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Synergistic activity of amenamevir (ASP2151) with nucleoside analogs against herpes simplex virus types 1 and 2 and varicella-zoster virus

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ABSTRACT

ASP2151 (amenamevir) is a helicase–primase complex inhibitor with antiviral activity against herpes simplex virus HSV-1, HSV-2, and varicella-zoster virus (VZV). To assess combination therapy of ASP2151 with existing antiherpes agents against HSV-1, HSV-2, and VZV, we conducted *in vitro* and *in vivo* studies of two-drug combinations. The combination activity effect of ASP2151 with nucleoside analogs acyclovir (ACV), penciclovir (PCV), or vidarabine (VDB) was tested via plaque-reduction assay and MTS assay, and the data were analyzed using isobolograms and response surface modeling. *In vivo* combination therapy of ASP2151 with valaciclovir (VACV) was studied in an HSV-1-infected zosteriform spread mouse model. The antiviral activity of ASP2151 combined with ACV and PCV against ACV-susceptible HSV-1, HSV-2, and VZV showed a statistically significant synergistic effect ($P < 0.05$). ASP2151 with VDB was observed to have additive effects against ACV-susceptible HSV-2 and synergistic effects against VZV. In the mouse model of zosteriform spread, the inhibition of disease progression via combination therapy was more potent than that of either drugs as monotherapy ($P < 0.05$). These results indicate that the combination therapies of ASP2151 with ACV and PCV have synergistic antiherpes effects against HSV and VZV infections and may be feasible in case of severe disease, such as herpes encephalitis or in patients with immunosuppression.

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.

HSV-1, HSV-2, and VZV are prevalent pathogens belonging to the human herpesvirus family (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 diseases in the world, have no seasonal variation, and naturally occur only in human beings (Whitley and Roizman, 2001), affecting 60%–95% of the adult human population globally (Fatahzadeh and Schwartz, 2007). Synthetic nucleoside analogs such as acyclovir (ACV), valaciclovir (VACV), penciclovir (PCV), and famciclovir, which target viral DNA polymerase, are the gold standard therapy against HSV and VZV infections in clinical settings (Brady and Bernstein, 2004). These analogs share similar mechanisms 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, 1982; Morfin and Thouvenot, 2003).

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

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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 (Chono et al., 2010), 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 (Chono et al., 2010). 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 (NCT: NCT00487682; manuscripts under preparation) 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.

2. Materials and methods

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.

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.

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 μM) by 3 passages at each concentration until the appearance of cytopathic effect (CPE) following plaque purification twice in the presence of 100 μM 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.

2.4. In vitro susceptibility test

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.

2.4.2. Antiviral assay of ASP2151 combination with ACV 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 μM. 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.

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.

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 which 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).

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 μM) in all possible combinations with five concentrations of ACV (200, 40, 8, 1.6, and 0.32 μM), PCV (200, 40, 8, 1.6, and 0.32 μM), or VDB (150, 30, 6, 1.2, and 0.24 μM). 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.

2.5. In vivo evaluation of combined therapy of ASP2151 with VACV 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.

2.6. Statistical analysis

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., 1995,1990). 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.

3. Results

3.1. In vitro susceptibility test against ACV-resistant or ACV–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 1 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.

3.2. In vitro antiherpes activity of ASP2151 in combination with ACV

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 Fig. 1. 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.

Table 1
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) (μM)[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.

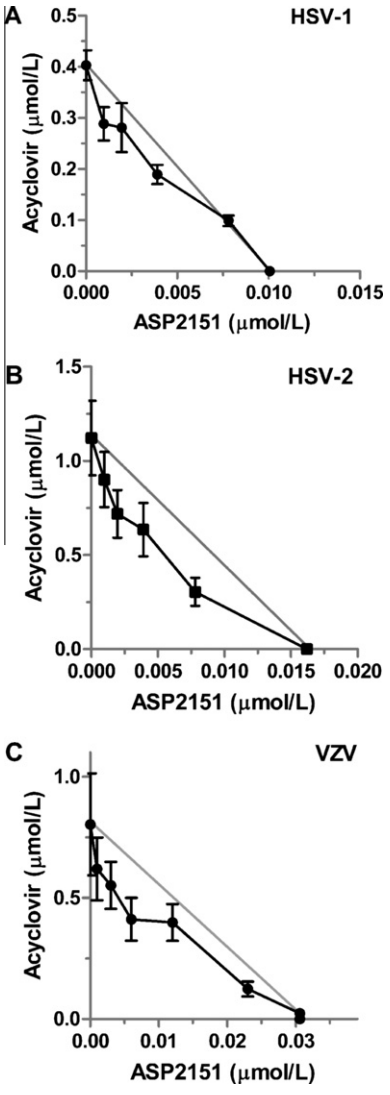

Fig. 1. Isobolograms for combination treatment of ASP2151 with ACV against HSV-1 KOS strain (A) and HSV-2 Genital isolate strain (B), and VZV Kawaguchi strain (C). The solid straight line (gray) indicates the theoretical additive antiviral activity in combination with ASP2151 and ACV. Each points show 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.

To statistically assess the combined antiherpes effect of ASP2151 with ACV on plaque formation *in vitro*, we analyzed the dataset plotted in the isobolograms (Fig. 1) 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 2. 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 μM) and ACV (highest concentration: 30 μM) 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 3). 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 μM) in combination with tested compounds (highest concentration: 200 μM ACV; 200 μM PCV; 200 μM VDB).

3.3. Combination therapy of ASP2151 with VACV 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 (Fig. 2). While ASP2151 1 mg/kg twice-daily monotherapy 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

Table 2
Analysis of antiviral efficacy of ASP2151 in combination with ACV (plaque reduction assay).

Virus (strain)	Test dose range (μM)		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 3
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, μM)	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.

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 (Fig. 3).

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 μM (VZV) or less. In contrast, the synergistic antiviral effect of ACV was relatively weak at low concentrations compared to ASP2151 (Fig. 1). Further, in the response surface model analysis, ASP2151 in combination with ACV showed significant synergistic antiviral effects (Table 2 and 3). 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 3), 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 μM). This is not unexpected because none of the compounds are cytotoxic within the concentration ranges evaluated (highest concentration: 200 μM ACV; 200 μM PCV; 200 μM 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

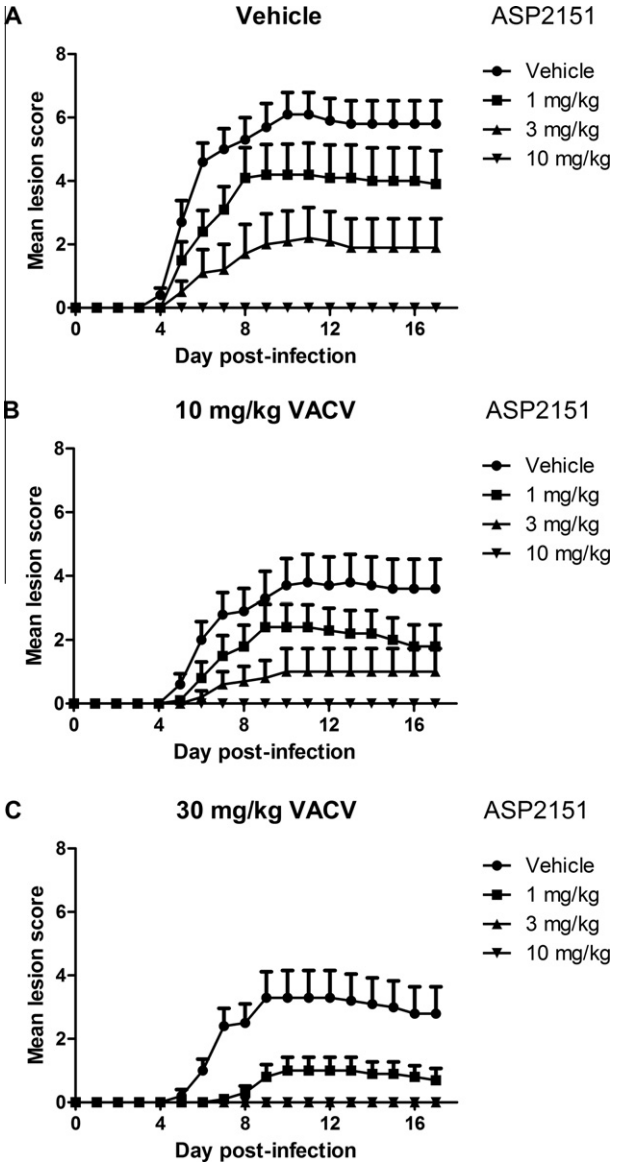

Fig. 2. Effects of combination of ASP2151 and VACV in HSV-1-infected hairless mouse model of zosteriform spread. Hairless mice infected with HSV-1 were orally administered vehicle only, or, 1, 3, or 10 mg/kg twice daily ASP2151 with vehicle (A); 10 mg/kg twice daily VACV (B); 30 mg/kg twice daily VACV (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.

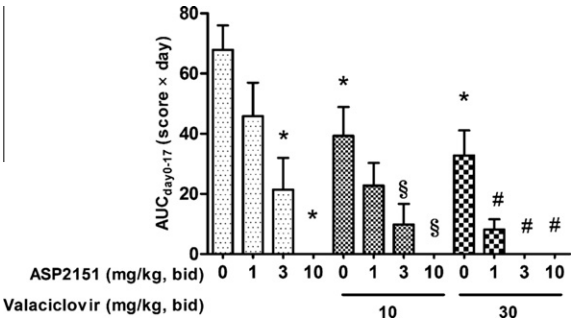

Fig. 3. Combination effect of ASP2151 with VACV in mouse model of zosteriform spread. The figure represents area under the disease score-time curve from Days 0 to 17 post-infection ($AUC_{Day0-17}$ score × day). Data are expressed as the mean + standard error of 10 mice per group. Results were significantly 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.

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.

In conclusion, combined therapy of ASP2151 with ACV and other nucleoside analog antiherpes drugs 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.

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Glossary

HSV-1: herpes simplex virus type 1
HSV-2: herpes simplex virus type 2
VZV: varicella-zoster virus
ACV: acyclovir
VCV: valaciclovir
VDB: vidarabine
PCV: penciclovir
TK: thymidine kinase
HPI: helicase–primase inhibitor