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ASP2151, a novel helicase–primase inhibitor, possesses antiviral activity against varicella–zoster virus and herpes simplex virus types 1 and 2

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Objectives: To evaluate and describe the anti-herpesvirus effect of ASP2151, amenamevir, a novel non-nucleoside oxadiazolylphenyl-containing herpesvirus helicase–primase complex inhibitor.

Methods: The 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 analysed viral DNA in cells infected with herpesviruses [herpes simplex virus (HSV), varicella–zoster virus (VZV) and human cytomegalovirus]. Sequencing analyses were conducted on an ASP2151-resistant VZV mutant. *In vitro* and *in vivo* antiviral activities were evaluated using a plaque reduction assay and an HSV-1-infected zosteriform-spread model in mice.

Results: 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 VZV, HSV-1 and HSV-2 through prevention of viral DNA replication. Further, the anti-VZV activity of ASP2151 (EC_{50}, 0.038–0.10 μM) was more potent against all strains tested than that of aciclovir (EC_{50}, 1.3–27 μM). ASP2151 was also active against aciclovir-resistant VZV. Amino acid substitutions were found in helicase and primase subunits of ASP2151-resistant VZV. In a mouse zosteriform-spread model, ASP2151 was orally active and inhibited disease progression more potently than valaciclovir.

Conclusions: ASP2151 is a novel herpes helicase–primase inhibitor that warrants further investigation for the potential treatment of both VZV and HSV infections.

Keywords: amenamevir, antiviral agents, *Alphaherpesvirinae* viruses

Introduction

Varicella–zoster virus (VZV), herpes simplex virus (HSV) type 1 (HSV-1) and HSV type 2 (HSV-2) are prevalent pathogens belonging to the human herpesvirus (HHV) family.[1] Both VZV and HSV establish a lifetime latent infection in sensory ganglia after the primary infection and eventually reactivate, leading to recurrent episodes. Infection with VZV leads to the development of two distinct disease episodes: varicella as the primary episode and herpes zoster as the recurrent episode.[2] 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.[3]

Since the late 1970s, synthetic nucleoside analogues targeting viral DNA polymerase, such as aciclovir, penciclovir, valaciclovir and famciclovir, have been developed for the treatment of VZV and HSV infections.[4,5] These nucleoside analogues represent safe and effective therapies for HSV and VZV infections. Given that the antiviral activities of aciclovir and penciclovir are more potent against HSV than against VZV,[6] treating a VZV infection with aciclovir or penciclovir necessitates more frequent and higher dosages to obtain therapeutic efficacy (e.g. aciclovir 800 mg, five times daily for herpes zoster[7]). Furthermore, the emergence of mutants that may be multiresistant to present treatment options is a growing concern, particularly among immunocompromised patients.[8] Nucleoside analogues share

the same mechanism of action, requiring phosphorylation by viral thymidine kinase (TK) and host kinases to inhibit viral DNA polymerase. 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 analogue drug class.[9] These limitations of the current standard of care highlight the need to develop novel anti-herpes drugs with potent antiviral activity based on alternative mechanisms of action.

Gene products essential for virus replication, such as the herpesvirus helicase–primase complex, are potential targets for novel antiviral agents. The herpesvirus helicase–primase complex is a heterotrimeric protein complex that possesses multiple enzymatic activities including DNA helicase, single-stranded DNA (ssDNA)-dependent ATPase and primase, all of which are essential for viral DNA replication and hence viral growth.[10] The helicase–primase complex is well conserved among members of the herpesvirus family. For instance, the genes encoding the HSV helicase subunit (UL5), primase subunit (UL52) and cofactor subunit (UL8) share homology with the UL105, UL70 and UL102 genes of the cytomegalovirus, and the ORF55, ORF6 and ORF52 genes of VZV.[11,12] Therefore, agents that target the helicase–primase complex have the potential to represent novel, broad-spectrum, anti-herpes agents. Indeed, the amino-thiazolylphenyl-containing compound, BILS 179 BS, and the thiazole urea derivative, BAY 57-1293, have been reported as helicase–primase inhibitors (HPIs) with anti-HSV activity.[13,14] BILS 179 BS has been shown to have 10-fold more potent activity against HSV than aciclovir *in vitro*, while its *in vivo* efficacy was comparable to that of aciclovir in animal studies.[13] BAY 57-1293 showed two orders of magnitude greater potency against HSV than aciclovir *in vitro*, and superior *in vivo* activities compared with valaciclovir in mouse and guinea pig models.[14] 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 drug, further investigation is warranted to optimize the antiviral spectrum, potency and *in vivo* efficacy of this class of drugs.

Here, we report that ASP2151, 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. Due to promising preclinical profiles of antiviral activity, safety, tolerability and pharmacokinetics, ASP2151 was selected as a development candidate and its clinical efficacy has been evaluated in two Phase II clinical studies for patients with herpes zoster[15] or genital herpes.[16]

Materials and methods

Antiviral compounds

ASP2151 (mol. wt, 482.55; international non-proprietary name, amenamevir; Figure 1a), BILS 179 BS and BAY 57-1293 were synthesized by Astellas Pharma Inc. (Tokyo, Japan). Aciclovir (Sigma-Aldrich, St Louis, MO, USA) and valaciclovir as Valtrex® film tablets (GlaxoSmithKline, Middlesex, UK) were purchased from commercial suppliers.

Viruses and cell lines

Four VZV strains clinically isolated in the USA 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 U/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.

Preparation of helicase–primase complex

Recombinant baculoviruses expressing HSV-1 UL5 (helicase), UL52 (primase) and N-terminally histidine-tagged UL8 (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 *Sf*9 cells triply infected with the baculoviruses, and then purified using Ni-NTA–agarose resin (Invitrogen) in accordance with a previously described method.[17–19]

ATPase assay

ssDNA-dependent ATPase activity of the HSV-1 helicase–primase complex was determined using an assay modified from a previously described method.[10] The reaction buffer contained 20 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 9 μg/mL ssDNA prepared using calf thymus DNA (Sigma-Aldrich), 90 μM 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 concentrations of 0.0001–3 μM was incubated for 75 min at 37°C. ATP hydrolysis was determined by adding 10 μL of Biomol® green according to the manufacturer's instructions (Enzo Life Science, Farmingdale, NY, USA).

DNA helicase assay

Forked DNA helicase substrate was prepared using the oligonucleotides 5′-CAGTCACGACGTTGTAAAACGACGGCCAGTGTTATTGCATGAAAGCCCGGCTG-3′ labelled at the 5′ end with Alexa Fluor® 488 (Invitrogen) and unlabelled 5′-GTCGGCCCACCTTCCTGTTATTGACTGGCCGTCGTTTTACAACGTC GTGACTG-3′ as previously reported.[20] The reaction mixture (10 μL) contained 20 mM HEPES (pH 7.6), 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 1 μg of helicase–primase complex, 20 nM forked DNA helicase substrate and a 200 nM concentration of a capture strand (5′-CAGTCACGACGTTGTAAA ACGACGGCCAGT-3′). Reactions containing ASP2151 were allowed to proceed for 60 min at 30°C, and then the products were electrophoresed through a 20% non-denaturing polyacrylamide gel. Fluorescence was detected using the ProXPRESS® 2D Proteomic Imaging System (PerkinElmer, Waltham, MA, USA).

Primase assay

Primase activity was measured by detecting synthesized RNA primers in the presence of fluorescence-labelled CTP using the 51-mer DNA oligonucleotide 5′-CTTCTTCGGTTCCGACTACCCCTCCCGACTGCCTATGATGTTTATCCTTT G-3′ as a template.[13,19] Reaction mixtures (10 μL) containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1 mM GTP, 1 mM UTP, 2 μM fluorescence-labelled CTP, 10 pmol 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 mM EDTA (pH 8.0) and 90% (v/v) formamide. The products were heat denatured at 95°C for 5 min and separated via denaturing PAGE (15% polyacrylamide–7 M urea), and then fluorescence was detected using the ProXPRESS® 2D Proteomic Imaging System (PerkinElmer).

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 VZV, HSV-1 or HSV-2 at a titre 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 PBS and stained with 0.02% Crystal Violet solution. The number of plaques present was counted under a microscope. An MTT assay or a Neutral Red assay was conducted using HEF cells to determine the cytotoxic concentration causing a 50% reduction in the number of viable cells (CC_{50}).

PAGE 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 US4, ORF31 or UL83 for HSV-1 and HSV-2, VZV or HCMV, respectively [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Each PCR was electrophoresed, stained and visualized. Human β-actin gene was used as an internal control.

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 the VZV glycoprotein B gene.[21] 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).

ASP2151-resistant VZV mutants

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 μM. 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 the 50% effective concentration (EC_{50}) of ASP2151 (0.1 μM) until cytopathic effects were visible. After cells were harvested, 10% of the collected cells were then dispersed on fresh monolayered HEF cells as a cell-associated VZV source and incubated in the presence of 1×, 2× or 4× the concentration of ASP2151 previously used. After incubation for 4–6 days, the cells from the flask in which cytopathic effects were evident for <50% of cells were used as viral source for the next passage. The procedure was continued until the ASP2151 concentration reached 60 μM (600 × EC_{50}). After the ASP2151 concentration reached 60 μM, virus passage was repeated five times in the presence of 60 μM ASP2151 to avoid contamination. The total number of passages and total period of the process were 16 and 72 days, respectively. Then cell-free VZV stock, designated C2151^{r}m, was prepared according to the method described previously.[22] DNA regions including the full-length open reading frame of the helicase (ORF55) and primase (ORF6) genes, were amplified via PCR by using the corresponding primer sets (ORF55, 5′-TGGTCATTTGGGTTACTTCCA-3′ and 5′-AGTGAAGAACCCGCCTAAC-3′; and 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 analysed using GENETYX® software (version 8.1.0; Genetyx, Tokyo, Japan).

In vivo antiviral activity

All animal experimental procedures were approved by the Animal Ethics Committee of Yamanouchi Pharmaceutical Co., Ltd, which is now known as Astellas Pharma, Inc. 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) on dorsolateral skin that had been scratched in a grid-like pattern with a 27-gauge needle under anaesthesia. ASP2151 at doses of 0.3, 1, 3, 10 and 30 mg/kg, or valaciclovir 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); and score 7, hind limb paralysis or death.

Statistical analyses

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

Results

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

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 HPI[23] to create more potent virus-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 relationships between ASP2151 and its related derivatives, will be published elsewhere.

The inhibitory activity of ASP2151 against helicase–primase complex was assayed using the recombinant UL5–UL52–UL8 complex of HSV-1 strain KOS. HSV helicase–primase complex has multiple enzymatic activities, namely DNA helicase and ssDNA-dependent ATPase activity catalysed by the UL5 helicase subunit[24,25] and primase activity catalysed by the UL52 primase subunit.[26] Results showed that ASP2151 inhibited the DNA helicase activity of the complex at concentrations of ≥0.1 μM (Figure 1b). Similarly, the ssDNA-dependent ATPase activity was inhibited in a concentration-dependent manner with a mean IC_{50} value of 0.078 μM [$n=3$, standard error (SE) 0.016 μM]. Interestingly, ASP2151 also inhibited the primase activity at concentrations of ≥0.03 μM (Figure 1c).

We then conducted a PRA to compare the potential antiviral activity and specificity of ASP2151 against herpes family

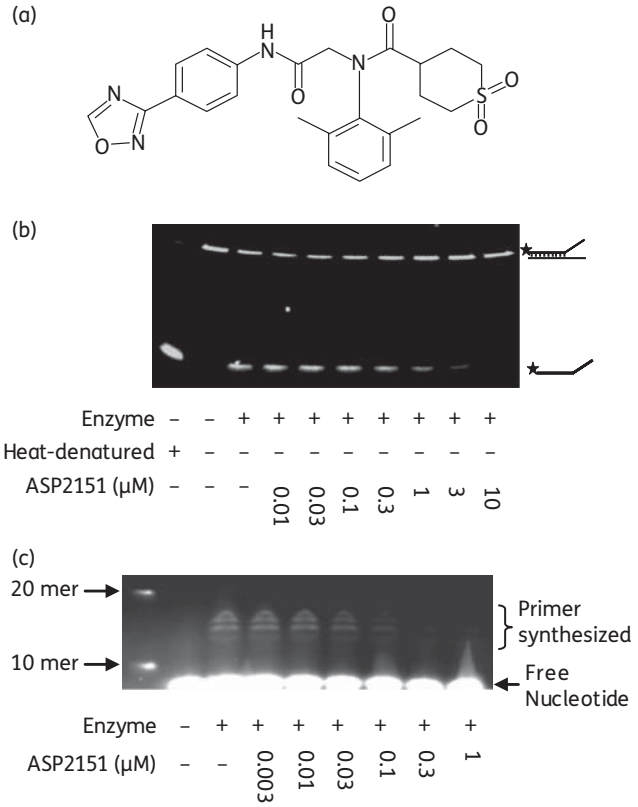

Figure 1. (a) Molecular structure of ASP2151. (b) ASP2151 inhibits the DNA helicase activity of the HSV-1 helicase–primase complex. Heat-denatured, forked DNA helicase substrate was heated at 95°C for 3 min and then cooled immediately on ice to denature; Enzyme, the recombinant HSV-1 strain KOS helicase–primase complex contained (+) or not contained (−) in the reaction mixture. The upper and lower schematic symbols at the side of the gel indicate the position of the forked duplex DNA helicase substrate with fluorescence label and the unwound labelled single-stranded DNA, respectively. (c) ASP2151 inhibits the primase activity of the HSV-1 helicase–primase complex. Arrows show the position of 10 mer and 20 mer fluorescence-labelled oligonucleotide markers in the leftmost lane. Enzyme, the recombinant HSV-1 strain KOS helicase–primase complex contained (+) or not contained (−) in the reaction mixture.

viruses *in vitro* with that of two known HSV HPIs: BILS 179 BS and BAY 57-1293.[13,14] The EC_{50} values of BILS 179 BS for HSV-1 and HSV-2 were 0.060 and 0.046 μM, respectively (Table 1). BAY 57-1293 also inhibited HSV-1 and HSV-2 replication at similar EC_{50} values of 0.014 and 0.023 μM, respectively (Table 1). In contrast, the EC_{50} values of BILS 179 BS and BAY 57-1293 for VZV were 4.1 and 11 μM, respectively. The EC_{50} value ratios for the anti-VZV activity of BILS 179 BS and BAY 57-1293 were ∼1/70 and 1/790 of those against HSV-1.

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

The anti-VZV activity of ASP2151 compared with aciclovir was further evaluated using several strains of VZV that included clinical isolates and an aciclovir-resistant mutant. ASP2151 inhibited the replication of all the VZV strains tested. The EC_{50} values of ASP2151 and aciclovir for aciclovir-susceptible VZV strains ranged from 0.038 to 0.10 μM and 1.3 to 5.9 μM, respectively (Table 2). ASP2151 was also active against the aciclovir-resistant mutant Kanno-Br, which showed reduced susceptibility to aciclovir (EC_{50} value, 27 μM), with an EC_{50} value of 0.082 μM. The CC_{50} of ASP2151 was determined to be >200 μM (the same as aciclovir) in HEF cells in a Neutral Red re-uptake assay, which provided an SI higher than 2000 (Table 2). No antiviral activity was observed for ASP2151 against HCMV, respiratory syncytial virus, influenza virus or HIV-1 for concentrations up to 25 μM (data not shown).

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

Table 1. Antiviral activities of herpes HPIs

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

[a]Data represent the mean EC_{50} and EC_{90} and SE of three independent experiments.
[b]Values for CC_{50} were determined using an MTT assay and a confluent monolayer culture of HEF cells.
[c]SI represents the smallest value among viruses tested.

Table 2. Anti-VZV and cytotoxic activities of ASP2151 and aciclovir

Strain	$EC_{50} \pm SE$ (μM)[a]	
	ASP2151	Aciclovir
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 USA		
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
Aciclovir-resistant mutant		
Kanno-Br	0.082 ± 0.016	27 ± 5
Cytotoxicity, CC_{50} (μM)[b]	>200	>200
SI, CC_{50}/EC_{50}[c]	>2000	>33.9

[a]Antiviral activity (EC_{50}) was determined using a PRA. The data represent the mean of four independent experiments using each strain.
[b]Data represent the mean of three independent experiments. Values for CC_{50} were determined using a Neutral Red assay in proliferating HEF cells.
[c]SI represents the smallest value among strains tested.

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 μM, 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 μM. 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 aciclovir reduced the quantity of VZV DNA in a concentration-dependent manner, with IC_{50} values of 0.057 and 0.44 μM, respectively (Figure 2b). The IC_{50} value of ASP2151 assessed via real-time PCR corresponded to the EC_{50} value of the anti-VZV effect assessed using PRA.

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 μM. 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. The other substitutions were Arg-446 to His (R446H) in ORF55 and Asn-939 to Asp (N939D) in ORF6.

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.[27] When compared with vehicle, oral administration of ASP2151 and valaciclovir significantly reduced mortality on day 17, the cumulative disease score and area under the disease score–time curve for the period days 0–17 post-infection ($AUC_{day0-17}$) at doses of ≥1 mg/kg twice daily and ≥10 mg/kg twice daily, respectively ($P<0.05$) (Table 3 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 more potent than valaciclovir in the model.

Discussion

Here, we showed that the novel oxadiazolylphenyl type herpesvirus HPI ASP2151 (amenamevir) possesses potent antiviral activity not only against 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 HPI (thiazole urea[14] and 2-amino-thiazolylphenyl[13,23] derivatives) have been 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,[10] 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 for ssDNA-dependent ATPase (0.078 μM) was found to be consistent with that of antiviral activity against HSV-1 strain KOS as assessed by PRA (EC_{50}, 0.036 μM; Table 1). In addition, the minimum concentration of ASP2151 at which DNA replication of HSV-1 was almost completely inhibited was 0.1 μM (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 1). 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.[13,14,23] Indeed, PRA results demonstrated that BILS 179 BS and BAY 57-1293 had similar potency against HSV-1 and HSV-2 (Table 1). The helicase–primase complex is an essential and well-conserved gene product among herpes family viruses; all herpesviruses are thought to have their own helicase–primase complex.[1] The amino acid sequences of the helicase and primase between HSV-1 and HSV-2 are highly homologous (89% and 82%, respectively; K. Chono, unpublished data). Moreover, the amino acid sequence of helicase motif IV

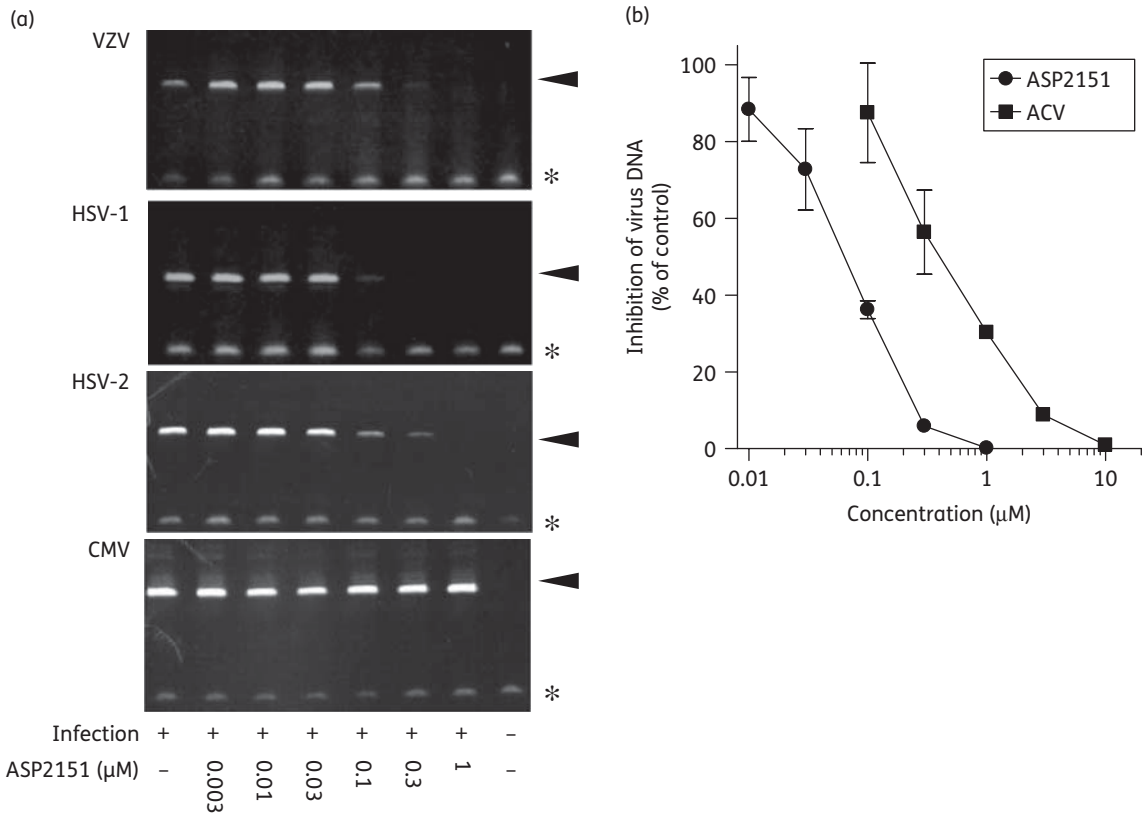

Figure 2. Effect of ASP2151 on the DNA replication of VZV, HSV-1, HSV-2 and HCMV. (a) PAGE was performed with amplified virus-specific PCR fragments from the total DNA extracted from virus-infected cells after incubation with ASP2151. Arrowheads and asterisks indicate the PCR fragments of each virus-specific region and human β-actin gene-specific region, respectively. (b) Effects of ASP2151 (circles) and aciclovir (squares) on VZV DNA replication in virus-infected HEF cells assessed by real-time PCR. Data were calculated from the percentage of control viral DNA extracted from vehicle-treated, virus-infected HEF cells, and are shown as mean ± SE from four independent experiments.

Table 3. 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.

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.[25,28–30] 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. ASP2151 was also active against both HSV-1 and HSV-2 with comparable EC_{50} and EC_{90} values as assessed in PRA (Table 1). 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 179 BS and BAY 57-1293 (K. Chono and H. Suzuki, unpublished data). 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.

Of particular interest, in addition to its activity with regard to HSV-1 and HSV-2, ASP2151 demonstrated potent antiviral activity against VZV. The anti-VZV activity of ASP2151 was carefully 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 μM for ASP2151 compared with 1.3–5.9 μM for aciclovir (Table 2). These PRA findings indicate that ASP2151 exerted more potent anti-VZV activity than did aciclovir in

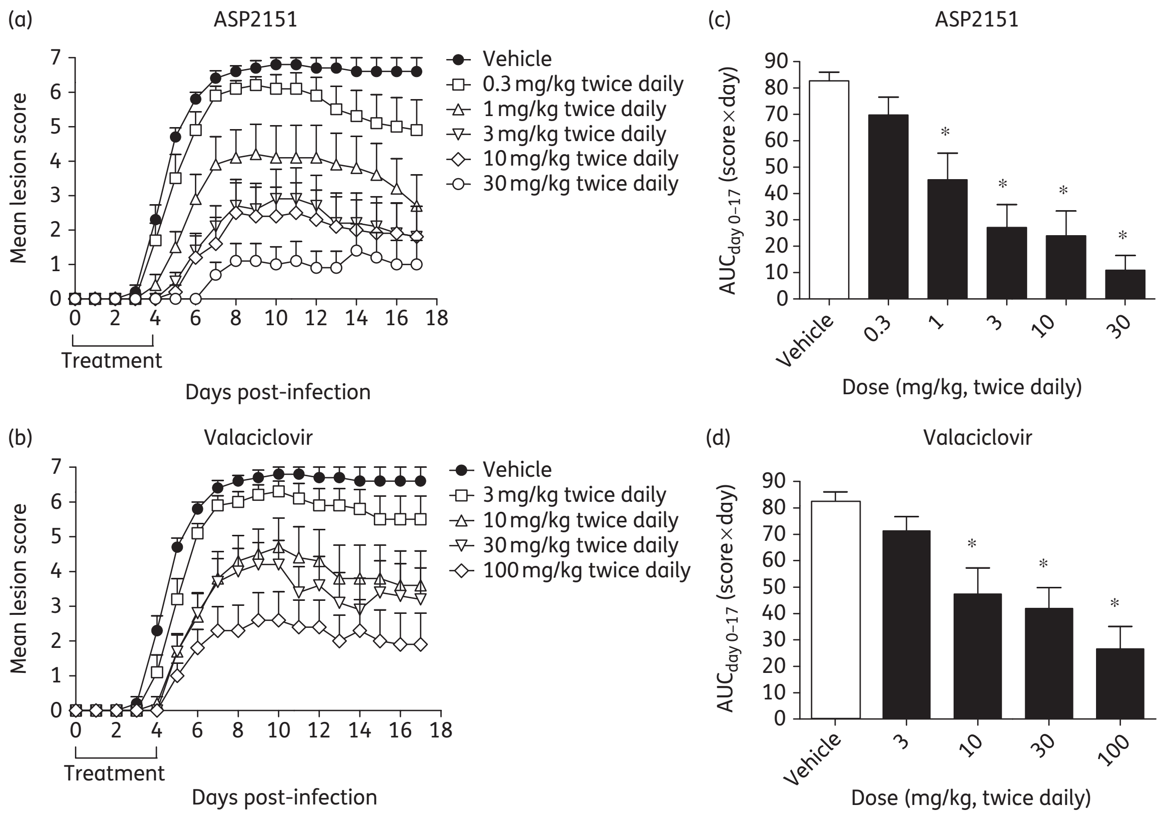

Figure 3. Antiviral activity of ASP2151 and valaciclovir in a HSV-1-infected hairless mouse zosteriform model. (a) and (b) The mean disease scores for each ASP2151 or valaciclovir administration group were calculated and plotted versus days post-infection. (c) and (d) Area under the disease score–time curve for the period from post-infection day 0 to post-infection day 17 [$AUC_{day0-17}$ (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 10 mice per group.

Virus	Strain		*	*	*	*	*	*	*		*			*	*	*	*		*		*	*	*	*
HSV-1	KOS	340	F	I	N	N	K	R	C	V	E	H	E	F	G	N	L	M	K	V	L	E	Y	G
	BILS 179 BS^{r}	340	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	**N**	·	·	·	·	·
	BAY 57-1293^{r}a	340	·	·	·	·	·	·	·	·	·	·	·	·	**V**	·	·	·	·	·	·	·	·	·
	BAY 57-1293^{r}b	340	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	**T**	·	·	·	·	·	·
	BAY 57-1293^{r}c	340	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	**Q**	·	·	·	·	·
	BAYr2	340	·	·	·	·	·	·	·	·	·	·	·	·	**R**	·	·	·	·	·	·	·	·	·
	BAY-Pr2	340	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	**T**	·	·	·	·	·
	BAY-pF-r3	340			**K**	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HSV-2		393	F	I	N	N	K	R	C	V	E	H	E	F	G	N	L	M	K	V	L	E	Y	G
VZV	CaQu	334	F	I	N	N	K	R	C	Q	E	D	D	F	G	N	L	L	K	T	L	E	Y	G
	C2151^{r}m	334	·	·	**K**	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
					Motif IV																			

Figure 4. Amino acid sequence adjacent to helicase motif IV in the helicase subunit of the helicase–primase complex. Amino acid sequences were aligned between HSV-1 strain KOS, HSV-2 strain HG52 and VZV strain CaQu; consensus residues are denoted using asterisks. The amino acid sequence of HSV-2 strain HG52 was from the RefSeq database (accession number, NP_044474). 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 BILS 179 BS- or BAY 57-1293-resistant HSV-1 mutants previously reported.[13,14,32,35,36] C2151^{r}m is a VZV mutant derived from its parent strain CaQu, which was prepared in the presence of stepwise increasing concentrations of ASP2151 up to 60 μM. Motif IV, one of the well-conserved six helicase motifs in the helicase subunit of the helicase–primase complex in *Herpesviridae* viruses.[25,28–30]

PRA, and this activity was also demonstrated in VZV DNA quantification using real-time PCR. It is known that particular series of thymidine analogues like sorivudine possesses extremely potent *in vitro* anti-VZV activity compared with aciclovir and showed clinical efficacy more potent than aciclovir in herpes zoster patients.[31] No HPI, however, has been reported to show

anti-VZV activity so far. To our knowledge, ASP2151 is the first HPI exerting more potent anti-VZV activity than aciclovir. As expected, ASP2151 was also active against an aciclovir-resistant VZV mutant (Table 2). Given that current nucleoside analogue drugs, such as aciclovir 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.[8,9] Although the prevalence of aciclovir-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.[9] ASP2151 may therefore offer a therapeutic option for treating aciclovir- or penciclovir-resistant virus infections.

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 to 60 μM. Of particular note is the fact that C2151^{r}m shows a marked defect in the viral replication profile [see Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. As a consequence, we were unable to obtain C2151^{r}m cell-free viral stocks with sufficient viral titre to characterize the mutant. 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.*[32] 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 UL5 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. 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.[32] Given the analogy with the BAY-pF-r3 mutation, the N336K substitution is also likely to confer resistance to ASP2151 on 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 C2151^{r}m, although their importance for C2151^{r}m remains to be evaluated. A genomic DNA sequence database of VZV strains revealing that amino acid at position 446 in helicase varies between either Arg or His[33,34] 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 UL52, 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.

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 mortality on day 17 post-infection due to HSV-1 infection were significantly improved. Since VZV is hard to infect into 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 more potent anti-HSV activity compared with 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 (Y. Aoki, Astellas Pharma Inc., personal communication). The safety profile of ASP2151 may be explained at least partly by the high SI (Tables 1 and 2). 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. Recently, ASP2151 was confirmed in its clinical efficacy and tolerability in Phase II clinical studies conducted in patients with herpes zoster[15] and recurrent genital herpes[16] (manuscripts in preparation).

In conclusion, ASP2151 is a novel viral HPI with potent activity against not only HSV-1 and 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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Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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