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Anti-herpes simplex virus (HSV-1) activity of oxyresveratrol derived from Thai medicinal plant: Mechanism of action and therapeutic efficacy on cutaneous HSV-1 infection in mice

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ABSTRACT

Oxyresveratrol, a major compound purified from *Artocarpus lakoocha*, a Thai traditional medicinal plant, was evaluated for its mechanism of action and therapeutic efficacy on cutaneous herpes simplex virus (HSV) infection in mice. The inhibitory concentrations for 50% HSV-1 plaque formation of oxyresveratrol, three clinical isolates, thymidine kinase (TK)-deficient and phosphonoacetic acid (PAA)-resistant HSV-1 were 19.8, 23.3, 23.5, 24.8, 25.5 and 21.7 μg/ml, respectively. Oxyresveratrol exhibited the inhibitory activity at the early and late phase of viral replication and inhibited the viral replication with pretreatment in one-step growth assay of HSV-1 and HSV-2. Oxyresveratrol inhibited late protein synthesis at 30 μg/ml. The combination of oxyresveratrol and acyclovir (ACV) produced synergistic anti-HSV-1 effect, as characterized by the isobologram of plaque inhibition. Mice orally treated with oxyresveratrol (500 mg/kg/dose) dose at 8 h before and three times daily had significant delay in herpetic skin lesion development ($P < 0.05$). Topical application of 30% oxyresveratrol ointment five times daily significantly delayed the development of skin lesions and protected mice from death ($P < 0.0001$).

1. Introduction

Herpes simplex virus (HSV) causes a variety of diseases in humans, with different degrees of severity, ranging from mild to severe, and in certain cases, it may even lead to life-threatening conditions, especially in immunocompromised patients. A normal sequela to a primary infection is the establishment of latency as the virus takes up permanent residence in the ganglia of the host. According to epidemiological surveys, the HSV infection rate has continuously increased in most countries. Nucleoside analogs, acyclovir (ACV) and other nucleoside derivatives, penciclovir, valaciclovir, famciclovir, and ganciclovir have been approved for treatment of HSV infections worldwide (Galasso et al., 1997; Leung and Sacks, 2000; De Clercq, 2001, 2004; Brady and Bernstein, 2004). However, the appearance of ACV-resistant virus is a current problem. The failure of treatment is also due to the recurrence of latent viruses. Consequently, there is still a need in the future to search for new and more effective antiviral agents that can substitute or complement currently used antiviral medicines.

Anti-herpetic activities of plant extracts have given interesting results for the search for new antiviral agents (Vanden Berghe et al., 1993; Namba et al., 1997; Kurokawa et al., 1998, 1999).

Traditional medicine is still the mainstay of health care in Thailand and many developing countries, and most of the drugs and cures used come from plants. The antiviral activities against HSV-1 of various extracts from Thai medicinal plants were reported (Yoosook et al., 2000; Lipipun et al., 2003). *Clinacanthus nutans* extract has been traditionally used in Thailand for the topical treatment of herpes simplex virus and varicella-zoster virus infections (Sangkitporn et al., 1993, 1995). For this indication, a 4% cream of *Clinacanthus nutans* extract (called Phya-Yaw cream) is commercially produced by Thai Government Pharmaceutical Organization. Oxyresveratrol (*trans*-2,4,3′,5′-tetrahydroxystilbene) (Fig. 1) was a major constituent previously purified from the heartwood of a Thai traditional plant, *Artocarpus lakoocha* Roxburgh (Moraceae) and shown to possess *in vitro* anti-HSV potential (Sritulaluk et al., 1998;

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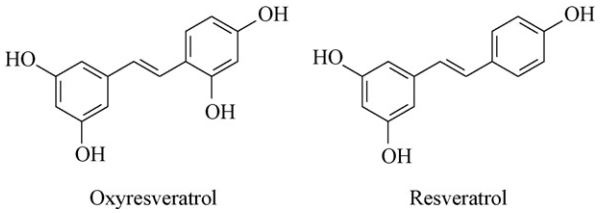

Fig. 1. Structure of oxyresveratrol (*trans*-2,3′,4,5′-tetrahydroxystilbene) as compared to resveratrol (*trans*-3,5,4′-trihydroxystilbene).

Likhitwitayawuid et al., 2005). Its potent tyrosinase inhibitory and antioxidant activities were reported (Sritulaluk et al., 1998; Kim et al., 2002; Lorenz et al., 2003; Likhitwitayawuid et al., 2006). It was also suggested to be neuroprotective and inhibit the apoptotic cell death in transient ischemia in a rat model (Andrabi et al., 2004). The inhibitory effects against HSV of several known and characterized antioxidants were reported (Palamara et al., 1995; Sheridan et al., 1997). The material has potential application as novel skin-whitening agent in cosmetic preparations (Tengamnuay et al., 2006; Likhitwitayawuid, 2008). It was reported that resveratrol (3,5,4′-trihydroxystilbene), the representative of stilbene group, was very active anti-HSV agent and showed inhibitory activity in viral replication and therapeutic activity in cutaneous HSV lesions in mice (Docherty et al., 1999, 2004). Resveratrol, however, is not readily available from Thai medicinal herbs, whereas oxyresveratrol can be abundantly obtained from the heartwood of *A. lakoocha*, a Thai medicinal plant widely distributed in the country. Therefore, we have focused on the mechanism of action and the therapeutic activity of oxyresveratrol against HSV.

In this study, the inhibitory activities of oxyresveratrol on the replication of HSV-1, HSV-2, clinical isolates, thymidine kinase (TK)-deficient and phosphonoacetic acid (PAA)-resistant strains of HSV-1 were investigated. Furthermore, oxyresveratrol was evaluated in a mouse model of HSV-1 infection.

2. Materials and methods

2.1. Viruses and cells

HSV strains used were HSV-1 (7401H) (Kurokawa et al., 1993), HSV-1 (KOS), HSV-2 (Baylor186) (Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand), TK-deficient HSV-1 strain (B2006 strain) (Dubbs and Kit, 1964; Kurokawa et al., 2001) and PAA-resistant strain (Kurokawa et al., 2001; Suzuki et al., 2006). Three clinical genital isolates of HSV-1 were provided by Dr. T. Kawana, Teikyo University, Japan from three patients with genital herpes (Yoshida et al., 2005). Virus stocks were prepared from infected-cultured cells as reported previously (Shiraki and Rapp, 1988; Kurokawa et al., 1993). African green monkey kidney cells (Vero cells, ATCC CCL81) were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 5% and 2% fetal bovine serum (FBS), respectively.

2.2. Drugs

ACV and PAA were purchased as powder from Sigma product. Vilerm (5% ACV cream) was purchased from Siam Bheasach Co. Ltd, Thailand.

2.3. Purification of oxyresveratrol from Artocarpus lakoocha

Oxyresveratrol was purified from the heartwood of *A. lakoocha* Roxburgh (Moraceae) and the method was previously reported (Sritulaluk et al., 1998; Likhitwitayawuid et al., 2005). Briefly, heartwood of *A. lakoocha* was extracted in methanol. The active fraction was isolated from the methanol extract using vacuum liquid chromatography. The purified compound was analyzed as *trans*-2,4,3′,5′-tetrahydroxystilbene (oxyresveratrol) by spectroscopy. The chemical was assayed by HPLC and found to be greater than 99% pure.

2.4. Effect of oxyresveratrol against wild-type and drug-resistant HSV by plaque reduction assay

Oxyresveratrol was examined for its inhibitory activity on plaque formation against wild-type, clinical isolates and drug-resistant HSV-1 strains. Duplicate cultures of Vero cells in 60-mm plastic dishes were infected with 100 plaque forming units (PFU)/0.2 ml of wild-type HSV-1 (7401H), three clinical isolates of HSV-1, TK-deficient HSV-1 or PAA-resistant HSV-1 strain for 1 h at room temperature. Cells were overlaid with 5 ml of nutrient agarose (1%) medium containing various concentrations of ACV, PAA, or oxyresveratrol and then cultured at 37 °C for 4–5 days. The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution. The number of plaques was counted under a dissecting microscope (Shiraki et al., 1991; Lipipun et al., 2003; Suzuki et al., 2006). The 50% inhibitory concentration for plaque formation (IC_{50}) was defined as the concentration at which the plaque number decreased to half of that in cells cultured without the addition of antiviral drugs. The IC_{50} was determined from the curve relating plaque formation (%) of the untreated culture to the concentration of the samples using the computer program Microplate Manager III (BioRad, Hercules, CA).

Antiviral activities of oxyresveratrol were also determined against HSV-1 (KOS), HSV-2, poliovirus type 1 (Sabin strain) and measles virus (Tanabe strain). Vero cells, in 60-mm tissue culture dishes, were infected with 100 PFU/0.2 ml of HSV-1, HSV-2, poliovirus type 1 or measles virus. After 1 h incubation for virus adsorption, the overlaid medium containing 12.5–100 μg/ml of oxyresveratrol was added. The infected cultures were incubated at 37 °C for 2 days for HSV and 3 and 5 days for poliovirus and measles virus, respectively. The infected cells were fixed, stained and the number of plaques was counted and the IC_{50} was determined as above.

Cytotoxicity was evaluated by the MTT reduction assay. Vero cells were seeded at a concentration of 2×10^5 cells/ml in 96-well tissue culture plates and grown at 37 °C for 1 day. The culture medium was replaced by fresh medium containing oxyresveratrol at various concentrations, 6.25–800 μg/ml and cells were further grown for 2 days. After incubation the media was replaced with 50 μl of a 1 mg/ml solution of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Co.) in media. Cells were incubated at 37 °C for 3 h, the untransformed MTT was removed and 50 μl of acid–isopropanol (0.04N HCl in isopropanol) was added to each well. After a few minutes at room temperature for dissolving the crystals, the plates were read on a microplate reader using a test wavelength of 570 nm and a reference wavelength of 620 nm. The concentration of oxyresveratrol reducing cell viability by 50% (CC_{50}) was determined.

2.5. Required treatment period for inhibition of plaque formation by oxyresveratrol

To determine the effect of treatment period for inhibition of plaque formation by oxyresveratrol, Vero cells were seeded at a concentration of 6×10^5 cells/ml in 24-well tissue culture plates and grown at 37 °C for 1 day. The cells were infected with HSV-1 (KOS) or HSV-2 (Baylor186), 50 PFU/0.5 ml/well. After 1 h of viral

adsorption, the cells were overlaid with 0.8% nutrient methylcellulose containing 12.5–50 μg/ml of oxyresveratrol. At each time of incubation of 3, 6, 24 and 48 h at 37 °C, the overlaid supplemented with compound was removed. The cells were washed and replaced with the drug-free medium and incubated thereafter up to 48 h, respectively. The percent plaque inhibition for each treatment was then determined.

2.6. Effect of oxyresveratrol treatment on the virus yield in the one-step growth assay

The anti-HSV activity of oxyresveratrol was examined by the virus yield assay. Vero cells were seeded at a concentration of 6×10^5 cells/ml in 25 cm^2 flasks and grown at 37 °C for 1 day. The cells were infected with HSV-1 (KOS) or HSV-2 (Baylor186) at a multiplicity of infection (MOI) of 1 PFU/cell for 1 h.

The experiment was designed to determine the efficacy of drug treatment after virus infection. The cells were infected as described above. After 1 h of viral adsorption, the cells were washed three times with compound-free medium and were treated with 50 μg/ml of oxyresveratrol in MEM supplemented with 5% FBS and incubated at 37 °C for 3 and 9 h after 1 h of viral adsorption and the cells were treated with oxyresveratrol for 6 h after 3 h of incubation in compound-free medium (indicated as 0–3 h, 0–9 h and 3–9 h in Fig. 3). At the end of 9 h after viral adsorption period, the compound-containing medium was removed. The cells were washed with compound-free medium three times and fed with compound-free medium. Then the cells were frozen and thawed for three cycles and cell debris was removed by centrifugation. The supernatants were titrated for the virus yield by the plaque formation assay. The infected cells incubated in medium were used as virus control (indicated as untreated in Fig. 3). Three separate independent experiments were performed.

The cells were also treated with oxyresveratrol 1 h prior to viral infection. Then the oxyresveratrol was removed and the cells were washed three times with compound-free medium. The cells were infected as described above. After 1 h of viral adsorption, the inoculums were removed and the cells were washed by compound-free medium three times. Then the compound at 50 μg/ml was added for 9 h (indicated as 1 h prior/0–9 h in Fig. 3). Also, cells were incubated in compound-free medium after viral adsorption period (indicated as 1 h prior in Fig. 3). At the end of 9 h after viral adsorption period, the cells were frozen and thawed, cell debris was removed, and the supernatants were titrated for the virus yield as above.

2.7. Inactivation of viral infectivity by oxyresveratrol

The various concentrations of oxyresveratrol, 25–100 μg/ml (final concentrations in the mixture), were incubated with HSV-1 (KOS) or HSV-2 (Baylor186) 10^5 PFU/ml for 1 h at 37 °C. The mixture of each concentration with virus was 10-fold serially diluted in complete media. Then the residual viruses were titrated in the monolayer of Vero cells by the plaque formation assay.

2.8. Effect of oxyresveratrol on viral protein synthesis

Oxyresveratrol was examined for its effect on viral protein synthesis in Vero cells infected with HSV-1 (7401H) strain. The cells were mock-infected or infected with HSV-1 and incubated in the absence (untreated infected cells) or presence (treated infected cells) of 30 and 50 μg/ml of oxyresveratrol or 200 μg/ml of PAA, at 37 °C. HSV-1-infected and mock-infected cells were labeled with 100 μCi/ml of [^{35}S]-methionine and [^{35}S]-cysteine (37 T Bq/mmol, GE Healthcare Bio-Science Corp., Piscataway, NJ) for 1–11 h after infection in the absence or presence of oxyresveratrol or PAA as described above. The labeled cells were lysed by sonication in RIPA buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) and centrifuged at 35,000 rpm for 30 min at 4 °C. The labeled viral proteins were immunoprecipitated with immunoglobulin for human use containing anti-HSV antibody (Rockland, Gilbertsville, PA). Immune complexes were analyzed by SDS-PAGE using 6% acrylamide gel followed by fluorography (Kurokawa et al., 1998; Phromjai et al., 2007). To quantitate the viral proteins in the treated cells, immunoprecipitation was performed in antibody excess condition and the amounts of viral proteins were assessed by densitometry.

2.9. Combined effect of oxyresveratrol with acyclovir on plaque formation

In order to analyze the combined effect of oxyresveratrol and acyclovir on plaque formation graphically, the IC_{50} of these agents in their various concentrations were plotted as an isobologram (Kurokawa et al., 2001; Suzuki et al., 2006). Synergy and antagonism are defined as deviations from an additive effect, which results when two drugs interact as if they were the same drug. Curves falling below the line of additivity indicate synergy, curves on the line indicate an additive reaction and curves above the line indicate an antagonistic reaction.

The combined effect of oxyresveratrol with ACV was examined for anti-HSV-1 activity in the plaque reduction assay. Duplicate cultures of Vero cells in 60-mm plastic dishes were infected with 100 PFU/0.2 ml of wild-type HSV-1 (7401H) for 1 h. The cells were overlaid with 5 ml of nutrient agarose (1%) medium containing various concentrations of oxyresveratrol and/or ACV, and then cultured at 37 °C for 5 days. The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution and then the number of plaques was counted under a dissecting microscope. The inhibitory concentrations for 50% plaque reduction (IC_{50}) were determined from a curve relating the plaque formation (%) of untreated culture to the concentration of drugs. The combined action of oxyresveratrol with ACV was evaluated by constructing an isobologram (Biron and Elion, 1980; Fraser-Smith et al., 1985; Kurokawa et al., 1995; Suzuki et al., 2006).

2.10. Therapeutic efficacy of oral and topical oxyresveratrol in cutaneous HSV-1 infection in mice

Female BALB/c mice (6 weeks old) were purchased from Sankyo Labo Service Co. Ltd., Tokyo, Japan. The right midflank of each mouse was clipped and depilated with a chemical depilatory, hair remover. Two or three days later, the naked skin was scratched using 27-guage needles and 5 μl of HSV-1 (7401H) suspension of 1×10^6 PFU was applied to the scarified area (Kurokawa et al., 1993; Lipipun et al., 2003). Oxyresveratrol was administered to mice (10 mice per group) as follows: Experiment 1 (oral treatment), oxyresveratrol (500 mg/kg/dose) or ACV (5 mg/kg/dose), dissolved in 2% DMSO in distilled water, was orally administered by using a gavage at 8 h before and three times daily for 7 days after HSV-1 infection. For control group, 2% DMSO in distilled water was used. Experiment 2 (topical treatment, the effect of dose), oxyresveratrol ointment prepared by grinding oxyresveratrol powder in petroleum jelly (Vaseline™) base at 15% or 30% was applied topically on the scratched area (5 mg/cm^2/dose) 1 h after HSV-1 infection and five times daily for 7 days. Experiment 3 (topical treatment, the effect of time interval), 30% oxyresveratrol ointment were applied topically on the scratched area (5 mg/cm^2/dose) 1 h after HSV-1 infection and twice, three, four or five times daily for 7 days in each group.

Experiment 4 (topical treatment of oxyresveratrol or ACV), 30% oxyresveratrol ointment or 5% ACV cream was applied topically on the scratched area (5 mg/cm^2/dose) 1 h before and five times daily for 7 days after HSV-1 infection. For control group, petroleum jelly base alone was used in topical treatment. In all experiments, the development of skin lesions and mortality were continuously observed three times daily in oral treatment and five times daily in topical treatment for 7–10 days after HSV-1 infection and scored as follows: 0, no lesion; 2, vesicles in local region; 4, erosion and/or ulceration in local region; 6, mild zosteriform lesion; 8, moderate zosteriform lesion; and 10, severe zosteriform lesion and death.

The toxicity of oxyresveratrol was assessed in the healthy mice by observing the loss of body weight compared with the control group (2% DMSO in sterile water). The mice were orally administered with oxyresveratrol 125 mg/kg/dose or 500 mg/kg/dose (dissolved in 2% DMSO in distilled water) three times daily for 7 days. The weight of mice was determined on day 0 and day 7. We conducted procedures in conforming to the National Institute of Health Guide for the Care and Use of Laboratory Animals with the approval of the Animal Care Committee at University of Toyama.

2.11. Statistical analysis

The repeated measure ANOVA was used to analyze the difference between oxyresveratrol-treated mice and control mice in mean skin lesions for 7–10 days after infection. The Student's *t*-test (one tailed test) was used to evaluate the significance of the differences in the mean weight between control and oxyresveratrol-treated mice. A *P*-value of less than 0.05 was defined as statistically significant.

3. Results

3.1. Effect of oxyresveratrol against wild-type and drug-resistant HSV-1 by plaque reduction assay

The antiviral activities of oxyresveratrol against wild-type HSV-1 (7401H), TK-deficient and PAA-resistant HSV-1 were examined by plaque reduction assays. As shown in Table 1, the IC_{50} of oxyresveratrol for wild-type HSV-1 (7401H), clinical isolate 1, clinical isolate 2, clinical isolate 3, TK-deficient strain and PAA-resistant strain were 19.8, 23.3, 23.5, 24.8, 25.5 and 21.7 μg/ml, respectively. There were no significant differences among the IC_{50} ($P > 0.05$) of various strains of HSV-1. ACV-resistant HSV-1 strains were similarly susceptible to oxyresveratrol, indicating that the mode of anti-HSV action of oxyresveratrol was different from that of ACV.

Antiviral activities of oxyresveratrol were determined by the plaque reduction assay against HSV-1 (KOS), HSV-1 (7401H), HSV-2 (Baylor186), poliovirus type 1 (Sabin strain) and measles virus (Tanabe strain). The compound exhibited the similar degree of inhibition against HSV-1 and HSV-2. No cytotoxic effect of oxyresveratrol was observed at the concentration tested (50 μg/ml). The CC_{50} was 237.5 μg/ml determined by the MTT reduction assay and the selective index (CC_{50}/IC_{50}) of 9.9–12.7 was indicated for HSV. The CC_{50} was also determined by the trypan blue exclusion method and it was correlated with the MTT reduction assay (data not shown). The antiviral activities of oxyresveratrol against poliovirus and measles virus were evaluated, and IC_{50} values were 65 and 90 μg/ml, respectively.

3.2. Required treatment period for inhibition of plaque formation by oxyresveratrol

The period of time for incubation of the infected cells with oxyresveratrol did affect the inhibitory activity of the compound.

Table 1
Antiviral activity determined by the plaque reduction assay

HSV-1 strains	IC_{50}[a] (μg/ml)		
	Acyclovir	Phosphonoacetic acid	Oxyresveratrol
A.			
Wild-type (7401H)	0.37 ± 0.01	16.0 ± 0.96	19.8 ± 3.3
Clinical isolate 1	0.16 ± 0.006	12.1 ± 0.64	23.3 ± 0.9
Clinical isolate 2	0.17 ± 0.008	12.6 ± 1.1	23.5 ± 1.2
Clinical isolate 3	0.09 ± 0.004	7.1 ± 0.49	24.8 ± 1.9
TK-deficient	>30	28.6 ± 2.0	25.5 ± 1.5
PAA-resistant	2.7 ± 0.52	112.6 ± 22.9	21.7 ± 2.6

Viruses	IC_{50} (μg/ml) oxyresveratrol	SI (CC_{50}/IC_{50})[b]
B.		
HSV-1 (KOS)	24.0 ± 0.4	9.9
HSV-1 (7401H)	19.8 ± 3.3	11.9
HSV-2 (Baylor186)	18.7 ± 0.9	12.7
Poliovirus type 1 (Sabin)	65.0 ± 2.9	3.7
Measles virus (Tanabe)	90.0 ± 3.2	2.6

(A) Susceptibility of HSV-1 (7401H), clinical HSV-1 isolates, TK-deficient, or PAA-resistant strains to ACV, PAA and oxyresveratrol. (B) Antiviral activity of oxyresveratrol against various viruses.

[a] The IC_{50} (50% inhibitory concentration) was expressed as the mean (μg/ml) ± S.E. of three to six independent experiments.

[b] SI = selectivity index, determined by the ratio of CC_{50} to IC_{50}, IC_{50} = 50% inhibitory concentration, mean ± S.E., determined by three independent experiments, CC_{50}, 50% cytotoxic concentration, mean ± S.E., determined by MTT reduction assay, CC_{50} = 237.5 ± 21.7 μg/ml.

Complete inhibitory activities against HSV-1 and HSV-2 were observed for the cells maintained in oxyresveratrol at 50 μg/ml for 24 and 48 h after infection. Viral inhibitions of 26.1% and 32.8% were indicated when the infected cells were maintained in the media with 50 μg/ml of oxyresveratrol for 3 and 6 h after HSV-2 infection, respectively and no inhibition of plaque formation was observed in HSV-1 infection under these conditions (Fig. 2).

3.3. Effect of oxyresveratrol treatment on the virus yield in the one-step growth assay

To evaluate the effect of oxyresveratrol on viral replication, the compound at 50 μg/ml was added for the various periods of time after 1 h of viral adsorption, and infected cells were harvested at 9 h after the viral adsorption period. The infected cells were treated with oxyresveratrol for 3 and 9 h after 1 h of viral adsorption and were treated with oxyresveratrol for 6 h after 3 h of incubation in compound-free medium. This result indicated that oxyresveratrol affected both the early stage (1 h) and the late stage (3 h) after viral adsorption period of HSV infection (indicated as 0–3 h, 0–9 h and 3–9 h in post-treatment, respectively in Fig. 3A).

The efficacy of compound treatment prior to viral infection was also examined. The cells were treated with 50 μg/ml oxyresveratrol 1 h prior to viral infection. After 1 h of viral adsorption, the inoculums were removed and the cells were washed by compound-free medium and incubated in compound-free medium or medium containing 50 μg/ml of oxyresveratrol for 9 h (indicated as 1 h prior and 1 h prior/0–9 h in pretreatment, respectively in Fig. 3B).

3.4. Inactivation of viral infectivity by oxyresveratrol

The result indicated that oxyresveratrol at concentration 25–100 μg/ml showed no inactivation effect on viral infectivity as indicated by the titration of the residual viral infectivity of HSV-1 (KOS) ($4.5–6.1 \times 10^4$ PFU/ml) and HSV-2 ($6.5–9.5 \times 10^4$ PFU/ml) determined in three independent experiments in Vero cells. There were no significant differences ($P > 0.05$) in the virus titer observed at various concentrations of oxyresveratrol compared with the

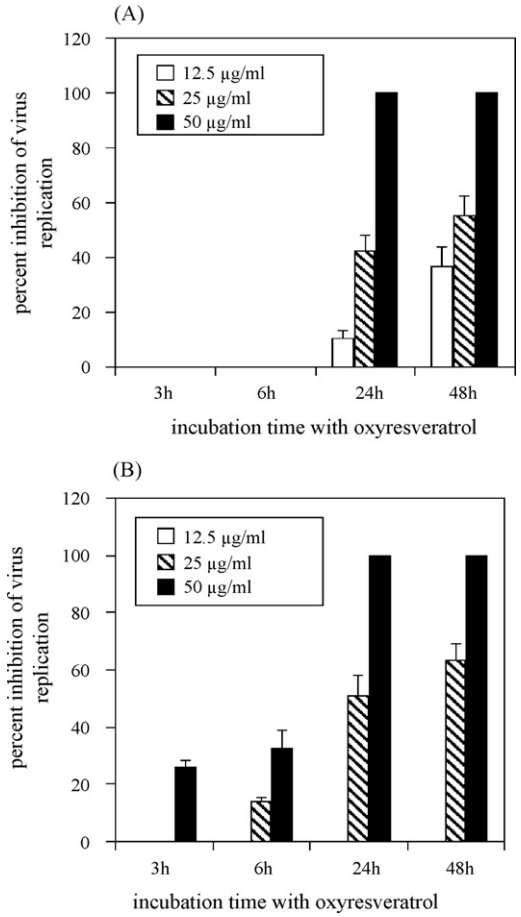

Fig. 2. Effect of treatment period for inhibition of plaque formation by oxyresveratrol: (A) anti-HSV-1 activity and (B) anti-HSV-2 activity, cells were incubated in overlaid medium containing 12.5–50 μg/ml of oxyresveratrol after 1 h of viral adsorption. At each time of incubation of 3, 6, 24 and 48 h at 37 °C, the overlaid medium supplemented with compound was removed. The cells were washed and replaced with the drug-free overlaid medium and incubated thereafter for up to 48 h, respectively. The data represented the average of plaque formation with the standard deviation from three independent experiments.

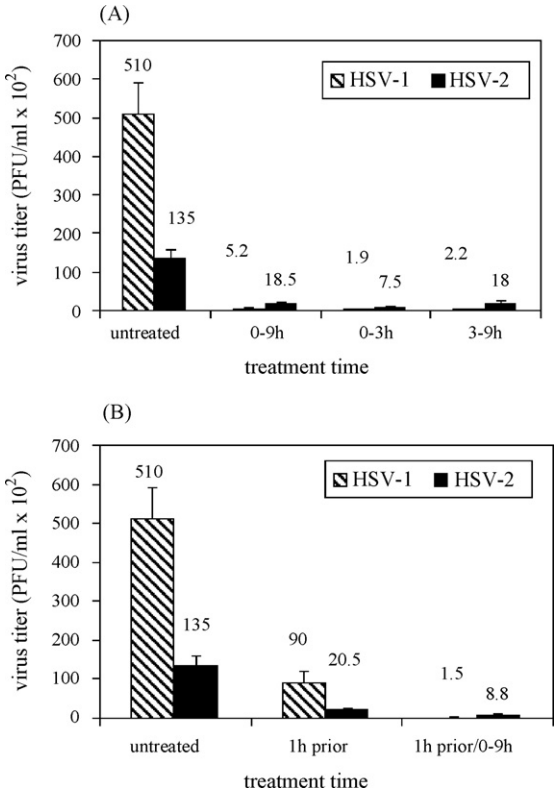

Fig. 3. Effect of oxyresveratrol treatment on virus yield: (A) Post-treatment; cells were incubated with the media containing oxyresveratrol for 9 h and 3 h after viral adsorption period (0–9 h, 0–3 h), cells were also incubated with compound-free medium for 3 h after viral adsorption period and then the medium containing oxyresveratrol for 6 h (3–9 h). (B) Pretreatment; cells were pretreated with oxyresveratrol for 1 h before viral adsorption period and were incubated with compound-free medium for 9 h (1 h prior), cells were also pretreated with oxyresveratrol for 1 h before viral adsorption period and were incubated with medium containing oxyresveratrol for 9 h (1 h prior/0–9 h). Infected cells incubated with compound-free medium (untreated) were used as control. The data represented the average of the virus yield with the standard deviation from three independent experiments.

titer of untreated HSV-1 (5.6×10^4 PFU/ml) and untreated HSV-2 (7.6×10^4 PFU/ml), respectively.

3.5. Effect of oxyresveratrol on viral protein synthesis

The synthesis of HSV proteins was examined in the presence of oxyresveratrol or PAA to compare the effects of oxyresveratrol and PAA on protein synthesis. HSV-1 proteins were labeled with [^{35}S]-methionine and [^{35}S]-cysteine for 1–11 h after infection. HSV proteins of 170, 150, 135, 130, 65, and 60 kDa were judged to be ICP4, ICP6, ICP8, gC, ICP27 and gD, respectively, by their molecular weights and PAA sensitivity (Honess and Roizman, 1973, 1974; Honess and Watson, 1977). The amounts of early gene products such as ICP6 and ICP8 were similar while the amounts of the late gene products such as gC and gD were decreased in the virus-infected cells treated with 30 μg/ml of oxyresveratrol or PAA as compared with untreated infected cells (Fig. 4B and C, lane 5).

3.6. Synergism of oxyresveratrol with acyclovir in anti-HSV activity

Combination of ACV with oxyresveratrol was investigated for their anti-HSV activity. The CC_{50} of oxyresveratrol was 237.5 μg/ml in Vero cells and the concentrations used in this assay for both drugs were not cytotoxic. The combined effect of oxyresveratrol with ACV on plaque formation of HSV-1 in Vero cells was analyzed by isobologram and the percent plaque formation of each combination was expressed as the representative data of three independent experiments. The data are shown in Fig. 5. The curve fell below the line of the additive effect at all concentrations indicating that the combination of acyclovir and oxyresveratrol exhibited synergism against HSV-1. The IC_{50} for ACV and oxyresveratrol, when combined with ACV, could be reduced by two- to fourfold compare to the IC_{50} when the drugs were used alone.

3.7. Therapeutic efficacy of oral and topical oxyresveratrol on cutaneous HSV-1 infection in mice

Oxyresveratrol was orally administered to mice by using a gavage at 8 h before and three times daily for 7 days after viral infection. Oxyresveratrol orally administered at the dose of 125 mg/kg/dose exhibited significant delays in the lesion development ($P < 0.05$) compared with control (data not shown). Oxyresveratrol at a 500 mg/kg/dose delayed the development and progression of skin lesions during days 5–8 after infection ($P = 0.04$) as compared with control, 2% DMSO in distilled water treatment (Fig. 6A). The survival time and percent mortality observed in oxyresveratrol-treated

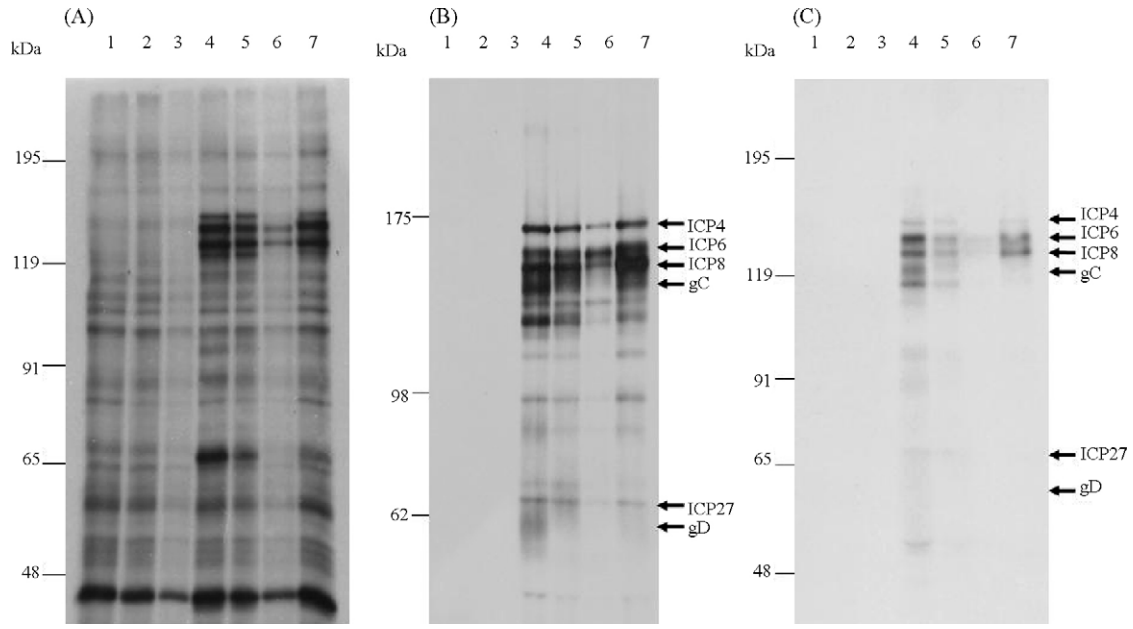

Fig. 4. Inhibition of protein synthesis of HSV-1 wild-type strain by oxyresveratrol. Oxyresveratrol was examined for its effect on viral protein synthesis in Vero cells infected with HSV-1: (A) cell lysates, (B) qualitative and (C) quantitative: immunoprecipitates, cells were mock-infected (lanes 1–3) or infected with HSV-1 (lanes 4–7); lane 1, untreated uninfected cells; lane 2, treated cells with 30 μg/ml, lane 3, 50 μg/ml of oxyresveratrol, lane 4, untreated infected cells, lane 5, treated infected cells with 30 μg/ml, lane 6, 50 μg/ml of oxyresveratrol, lane 7, 200 μg/ml of PAA. The molecular weights and HSV proteins were indicated in the left and right margins, respectively.

mice were not significantly different ($P > 0.05$) compared with the control group. The infected mice receiving ACV (5 mg/kg/dose) as positive control showed significant retardation ($P < 0.001$) in the development of lesion score and time survival and all the mice survived. The percent death of mice observed at day 8 after infection were 100, 75 and 0% for the untreated control, oxyresveratrol-treated and ACV-treated group, respectively. To evaluate the toxicity of oxyresveratrol, the mean of body weight of mice in each group was statistically analyzed. The mean weights of mice administered at the 125 mg/kg/dose and 500 mg/kg/dose of oxyresveratrol three times daily for 7 days were not significantly different compared with the control mice on day 0 and day 7, showing no toxicity of the compound ($P > 0.05$).

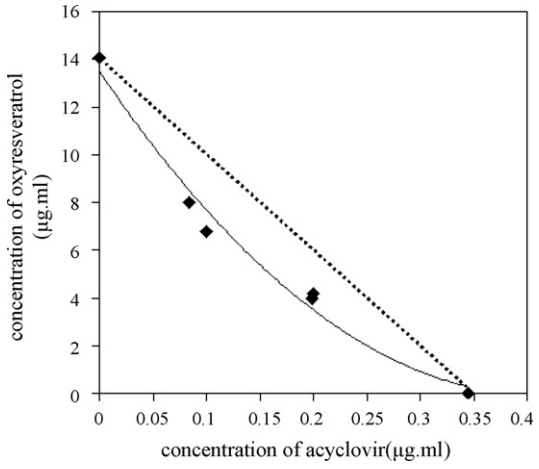

Fig. 5. Combination of oxyresveratrol and acyclovir on wild-type HSV-1 plaque formation analyzed by isobologram. The figure was one of the representative data from three independent experiments using HSV-1. The dotted lines indicated the theoretical additive activity. The measured line (■) was significantly lower than the dotted line ($P < 0.001$) by the paired *t*-test, indicating synergy.

Comparison of oxyresveratrol efficacy between oral administration and topical treatment was concomitantly investigated. To evaluate antiviral efficacy of oxyresveratrol for cutaneous treatment, oxyresveratrol ointment was prepared by grinding oxyresveratrol powder in petroleum jelly (Vaseline™) base. Oxyresveratrol ointment at 15% or 30% applied five times a day for 7 days were compared with control groups including no treatment or vaseline base-treatment. The results showed that lesion scores of the no treatment group and the vaseline base-treatment group were not significantly different ($P = 0.70$). Oxyresveratrol ointment at 15% and 30% showed significant antiviral activity compared with the no treatment control groups ($P = 0.03$ and <0.0001, respectively) and also exhibited significant antiviral activity compared with the vaseline base-treatment groups ($P = 0.01$ and <0.0001, respectively) (Fig. 6B). Oxyresveratrol, 15% ointment showed less therapeutic efficacy than that of 30% concentration with significant difference ($P < 0.0001$). The percent death of mice observed at day 10 after infection were 100, 89, 89 and 44% for the untreated control, vaseline base-treated, 15% oxyresveratrol-treated, and 30% oxyresveratrol-treated group, respectively. The petroleum jelly base used to prepare oxyresveratrol ointment did not possess antiviral activity.

To determine time interval or optimal frequency of oxyresveratrol ointment treatment, 30% oxyresveratrol ointment was applied twice, three, four or five times a day for 7 days. The results showed that oxyresveratrol ointment acquired frequency-dependent efficacy, and four and five applications a day provided the best therapeutic effect with significant difference compared with no treatment control ($P < 0.0001$) (Fig. 6C). Three times a day of application indicated significant therapeutic efficacy ($P = 0.004$) but twice a day of application showed no significant efficacy ($P = 0.10$) compared with the control group. The percent death of mice observed at day 10 after infection were 100, 44, 25, 89 and 100% for the untreated control, five, four, three and twice oxyresveratrol-treatment groups, respectively. In topical treatment with the application of 1 h before and five times daily after viral infection, 30% oxyresveratrol ointment showed no signifi-

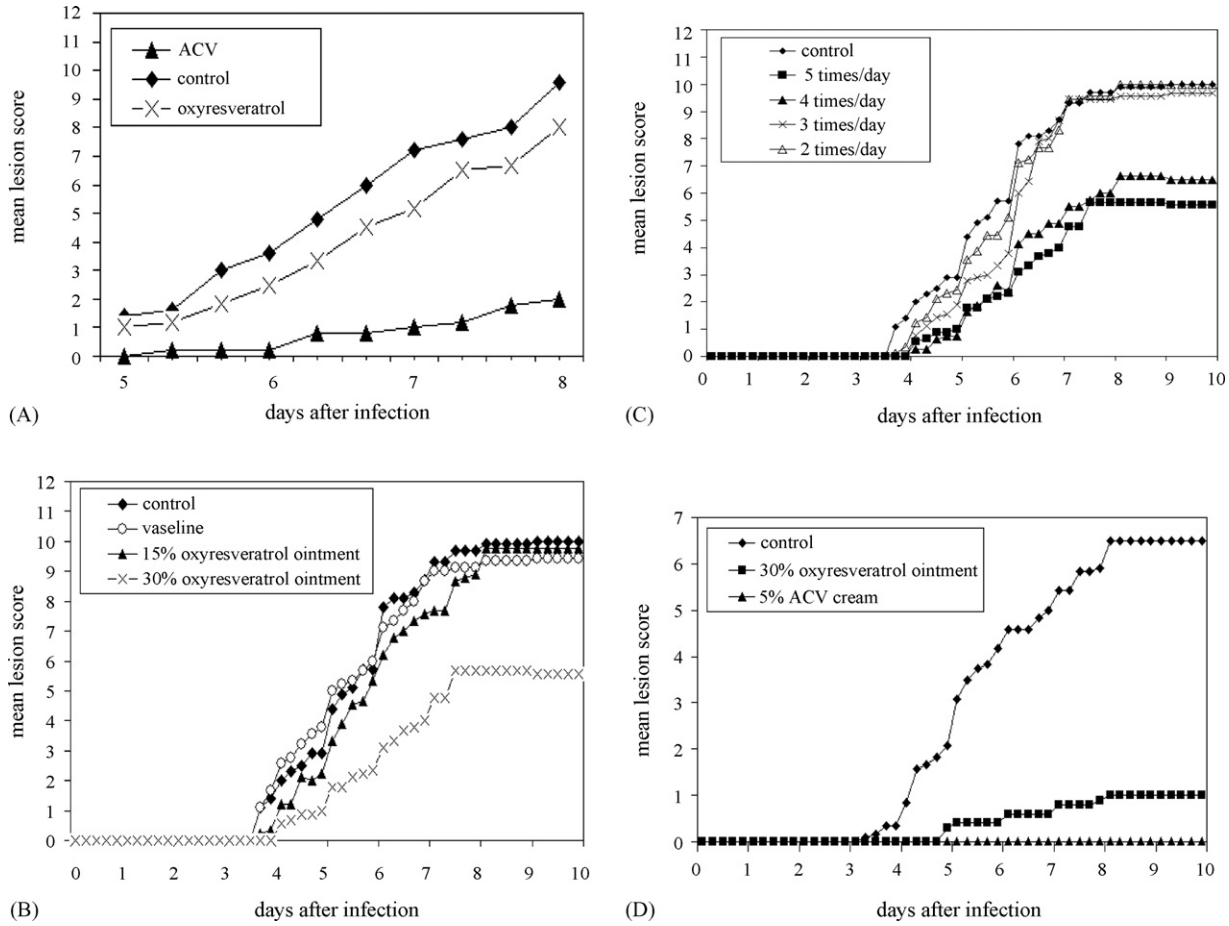

Fig. 6. Effect of administration of oxyresveratrol on mice cutaneously infected with HSV-1. (A) Oral administration. (Oxyresveratrol (500 mg/kg) or ACV (5 mg/kg) was orally administered at 8 h before and three times daily for 7 days after viral infection, 2% DMSO in distilled water was used as control. (Oxyresveratrol and ACV were significantly effective ($P = 0.04$ and <0.001, respectively) compared with control, by the repeated measure ANOVA). (B) Effect of dose. 15% or 30% oxyresveratrol ointment was topically applied 1 h after HSV-1 infection and five times daily for 7 days, mice without treatment or vaseline base alone was used as control (15% and 30% oxyresveratrol ointment were significantly effective ($P = 0.03$ and <0.0001) compared with mice without treatment control, by the repeated measure ANOVA). (C) Effect of time of application. 30% oxyresveratrol ointment was topically applied 1 h after HSV-1 infection and twice, three, four or five times daily for 7 days, mice without treatment was used as control (five times daily, four times daily and three times daily of treatment were significantly effective compared with control; $P < 0.0001$, <0.0001 and <0.004, respectively, by the repeated measure ANOVA). (D) Effect of pretreatment. 30% oxyresveratrol ointment or 5% ACV cream was topically applied 1 h before and five times daily for 7 days after viral infection, mice without treatment was used as control (30% oxyresveratrol ointment and 5% ACV cream were significantly effective ($P < 0.0001$) compared with control, by the repeated measure ANOVA).

cantly different efficacy ($P = 0.62$) compared with 5% ACV cream (Fig. 6D).

The application of vaseline base alone (no drug) five times a day with the pretreatment dose (1 h before infection) did not provide a significant difference from the untreated control group ($P = 0.31$) (data was not shown). The percent death of mice observed at day 10 after infection were 58, 10 and 0% for the untreated control group, oxyresveratrol-treated group and ACV-treated group, respectively. Mortality rate of oxyresveratrol topical-treated (four to five times daily for 7 days) group (25–44%) was lower than that of orally administered (1 h before and three times daily after viral infection for 7 days) group (75%). Overall, the results indicated that topical administration of oxyresveratrol is suitable as an anti-HSV therapeutic route for cutaneous HSV infection in mice.

4. Discussion

In this study the anti-HSV activity of oxyresveratrol purified from *A. lakoocha* was evaluated. Oxyresveratrol was shown to exhibit anti-HSV activity in the plaque reduction assay against TK-deficient (ACV-resistant) and PAA-resistant HSV-1 strains as well as wild-type HSV-1 *in vitro*. This suggested that its antiviral activity may involve a mode of action different from that of ACV or PAA. Oxyresveratrol had efficacy on ACV-resistant mutant strains and might be useful for use against ACV-resistant mutant virus strains.

The result demonstrated that plaque formation of HSV-1 and HSV-2 in the presence of oxyresveratrol at a noncytotoxic concentration (50 μg/ml) was reduced by 100%. Oxyresveratrol showed anti-poliovirus activity and anti-measles virus activity with higher IC_{50} in the plaque reduction assay as compared to its anti-HSV activity. Since these viruses have different structure and different modes of replication, their differences in sensitivity to the compound may be due to the different target(s) of antiviral action. Oxyresveratrol and resveratrol differ by only one hydroxyl group; therefore the observed inhibitory effect of oxyresveratrol and resveratrol (Docherty et al., 1999, 2004) on HSV replication may be mediated by a similar mechanism.

The effect of treatment period for the antiviral activity of oxyresveratrol was studied and time-dependent activity of the compound on HSV growth inhibition was demonstrated. A complete inhibitory effect was observed after 24–48 h incubation of the infected cells in the medium containing oxyresveratrol at 50 μg/ml (Fig. 2). The decrease in inhibitory effect was observed when the infected cells were incubated in medium containing oxyresvera-

trol at 12.5–25 μg/ml and/or incubated for 3 or 6 h. The reduction in virus yield did not seem to be caused by a direct inactivation of HSV by oxyresveratrol. Oxyresveratrol effectively reduced the virus yield when it was added for 3 and 9 h after 1 h of viral adsorption and for 6 h after 3 h of incubation of infected cells in compound-free medium. The compound was also effective in reducing virus yield when the cells were treated with the compound 1 h prior to viral infection and incubated further either in compound-free medium or compound-supplemented medium. However, the compound did show less efficacy in the cells pretreated with the compound 1 h before viral adsorption and further incubated with compound-free medium. We have shown the inhibitory activity of oxyresveratrol in replication of HSV-1 and HSV-2. It exhibited inhibitory activity with 1-h treatment prior to viral infection and further treatment in the early phase and late phase of viral replication. Anti-HSV activity was specific without cellular toxicity.

The mechanism of its anti-HSV action in inhibition of protein synthesis was analyzed by immunoprecipitation and SDS-PAGE, and the data indicated a similar pattern of inhibition in late protein synthesis compared with PAA. Synthesis of late protein was decreased in the presence of 30 μg/ml of oxyresveratrol and of 200 μg/ml of PAA (Fig. 4).

In an isobologram analyzing the combined effect of oxyresveratrol with ACV in Vero cells (Fig. 5), oxyresveratrol enhanced the anti-HSV-1 activity of ACV synergistically. The IC_{50} for ACV and oxyresveratrol, when combined with ACV, could be reduced by two- to fourfold compared to the IC_{50} when the drugs were used alone. Thus, the mode of anti-HSV-1 action of oxyresveratrol was different from that of ACV. ACV is a nucleoside analog that exhibits anti-herpetic activity after phosphorylation by viral TK. Acyclovir triphosphate then interferes with viral DNA polymerization through competitive inhibition with guanosine triphosphate and obligatory DNA chain termination (De Clercq, 2001, 2004). It was shown that oxyresveratrol affected the viral replication by inhibiting the late viral protein synthesis, while acyclovir did so, at a different site, by inhibiting viral DNA synthesis. The combined application of oxyresveratrol with ACV had synergistic activity and should delay the development of drug (ACV) resistance. If simultaneous application of antiviral compounds results in smaller doses being used, any cytotoxic effects of the drugs may also be reduced. The combination of ACV with other drugs should be more effective than either compound alone, if the second drug has a different mechanism of activity.

In the mouse cutaneous infection model, the dose of oxyresveratrol orally administered at 8 h before and three times daily after viral infection at 500 mg/kg significantly retarded the development of skin lesions ($P = 0.04$). The survival time and mortality rate of oxyresveratrol-treated mice were not significantly different from 2% DMSO-treated control mice. As oral administration of oxyresveratrol at 125 mg/kg/dose exhibited significant delay in the development of skin lesions versus control ($P < 0.05$) (data not shown), this indicated that there was no difference in the efficacy of treatment between the 125 and 500 mg/kg/dose. The doses of oxyresveratrol used in HSV-1-infected mice were not toxic. The effectiveness of oxyresveratrol in vivo may be influenced by the number of administration and the limitation of active metabolites after oral absorption, the subsequent distribution and the rate of excretion. The phenolic compounds, as reported for resveratrol have generally been assumed to be rapidly excreted in the form of glucuronide conjugate in vivo and to be pharmacologically inactive (Kuhnle et al., 2000). However, resveratrol glucuronides could be converted back locally or systematically in vivo to resveratrol by existing human beta-glucuronidase (Wang et al., 2004).

The results from topical treatment in mice showed that efficacy of oxyresveratrol was dose- and frequency-dependent. Oxyresveratrol ointment at 30% applied four to five times a day was the most significantly effective in delaying the skin lesion development and in protection against death. The schedule of administration of 30% oxyresveratrol applied two times a day was not significantly effective ($P = 0.10$, Fig. 6C), while this schedule of application with treatment prior to infection showed a significant difference ($P < 0.0001$) compared with the control group (data not shown). The schedule of treatment of 30% oxyresveratrol with 1 h before and five times daily after infection showed similar efficacy with no significant difference ($P = 0.62$, Fig. 6D) compared to 5% ACV cream. Oxyresveratrol strongly inhibited HSV replication by inhibition of late viral protein synthesis at a concentration of 30 μg/ml and the inhibition of late viral protein synthesis was similar to that of PAA. Ooxyresveratrol showed an antiviral effect with 1 h pretreatment of the cells. This may be due to interference of oxyresveratrol with virus adsorption. These anti-HSV activities were also related to the greater efficacy with pretreatment of oxyresveratrol (prior to viral infection) in cutaneous HSV-1 infection in mice. Resveratrol, the prototype of this class of compounds, has been reported as inhibitory towards HSV in topical treatment in animal experiments (Docherty et al., 1999, 2004). From our data oxyresveratrol by cutaneous application appears to be more effective than oral administration. Oxyresveratrol may be appropriate for topical HSV-1 treatment.

In conclusion, oxyresveratrol was shown to be effective against both wild-type and ACV-resistant HSV strains. It exhibited the inhibitory activity at the early phase and late phase of replication of HSV-1 (KOS) and HSV-2. It demonstrated synergistic activity with acyclovir and inhibited late protein synthesis. A strong anti-HSV activity following pretreatment was observed. The results provided evidence that oxyresveratrol showed better therapeutic efficacy by topical treatment than oral treatment in cutaneous HSV infection in mice. Therefore, oxyresveratrol was verified to be a promising anti-HSV drug candidate for the topical treatment of HSV infections. Further investigations will focus on the development of topical formulations with, hopefully, higher effectiveness of oxyresveratrol as an anti-HSV agent.

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