, safety, and pharmacology profile of ASP2151 in preclinical and clinical studies before properly appraising this new anti-HSV and -VZV candidate.

Chapter 3 Characterization of Herpes Simplex Virus Type 1, 2, and Varicella-zoster Virus Strains Resistant to ASP2151

3.1. Introduction

Synthetic nucleoside analogs such as acyclovir (ACV), penciclovir (PCV), valaciclovir (VACV), and famciclovir targeting viral DNA polymerase have been utilized as the gold standard therapy against HSV and VZV infections since the late 1970s in clinical settings. However, these analogs share the same mechanism of action, requiring phosphorylation by viral thymidine kinase (TK) and host kinases. After triphosphorylation of nucleoside analogs, these analogs interfere with viral DNA polymerization through competitive inhibition with guanosine triphosphate and obligatory chain termination [Biron and Elion, 1980; Elion, 1993; Elion et al., 1977; Miller and Miller, 1980; Miller and Miller, 1982]. As viral TK is not essential for viral replication, HSV and VZV lacking a functional TK (TK-deficient or TK-altered viruses) are still viable and therefore are imbued with cross-resistance to the nucleoside analog drug class [Coen and Schaffer, 1980; Larder and Darby, 1986; Piret and Boivin, 2011].

Helicase-primase inhibitors (HPIs) could supplement the currently available therapies [Field and Biswas, 2011; Kleymann, 2004]. ASP2151 (amenamevir) is an HPI with an oxadiazolephenyl moiety that possesses antiviral activity against not only HSV-1 and HSV-2 but also VZV, differing from previously reported HPIs (BAY 57-1293 [Kleymann et al., 2002] and BILS 179 BS [Crute et al., 2002]), which inhibit HSV replication but not VZV, suggesting its application against diseases caused by both HSV (genital herpes, herpes labialis, and herpetic keratitis) and VZV infections (varicella and herpes zoster) [Levin and Schmader, 2007].

Mutants resistant to HSV-specific HPIs BAY 57-1293 and BILS 179 BS were previously characterized in terms of prevalence of pre-existing resistance, ability of replication *in vitro*, and pathogenicity in mice [Biswas et al., 2009; Crute et al., 2002; Levin and Schmader, 2007; Sukla, Biswas, Birkmann, Lischka, Ruebsamen-Schaeff, et al., 2010; Sukla, Biswas, Birkmann, Lischka, Zimmermann, et al., 2010]. However, few studies have been conducted to characterize ASP2151-resistant HSV and VZV mutants.

Here, to analyze the effect of amino acid mutations in helicase and primase on *in vitro* and *in vivo* growth characteristics, we characterized the ASP2151-resistant HSV-1, HSV-2, and VZV variants or mutants based on findings for sequencing analysis, growth, pathogenicity, and susceptibility testing.

3.2. Materials and Methods

3.2.1. Ethics Statement

All animals were housed and handled according to Astellas Pharma Inc.'s Institutional Animal Care and Use Committee guidelines and the Animal Ethical Committee guidelines of Yamanouchi Pharmaceutical Co., Ltd., which is now known as Astellas Pharma Inc.

3.2.2. Compounds

ASP2151 (molecular weight, 482.55; international non-proprietary name, amenamevir) was synthesized at Astellas Pharma Inc. (Tokyo, Japan). Acyclovir (ACV), vidarabine (araA), and idoxuridine (IDU) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Penciclovir (PCV) was purchased from LKT Laboratories Inc. (St. Paul, MN, USA).

3.2.3. Viruses and Cell Lines

HSV-1 strains (KOS, A4-3, and WT-51), HSV-2 strains (G, Lyon, and Kondo), and human embryonic fibroblast (HEF) cells were provided by Rational Drug Design Laboratories (Fukushima, Japan). HSV-1 US clinical isolates CI-25 and CI-116 and HSV-2 US clinical isolate CI-5243 were kindly provided by Dr. Sawtell from Cincinnati Children's Hospital Medical Center (Cincinnati, OH, USA). HSV-2 MS strain and Vero cells (derived from African green monkey kidney cells) were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were grown in growth medium consisting of Eagle's minimum essential medium (Sigma-Aldrich, St.

Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Maintenance medium containing 2% FBS was used for infection and propagation of viruses. HSV-1 and HSV-2 strains were propagated using HEF cells in maintenance medium.

3.2.4. Selection of ASP2151-resistant Mutants of HSV-1, HSV-2, and VZV

ASP2151-resistant mutants of HSV-1 or HSV-2 were derived from KOS- or Lyon-infected HEF cells through serial passages in the presence of ASP2151. ASP2151-resistant HSV-1 mutants were selected by 14 serial passages of supernatant of parent HSV-1 strain KOS starting from a single stock clone in the presence of stepwise increasing concentrations of ASP2151 from 0.1 to 160 μmol/L. ASP2151-resistant HSV-2 mutants were selected by three serial passages of stepwise increasing concentrations of ASP2151 from 1.0 to 10 μmol/L. The cell-free ASP2151-resistant mutants of HSV-1 and HSV-2 stock, respectively, designated as K2151^{r}m and L2151^{r}m, were stored at -80 °C.

ASP2151-resistant VZV mutant, "C2151^{r}m", was selected by serial passage of VZV strain CaQu using HEF cells in the presence of stepwise-increasing concentrations of ASP2151 from 0.1 to 60 μmol/L. In brief, monolayered HEF cells in a 25 cm^2 flask were initially infected with cell-free VZV stock of parental strain CaQu and cultured in the presence of EC_{50} concentration of ASP2151 (0.1 μmol/L) until cytopathic effects were visible. After cells were harvested, 10% of the collected cells were then dispersed on fresh monolayered HEF cells as cell-associated VZV source

and incubated in the presence of 1-, 2-, or 4-times concentration of ASP2151 than previously used. After 4 to 6 days incubation, the cells from the flask, in which cytopathic effects were evident for over 50% of cells, were used as viral source for next passage. The procedure was continued until ASP2151 concentration reached at 60 μmol/L (600 times EC_{50} value). After the ASP2151 concentration reached at 60 μmol/L, virus passage was repeated 5 times in the presence of 60 μmol/L ASP2151 to avoid a contamination. Total number of passages and total period of the process were 16 and 72 days, respectively. Then cell-free VZV stock, designated as C2151^{r}m, was prepared according to the method described previously [Baba et al., 1984].

3.2.5. Plaque-purified ASP2151-resistant HSV Mutants

Plaque-purified ASP2151-resistant HSV-1 and HSV-2 mutants were obtained via single plaque purification from K2151^{r}m and L2151^{r}m, respectively, using HEF cells in 24-well plates. To prepare the plaque-purified stocks, a single plaque was picked up from one of the plaques of K2151^{r}m or L2151^{r}m present in infected HEF cells using a micropipette under a microscope (repeated three times). Large-volume stocks isolated from the third passage were then prepared in ASP2151-free medium by infecting HEF cells in a 75-cm^2 tissue culture flask.

3.2.6. Sequencing Analyses

DNA regions, including the full-length open reading frame of the helicase (U_L5) and primase (U_L52) genes of HSV-1 and HSV-2, were amplified via polymerase chain reaction (PCR) using the

corresponding primer sets (Life Technologies, Carlsbad, CA, USA) (HSV-1 U_L5, 5'-ATCTCGCGGAACAGCATCGT-3' and 5'-GCTATACGCGATGGTCGTCTGT-3'; HSV-2 U_L5, 5'-AGGAGAATCTCGCGGAACA-3' and 5'-TGACCCAGGAGTCAAAGGG-3'; HSV-1 U_L52, 5'-GGATCATCTCATATTGTTCCTC-3' and 5'-GAAACAATAGGGTCTGGTTC-3'; HSV-2 U_L52, 5'-TGTCGTCCAGCGACTCTAGG-3' and 5'-CCAGAAACAACAGCGTCTGGT-3') and viral DNA extracted from cell-free ASP2151-resistant HSVs mutant stocks and the parent strain using TE-saturated phenol (Wako Pure Chemical Industries, Osaka, Japan) and chloroform (Kanto Chemical, Tokyo, Japan) extraction methods. In a similar way, DNA regions, including the full-length open reading frame of the helicase (ORF55) and primase (ORF6) genes of VZV, were amplified via PCR by using the corresponding primer sets (ORF55; 5′-TGGTCATTTGGGTTACTTCCA-3′ and 5′-AGTGAAGAACCCGCCTAAC-3′, ORF6; 5′-CAGCGGTTAAAGCCTCTTG-3′ and 5′- CGGTCCACCATTAATCACC-3′) and viral DNAs extracted from cell-free stock of the C2151^{r}m and its parent CaQu. Each PCR product was used as a template for direct sequencing (BigDye® Terminator v3.1 Cycle Sequencing kit; Applied Biosystems). Amino acid substitutions were analyzed using GENETYX® software (version 8.1.0; Genetyx, Tokyo, Japan).

3.2.7. Plaque Reduction Assay

Vero cells were seeded into 48-well cell culture plates and incubated until the cells formed a confluent monolayer. After the medium was removed, the cells were infected with HSV-1 and

HSV-2 strains at approximately 40 plaque-forming units (pfu)/well in maintenance medium followed by centrifugation at 2,000 rpm for 20 min at room temperature. After incubation for approximately 1 h at 37 °C, cells were washed twice with maintenance medium and treated with the test compound in maintenance medium containing 0.8% methylcellulose (Dow Chemical Company, Midland, MI, USA) until plaques appeared under a microscope. After fixing cells with 10% formalin in phosphate-buffered saline (Wako Pure Chemical Industries) and staining them with 0.02% crystal violet solution (Wako Pure Chemical Industries), the number of plaques was counted under a microscope.

3.2.8. One-step Growth Experiment

One-step growth of ASP2151-resistant mutants of HSV-1 or HSV-2 K2151^{r}m and L2151^{r}m8#C1 was compared with that of the respective parental wild-type virus strains KOS and Lyon. Vero cells were infected at a multiplicity of infection (MOI) of 1 pfu/cell and incubated at 37 °C for 26 h post-infection (pi). Infected cells and supernatant were collected at 0, 3, 6, 8, 10, 15, and 26 h pi and simultaneously harvested from 2 separate wells at each collection-time point, stored at -80 °C, and then thawed for virus titration via a plaque assay on Vero cells in 48-well plates.

3.2.9. Multi-step Growth Experiment of HSV-1 and HSV-2

Vero cells were seeded into a 75-cm^2 culture flask at 4×10^6 cells/flask with 40 mL growth

medium and incubated at 37 °C in a humidified atmosphere with 5% CO_2 until the cells formed a confluent monolayer. After medium was removed, the cells were inoculated with maintenance medium containing HSV-1 strains (KOS or K2151^{r}m) or HSV-2 strains (Lyon or L2151^{r}m8#C1) at 1.0×10^4 pfu/flask. Cells were incubated at 37 °C for 2 h, followed by 3 washes with maintenance medium (defined as 0 h pi) and incubation for 8 or 6 days with 50 mL/flask of maintenance medium for HSV-1 or HSV-2, respectively. Supernatant (200 µL) was collected every 12 h for 5 days and then every 24 h for 3 days for HSV-1 strains or at 4, 12, 20, 32, 48, 72, 96, and 144 h after inoculation for HSV-2 strains. All collected supernatants were stored at -80 °C until titration. Virus titers in the supernatant at each collection time point were titrated via plaque assay on Vero cells in 48-well plates. The titration assay was performed in duplicate using 200 µL of supernatant after serial 10-fold dilution (10- to 10^8-fold). The lower limit of detection in this assay was 25 pfu/mL when the mean number of plaques in the samples diluted 10-fold in duplicate was 0 or 0.5.

3.2.10. Replication Profile Analysis of ASP2151-resistant VZV Mutant

Replication kinetics of wild type VZV (CaQu) strain and ASP2151-resistant VZV mutant (C2151^{r}m) was analyzed by a conventional infectious center assay consisted of infecting HEF cells with 50 pfu of cells infected with C2151^{r}m or CaQu that were seeded in 24-well tissue culture plates. After indicating hours post-inoculation, two wells of cells were collected and dispersed with serial dilution on fresh HEF cells for titration of infectious cell number. Since C2151^{r}m needs longer incubation time than CaQu to form visible plaque, the cells for titration were then incubated

for 3 days or 5 days to count plaques of CaQu or C2151^{r}m, respectively.

3.2.11. *In Vivo* Pathogenicity Test of ASP2151-resistant HSV-1 Mutants

Hairless mice (HOS:HR-1, female, aged 7 weeks at infection; Japan SLC, Shizuoka, Japan) under anesthesia were inoculated with 15 μL of virus suspension containing 5.25×10^7 pfu/mL of HSV-1 K2151^{r}m or KOS parent strain on a strip of dorsolateral skin, where a grid-like pattern had been scratched using a needle before inoculation. Disease course was observed daily for 18 days and scored on a 0 to 7 composite scale based on the severity of zosteriform lesions and clinical symptoms according to the following criteria: 0, no sign of infection; 1, localized, barely perceptible small vesicles or crust; 2, a few vesicles or spreading crust; 3, large patches of vesicles or crust; 4, zosteriform vesicles or crust; 5, large patches of ulcers; 6, large, severe zosteriform ulcers; 7, hind limb paralysis or death. Lesions and neurological scores for each animal were combined to produce a composite score.

3.2.12. Detection Frequency of ASP2151- or ACV-resistant Variants

Vero cells were seeded into 6-well tissue culture plates at 6×10^5 cells/well and incubated at 37 °C in a humidified atmosphere with 5% CO_2 until the cells formed a monolayer. After the growth medium was removed, Vero cells were infected with HSV-1 strain KOS or HSV-2 strains G, Lyon, or Kondo in maintenance medium and then centrifuged at 2,000 rpm for 20 min at room temperature. After incubation for 1 to 2 h at 37 °C, the cells were washed with maintenance

medium and then incubated for 7 days in maintenance medium containing 0.8% methyl cellulose and a specified concentration of ASP2151 or ACV.

We defined HSV variants resistant to ASP2151 or ACV as those viruses able to replicate in the presence of those compounds. Concentrations of ASP2151 and ACV used were 20 times the 50% effective concentration (EC_{50}) values as determined in advance by a plaque reduction assay in Vero cells (Table 7). The cells were fixed with 10% formalin in phosphate-buffered saline and then stained with 0.02% crystal violet. Numbers of plaques formed by infection with compound-resistant variants were counted, and the frequency of ASP2151-resistant HSV variants was determined via plaque assay as the ratio of the number of plaques formed by variants plaques in the presence of a compound to the total number of plaques formed in infected cells under no-compound conditions. Frequency of compound-resistant HSV variants was then calculated using the formula: Frequency of compound-resistant variants = (Number of plaques formed in the presence of a compound) / (Total plaques formed under no-compound conditions)

3.2.13. Emergence of drug-resistant HSV Mutants under Long-term Treatment of ASP2151 and ACV

Vero cells were seeded into 75-cm^2 culture flasks at 4×10^6 cells/flask and then incubated at 37 °C in a humidified atmosphere with 5% CO_2 until a confluent monolayer was formed. After removal of the culture medium, the cells were infected with HSV-1 or HSV-2 strains at an MOI of 0.01 pfu/cell (0 h pi). After incubation at 37 °C for 2 to 3 h, the cells were washed with maintenance medium and incubated for 7 days in the presence of a test compound or vehicle (0.1%

dimethylsulfoxide; Sigma-Aldrich) in maintenance medium (40 mL/flask). The concentrations of ASP2151 and ACV used in the study were set at 20 times the EC_{50} value previously reported for each strain and almost completely inhibited the virus growth, efficacy much greater than that achieved with the 90% effective concentration for each strain (Table 6). Supernatants were harvested at 8, 24, 48, 72, 96, 120, 144, and 168 h pi with a volume of 200 μL each. Virus titer in the supernatants was determined using a plaque assay in Vero cells.

3.2.14. Statistical Analyses

Statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA). The EC_{50} values for plaque reduction assay were calculated using non-linear regression analysis with a sigmoid-E_{max} model. In determining the frequency of compound-resistant variants, results were analyzed using Student's t-test for comparison between ASP2151-resistant HSVs variants and ACV-resistant HSVs variants in four strains, and $P < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. Sequencing analysis and Susceptibility Test in ASP2151-resistant Mutants of HSV-1 K2151^{r}m and HSV-2 L2151^{r}m

DNA sequence analyses identified several single-base-pair substitutions that resulted in amino acid changes in helicase and primase of ASP2151-resistant mutants from parental HSV-1 and HSV-2 strains (Table 4 and Figure 4). The U_L5 helicase gene of HSV-1 K2151^{r}m and its

plaque-purified isolates contained substitutions from the parent KOS strain at nucleotide 1055 to generate G352V (G-to-T in the second base pair of parental 352$^{nd.}$ glycine to valine) and nucleotide 1065 to generate M355I (G-to-A in the third base pair of the 355th methionine to isoleucine) (Figure 4). The U_L52 primase gene of HSV-1 K2151^{r}m and its single plaque isolates contained a substitution at nucleotide 1090 to create S364G (A-to-G in the first base pair of the 364th serine to glycine). A mixed base-pair signal (A or G) was also found at nucleotide 1100 in the U_L52 primase gene of K2151^{r}m, wherein the second base pair of the 367th arginine codon was substituted with histidine or arginine. The R367H substitution was evident in K2151^{r}m single plaque isolates K2151^{r}m#B9 and K2151^{r}m#H10 but not in K2151^{r}m#D9 or K2151^{r}m#G11 (Table 4 and Figure 5).

In the ASP2151-resistant HSV-2 mutant L2151^{r}m#C1, DNA sequence analyses identified two single-base-pair substitutions that resulted in amino acid changes in the helicase gene but not the primase gene, distinct from the HSV-2 parental strain Lyon (Table 4 and Figure 4). The U_L5 helicase gene of L2151^{r}m#C1 contained substitutions from the parent strain at nucleotide 1065 to generate K355N (G-to-T in the third base pair of parental 356th lysine to asparagine) (Figure 4) and nucleotide 1352 to generate K451R (A-to-G in the second base pair of the 451st lysine to arginine).

3.3.2. Sequencing Analyses of the ASP2151-resistant VZV Mutant

To confirm that the anti-VZV activity of ASP2151 was due to targeting of the VZV helicase-primase complex, we isolated the VZV strain CaQu-derived mutant "C2151^{r}m", which is capable of replicating in the presence of ASP2151 at higher concentrations up to 60 μmol/L.

Sequencing analysis of ORF55 (helicase gene) and ORF6 (primase gene) of C2151^{r}m indicated three amino acid changes from the parent strain. Substitution of Asn-336 to Lys (N336K) was found in helicase motif IV, one of the six well-conserved sequence motifs in ORF55 (Figure 4). The other substitutions were Arg-446 to His (R446H) in ORF55, and Asn-939 to Asp (N939D) in ORF6.

3.3.3. Susceptibility of ASP2151-resistant Mutants of HSV-1 and HSV-2 to ASP2151

We conducted a plaque reduction assay to compare susceptibility to ASP2151 between the ASP2151-resistant mutants of HSV-1 and HSV-2 and to examine whether or not substitution of R367H in the HSV-1 U_L52 primase affects susceptibility of mutant strains to ASP2151. ASP2151-resistant mutants of both HSV-1 and HSV-2 showed less susceptibility to ASP2151 than either parent strain (Table 4). Mean EC_{50} values against K2151^{r}m#D9 and K2151^{r}m#G11, which possessed the S364G substitution in U_L52, were 19.6 and 28.2 μmol/L, respectively. In addition, the ASP2151-resistant mutants K2151^{r}m#B9 and K2151^{r}m#H10, with both the R367H and S364G substitutions in U_L52 were much less susceptible to ASP2151 than mutants with only the S364G substitution in U_L52 (105.4 and 118.0 μmol/L, respectively).

3.3.4. Growth of ASP2151-resistant Mutants in One-step Growth Experiment

Growth rates of wild-type virus and ASP2151-resistant mutants were compared in a one-step growth experiment (Figure 6). In the experiments with ASP2151-resistant HSV-1 mutants,

K2151^{r}m showed attenuated growth in Vero cells compared with its parental strain and exhibited relatively low titers through the exponential phase of growth (6-10 h pi), reaching a value 3.12 $\log_{10}$ (pfu/mL) lower than that of the parental strain at the final sampling point (26 h pi) (Figure 6A). Although the ASP2151-resistant HSV-2 mutant L2151^{r}m8#C1 showed a growth curve almost identical to its parental strain in the rapid growth phase (6-10 h pi), its titer was 1.31 $\log_{10}$ (pfu/mL) lower than that of its parental strain in the plateau phase (Figure 6B).

3.3.5. Multi-step Growth Experiment

A one-step growth experiment can usually be done at high MOI given the time course of virus replication and the virus yield per cell for a single cycle of infection. While the time from infection to plateau does not accurately reflect the time required for a single cycle of infection at low MOI, growth experiments conducted at relatively low MOI are useful for measuring both growth and spread of a virus in culture.

Multi-step growth ability of ASP2151-resistant K2151^{r}m or L2151^{r}m mutants was examined and compared with that of parental strains using Vero cells. Figure 7 shows the time course of the virus titer during the experimental period. In the titration assay of supernatants for HSV-1, the viruses were initially detected above the limit of detection level at 24 and 36 h pi for KOS and K2151^{r}m, respectively, with respective maximum titers of 7.03 and 5.84 $\log_{10}$ (pfu/mL). The virus titers of plaque-purified L2151^{r}m strains were lower than those of the parental strain at all sampling points. The titer of HSV-2 strain Lyon was 6.11 $\log_{10}$ (pfu/mL) at 144 h pi, whereas those of

L2151^{r}m8#C1, L2151^{r}m8#C2, L2151^{r}m10#B1, and L2151^{r}m10#B3 were 4.48, 4.52, 4.54, and 4.47, respectively.

Replication profile of ASP2151-resistant VZV C2151^{r}m in comparison with parent strain CaQu. Replication kinetics was analysed by a conventional infectious centre assay consisted of infecting HEF cells with 50 pfu of cells infected with C2151^{r}m or CaQu that were seeded in 24-well tissue culture plates. After indicating hours post-inoculation, two wells of cells were collected and dispersed with serial dilution on fresh HEF cells for titration of infectious cell number. Since C2151^{r}m needs longer incubation time than CaQu to form visible plaque, the cells for titration were then incubated for 3 days or 5 days to count plaques of CaQu or C2151^{r}m, respectively. Of particular note is the fact that C2151^{r}m shows marked defect in viral replication profile (Figure 8).

3.3.6. Pathogenicity of ASP2151-resistant HSV-1 Mutants

To examine whether or not the pathogenicity of K2151^{r}m differs from its parent strain *in vivo*, hairless mice were infected with equal titers of K2151^{r}m or KOS. Mean disease score for each group was plotted versus time post-infection (Figure 9). While no symptoms were observed during the experimental period in the K2151^{r}m-infected group, 8 of 10 animals receiving the KOS parent strain developed cutaneous lesions, with 4 of the 8 symptomatic animals dying. The result indicates that the ASP2151-resistant HSV-1 mutant K2151^{r}m have attenuated pathogenicity compared to the parent strain in hairless mice.

3.3.7. Activities of Existing Antivirals against ASP2151-resistant HSV-1 Mutants

Antiviral activities of ASP2151, ACV, PCV, araA, and IDU against KOS and K2151^{r}m strains were examined using the plaque reduction method. Mean EC_{50} values and 95% confidence intervals are summarized in Table 7. K2151^{r}m used in this study showed approximately 770-fold less susceptibility to ASP2151 than its parent strain, based on the difference in EC_{50} value. In contrast, mean EC_{50} values of existing antiviral drugs against KOS and K2151^{r}m strains were 0.31 and 0.28 μmol/L for ACV, 1.0 and 1.0 μmol/L for PCV, 9.9 and 11 μmol/L for araA, and 0.40 and 0.44 μmol/L for IDU, respectively, and 95% confidence intervals of those mean values against K2151^{r}m and KOS were extremely similar.

3.3.8. Frequency of ASP2151-resistant Variants of HSV-1 or HSV-2

We determined the frequency of naturally existing ASP2151-resistant variants in stocks of wild-type HSV-1 strain KOS or HSV-2 strains G, Lyon, and Kondo in comparison with that of ACV-resistant variants. The frequencies of the compound-resistant variants are shown in Table 8. The ASP2151-resistant HSV variants in all tested strains were significantly less frequent in virus stocks than ACV-resistant HSV variants from 8.77×10^{-7} to 1.04×10^{-6} ($P = 0.0002$). The fold frequency between ASP2151- and ACV-resistant HSV variants was 1389 (geometric mean value of all tested strains), indicating that the frequency of pre-existing ASP2151-resistant HSV variants was lower than that of ACV-resistant ones.

3.3.9. Emergence of ASP2151-resistant HSV Mutants *In Vitro*

Increases in the HSV-1 or HSV-2 titers in vehicle-treated samples were suppressed for a longer duration when treated with ASP2151 than with ACV (Figure 10). Treatment with ASP2151 held the viral titers of seven of eight strains tested at or under the detection limit during the experimental period. Although the viral titers of HSV-1 strain CI-25 treated with ASP2151 increased after 120 h pi, the level was approximately 3 log less than that noted in vehicle-treated cells at 168 h pi. In contrast, the titers of all strains treated with ACV increased at or after 48 h pi, and the yields of seven of eight strains reached a level comparable to that for vehicle treatment at 168 h pi.

The susceptibility test conducted using HSV-1 and HSV-2 in the supernatants of each strain obtained at 168 h pi revealed that the EC_{50} values of ACV against ACV-treated HSV strains were higher than values against the vehicle-treated strain. The EC_{50} values (μmol/L) of ACV against ACV- and vehicle-treated HSV-1 stains were 61 and 1.3 for KOS, 29 and 2.1 for WT51, 43 and 0.93 for CI-25, and 12 and 0.89 for CI-116, respectively, while values against ACV- and vehicle-treated HSV-2 strains were 180 and 5.6 for G, >200 and 4.1 for Lyon, 170 and 3.6 for Kondo, and >200 and 1.0 for CI-5243, respectively. ASP2151-treated HSV-1 strain CI-25 had an ASP2151 EC_{50} value approximately six times that for the vehicle. However, ACV-treatment resulted in no obvious changes in susceptibility to ASP2151.

3.4. Discussion

Sequencing analyses revealed several single-base-pair substitutions resulting in amino acid changes in helicase and primase of the ASP2151-resistant HSV mutants. Amino acid alterations in the helicase subunit were clustered near helicase motif IV in the U_L5 helicase gene of both HSV-1 and HSV-2, while the primase subunit substitution associated with reduced susceptibility was found only in ASP2151-resistant HSV-1 mutants. Interestingly, we found that R367H with S364G substitution in the U_L52 primase gene (double mutation) enhanced resistance to ASP2151 compared with S364G substitution alone (single mutation). ASP2151-resistant HSV mutants showed attenuated growth in tissue culture compared to the parent strains in both single- and multi-step growth experiments. In addition, ASP2151-resistant HSV-1 mutant K2151^{r}m had attenuated pathogenicity compared to the parent strain *in vivo*. Further, ASP2151-resistant HSV-1 mutant K2151^{r}m was susceptible to nucleoside analog antivirals tested in this study. Taken together, results from the present study suggest that mutations in the helicase-primase complex found in ASP2151-resistant HSV mutants reduce viruses' growth capability and pathogenicity but left them with susceptibility to existing antiherpetic agents similar to that of parent and wild-type viruses.

Sequence analyses of U_L5 helicase and U_L52 primase genes of ASP2151-resistant HSV mutants K2151^{r}m and L2151^{r}m have helped to characterize ASP2151-resistant HSV mutants. Amino acid changes in the primase of K2151^{r}m single plaque-purified isolates were found at S364G, with or without similar changes at R367H. Results of susceptibility tests (Table 4) indicated that the R367H substitution in U_L52 primase contributes to HSV-1 acquiring ASP2151-resistance,

suggesting that ASP2151 might interact with not only helicase but also primase in the HSV-1 helicase-primase complex.

The amino acid sequence of the helicase and primase between HSV-1 and HSV-2 are highly homologous (89% and 82%, respectively). Moreover, the amino acid sequence of helicase motif IV and its adjacent regions are identical between HSV-1 and HSV-2 (Figure 4). The helicase motif IV is one of the six helicase motifs that form the functional active site; these motifs are known to be essential for the activity of the HSV-1 helicase-primase complex [Biswas, Kleymann, et al., 2008; Biswas, Tiley, et al., 2008; Field and Darby, 1980; Sukla, Biswas, Birkmann, Lischka, Ruebsamen-Schaeff, et al., 2010]. It has been reported that amino acid mutations were identified at the region close to the helicase motif IV in HSV-1 mutants resistant to BILS 179 BS and BAY 57-1293 (Figure 4), suggesting the presence of a putative binding region of HPIs to the helicase-primase complex. In analyses of ASP2151-resistant HSV-1 and HSV-2 mutants selected by serial passage in the presence of ASP2151, amino acid mutations were noted at sites close to the helicase motif IV, and the ASP2151-resistant HSV also showed relatively low susceptibility to BILS 179BS and BAY 57-1293. Thus, it is reasonable to speculate that ASP2151 and other HPIs may target, at least in part, an indistinguishable binding site structurally close to the helicase motif IV accounting for the equipotent antiviral effects against HSV-1 and HSV-2.

Considering the physiochemical properties of individual amino acid residues, S364G substitution of U_L52 in K2151^{r}m mutants is believed to cause a physiochemical change from an uncharged polar to a nonpolar residue at the substitution point. Similarly, K355N substitution of

U_L5 in the L2151^{r}m mutant is believed to induce a physiochemical change from a basic side chain to an uncharged polar side chain. These physiochemical changes may be the reason for the failed interaction between ASP2151 and helicase-primase complex resulting in emergence of ASP2151-resistant HSV mutants. In addition, while other amino acid substitutions in ASP2151-resistant mutants (G352V and M355I in U_L5 of HSV-1, nonpolar to nonpolar; R367H in U_L52 of HSV-1, basic to basic; K451R in U_L5 of HSV-2, basic to basic) do not evoke marked changes in physiochemical property, these substitutions may induce conformational changes in the helicase-primase complex, thereby reducing the interaction between ASP2151 and the helicase-primase complex. However, conformational interactions between ASP2151 and the helicase-primase complex remain to be elucidated, and X-ray structural analysis and enzymatic activity analysis may reveal the mechanism of ASP2151 action.

Drug-enzyme interaction between the HSV helicase-primase complex and ASP2151 with respect to involvement of amino acid residues of U_L5 and U_L52 remain unclear. While the effect of amino acid substitutions in the U_L5 of BAY 57-1293-resistant mutants on the growth of HSV-1 in tissue culture and pathogenicity in a mouse model of skin infection has been investigated [Biswas et al., 2007; Biswas et al., 2009; Biswas, Tiley, et al., 2008; Sukla, Biswas, Birkmann, Lischka, Ruebsamen-Schaeff, et al., 2010], the effects of amino acid substitution in U_L52 of these mutants have not been well elucidated. Biswas et al. previously suggested that BAY 57-1293 may interact with both the helicase and primase subunits of the helicase-primase complex in HSV-1 [Biswas, Kleymann, et al., 2008] but provided no concrete data of resistant amino acid substitutions in U_L52

directly related to reduced susceptibility over that of wild type strains. Our present findings showed that positional differences of resistant substitution in U_L52 existed between BAY 57-1293- and ASP2151-resistant HSV-1 mutants, suggesting that these two HPIs might interact differently with the helicase-primase complex of HSV-1. Further investigation is needed to elucidate whether or not particular drug-resistant HSV mutants show cross-resistance.

Sequence analysis of the ORF55 helicase and ORF6 primase genes of the ASP2151-resistant VZV mutant C2151^{r}m identified amino acid changes from the parent strain at N336K and R446H in the helicase gene and at N939D in the primase gene. The C2151^{r}m was prepared as a VZV mutant strain by passaging the parent CaQu strain in the presence of stepwise ASP2151 concentrations from 0.1 μmol/L to 60 μmol/L. C2151^{r}m shows marked defect in viral replication profile. As a consequence, we were unable to obtain the C2151^{r}m cell-free viral stocks with sufficient viral titre to characterize the mutant for *in vitro* test. An amino acid substitution in the mutant that conferred resistance to ASP2151 was speculated to result in the observed poor growth of C2151^{r}m. Recently, Biswas et al. reported that the BAY 57-1293–resistant HSV-1 mutant strain BAY-pF-r3 contained a single amino acid substitution of Asn to Lys at position 342 (N342K) located in the U_L5 helicase motif IV, although substitutions at amino acid positions downstream of the helicase motif IV had been found in all HPI-resistant HSVs previously reported [Biswas et al., 2009]. Interestingly, based on amino acid alignment, the 342nd amino acid position in HSV-1 corresponds to the 336th position in VZV (Figure 4). The BAY-pF-r3 mutant showed decreased growth property in comparison with the wild-type virus. Given the analogy with the BAY-pF-r3

mutation, the N336K substitution is also likely to confer resistance to ASP2151 for VZV. In addition to the N336K substitution, we found other amino acid changes such as R446H in the helicase gene and N939D in the primase gene in the C2151^{r}m, although their importance for C2151^{r}m remains to be evaluated. Genomic DNA sequence database of VZV strains revealing that amino acid at position 446 in helicase varies between either Arg or His has suggested that R446H may be a naturally occurring polymorphism. Interestingly, BAY 57-1293 has been suggested to interact with both the HSV helicase-primase subunits, since an amino acid mutation in the primase subunit, A899T in U_L52, was found in a BAY 57-1293–resistant HSV-1.35 Further analyses of mutations in ASP2151-resistant VZV mutants will aid in determining how ASP2151 targets the VZV helicase-primase complex.

Although TK-deficient HSV mutants resistant to ACV exhibit reduced virulence in animal models [Field and Darby, 1980], some TK-deficient HSVs cause progressive diseases in humans [Shimada et al., 2007], and ACV-resistant pathogenic HSV infections pose a growing problem in immunocompromised patients [Erlich et al., 1989], particularly among those receiving bone marrow transplantation [Danve-Szatanek et al., 2004; Morfin and Thouvenot, 2003; Saijo et al., 2002; Stránská et al., 2005]. In a recent study using a mouse model of wild type and TK-deficient HSV-2 ear pinna inoculation, ASP2151 showed therapeutic efficacy in mice infected with both wild-type and TK-deficient HSV-2 [Himaki et al., 2012]. In both one- and multi-step growth experiments and the *in vivo* pathogenicity test in the present study, we observed attenuated growth and pathogenicity in ASP2151-resistant mutants compared with the parent strain, ostensibly due to

impairment of enzyme activity in the mutant helicase-primase complex. Even when ASP2151-resistant mutants emerged after treatment with a concentration insufficient to inhibit virus replication, these mutants showed attenuated growth and pathogenicity lower than or, at most, equivalent to that in ASP2151-susceptible strains. Because of the difference in targets between ASP2151 and existing antiherpes agents, existing agents will be effective against any potentially emerging ASP2151-resistant HSV mutants (Table 7).

Frequencies of ACV-resistant HSV variants have been reported to range from 7.5×10^{-4} to 1.5×10^{-3} in both clinical specimens and laboratory stock strains [Shin et al., 2001], and those frequencies for ACV-resistant HSV-1 and HSV-2 variants observed in the present study were comparable to these published results. TK-deficient HSV mutants resistant to ACV can potentially replicate in the presence of ACV, as the TK gene is not essential for virus replication. In contrast, because the protein subunits comprising the helicase-primase complex are all essential for replication of HSV DNA, HSV mutants deficient in helicase, primase, or cofactor and resistant to ASP2151 cannot exist. As such, while HSV variants with reduced susceptibility to ASP2151 might emerge, such ASP2151-resistant variants could not grow to occupy the majority in a virus population. These hypotheses are supported by the lack of observed elevation in virus titers of ASP2151-resistant HSV mutants in seven of eight strains during the test period (Figure 10). Although ACV suppressed increases in virus titers compared to the vehicle, the virus titers of all HSV strains treated with ACV eventually rose due to the emergence and increased proportion of drug-resistant viruses. In contrast, ASP2151 continuously suppressed HSV-1 replication *in vitro*

with a lower risk of emergence of viruses resistant to HSV than to ACV.

Chapter 4 Synergistic Activity of ASP2151 with Acyclovir against Herpes Simplex Virus Type 1, 2 and Varicella-zoster Virus

4.1. Introduction

In evaluation of multi-drug combination therapy for infectious diseases, determining whether or not the therapy is more effective than either monotherapy is of great importance. While multi-drug therapy can potentially improve treatment efficacy, it also presents the risks of increased harm and resource utilization. Compounding of harms related to pharmacotherapy may be particularly pronounced when combining multiple drugs that act in a similar manner. One strategy in designing multi-drug combination therapy for increased efficacy is to select agents with different mechanisms of action. However, for the treatment of herpesvirus infections caused by herpes simplex virus (HSV) type 1 (HSV-1), HSV type 2 (HSV-2) and varicella-zoster virus (VZV), limited data are available on multi-drug combination therapy using antiherpes drugs with different mechanisms of action.

Although nucleoside analogs used as standard monotherapy for HSV and VZV share similar mechanism of actions, combined antiviral treatment with a drug which has a different mechanism of action is expected to show synergistic antiviral activity [Safrin et al., 1994; Suzuki et al., 2006]. Indeed, combination administration of ACV and foscarnet showed a subadditive antiviral effect, which is less than the calculated additive combination effect but greater than the most effective

agent alone, activity against HSV-1 and additive antiviral activity against HSV-2 [Schinazi and Nahmias, 1982]. However, this combination of nucleoside analog antiherpes drugs may be difficult to administer effectively due to similar toxicity and differences in dose and route of administration [Brady and Bernstein, 2004].

Helicase-primase inhibitors (HPIs) are antiherpetic agents that may be used to supplement currently available antiherpes therapies [Field and Biswas, 2011; Kleymann, 2004]. The oxadiazolephenyl derivative ASP2151 (amenamevir) is an HPI with antiviral activity against not only HSV-1 and HSV-2 but also VZV, setting it apart from other HPIs reported to date—BAY 57-1293 [Kleymann et al., 2002] and BILS 179 BS [Crute et al., 2002]—which inhibit only HSV replication. Due to promising preclinical profiles on antiviral activity, safety, tolerability and pharmacokinetics, ASP2151 was selected as a development candidate and the clinical efficacy of ASP2151 has been evaluated in two phase-2 clinical studies for patients with herpes zoster (ClinicalTrials.gov Identifier: NCT00487682) and genital herpes [Tyring et al., 2012]. Given its different mechanism of action compared with nucleoside analog drugs, ASP2151 is expected to exhibit a combination effect with existing nucleoside analog antiherpes drugs against HSVs and VZV strains and nucleoside analog drug-resistant mutants, as well as to avoid increased toxicity and complication of administration caused by the combination therapy of nucleoside analog drugs.

However, while combination therapy of ASP2151 with nucleoside analog antiherpes drugs may be a promising option in cases of severe disease in which superior antiviral treatments are required, such as herpes encephalitis or disseminated HSV and VZV infections in patients with

immunosuppression [Tyler, 2004; Whitley and Lakeman, 1995], whether or not such combination therapy will have synergistic, additive, or antagonistic effects compared to either drug in monotherapy remains unclear.

Here, to assess the combination therapy of ASP2151 and existing nucleoside analog antiherpes drugs, we tested the antiviral activity of ASP2151 combined with ACV and other nucleoside analogs, PCV and vidarabine (VDB), *in vitro* and a combination therapy with ASP2151 and VACV in a mouse model of zosteriform spread.

4.2. Materials and Methods

4.2.1. Ethics Statement

All animals were housed and handled in accordance with the Animal Ethical Committee guidelines of Yamanouchi Pharmaceutical Co., Ltd., which is now known as Astellas Pharma Inc., and the Astellas Pharma's Institutional Animal Care and Use Committee guidelines.

4.2.2. Antiviral Compounds

ASP2151 (molecular weight, 482.55; international non-proprietary name, amenamevir) was synthesized by Astellas Pharma Inc. (Tokyo, Japan). ACV (Sigma-Aldrich, St. Louis, MO, USA), PCV (LKT Laboratories, St. Paul, MN, USA), VDB (Sigma-Aldrich, St. Louis, MO, USA), and VACV as Valtrex® film tablets (GlaxoSmithKline, Middlesex, UK) were purchased from commercial suppliers.

4.2.3. Cells and Viruses

Two HSV-2 strains, "Genital isolate" and "Whitlow 2," were isolated from genital ulcer and recurrent whitlow clinical specimens, respectively [Shimada et al., 2007]. Five ACV-resistant VZV mutants (TK-deficient mutant, A2, A3, A7, and A8) were isolated in the presence of increasing concentrations of ACV (20, 50, or 100 μmol/L) by 3 passages at each concentration until the appearance of cytopathic effect (CPE) following plaque purification twice in the presence of 100 μmol/L of ACV.

A2, A3, A7, and A8 (ACV-resistant DNA polymerase mutants of VZV) are divided into two phenotype groups of resistance against other antiherpes drugs. A2 and A3 are both foscarnet- and VDB-resistant mutants, while A7 and A8 are both foscarnet- and VDB-hypersensitive mutants [Kamiyama et al., 2001; Shiraki et al., 1983]. HSV-1 strains, KOS, A4-3, and WT-51, and human embryonic fibroblast (HEF) cells were provided by Rational Drug Design Laboratories (Fukushima, Japan). HSV-2 MS strain (ATCC number: VR-540), VZV Ellen strain (ATCC number: VR-1367), Vero cells derived from African green monkey kidney (ATCC number: CCL-81), and MRC-5 cells derived from human embryonic lung fibroblast (ATCC number: CCL-171) were purchased from American Type Culture Collection (Manassas, VA, USA). HEF, Vero, and MRC-5 cells were grown in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). HSV-1, HSV-2, and VZV were propagated using HEF or MRC-5 cells in maintenance medium containing

2% FBS.

4.2.4. *In Vitro* Susceptibility Test.

4.2.4.1. Plaque Reduction Assay (PRA)

Vero cells and HEF cells were seeded into 48- or 96-well cell culture plates and incubated until the cells formed a monolayer. After the medium was removed, the cells were infected with HSV-1, HSV-2, or VZV at an inoculum of approximately 40 plaque-forming units (pfu)/well. The viruses were allowed to absorb onto the cells for 1 h at 37 °C, and after being washed twice with maintenance medium, cells were treated with the test compound in maintenance medium with 0.8% methylcellulose for HSV-1 and HSV-2 or without 0.8% methylcellulose for VZV until apparent plaques appeared. The cells were then fixed with 10% formalin in PBS and stained with 0.02% crystal violet solution. After gently rinsing the wells with water and allowing the plates to dry, we counted numbers of plaques using a microscope.

4.2.4.2. Antiviral Assay of ASP2151 Combination with Acyclovir Using PRA

A checkerboard plate format was used to test 11 concentrations of ASP2151 or ACV including vehicle wells. Solutions of ASP2151 and ACV were prepared by 2-fold serial dilution, with concentrations of ASP2151 ranging from 1.0 to 0.0001 and concentrations of ACV ranging from 30 to 0.003 μmol/L. We selected a range of test compounds for evaluation of combined antiherpes efficacy based on dose-response curve plots of ASP2151 and ACV against HSV-1

(KOS), HSV-2 (Genital isolate), and VZV (Kawaguchi). The combined antiherpes effect of ASP2151 with ACV *in vitro* was assessed based on graphical evaluation by isobologram and statistical analysis by response surface modeling.

4.2.4.3. Combined Antiviral Assay of VZV Strain Ellen

MRC-5 cells were seeded at 7.5×10^4 cells/well in 24-well plates using growth medium. The plates were incubated overnight at 37 °C and 5% CO_2. The following day, media was aspirated and approximately 90 pfu of VZV strain Ellen was added to 21 wells of each plate in a volume of 200 µL of assay medium. The remaining 3 wells of each plate served as cellular control wells and received 200 µL of assay medium without virus. The virus was allowed to adsorb onto the cells for 1 h at 37 °C and 5% CO_2.

Compounds were prepared by dilution in overlay medium. After incubation, 1 mL of each drug dilution (or combination of two drugs) was added to the assay wells (without aspirating the virus inoculums). A checkerboard plate format across two 24-well plates was used to test 5 concentrations of ASP2151 in all possible combinations with 5 concentrations of ACV, PCV, or VDB. Overlay medium (without drug) was added to the three cell control wells and to three virus control wells on each plate. The plates were incubated for five days to allow for plaque formation. Cultures were examined microscopically, and compound precipitation and toxicity were noted if present. The media was then aspirated from the wells and the cells fixed and stained using 20% methanol containing crystal violet. Plaques were counted via microscope.

4.2.4.4. MTS Assay

After incubating plates at 37 °C and 5% CO_2 for 5 days, the assay plates were stained with the soluble tetrazolium-based dye MTS (CellTiter 96 Aqueous One Solution; Promega, Madison, WI, USA) to determine cell viability and evaluate virus replication. At assay termination, 10 µL/well of MTS reagent was added to 96-well microtiter plates that were then incubated at 37 °C for 4 h. The MTS formazan was then measured spectrophotometrically at 490/650 nm using a Vmax (Molecular Devices, Sunnyvale, CA, USA) or SpectraMaxPlus plate reader (Molecular Devices).

4.2.4.5. Combined Antiviral Assay of HSV-2 using MTS Assay

Compounds were evaluated for antiviral activity against HSV-2 strain MS in Vero cells using a virus-induced CPE-inhibition assay procedure. Antiviral assays were performed at an FBS concentration of 2%. On the day of the assay, growth medium was removed from the pre-plated cells and replaced with test drugs (50 µL) and virus suspension (50 µL). A checkerboard plate format was used to test 8 concentrations of ASP2151 (1000, 320, 102, 32.8, 10.5, 3.38, 1.07, and 0.34 µmol/L) in all possible combinations with five concentrations of ACV (200, 40, 8, 1.6, and 0.32 µmol/L), PCV (200, 40, 8, 1.6, and 0.32 µmol/L), or VDB (150, 30, 6, 1.2, and 0.24 µmol/L). Combination antiviral efficacy was evaluated in triplicate on identical assay plates that included cell control wells (cells only) and virus control wells (cells plus virus). Antiviral efficacy was

monitored via MTS staining at the experimental endpoint.

4.2.5. *In Vivo* Evaluation of Combined Therapy of ASP2151 with Valaciclovir in HSV-1 Infected Mouse Model of Zosteriform Spread

Hairless mice (HOS:HR-1, female, aged 7 weeks at virus infection; Hoshino Laboratory Animals, Saitama, Japan) were infected (designated as Day 0 post-infection) with HSV-1 strain WT51 (15 μL/body of virus suspension at a titer of 8.0×10^5 pfu/mL) on a dorsolateral strip of skin that had been scratched in a grid-like pattern with a 27-gauge needle under anesthesia [Nagafuchi et al., 1979]. ASP2151 was suspended in 0.5% methylcellulose (MC) solution to prepare 1, 3, and 10 mg/10 mL suspensions, while VACV was suspended in 0.5% MC solution to prepare 10 and 30 mg/10 mL suspensions. ASP2151 and VACV were then suspended together in 0.5% MC to make combination solutions of intended concentrations (10 mg/kg twice daily VACV with 1, 3, or 10 mg/kg twice daily ASP2151; 30 mg/kg twice daily VACV with 1, 3, or 10 mg/kg twice daily ASP2151). The compounds were orally administered at 10 mL/kg body weight twice daily for 5 days starting 3 h after virus inoculation.

Disease course was monitored daily for 17 days and scored on a composite scale from 0 to 7 based on the severity of zosteriform lesions and general symptoms according to the following criteria: 0, no sign of infection; 1, localized, barely perceptible small vesicles; 2, slight vesicle spread; 3, large patches of vesicles formed; 4, zosteriform vesicles; 5, large patches of ulcers formed; 6, large zosteriform ulcers (severe); 7, hind limb paralysis or death. The mean composite

disease scores of zosteriform lesions and neurological deficits for individual animals were determined daily after infection. The extent of disease was measured from the area under the curve (AUC) of the mean daily composite disease score each day after viral inoculation.

4.2.6. Statistical Analyses

The 50% effective concentration (EC_{50}) values for PRA were calculated using non-linear regression analysis with a sigmoid-E_{max} model (GraphPad Prism 5; GraphPad Software, La Jolla, CA, USA). In graphical evaluation of synergy, the EC_{50} values of the assessed agents in their various concentrations were plotted using an isobologram. Synergy and antagonism were defined as deviations from dose-wide additivity, which results when two drugs interact as if they were the same drug. Curves falling below the line of additivity indicate synergy, while those on the line indicate an additive reaction, and those above the line indicate an antagonistic reaction [Kuramoto et al., 2010; Kurokawa et al., 2001; Suzuki et al., 2006]. In statistical analysis of combined antiviral activity, a response surface model was used [Meletiadis et al., 2003; Tallarida, 2001]. An interaction parameter, alpha (α) was calculated using Greco's response surface model estimation [Greco et al., 1995; Greco et al., 1990]. When α and the lower 95% confidence limit are positive, synergism is indicated; when α and the upper 95% confidence limit are negative, antagonism is indicated; and when α is zero or the 95% confidence interval includes zero, no interaction or additivity is indicated. Response surface model analysis was performed using SAS software (SAS Institute Inc., Cary, NC, USA).

The effect of ASP2151 or VACV monotherapy *in vivo* was analyzed in terms of reduction in

AUC of the disease score with placebo using Dunnett's multiple range test, with $P<0.05$ considered statistically significant. To assess significant differences in the VACV monotherapy group, Dunnett's multiple range test was used to compare AUCs between VACV alone and the combination of corresponding doses of VACV with every dose of ASP2151 at a significance level of $P<0.05$ using SAS software.

4.3. Results

4.3.1. *In Vitro* Susceptibility Test against Acyclovir-resistant or Acyclovir–susceptible HSV-1, HSV-2 and VZV Strains

Two HSV-2 strains, "Genital isolate" and "Whitlow 2," were obtained from the genital ulcer and recurrent whitlow in a 40-year-old man with acute myelogenous leukemia. Genital isolate is an ACV-susceptible variant, and Whitlow 2 is an ACV-resistant variant [Shimada et al., 2007]. Table 9 shows the antiviral activities of ASP2151 and ACV against ACV-resistant and ACV-susceptible HSV-1, HSV-2, and VZV strains tested using PRA. Although EC_{50} values of ACV against ACV-resistant HSV-1, HSV-2, and VZV strains were higher than those of ACV-susceptible strains, EC_{50} values of ASP2151 against ACV-resistant strains were similar to those for susceptible strains.

4.3.2. *In Vitro* Antiherpes Activity of ASP2151 in Combination with Acyclovir

Results of graphic evaluation of combined antiviral activity of ASP2151 and ACV on plaque

formation of the HSV-1 KOS strain, the HSV-2 Genital isolate strain, and the VZV Kawaguchi strain, which are all ACV-susceptible virus strains, are shown in Figure 11. In isobologram analysis, the curves connected with EC_{50} values fell on or below the line of the additive effect at all concentrations in HSV-1, HSV-2, and VZV, indicating that combined treatment of ASP2151 with ACV worked synergistically against tested virus strains.

To statistically assess the combined antiherpes effect of ASP2151 with ACV on plaque formation *in vitro*, we analyzed the dataset plotted in the isobolograms (Figure 11) using the response surface model. Dose ranges against each virus and Greco's response surface model interaction parameter for each virus are shown in Table 10. The interaction parameter and its lower 95% confidence limit for ASP2151 and ACV were positive in all viruses, indicating that combination treatment of ASP2151 with ACV exhibited statistically significant synergy against tested strains of HSV-1, HSV-2, and VZV ($P<0.0001$, $P=0.0009$, $P=0.0005$, respectively). In addition to antiviral activity, we microscopically examined cytotoxicity for monotherapy and combination therapy. We observed no notable cytotoxicities of monotherapy and combination therapy of ASP2151 (highest concentration: 1.5 μmol/L) and ACV (highest concentration: 30 μmol/L) at concentrations that substantially inhibit virus plaque formation.

Response surface model analysis was also applied to other combination therapies of ASP2151 with nucleoside analog antiherpes drugs using MTS assay for HSV-2 and PRA for VZV. The combination effect of ASP2151 with ACV, PCV, or VDB against HSV-2 strain MS and VZV strain Ellen on virus replication *in vitro* showed synergistic efficacy against all tested virus strains in each

drug combinations except for combination of ASP2151 with VDB against HSV-2 strain MS, which showed only an additive effect (Table 11). In addition, we tested cell viability and compound toxicity in Vero cells and MRC-5 cells using MTS assay and analyzed its synergistic toxicity. There was no evidence of synergistic cytotoxicity within the concentrations examined for ASP2151 (highest concentration: 1 μmol/L) in combination with tested compounds (highest concentration: 200 μmol/L ACV; 200 μmol/L PCV; 200 μmol/L VDB).

4.3.3. Combination Therapy of ASP2151 with Valaciclovir in HSV-1-Infected Mouse Model

The combined effect of ASP2151 with VACV was studied in an HSV-1-infected mouse model of zosteriform spread. The mean disease score for each treatment group was calculated and plotted versus the time post-infection (Figure 12). While 1 mg/kg twice-daily monotherapy of ASP2151 appeared to have a modest but consistent effect on composite disease scores, the difference was not statistically significant compared with the vehicle group ($P = 0.191$). However, ASP2151 reduced composite disease scores by 68% at 3 mg/kg twice daily ($P=0.0015$) and 100% at 10 mg/kg twice daily ($P<0.001$) when compared to the AUC of the mean disease curve. VACV monotherapy significantly reduced the AUC by 42% at 10 mg/kg twice daily ($P<0.05$) and 52% at 30 mg/kg twice daily $P=0.015$). While combination therapy with 10 mg/kg VACV and 1 mg/kg ASP2151 induced no significant changes compared with 10 mg/kg VACV alone, combination therapy with 3 mg/kg ASP2151 reduced composite disease scores by 75% ($P=0.014$), and 10 mg/kg ASP2151 reduced composite disease scores by 100% ($P=0.0009$) compared to 10 mg/kg VACV monotherapy.

Further, combination of 30 mg/kg VACV with 1, 3, or 10 mg/kg ASP2151 was significantly more effective at reducing composite disease scores by 75%, 100%, and 100%, respectively, compared to 30 mg/kg VACV monotherapy (Figure 13).

4.4. Discussion

We demonstrated that ASP2151 exerted antiviral activity against ACV-susceptible HSV-2 strain, ACV-susceptible VZV strain, and ACV-resistant VZV mutants, and that ASP2151 combined with ACV, PCV, or VDB exerted a synergistic or additive antiviral effect against HSV-1, HSV-2, and VZV replication *in vitro*. Combination therapy with ASP2151 and VACV significantly conferred additional antiviral benefit over VACV monotherapy in an HSV-1 infected mouse model of zosteriform spread.

In the herpes virus genome DNA replication cascade, helicase unwinds double-stranded DNA while primase synthesizes primers prior to DNA polymerization [Liptak et al., 1996; Muylaert et al., 2011]. Studies of HSV-1 helicase activity have shown that leading strand synthesis by DNA polymerase does not stimulate the rate of unwinding of a DNA duplex by the helicase-primase complex, and DNA polymerization by the HSV-DNA polymerase is a rate-limiting step during leading strand synthesis [Falkenberg et al., 1998]. Combined treatment of ASP2151 with nucleoside analogs can inhibit both helicase-primase activity and DNA polymerization activity in virus genome DNA replication. Taken together, these results suggest that inhibition of either or both helicase or primase activity decreases the substrate of DNA polymerase—a single-stranded

DNA template—thereby affecting the rate-limiting step in the course of virus genome DNA replication under combination therapy.

Under this hypothesis, partial inhibition of helicase-primase activity could enhance the inhibitory effect of ACV against HSV DNA polymerization, even at concentrations of ASP2151 much lower than the EC_{50} value. Our isobologram analysis showed the synergistic antiviral effect of ASP2151 at concentrations of 0.0039 (HSV-1), 0.0078 (HSV-2), and 0.023 μmol/L (VZV) or less. In contrast, the synergistic antiviral effect of ACV was relatively weak at low concentrations compared to ASP2151 (Figure 11). Further, in the response surface model analysis, ASP2151 in combination with ACV showed significant synergistic antiviral effects (Table 10 and Table 11). Taken together, these findings suggest that combination therapy of ASP2151 with a nucleoside analog antiherpes drug may more effectively inhibit herpes viral DNA replication than therapy with either agent alone.

However, unlike ACV and PCV, VDB exhibited only additive interaction for antiviral efficacy in combination with ASP2151 against HSV-2 strain MS (Table 11), a result which did not reflect any antagonistic or non-synergistic interaction and was instead attributable to the mild cytotoxicity observed at high concentrations of VDB in MTS assays used to determine the number of viable cells in proliferation. VDB is phosphorylated to its active VDB-triphosphate form by cellular kinases without viral TK and is able to inhibit the DNA synthesis of ACV-resistant/TK-deficient mutants of HSV and VZV [Schwartz et al., 1984]. The differences in antiviral activity of ASP2151 in combination with VDB between HSV-2 (additive) and VZV

(synergistic) may reflect the characteristics and proposed mechanisms of antiviral activity of VDB [Suzuki et al., 2006]. One of the proposed mechanisms of antiviral activity of VDB is inhibition of viral DNA polymerase [Muller et al., 1977]. The differences in viral DNA polymerase cause the alteration of susceptibility of each virus to VDB. VZV was most susceptible to VDB, followed by HSV-1 and HSV-2 [Gephart and Lerner, 1981]. These indicate that VDB inhibits the DNA polymerase of HSV-2 less sufficiently than that of VZV. In the combination test against VZV, the contribution of VDB to antiviral activity was higher than that of ACV [Miwa et al., 2005]. Even if ASP2151 inhibits helicase-primase activity, the weak inhibitory effect of VDB on the viral DNA polymerase may not enhance the antiviral activity in the course of virus genome DNA replication under combination therapy. As a consequence, ASP2151 with VDB may show a synergistic antiviral effect on VZV that is not observed in HSV-2.

There was no evidence of synergistic cytotoxicity within the concentrations examined for ASP2151 (highest concentration:1 μmol/L). This is not unexpected because none of the compounds are cytotoxic within the concentration ranges evaluated (highest concentration: 200 μmol/L ACV; 200 μmol/L PCV; 200 μmol/L VDB). Much higher concentrations of all compounds would be required to correctly examine potential synergistic cytotoxicity interactions. However, there are no notable synergistic cytotoxicities among the test compounds observed at concentrations used in this study.

Evaluating efficacy in combination therapy of multi-drug administration using not only isobologram analysis but also response surface model analysis typically requires a large

checkerboard format to perform the assay in all possible combinations with tested concentrations of each drug. In the present study, we used PRA and MTS assays in testing against HSV-2. The PRA requires more labor intensive and well-trained skills than a colorimetric method using a plate reader, such as MTS assay. While endpoints differ between the PRA, which evaluates inhibitory effect on plaque formation, and the MTS assay, which determines the number of viable cells in proliferation, the obtained results were similar between the two methods in evaluating two-drug combination therapy. The high-throughput colorimetric method is efficient and useful in multi-drug therapy testing *in vitro*, which typically requires a larger number of combinations than PRA, particularly against HSV-1 and HSV-2, which can grow rapidly and effectively in cell culture.

Here, using a zosteriform spread model, we demonstrated that combination therapy with ASP2151 and VACV more effectively inhibited disease progression than monotherapy with either agent. However, the number of doses of each drug during therapy and combinations of treatment regimens were limited by resource considerations. Evaluation of multi-drug combination therapies is commonly restricted to *in vitro* testing, as evaluating efficacy of two-drug combination therapies *in vivo* would require a huge number of animals. Therefore, we were not able to statistically determine whether or not the combination therapy was additive or synergistic in the zosteriform spread model in a manner similar to *in vitro* study. While the HSV-1-infected mouse model of zosteriform spread is a well-characterized test system, the host-specific property of viruses, particularly VZV, limits our use of animal challenge models in evaluation, except for already established models. Identifying the optimum combination therapy regimen (frequency, dosing

period, dosage of each compound, and route) of ASP2151 with other antiherpes drugs will require further study.

Chapter 5 Conclusion

The novel oxadiazolyl-phenyl type herpesvirus helicase-primase inhibitor ASP2151 (amenamevir) possesses potent antiviral activity against not only HSV-1 and HSV-2 but also against VZV.

Characterization studies of HSV-1, HSV-2, and VZV strains resistant to ASP2151 showed that important amino acid substitutions associated with the susceptibilities of HSV-1, HSV-2, and VZV to ASP2151 exists in not only the helicase subunit but also the primase subunit of the helicase-primase complex and that mutations in the helicase-primase complex against ASP2151 have little influence on susceptibility to existing antiherpetic agents. In addition, we observed reduced frequencies and growth rates of ASP2151-resistant HSV-1, HSV-2, and VZV variants in laboratory stocks. Taken together, these results indicate that ASP2151 targeting the essential virus proteins of HSV and VZV is effective in preventing resistant mutants from emerging and becoming the majority in a virus population.

To assess the combination therapy of ASP2151 and existing nucleoside analog antiherpes drugs, we tested the antiviral activity of ASP2151 combined with ACV and other nucleoside analog antiherpes drugs. Combined therapy of ASP2151 with ACV and other nucleoside analogs demonstrated a synergistic/additive antiherpes effect against HSV and VZV infections. Such

combination therapy may be a useful approach for treating herpes infections suspected to be caused by nucleoside analog drug-resistant virus variants and represent more effective therapeutic options than monotherapy with either of the involved drugs, particularly for severe diseases conditions, such as herpes encephalitis or in patients with immunosuppression.

ASP2151 is a novel viral helicase-primase inhibitor with potent activity against not only HSV-1, HSV-2 but also VZV. Based on our results, ASP2151 warrants further investigation for the treatment of VZV, HSV-1, and HSV-2 infections.

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Figures and Tables

Figures

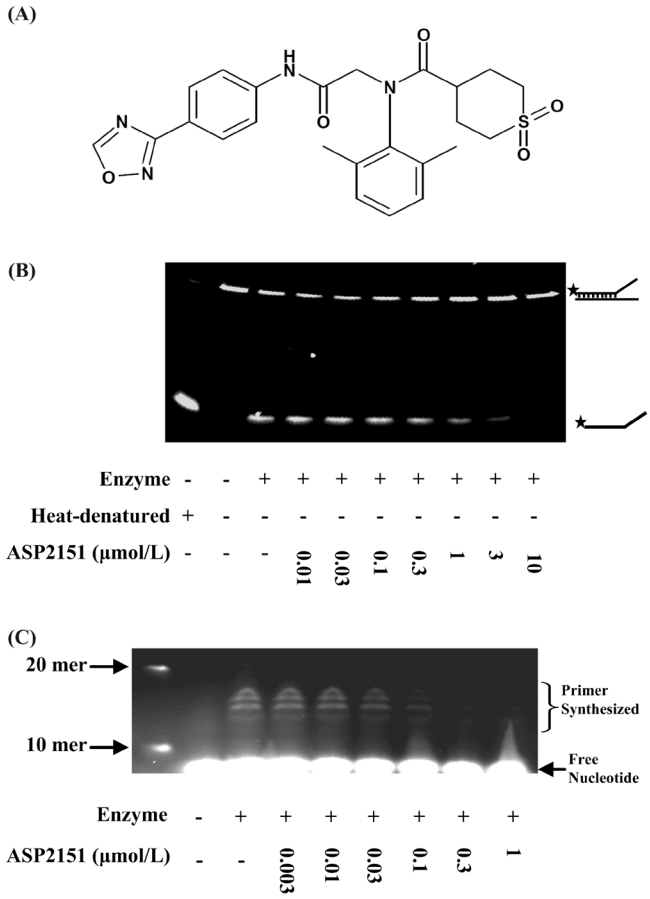

Figure 1 (A) Molecular structure of ASP2151. (B) ASP2151 inhibits the DNA helicase activity of herpes simplex virus type 1 (HSV-1) helicase-primase complex. Heat-denatured, forked DNA helicase substrate was heated at 95 °C for 3 min and then cooled immediately on ice to denature; Enzyme, the recombinant HSV-1 strain KOS helicase-primase complex containing (+) or not (-) in reaction mixture. The upper and lower schematic symbol at the side of the gel indicates the position of the forked duplex DNA helicase substrate with fluorescence-label and

the unwound labeled single-stranded DNA, respectively. (C) ASP2151 inhibits the primase activity of HSV-1 helicase-primase complex. Arrows show the position of 10- and 20-mer fluorescence-labeled oligonucleotide markers in the leftmost lane. Enzyme, the recombinant HSV-1 strain KOS helicase-primase complex containing (+) or not (-) in reaction mixture.

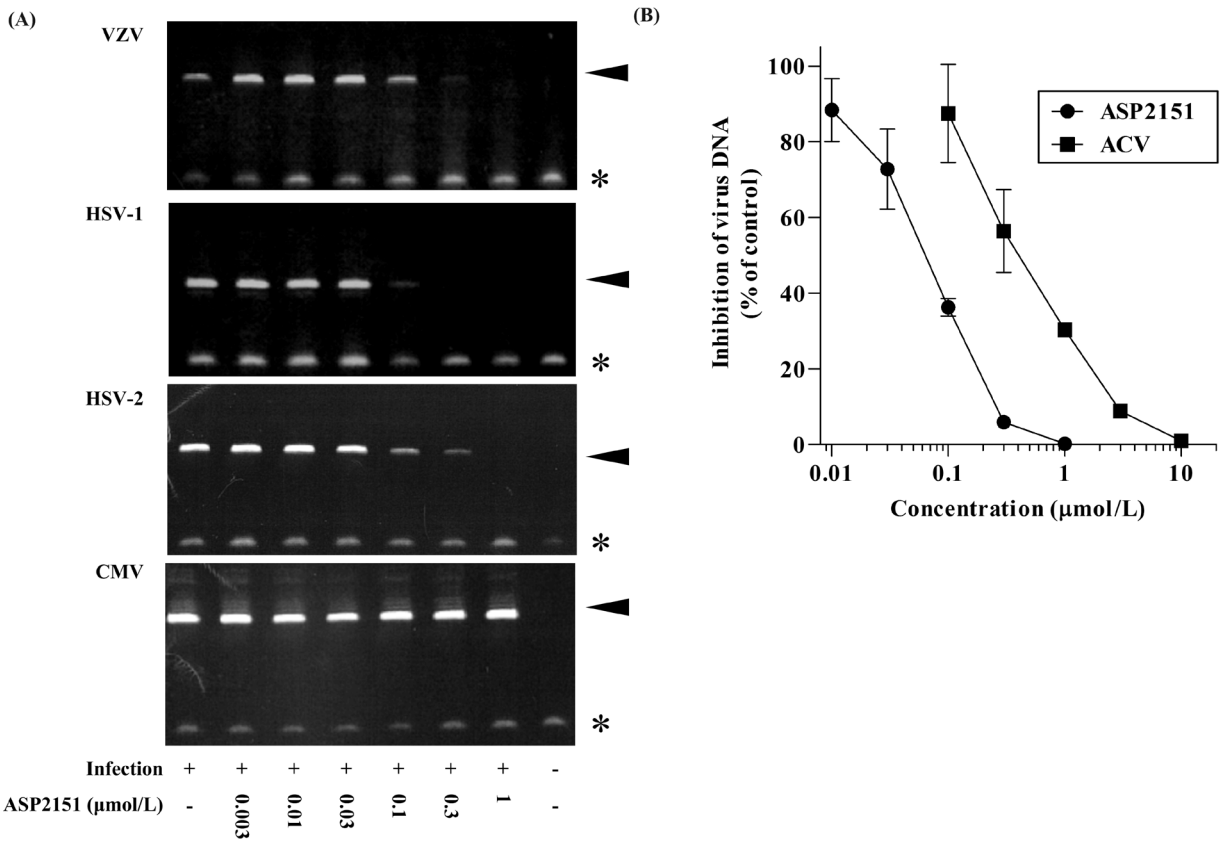

Figure 2 Effect of ASP2151 on the DNA replication of varicella-zoster virus (VZV), herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2) and human cytomegalovirus (HCMV). (A) Polyacrylamide gel electrophoresis was performed with amplified virus-specific PCR fragments from the total DNA extracted from virus-infected cells after incubation with ASP2151. Arrowheads and asterisks indicate the PCR fragments of each virus-specific region and human β-actin gene-specific region, respectively. (B) Effects of ASP2151 (closed circle) and acyclovir (closed square) on VZV DNA replication in virus-infected human embryonic fibroblast (HEF) cells assessed by real-time PCR. Data were calculated from the percent of control viral DNA extracted from vehicle-treated, virus-infected HEF cells, and are shown as mean ± standard error from four independent experiments.

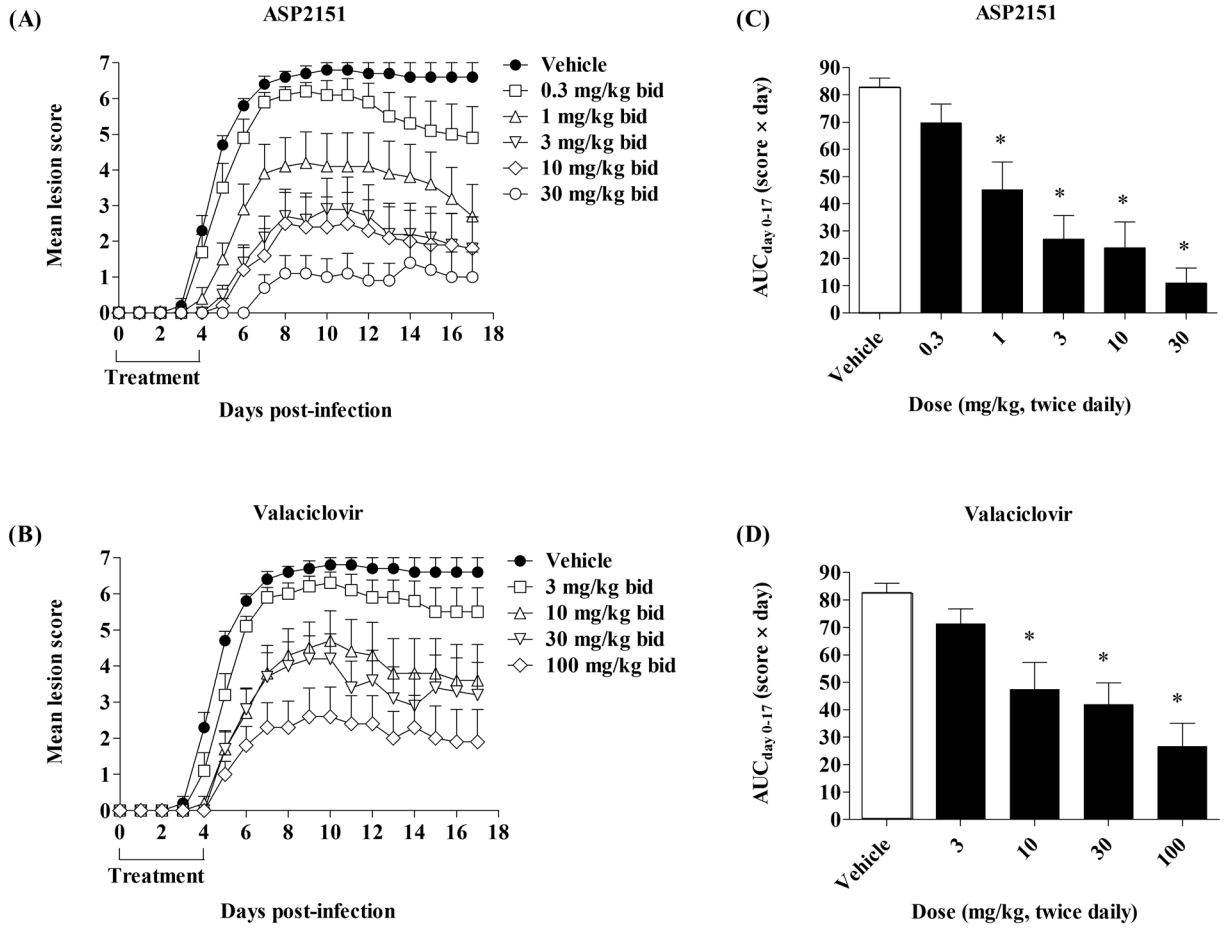

Figure 3 The antiviral activity of ASP2151 and valaciclovir in a herpes simplex virus type 1 (HSV-1)-infected hairless mouse zosteriform model. (A) and (B) The mean disease scores for each ASP2151 or valaciclovir administration group were calculated and plotted versus the days post-infection. (C) and (D) Area under the disease score-time curve for the period from post-infection day 0 to day 17 post-infection ($AUC_{day\ 0–17}$ [score × day]) in HSV-1-infected hairless mice. *, Significantly different ($P<0.05$, Dunnett's multiple comparison test) from the vehicle group. Data are expressed as the mean + standard error of ten mice per group.

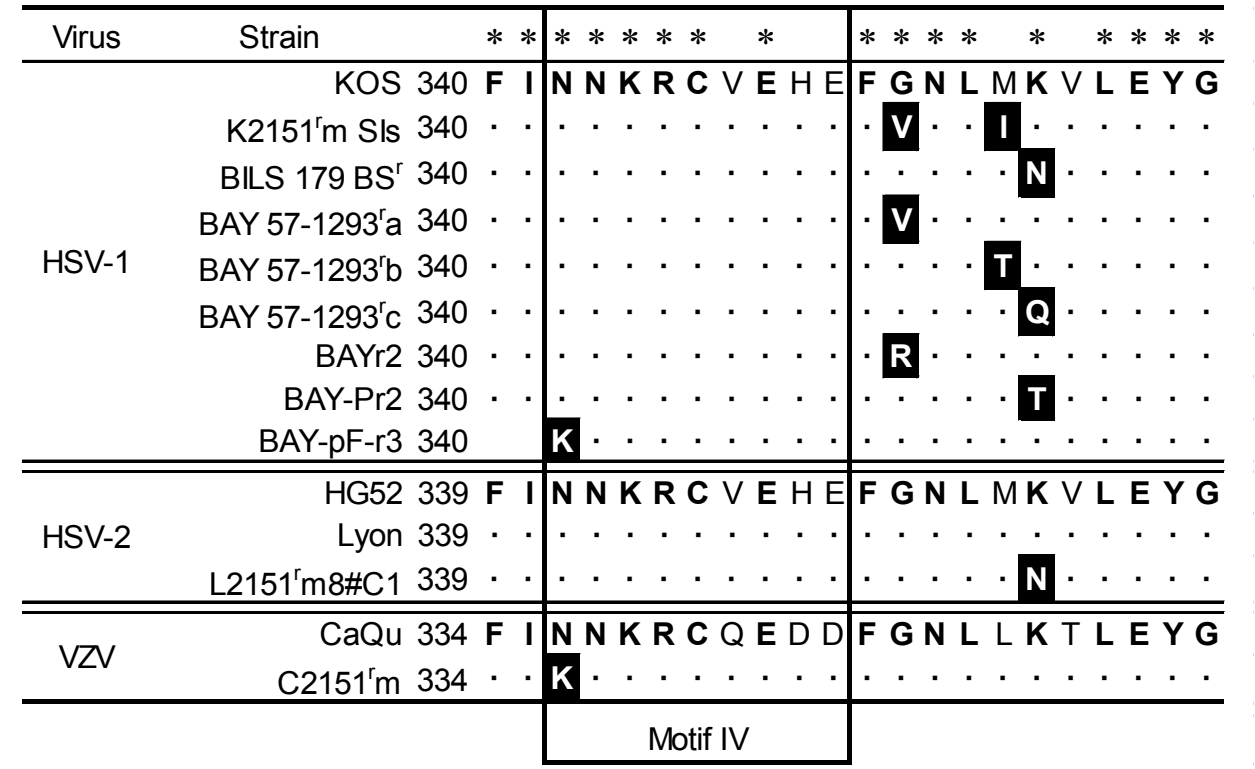

Figure 4 Comparison of amino acid sequences near helicase motif IV in helicase-primase inhibitor-resistant HSV mutants. Amino-acid sequences adjacent to helicase motif IV in the helicase subunit of the helicase-primase complex of HPI-resistant mutants are shown here. Amino-acid sequences were aligned, and consensus residues are denoted using asterisks between HSV-1 (KOS strain), HSV-2 (HG52 and Lyon strains), and VZV (CaQu strain). BILS 179 BS^r, BAY 57-1293^{r}a, BAY 57-1293^{r}b, BAY 57-1293^{r}c, BAYr2, BAY-Pr2, and BAY-pF-r3 are previously reported BILS 179 BS- or BAY 57-1293-resistant HSV-1 mutants. K2151^{r}m single-plaque isolates (SIs) are single plaque purified ASP2151-resistant HSV-1 mutants derived from the parent strain KOS (K2151^{r}m#B9, K2151^{r}m#D9, K2151^{r}m#G11and K2151^{r}m #H10). L2151^{r}m8#C1 is an ASP2151-resistant HSV-2 mutant derived from the parent strain Lyon. C2151^{r}m is an ASP2151-resistant VZV mutant derived from the

parent strain CaQu. Motif IV is one of six well-conserved helicase motifs in the helicase subunit of the helicase-primase complex in *Herpesviridae* viruses. HSV-2 strain HG52 amino acid sequence data were derived from the RefSeq (RefSeq ID: NP_044523). HSV-1, herpes simplex type 1; HSV-2, herpes simplex type 2; VZV, varicella-zoster virus.

Virus	Strain																		
	KOS	358	A	H	F	E	C	F	S	P	P	R	L	A	T	H	L	R	A	V
	K2151^{r}m	358	·	·	·	·	·	·	**G**	·	·	**X**	·	·	·	·	·	·	·	·
HSV-1	K2151^{r}m#B9	358	·	·	·	·	·	·	**G**	·	·	**H**	·	·	·	·	·	·	·	·
	K2151^{r}m#D9	358	·	·	·	·	·	·	**G**	·	·		·	·	·	·	·	·	·	·
	K2151^{r}m#G11	358	·	·	·	·	·	·	**G**	·	·		·	·	·	·	·	·	·	·
	K2151^{r}m#H10	358	·	·	·	·	·	·	**G**	·	·	**H**	·	·	·	·	·	·	·	·

Figure 5 Comparison of amino acid sequences of primase gene products. Amino acid substitutions found in U_L52 of ASP2151-resistant HSV-1 are indicated by filled boxes. U_L52 is composed of 1058 amino acids. 'X' represents Arg (R) or His (H), given detection of a mixed-base signal at the 367th Arg codon. K2151^{r}m was an ASP2151-resistant HSV-1 mutant derived from HSV-1 strain KOS. K2151^{r}m#B9, K2151^{r}m#D9, K2151^{r}m#G11, and K2151^{r}m#H10 were derived from K2151^{r}m by single plaque isolation.

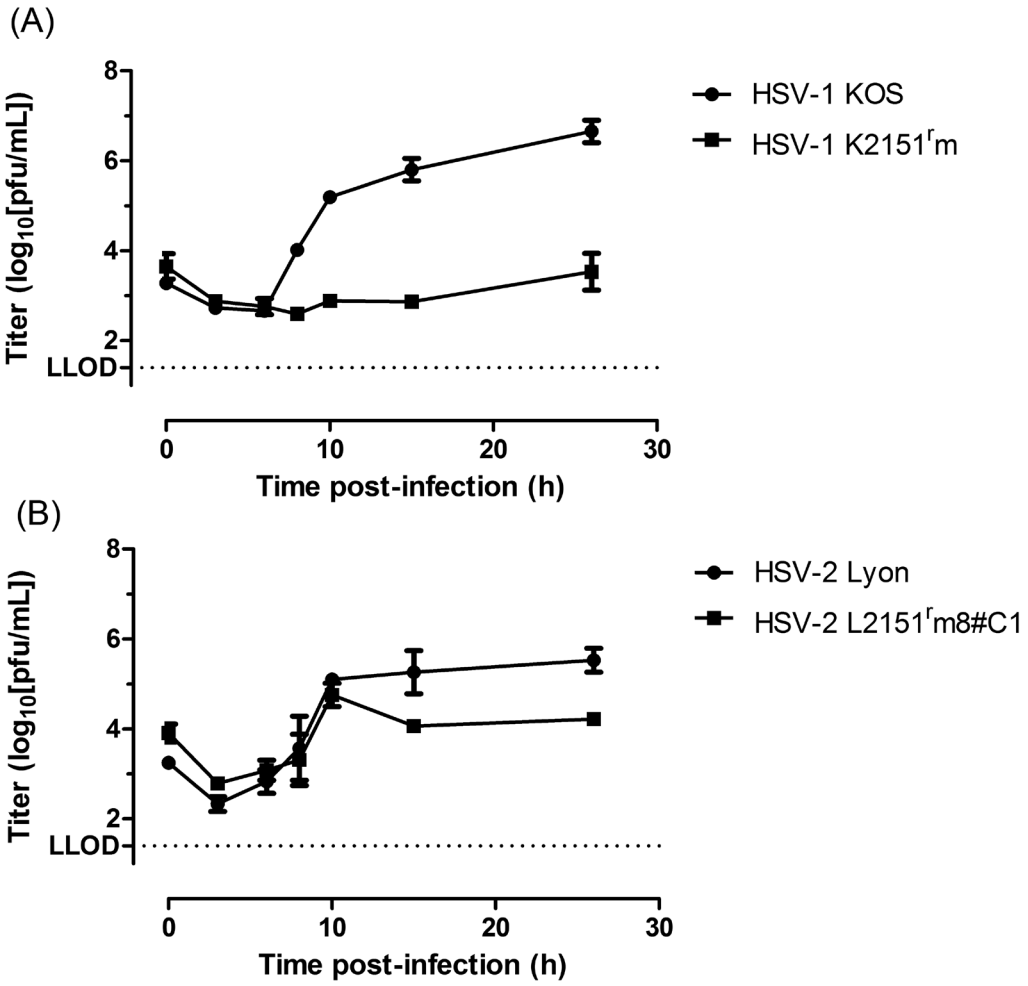

Figure 6 One-step growth experiment of virus in culture supernatant of Vero cells infected with ASP2151-resistant HSV-1 or HSV-2 mutants compared with parental strains. One-step growth curves of ASP2151-resistant mutants HSV-1 K2151^{r}m (A) and HSV-2 L2151^{r}m8#C1 (B) in Vero cells. Each symbol represents the log-transformed mean ± standard error of four independent experiments. LLOD, lower limit of detection (1.40 log [pfu/mL]); pfu, plaque-forming unit.

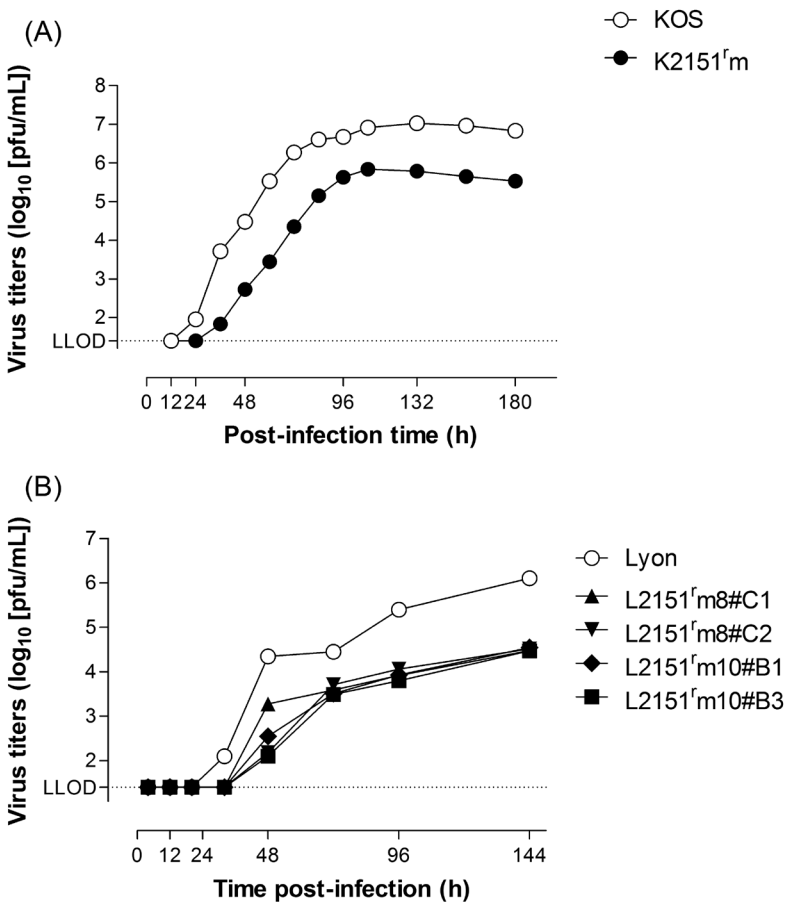

Figure 7 Multi-step growth experiment of virus in culture supernatant of Vero cells infected with ASP2151-resistant HSV-1 or HSV-2 mutants compared with parental strains. Virus titers of ASP2151-resistant mutants and respective parental strains in tissue culture supernatants were measured using a plaque assay in Vero cells. The dashed line represents the lower limit of detection (LLOD, 1.4 $\log_{10}$ [plaque-forming unit (pfu)/mL]) of virus titer for the assay. Each symbol represents the log-transformed mean ± standard error of four independent experiments (A) and the value of one independent experiment performed in duplicate (B).

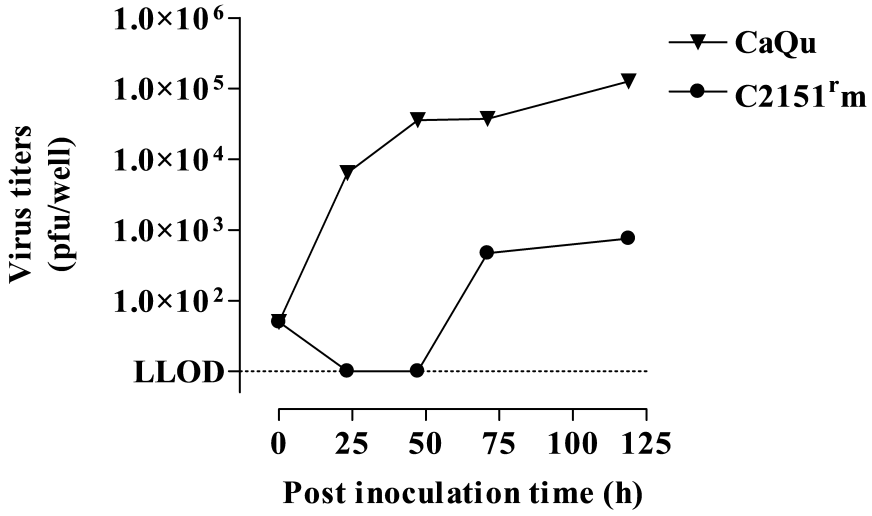

Figure 8 Replication profile of ASP2151-resistant VZV C2151^{r}m in comparison with parent strain CaQu. The dashed line represents the lower limit of detection (LLOD, 10 [plaque-forming unit (pfu)/well]) of virus titer for the assay. The number of plaques from each day was averaged to generate a growth curve.

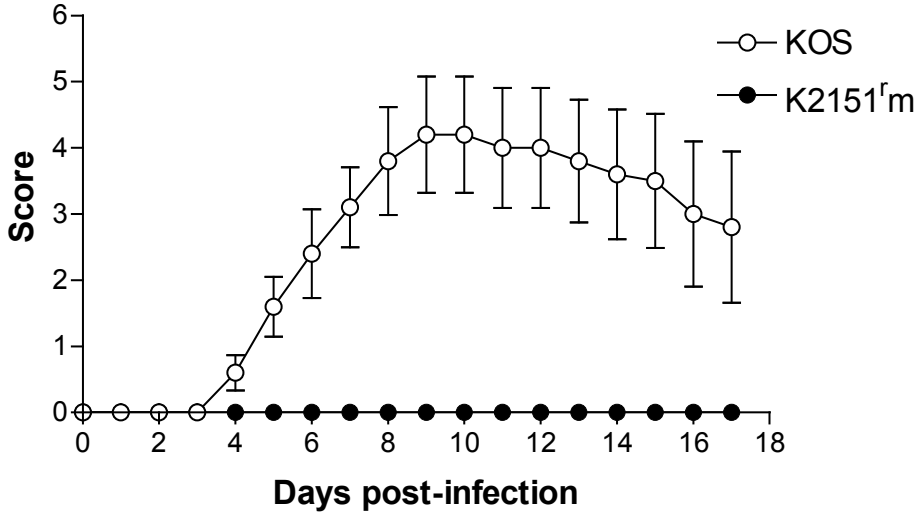

Figure 9 Pathogenicity of HSV-1 KOS strain and ASP2151-resistant HSV-1 mutant K2151^{r}m. Hairless mice (HOS:HR-1, female, aged 7 weeks at infection) were infected intradermally with 5.25×10^{7} pfu/mL of HSV-1 strain KOS or ASP2151-resistant HSV-1 mutant (K2151^{r}m). Disease scores for each animal were combined to produce a composite score. Each symbol represents the mean composite score ± standard error of 10 mice per group.

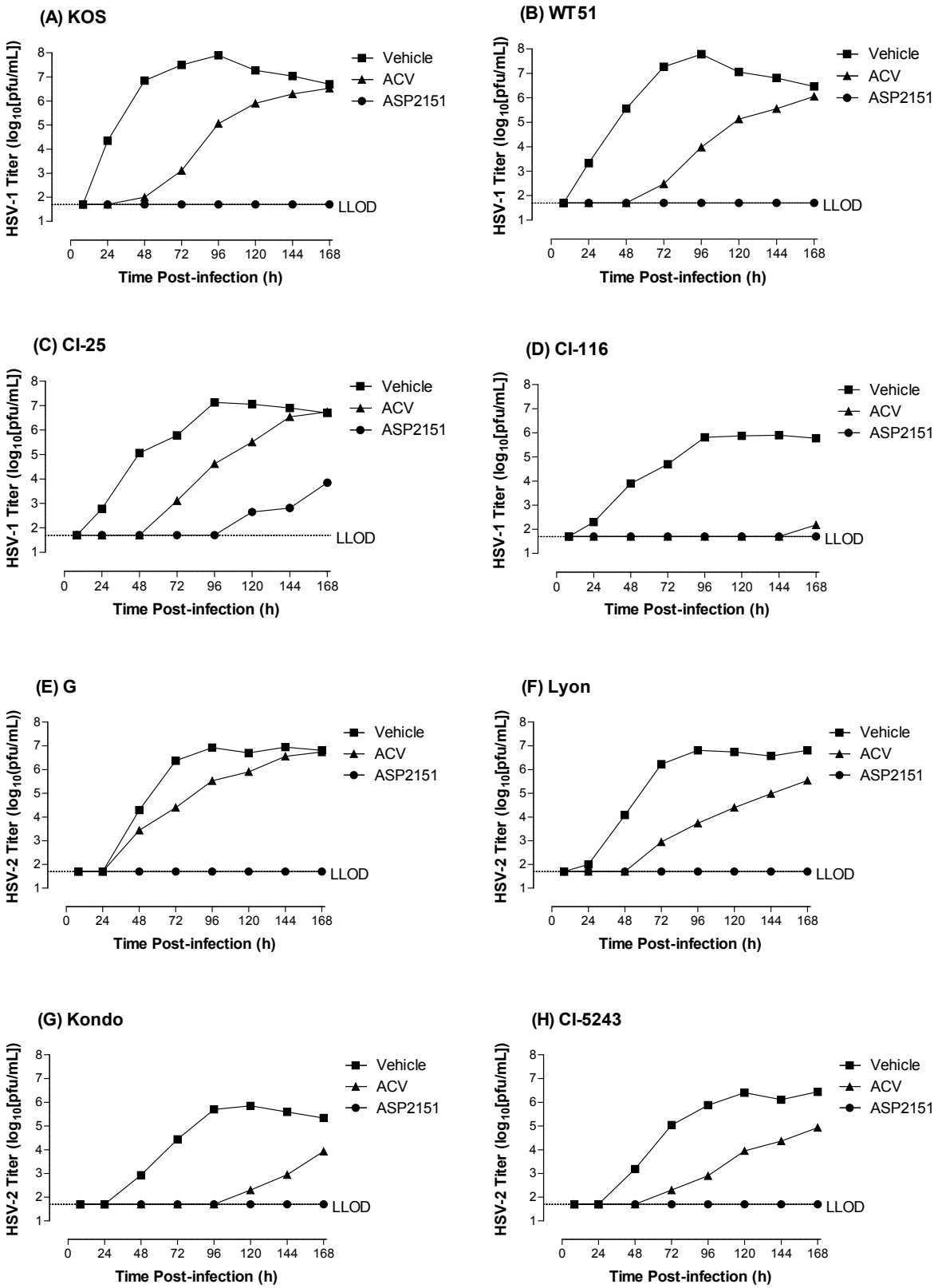

Figure 10 Emergence of drug-resistant HSV-1 or HSV-2 mutants in Vero cells under long-term

treatment with ASP2151 or acyclovir. Vero cells were infected with herpes simplex virus type 1 (HSV-1) strains (A)-(D) (KOS, WT51, CI-25, or CI-116, respectively) or herpes simplex virus type 2 (HSV-2) strains (E)-(H) (G, Lyon, Kondo, or CI-5243, respectively) and incubated in the presence of vehicle (0.1% dimethylsulfoxide), acyclovir (ACV), or ASP2151. Virus titer expressed as $\log_{10}$ (plaque-forming unit [pfu]/mL) in culture supernatants was determined by plaque assay using Vero cells. Data represent values at each time point of one experiment. The dotted line indicates the lower limit of detection (LLOD; 1.7 $\log_{10}$ [pfu/mL]) for the titration.

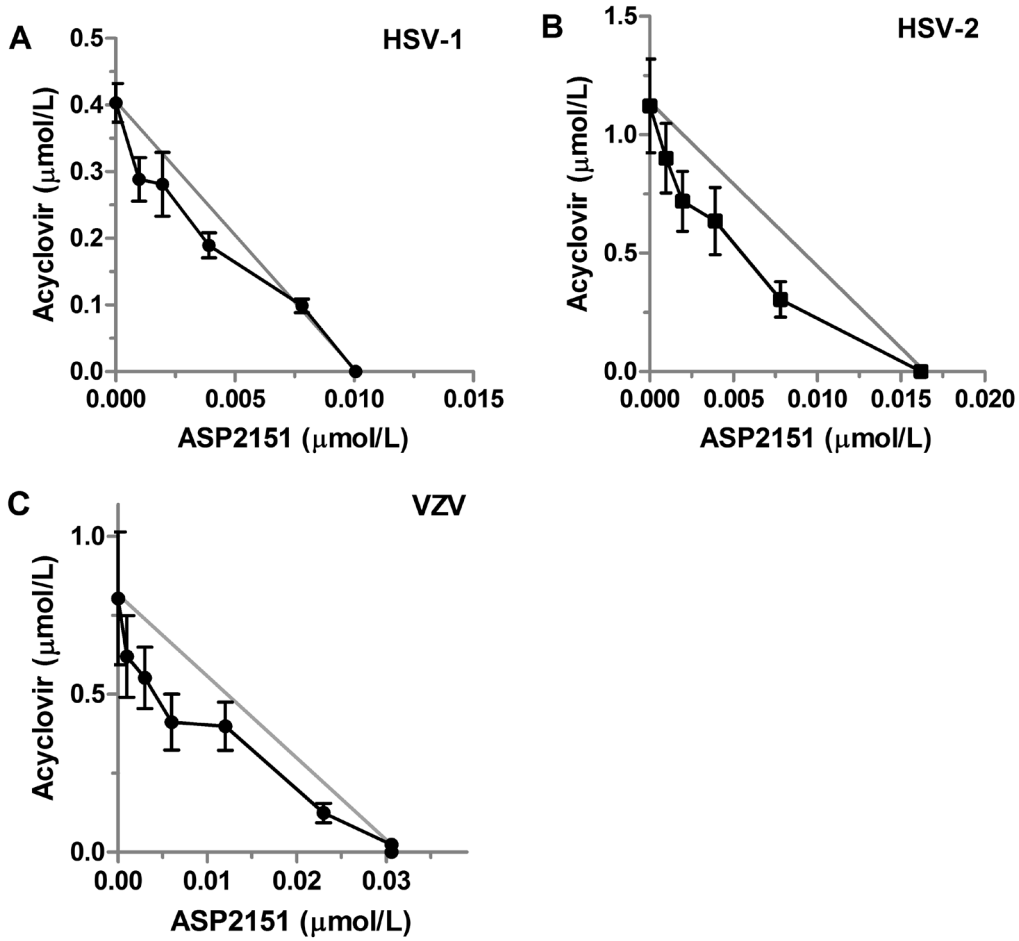

Figure 11 Isobolograms for combination treatment of ASP2151 with ACV against HSV-1 KOS strain (A) and HSV-2 Genital isolate strain (B), and VZV Kawaguchi strain (C). The solid straight line (gray) indicates the theoretical additive antiviral activity in combination with ASP2151 and ACV. Each points show EC_{50} values of ACV corresponding to concentrations of ASP2151 analyzed by PRA in HEF cells and are shown as mean ± standard error from four independent experiments.

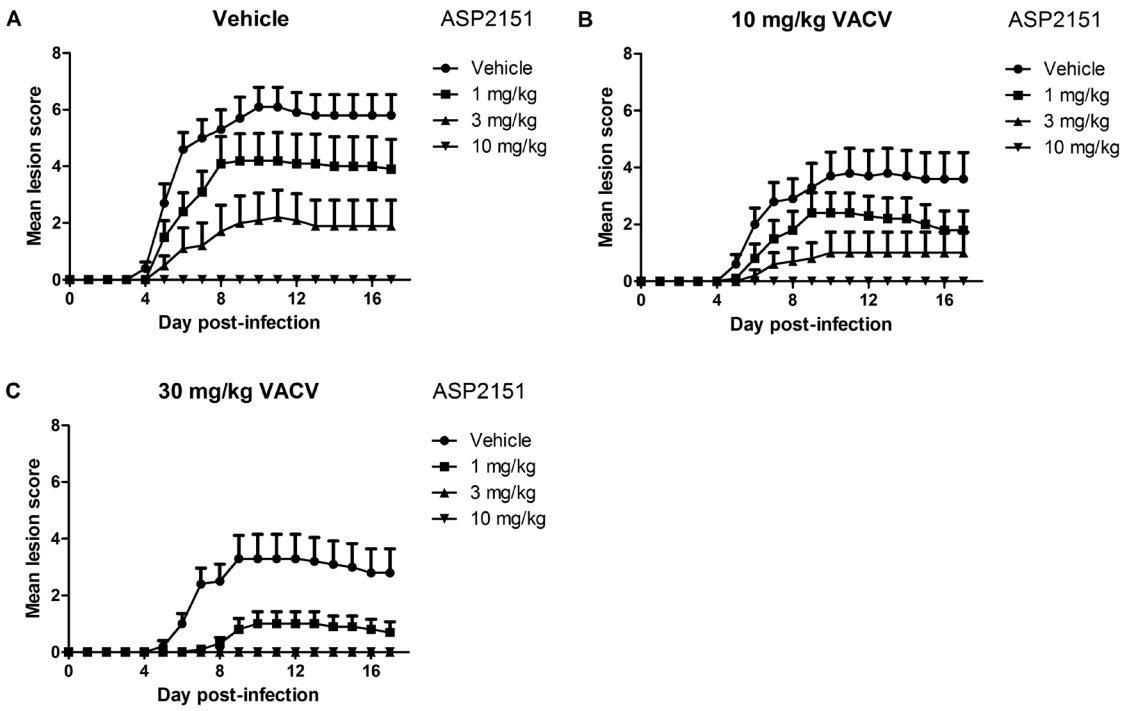

Figure 12 Effects of combination of ASP2151 and valaciclovir (VACV) in HSV-1-infected hairless mouse model of zosteriform spread. Hairless mice infected with HSV-1 were orally administered vehicle only, or, 1, 3, or 10 mg/kg twice daily ASP2151 with vehicle (A); 10 mg/kg twice daily VACV (B); 30 mg/kg twice daily VACV (C) from Days 0 (infection day) to 4 post-infection. The mean disease score for each treatment group was calculated at different times post-infection and plotted versus the time post-infection. Data are expressed as the mean + standard error of 10 animals per group. Vehicle, 0.5% MC solution.

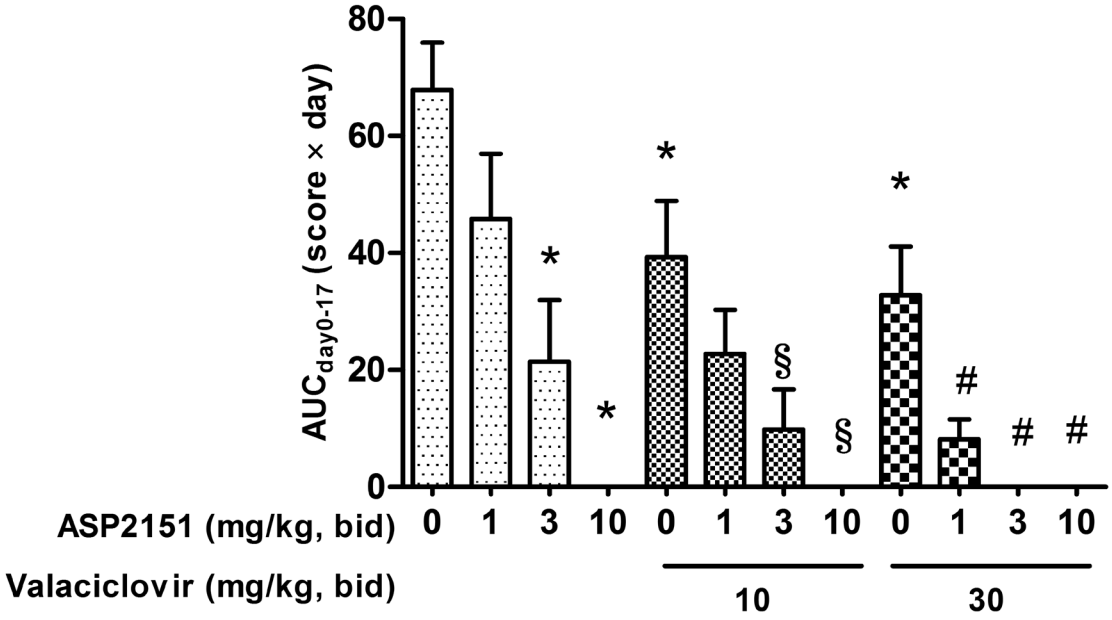

Figure 13 Combination effect of ASP2151 with valaciclovir (VACV) in mouse model of zosteriform spread. The figure represents area under the disease score-time curve from Days 0 to 17 post-infection ($AUC_{Day0\text{-}17}$ score × day). Data are expressed as the mean + standard error of 10 mice per group. Results were significantly different ($P<0.05$, Dunnett's multiple comparison test) from the vehicle group (*), 10 mg/kg VACV monotherapy group (§), or 30 mg/kg VACV monotherapy group (#). Vehicle, 0.5% methylcellulose solution; bid, twice daily.

Tables

Table 1 Polymerase chain reaction primer sequences

Virus	Gene	Primer Name[a]	Sequence (5'-NNN-3')	Position[b]
VZV	ORF31	VZV-gB-F	GGGATGGTGCATACAGAGAAC	929 - 949
		VZV-gB-R	GTGCATACTCATCGCGAACT	1140 - 1159
HSV-1	U_S4	HHV1-gG-F	CATTATCGGGCCGCTGGCAA	447 - 466
		HHV1-gG-R	AACCGCCACACAGGTGTGTC	649 - 630
HSV-2	U_S4	HHV2-gG-F	TCCGAACCCCAACAAACC	1758 - 1775
		HHV2-gG-R	GGCGACCAGACAAACGAA	2007 - 1990
HCMV	U_L83	HCMV-UL83-F	GTCAGCGTTCGTGTTTCCCA	549 - 568
		HCMV-UL83-R	GGGACACAACACCGTAAAGC	831 - 812

VZV, varicella-zoster virus; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; HCMV, human cytomegalovirus.

[a] All primer sets were designed using HSV-1 complete genome sequence (accession, NC_001806), HSV-2 complete genome sequence (accession, NC_001798), VZV complete genome sequence (accession, NC_001348) and HCMV complete genome sequence (accession, NC_001347) acquired from the Nucleotide database of NCBI Entrez. The primer sets were designed using GENETYX software (version 8.2.2, Genetyx, Tokyo, Japan)

[b] 'Position' indicates nucleotide number targeted by each primer when A of the 1st Met codon (ATG) in each gene is designated one.

Table 2 Antiviral activities of herpes helicase-primase inhibitors

	Upper: EC_{50} ± SE (µmol/L)[a] **Lower: EC_{90} ± SE (µmol/L)**		
Virus (Strain)	**ASP2151**	**BILS 179 BS**	**BAY 57-1293**
HSV-1 (KOS)	0.036 ± 0.0047	0.060 ± 0.016	0.014 ± 0.0018
	0.23 ± 0.037	0.63 ± 0.13	0.082 ± 0.013
HSV-2 (G)	0.028 ± 0.0013	0.046 ± 0.016	0.023 ± 0.0018
	0.46 ± 0.30	2.4 ± 0.99	0.91 ± 0.71
VZV (Ellen)	0.047 ± 0.013	4.1 ± 0.56	11 ± 0.92
	0.46 ± 0.11	23 ± 7.2	>100
CC_{50} (µmol/L)[b]	>30	>30	>30
Selectivity index: CC_{50} /EC_{50} [c]	>638	>7.3	>2.7

[a] Data represent the mean 50% effective concentration (EC_{50}) and 90% effective concentration (EC_{90}) values and standard error (SE) of three independent experiments.

[b] Values for the cytotoxic concentration causing 50% reduction in the number of viable cells (CC_{50}) were determined using MTT assay in confluent monolayer culture of HEF cells.

[c] Selectivity index represents the smallest value among viruses tested.

Table 3 Anti-varicella-zoster virus and cytotoxic activities of ASP2151 and acyclovir

	EC_{50} ± SE (μmol/L)[a]	
Strain	**ASP2151**	**Acyclovir**
Laboratory-stocked		
CaQu	0.10±0.00	4.1±0.2
Clinical isolates from Japan		
Saitou	0.065±0.12	4.4±0.6
Takahashi	0.078±0.013	5.9±2.0
Housen	0.10±0.03	5.2±0.8
Tokumaru	0.055±0.011	3.0±0.2
Clinical isolates from the United States		
Hunter	0.042±0.010	1.3±0.3
Klein	0.050±0.006	1.6±0.2
Mazzola	0.038±0.005	1.8±0.4
Negg	0.043±0.008	1.7±0.4
Acyclovir-resistant mutant		
Kanno-Br	0.082±0.016	27±5
Cytotoxicity: CC_{50} (μmol/L)[b]	>200	>200
Selectivity index: CC_{50} /EC_{50}[c]	>2000	>33.9

[a] Antiviral activity (EC_{50}, 50% effective concentration) was determined using plaque reduction assay. The data represent the mean of four independent experiments using each strain.

[b] Data represent the mean of three independent experiments. Values for the cytotoxic concentration causing 50% reduction in the number of viable cells (CC_{50}) were determined using neutral red assay in proliferating HEF cells.

[c] Selectivity index represents the smallest value among strains tested.

Table 4 Amino acid substitutions in helicase and primase for ASP2151-resistant HSV-1 and HSV-2 mutants, and susceptibility to ASP2151

Virus	Strains or Mutants[a]	Helicase gene (U_L5)	Primase gene (U_L52)	EC_{50} (μmol/L)[b]	Fold increase
HSV-1	KOS	-[c]	-	0.037	-
	K2151^{r}m	G352V, M355I	S364G, R367X[d]	131.8	3562
	K2151^{r}m#B9	G352V, M355I	S364G, R367H	105.4	2849
	K2151^{r}m#D9	G352V, M355I	S364G	19.6	530
	K2151^{r}m#G11	G352V, M355I	S364G	28.2	762
	K2151^{r}m#H10	G352V, M355I	S364G, R367H	118.0	3189
HSV-2	Lyon	-	-	0.12	-
	L2151^{r}m8#C1	K355N, K451R	-	>150	>1250

[a] K2151^{r}m#B9, K2151^{r}m#D9, K2151^{r}m#G11, and K2151^{r}m#H10 were derived from K2151^{r}m and L2151^{r}m8#C1 from L2151^{r}m by single plaque isolation.

[b] The 50% effective concentration (EC_{50}) was calculated via nonlinear regression analysis using a sigmoid-E_{max} model from one (HSV-2) or three (HSV-1) independent experiments performed in triplicate.

[c] Same as parental sequence, or no substitutions were observed.

[d] 'X' means Arg (R) or His (H) due to detection of mixed-base signal at the 367th Arg codon.

Table 5 Effect of ASP2151 and valaciclovir on the mortality in zosteriform-spread model.

Treatment	Dose (mg/kg twice daily)	Survival on day 17 post-infection (no. surviving/no. tested)
Vehicle[a]	-	1/10
ASP2151	0.3	4/10
	1	8/10
	3	8/10
	10	8/10
	30	9/10
Valaciclovir	3	4/10
	10	6/10
	30	7/10
	100	8/10

[a] 0.5% methyl cellulose solution.

Table 6 ASP2151- or acyclovir-concentrations assayed to detect compound-resistant mutants in herpes simplex virus types 1 and 2 strains

Virus	Strain	$20EC_{50}$ (μmol/L)[a]		EC_{90} (μmol/L)[b]	
		ASP2151	Acyclovir	ASP2151	Acyclovir
HSV-1	KOS	0.52	42	0.062	7.0
	WT51	0.60	15	0.075	1.4
	CI-25	0.18	16	0.035	5.6
	CI-116	0.20	19	0.034	4.0
HSV-2	G	0.50	32	0.075	7.3
	Lyon	0.68	52	0.12	8.0
	Kondo	0.46	28	0.064	4.3
	CI-5243	0.28	22	0.057	6.0

Data are calculated from four independent experiments performed in triplicate.

[a] Twenty times the 50% effective concentrations ($20EC_{50}$) of ASP2151 or acyclovir determined by plaque reduction assay in Vero cells.

[b] Ninety percent effective concentrations (EC_{90}) of ASP2151 or acyclovir determined by plaque reduction assay in Vero cells.

Table 7 Antiviral activity of ASP2151 and existing antivirals against HSV-1 KOS and K2151^{r}m

Virus Strain	EC_{50} (µmol/L; mean [95% confidence interval])[a]				
	ASP2151	**ACV**	**PCV**	**araA**	**IDU**
KOS	0.0097 (0.0064-0.015)	0.31 (0.21-0.46)	1.0 (0.54-1.9)	9.9 (6.9-14)	0.40 (0.32-0.52)
K2151^{r}m	7.5 (4.2-13)	0.28 (0.24-0.33)	1.0 (0.65-1.6)	11 (7.4-17)	0.44 (0.35-0.55)

[a] Plaque reduction assays were performed to determine EC_{50} values and 95% confidence intervals of ASP2151, acyclovir (ACV), penciclovir (PCV), vidarabine (araA), and idoxuridine (IDU) against HSV-1 KOS strain and ASP2151-resistant HSV-1 (K2151^{r}m). Data are calculated from four independent experiments performed in triplicate.

Table 8 Frequency of ASP2151- or Acyclovir-resistant variants in herpes simplex virus types 1 and 2

Viruses	Strains	Frequency of compound-resistant variants[a]		Fold of frequency ($ACV^r/ASP2151^r$)
		$ASP2151^r$	ACV^r	
HSV-1	KOS	1.3×10^{-6}	0.86×10^{-3}	662
	G	1.04×10^{-6}	2.11×10^{-2}	20288
HSV-2	Lyon	1.67×10^{-6}	1.56×10^{-3}	934
	Kondo	8.77×10^{-7}	2.60×10^{-4}	296
Geometric mean value		1.19×10^{-6}*	1.65×10^{-3}	1389

[a] Values were determined for four independent experiment sets, and the result represents the mean value. ASP2151- or acyclovir-concentrations were 20 times the 50% effective concentration determined by plaque reduction assay against indicated strains in Vero cells (Table 2).

* Comparison with common logarithm of the frequency values of ACV-resistant HSVs variants was statistically analyzed using Student's t-test (P = 0.002).

ACV^r, ACV-resistant HSV-1, or HSV-2 variants; $ASP2151^r$, ASP2151-resistant HSV-1, or HSV-2 variants

Table 9 EC_{50} values of ASP2151 and ACV against ACV-resistant or ACV-susceptible HSV-1, HSV-2, and VZV strains (plaque reduction assay)

Virus	**Strain**	**EC_{50} (95% confidence interval) (μmol/L)[a]**		**Susceptibility (ASP2151/Acyclovir)[d]**
		ASP2151	***Acyclovir***	
HSV-1	KOS	0.010 (0.0082-0.012)	0.400 (0.32-0.50)	+ / +
	A4-3	0.067 (0.049-0.091)	115 (98.8-133)	+ / -
HSV-2	Genital isolate	0.012 (0.006-0.023)	1.34 (0.51-3.56)	+ / +
	Whitlow 2	0.012 (0.006-0.022)	65.9 (31.9-136)	+ / -
VZV	Kawaguchi[b]	0.064 (0.043-0.094)	1.61 (0.99-2.63)	+ / +
	TK-deficient mutant	0.068 (0.052-0.088)	12.8 (9.5-17.3)	+ / -
	A2[c]	0.11 (0.078-0.16)	11.5 (6.5-20.3)	+ / -
	A3[c]	0.11 (0.049-0.26)	19.2 (11.1-33.1)	+ / -
	A7[c]	0.065 (0.045-0.093)	41.4 (21.6-79.2)	+ / -
	A8[c]	0.10 (0.062-0.162)	82.2 (72.7-92.9)	+ / -

[a] Means of four independent experiments.

[b] Parental strain of TK-deficient mutant, A2, A3, A7, and A8.

[c] DNA polymerase mutant.

[d] Susceptibility of virus strains to each compound: +, susceptible; -, resistant

Table 10 Analysis of antiviral efficacy of ASP2151 in combination with ACV (plaque reduction assay)

Virus (Strain)	**Test dose range (μmol/L)**		**α (95% CI)[a]**	**P value**	**Antiviral efficacy**
	ASP2151	***Acyclovir***			
HSV-1 (KOS)	0.001-1	0.03-30	0.56 (0.32-0.79)	< 0.0001	Synergistic
HSV-2 (Genital isolate)	0.001-1	0.03-30	0.62 (0.26-0.98)	0.0009	Synergistic
VZV (Kawaguchi)	0.0015-1.5	0.03-30	0.56 (0.24-0.87)	0.0005	Synergistic

[a] Means of four independent experiments.

Table 11 Combination effects of ASP2151 and antiviral drugs on replication of HSV-2 in Vero cells (MTS assay) and VZV in MRC-5 cells (plaque reduction assay)

Virus (Strain)	Test compounds combined with ASP2151 (range, μmol/L)	α (95% CI)[a]	P value	Antiviral efficacy
HSV-2 (MS)	Acyclovir (0.32-200)	14.6 (10.2-19.1)	< 0.01	Synergistic
	Penciclovir (0.32-200)	8.9 (6.9-11.0)	< 0.01	Synergistic
	Vidarabine (0.24-150)	0.67 (−0.66-1.99)	0.320	Additive
VZV (Ellen)	Acyclovir (0.32-200)	11.4 (7.7-15.0)	< 0.01	Synergistic
	Penciclovir (0.32-200)	11.7 (5.9-17.5)	< 0.01	Synergistic
	Vidarabine (0.32 - 200)	13.2 (6.8-19.6)	< 0.01	Synergistic

[a] Means of two independent experiments.

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