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ORIGINAL ARTICLE

Anti-herpes virus activity of polyether antibiotic CP-44161 *in vivo*

Yukiko Yamagishi[1,7], Motoi Ueno[1,7], Chihoko Ueno[1,7], Akemi Kato[1], Ryuichi Kanasaki[1], Bunji Sato[1], Koji Takakura[2], Akihiko Fujie[1], Hidenori Nakajima[3], Takashi Fujii[4], Motohiro Hino[5] and Eisaku Tsujii[6]

In the previous study, we discovered a polyether antibiotic CP-44161, which was reported earlier as an anticoccidal agent, as an anti-varicella zoster virus compound. In this study, we demonstrated that CP-44161 had a very strong and broad anti-herpes virus activities against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) *in vitro*. To determine the antiviral activity of CP-44161 *in vivo*, we examined its effect on the cutaneous HSV-2 infection model in Balb/c mice. CP-44161 showed inhibitory effect on lesion development as well as acyclovir (ACV) when the treatment was started from day 3. Meanwhile, in case the start of treatment was delayed until day 4, when ACV was no longer effective, the effectiveness of CP-44161 still remained. In this model, CP-44161 also showed inhibitory effect on the proliferation of HSV-2 DNA in dorsal root ganglia. This is the first article to report that polyether antibiotics can be effective on viral infection *in vivo*.

INTRODUCTION

There are eight human herpesvirus that cause various diseases in humans. Herpes simplex virus type 1 (HSV-1), a causative agent of cold sore and corneal herpes, HSV type 2 (HSV-2), a causative agent of genital herpes, and varicella zoster virus (VZV), a causative agent of varicella (chickenpox) and herpes zoster (shingles), belong to alphaherpesvirinae. They usually infect mucosal epithelial cells and travel along the neuron (by a process called retrograde transport) to trigeminal ganglia and then establish latent infection.[1] Most of the problems with herpesvirus infections are due to reactivation that may lead to recurrent infections. The risk of diseases increases with age, and also frequently occurs in immunocompromised hosts, such as cancer patients, transplantation patients or acquired immunodeficiency syndrome patients.[2]

Standard antiviral drugs at present used in the treatment of HSV and VZV infections include acyclovir (ACV), valaciclovir (VACV, the oral prodrug of ACV), famciclovir (the oral prodrug of penciclovir) and vidarabine (Ara-A).[3,4] Despite a number of recent therapeutic advancements, there remains an urgent need to develop a new class of therapy, especially novel anti-herpes virus agent with a strong potency against HSV-2 and VZV, because anti-herpes virus agents used at present exhibit lower potency against HSV-2 and VZV. Furthermore, as they had some problems, such as cross-resistance and mutagenicity, the development of new agents with different mechanism of action from nucleoside analogs was required.

To discover a new candidate for anti-herpes virus agent, we screened microbial fermentation samples. In the preceding article,[5] we have reported the discovery of anti-VZV activity of CP-44161 (Figure 1; refs 6, 7), from an extract of fermentation broth of *Dactylosporangium* sp. no. 642788. CP-44161, which was a polyether reported earlier as an anticoccidal compound, exhibited a strong anti-VZV activity and lower cytotoxicity than other antiviral polyethers, such as nigericin and monensin.

In this study, we showed strong and broad antiviral activities of CP-44161 against HSV-1 and HSV-2 *in vitro*. Moreover, we demonstrated the antiviral activity of CP-44161 *in vivo* on the cutaneous HSV-2 infection model in Balb/c mice.

MATERIALS AND METHODS

Cells and viruses

African green monkey kidney cell line, Vero (CCL-81), was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were grown and maintained in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.075% $NaHCO_3$, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin sulfate. Cells were grown at 37 °C in an atmosphere of 5% CO_2.

Herpes simplex virus type 1 (VR-3 strain) was kindly provided by Dr T Suzutani (Fukushima Medical University) and HSV-2 (186 strain)[8] was kindly provided by Dr Y Nishiyama (Nagoya University). Vero cells were
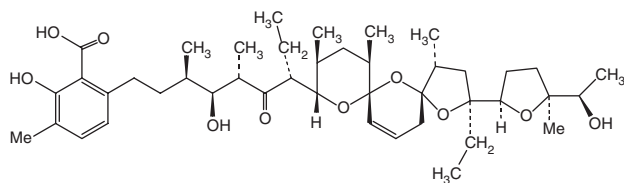
Figure 1 The structure of CP-44161.

used to produce virus pools, quantification of viral titers of stocks by the plaque assay. Viral replication studies of HSV were also performed in Vero cells.

Reagents and antiviral compounds

CP-44161 was prepared from a culture broth of *Dactylosporangium* sp. no. 642788 as described in our earlier report.[5]

Adenine 9-β-D-arabinofuranoside (Ara-A), DMSO and 3-(4,5-dimethylthia-zol-2-yl)-2,5,-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St Louis, MO, USA). ACV was purchased from GlaxoSmithKline (Tokyo, Japan). All compounds were dissolved in DMSO and then diluted with cell culture medium to yield 0.1% DMSO.

In vitro antiviral assays

The antiviral effects of compounds were determined by plaque reduction assay. Vero cells were grown to confluence in 24-well tissue culture plates and then infected with HSV-1 VR-3 strain or HSV-2 186 strain (40 plaque forming units (PFU) per well). After 1 h incubation, media containing 1.5% agarose in the presence or absence of various concentrations of the compounds were added. After 2 days, the cells were stained overnight with 0.01% neutral red and the number of viral plaques was counted.

Cytotoxicity assay

The cytotoxicity of compounds against growing Vero cells (5×10^3 cells per well in 96-well tissue culture plates) for 48 h was determined with an MTT assay as the procedure described earlier.[9]

In vivo studies

All animal experimental procedures were approved by the Animal Experiment Committee of Fujisawa Pharmaceutical Co. Ltd., the company that is now known as Astellas Pharma Inc.

Antiviral effect on cutaneous HSV infection in Balb/c mice

Seven-week-old female Balb/c mice (Charles River Laboratories, Yokohama, Tokyo, Japan) were used. Each group of animals, controls or treated, contained 10 mice.

Mice were anaesthetized with pentobarbital ($50 \, \mathrm{mg \, kg^{-1}}$, i.p.), and then the mid-flank and right foot were clipped and depilated with a chemical depilatory, Hair Remover Milkycream (Kanebo Co. Ltd., Tokyo, Japan). Three days later, HSV-2 (3×10^3 PFU in 10 µl) was inoculated on the shin of the right hind paw.

For topical treatments of CP-44161, 3% olive oil solution was formulated. The olive oil without CP-44161 was used as the control. Drug treatment of animals began 3 or 4 days post-infection, and drugs were applied twice a day up to 8 days post-infection with a sterile cotton swab. ACV was dissolved in physiological saline and was administered orally at a dose of $100 \, \mathrm{mg \, kg^{-1}}$.

Beginning 1 day after infection, the inoculated site was evaluated and scored daily for 8 days: 0, no visible change in the inoculation or surrounding tissue; 1–2, one or two small vesicles on the inoculation site; 3–4, many vesicles on the inoculation or surrounding tissue; 5–6, mild zosteriform; 7–8, severe zosteriform.

Quantification of HSV DNA in the dorsal root ganglia

Seven-week-old female Balb/c mice (Charles River Laboratories, Yokohama, Japan) were infected as described above. Drug treatment of animals began 3 days after infection, and drugs were applied twice a day for the subsequent 3 days. Each group of animals, controls or treated, contained nine mice.

Five days post-infection, the dorsal root ganglia (DRG) were dissected from decapitated mice, immediately frozen by dry ice and stored at $-80 \, ^\circ\mathrm{C}$ until

Table 1 Antiviral activities against HSV-1,2 and cellular cytotoxicity of CP-44161

Compound	HSV-1 VR-3 EC_{50} (µg ml^{-1})[a]	HSV-2 186 EC_{50} (µg ml^{-1})[a]	Vero CC_{50} (µg ml^{-1})[b]
CP-44161	0.025	0.018	0.58
ACV	0.025	1.5	>50
Ara-A	1.9	5.6	25

Values provided in this table represent the mean of three independent experiments.
[a]Antiviral activity was determined by plaque assay. EC_{50} was the concentration that inhibited 50% of HSV replication in Vero cells.
[b]Cytotoxic effect was determined by MTT assay. CC_{50} was the concentration that showed 50% cytotoxic effects in Vero cells.
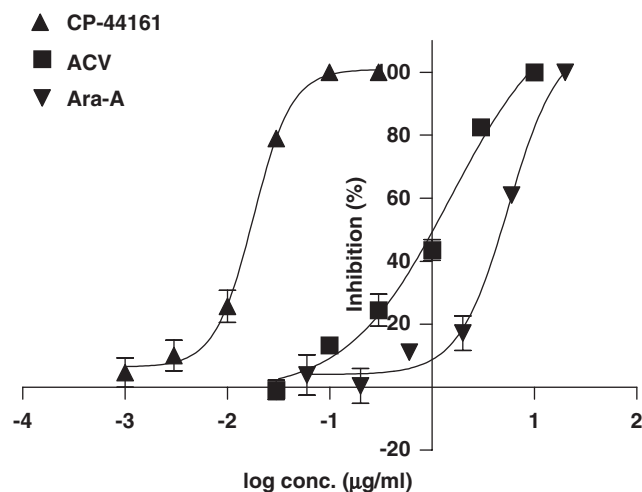
Figure 2 Dose-dependent antiviral activity of CP-44161 against HSV-2. Data are presented as the mean ± standard deviation of triplicate experiments.

assay. Total DNA in the tissue was extracted with DNeasy tissue kit (Qiagen, Tokyo, Japan) according to a manufacturer's protocol.

The determination of HSV-2 DNA in the total DNA was quantified by real-time PCR using an ABI prism 7900HT sequence detection system (Applied Biosystems, Tokyo, Japan). Primers were designed for the detection of HSV-2 thymidine kinase (TK) gene. The sequences of the primers were as follows: TK (forward), 5′-ggctcctcatatcgggggggg-3′; TK (reverse), 5′-cgcggaacagggcaaacagc-3′. PCR was performed using the SYBR-green technology. The PCR program consists of activation step at $95 \, ^\circ\mathrm{C}$ for 10 min and 40 cycles of denaturation at $94 \, ^\circ\mathrm{C}$ for 0.5 min, annealing and extension at $67 \, ^\circ\mathrm{C}$ for 1 min; HSV-2 genome was used as a standard. On each amplification curve, the threshold cycle was determined and used for the quantification of DNA using the ABI prism 7900 software.

Statistical analysis

Statistical significance was estimated according to *t*-tests using GraphPad Prism 4 software (GraphPad Software Inc.). A *P*-value of <0.05 was judged significant.

RESULTS

Antiviral activities of CP-44161 against HSV-1 and HSV-2

We obtained antiviral activities of CP-44161 against HSV-1 VR-3 strain and HSV-2 186 strain in plaque reduction assay. As shown in Table 1 and Figure 2, CP-44161 inhibited the replication of HSV-1 and HSV-2 in a dose-dependent manner. Of particular interest is the anti-HSV-2 activity (Figure 2). CP-44161 gave an EC_{50} value of $0.018 \, \mathrm{µg \, ml^{-1}}$ compared with an EC_{50} value of $1.5 \, \mathrm{µg \, ml^{-1}}$ for ACV

and of $5.6\,\mu\mathrm{g}\,\mathrm{ml}^{-1}$ for Ara-A at 48 h. These results showed that CP-44161 had stronger anti-HSV-2 activity than those of ACV and Ara-A, which are in clinical use.

Antiviral effect of CP-44161 on cutaneous HSV-2 infections in Balb/c mice

Next, we tried to evaluate the antiviral activity of CP-44161 *in vivo* on the cutaneous HSV-2 infection model in Balb/c mice.

In the preliminary experiment, we found that mice cutaneously infected with HSV-2 showed lesion, but the lesion score began to decrease after day 8 in the control group. Therefore, the therapeutic effect was judged at day 8. As the oral bioavailability of CP-44161 was poor (data not shown), we evaluated antiviral activity of CP-44161 *in vivo* using the topical ointment. CP-44161 was administered topically with 3% olive oil solution (equivalent to $30\,\mathrm{mg}\,\mathrm{kg}^{-1}$). ACV was administered orally at a dose of $100\,\mathrm{mg}\,\mathrm{kg}^{-1}$.

In this model, the lesion started to develop on the skin on day 4 (Figures 3a and b). When the treatment was started from day 3, both CP-44161 and ACV significantly suppressed lesion development in the skin of mice with equivalent efficacy on day 8 (Figure 3c, $P < 0.01$), but the olive oil control was ineffective in preventing lesion formation. Unlike the case of the treatment started from day 3, when the treatment was started from day 4, ACV was no longer able to inhibit the development of skin lesions. However, CP-44161 showed a significant effect in equivalent efficacy of the results that was started from day 3 (Figure 3c, $P < 0.01$). These results indicated that CP-44161 could be given after the development of symptoms when ACV was no longer effective.

Effect of CP-44161 on HSV-2 DNA in the DRG

In the above cutaneous infection model, we ascertained the antiviral effect of CP-44161 *in vivo* by lesion score. To elucidate whether CP-44161 could inhibit viral proliferation *in vivo*, we quantified the HSV-2 DNA in DRG. We modified the method for the detection of HSV TK gene by using real-time PCR instead of the PCR analysis.[10]

In the preliminary experiment, we found that HSV proliferation in DRG peaked at day 5. Therefore, the therapeutic effect was judged at day 5. As shown in Figure 4, 3% ointment of CP-44161 significantly inhibited the replication of HSV-2 DNA in DRG (Figure 4, $P < 0.01$). It was administered from day 3 to day 5. On day 5, DRG were dissected from treated mice, and the amount of HSV-2 DNA was quantified. When we made a comparison of the effect between CP-44161 and ACV on the same condition, CP-44161 exhibited an equivalent efficacy to the oral treatment of ACV ($100\,\mathrm{mg}\,\mathrm{kg}^{-1}$) (data not shown).

DISCUSSION

We demonstrated earlier that the polyether compound CP-44161, discovered from one of the microbial fermentation samples produced by *Dactylosporangium* sp. no. 642788, showed anti-VZV activities.[5]

In this study, we first demonstrated that CP-44161 exhibited a broad anti-herpes virus activity against alphaherpesvirinae, such as HSV-1 and HSV-2, *in vitro* in a dose-dependent manner (Table 1 and Figure 2). CP-44161 inhibited replications of HSV-1 and HSV-2, similar to other polyethers, such as nigericin and monensin (data not shown). Especially in terms of anti-HSV-2 activity, this compound exhibited stronger potency than anti-HSV agents used at present, ACV and Ara-A (Figure 2).

Next we demonstrated that CP-44161 exhibited anti-herpes virus activity *in vivo*. Owing to its poor oral bioavailability, we evaluated antiviral activity of CP-44161 topically. As the solvent for CP-44161,
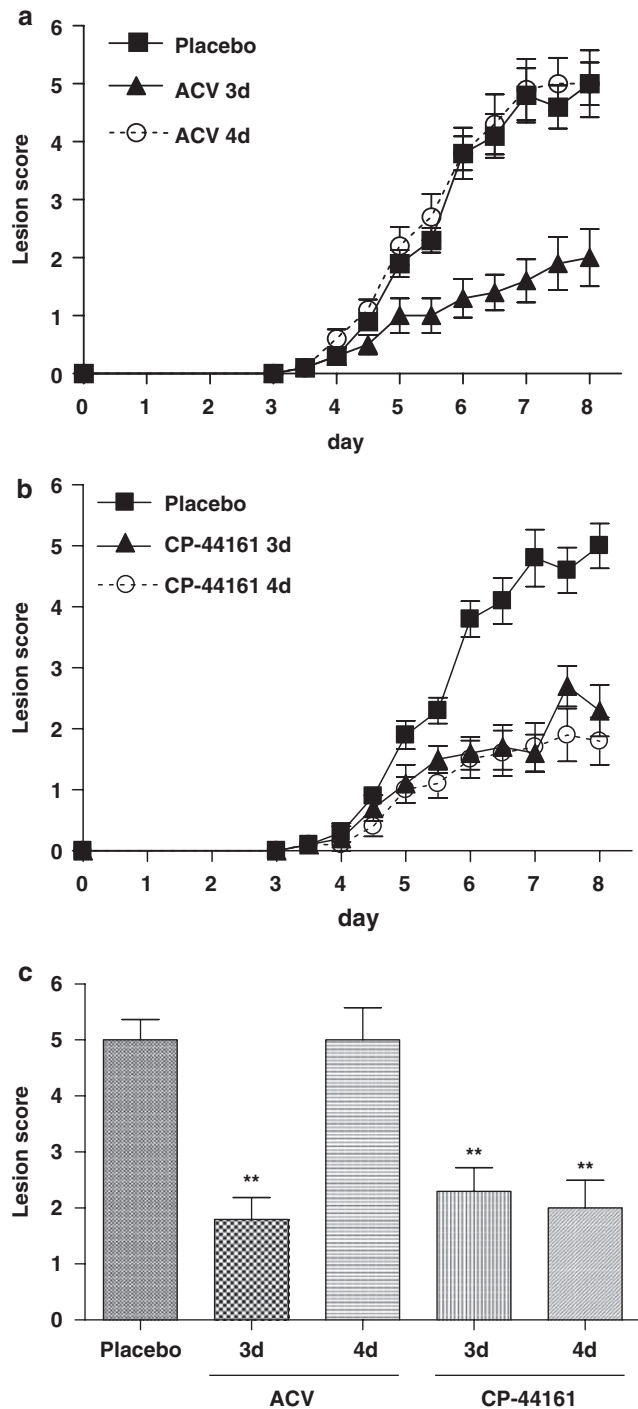
Figure 3 Effects of treatment with CP-44161 and ACV on the development of skin lesions on cutaneous HSV infection in Balb/c mice. Mice were inoculated with 3×10^3 PFU of HSV-2 strain 186 on the right hind paw on day 0. (**a**) ACV ($100\,\mathrm{mg}\,\mathrm{kg}^{-1}$) was administered orally twice a day from 3 or 4 days after HSV-2 inoculation. Placebo (saline) was administered orally twice a day from 3 days after HSV-2 inoculation. (**b**) CP-44161 (3% ointment) was administered topically twice a day from 3 or 4 days after HSV-2 inoculation. Placebo (olive oil) was administered topically twice a day from 3 days after HSV-2 inoculation. (**c**) Lesion score on day 8. Data points represent the average score of 10 mice per group ± s.e.
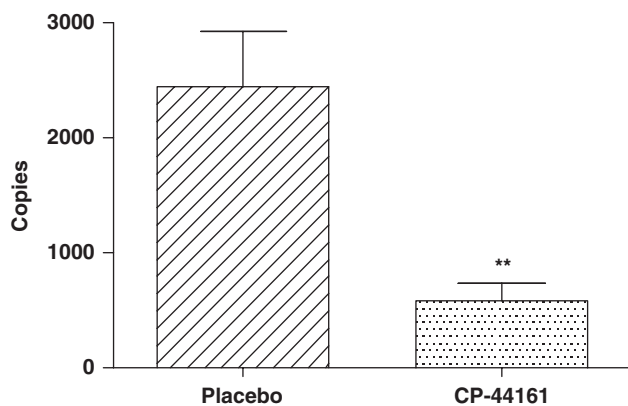
Figure 4 Effects of treatment with CP-44161 on the levels of HSV-2 gene in the dorsal root ganglia (DRG) on cutaneous HSV infection in Balb/c mice. Mice were inoculated with 3×10^3 PFU of HSV-2 strain 186 on the right hind paw on day 0. CP-44161 (3% ointment) and placebo (olive oil) was administered topically twice a day from 3 days after HSV-2 inoculation. On day 5, DRG were dissected and DNA was extracted. The copy number of HSV-2 in DRG was quantified by real-time PCR. Data points represent the average score of 9 mice per group ± s.e.

olive oil was selected, as CP-44161 was dissolved in olive oil completely and the olive oil formulation gave us the best antiviral effect (data not shown). Topical treatment of cutaneous HSV infection has the advantages of convenience and target site selection along with the prospect for maintenance of high local dose rates. However, in herpes zoster, topical monotherapy with ACV is less effective at preventing virus shedding than oral therapy and has little effect on symptoms.[11] So we selected oral administration of ACV.

The development of a zosteriform rash in this Balb/c mouse model is analogous to the final step in the development of a recrudescent lesion, which might occur after reactivation of latent virus.[12] In this system, virus traveled from the inoculation site, where virus replicated primarily, to the sensory nervous system and then returned to the skin, resulting in the zosteriform rash. When we started the treatment from day 3, after the onset of lesion development on the skin, both CP-44161 (3% ointment) and ACV ($100 \, \text{mg} \, \text{kg}^{-1}$) showed significant effects in lesion score, with equivalent efficacy on day 8 (Figure 3c). However, CP-44161 was effective even if the start of the treatment delayed, when ACV was no longer effective. Early treatment with antiviral agents, such as ACV and vidarabine, needs to shorten the skin lesions and complications related to herpes zoster.[13] In clinical practice, the start of administration of VACV limited within the first 72 h after the onset of symptoms. These results indicated that CP-44161 could be given over 72 h after the onset; that is, the recalcitrant cases against ACV.

Then we demonstrated that CP-44161 could inhibit viral proliferation *in vivo* by the quantified detection of the HSV-2 DNA in DRG. The results in Figure 4 confirmed that CP-44161 showed significant inhibition of the replication of HSV-2 DNA in DRG. The result indicated that the inhibition on the proliferation of HSV-2 in DRG may be responsible for the inhibition of zosteriform development of this compound.

Tissue culture studies have demonstrated antiviral activity of polyethers against a wide range of viruses.[14–17] In terms of the

in vivo antiviral activities of polyethers, monensin, narasin, septamycin and nigericin were effective in treating an infection of transmissible gastroenteritis virus in piglets at doses of $0.1–100 \, \text{mg} \, \text{kg}^{-1}$ (ref. 18). In Bulgaria, a preparation containing a nigericin derivative (named Pandavir) was used topically as an anti-herpes drug.[19] However, the mechanism of antiviral activities *in vivo* has not been characterized. Here, we showed antiviral activity of polyether on murine model for the first time. This enabled us to analyze the mechanism of action of polyethers *in vivo* easily. Further experimental studies concerning the mode of action of CP-44161 *in vitro* and *in vivo* are under investigation.

According to this study, our data indicated that CP-44161 might be a good candidate for a new anti-herpes virus agent. It could be used on not only HSV but also VZV and also might have a potential against post-herpetic neuralgia.

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