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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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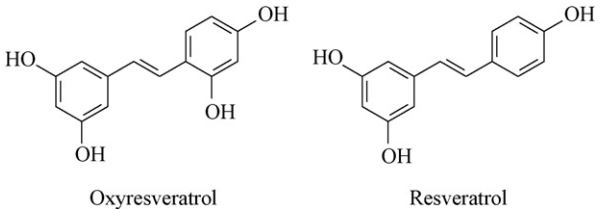

Fig. 1. Structure of oxyresveratrol (*trans*-2,3′,4,5′-tetrahydroxystilbene) as compared to resveratrol (*trans*-3,5,4′-trihydroxystilbene).

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