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The antiviral activity of arctigenin in traditional Chinese medicine on *porcine circovirus* type 2

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ABSTRACT

Arctigenin (ACT) is a phenylpropanoid dibenzylbutyrolactone lignan extracted from the traditional herb *Arctium lappa* L. (Compositae) with anti-viral and anti-inflammatory effects. Here, we investigated the antiviral activity of ACT found in traditional Chinese medicine on *porcine circovirus* type 2 (PCV2) in vitro and in vivo. Results showed that dosing of 15.6–62.5 μg/mL ACT could significantly inhibit the PCV2 proliferation in PK-15 cells ($P < 0.01$). Dosing of 62.5 μg/mL ACT 0, 4 or 8 h after challenge inoculation significantly inhibited the proliferation of 1 MOI and 10 MOI in PK-15 cells ($P < 0.01$), and the inhibitory effect of ACT dosing 4 h or 8 h post-inoculation was greater than 0 h after dosing ($P < 0.01$). In vivo test with mice challenge against PCV2 infection demonstrated that intraperitoneal injection of 200 μg/kg ACT significantly inhibited PCV2 proliferation in the lungs, spleens and inguinal lymph nodes, with an effect similar to ribavirin, demonstrating the effectiveness of ACT as an antiviral agent against PCV2 in vitro and in vivo. This compound, therefore, may have the potential to serve as a drug for protection of pigs against the infection of PCV2.

1. Introduction

Porcine circovirus (PCV) is a single-stranded circular DNA virus (Family Circoviridae, Genus *Circovirus*) that has attracted great attention in recent years due to the causes of severe economic losses to the pig industry. According to the antigenicity and genetic composition, the PCV can be divided into two serotypes, i.e., PCV1 and PCV2. PCV1, first discovered in 1974 by Tischer et al. (1987), is a non-pathogenic virus, whereas PCV2 identified in 1997 has been known to cause various animal diseases such as postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), congenital tremors (CT) and reproductive disorders. In China, the PCV2 infection rate varies from 20%–85% (Ge et al., 2012). While swine can be infected by PCV2 at all ages, in general, 5–12 week old piglets are the most susceptible to infection. PCV2 is excreted through feces, nasal fluid, and semen of infected swine. Vertical transmission of PCV2 can occur between infected sows and piglets through the placental barrier. In addition to causing serious symptoms, porcine infection with PCV2 can also lead to secondary infection by other bacteria or viruses (Ge et al., 2012). Upon infection, PCV2 inhibits the delivery of viral antigens to T lymphocytes through an overall reduction in dendritic cell number which also causes a substantial decrease in the number of immunocompetent T and B lymphocytes (Ge et al., 2012). This prevents effective identification and removal of the thymus-dependent and non-thymus-dependent antigens, causing reduction of immune functions and ultimately an increased propensity for secondary infection (i.e. *porcine parvovirus*, *classical swine fever virus* and *porcine reproductive and respiratory syndrome virus*). Now the pig-producers used a commercialized vaccine (CircoFlex) and some killed vaccines derived from whole virus of Chinese PCV2 strains and some other comprehensive measures to control PCV2 infection in China. Based on the immunosuppressive characteristics of *porcine circovirus* diseases (PCVD), we are now considering looking for specific medicines or medicine ingredients from traditional Chinese medicines, which have a direct effect on the virus as well as an inhibitory effect on viral replication through the modulation of immune function, further promoting immune reconstitution.

Arctium, also called Shunianzi and Dalizi, was initially documented in the Ming-Yi-Bie-Lu, an ancient Chinese medical book and formerly known as the E'shi. It comes from the dried ripe fruit of the Asteraceae biennial herb burdock, *Arctium lappa* L., and is a commonly used traditional Chinese medicine. Arctigenin (ACT), which was widely used in China and some other countries, is the primary component of *Arctium* with physiological and pharmacological activities and significant anti-inflammatory (Cho et al., 1999; Zhao et al., 2009) and antiviral effects (Vlietinck et al., 1998; Gao et al., 2002; Yang et al., 2005; Zhao et al.,

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2009; Hayashi et al., 2010). The effect of ACT on PCV2 is currently unknown, so answering this question may have beneficial effects on the local swine. This study examined the effect of ACT on PCV2 proliferation in vitro and the impact of this traditional Chinese medicine ingredient on PCV2 proliferation in the mouse inguinal lymph nodes, lung, and spleen. The results showed that ACT maybe as an antiviral drug for development.

2. Materials and methods

2.1. Cell line

The PK-15 cell line was provided by the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University.

2.2. Virus

The PCV-2 WH strain (GenBank: FJ 598044) was provided by the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University and stored at −80 °C. The viral load was measured by quantitative PCR (qPCR) (3.32×10^7 copies/μL), and diluted with growth medium to the concentration of 1 and 10 MOI.

2.3. Drug

ACT (99.3% purity) was extracted and prepared in a 25 mg/mL solution using dimethyl sulfoxide (DMSO) (Sigma, America) and then diluted to the desired concentration using the cell growth medium or saline (Ye et al., 2011). Briefly, a mixture of degreased fruit powder of *Fructus arctii* and 50% alcohol solution containing 5% hydrochloric acid was refluxed for 5 h. After filtration, the solid residue was re-suspended in 50% alcohol solution, and refluxed for 1 h again. The combined filtrate was basified (pH 7) with 5 mol L^{-1} NaOH solution, and then extracted with chloroform twice. Further, the combined organic phase was evaporated using a rotary evaporator under reduced pressure. The crude extract was purified by silica gel column chromatography twice, and finally gave the off-white powder. The pH was adjusted to 7.2 using 7% $NaHCO_3$, and the solution was stored at 4 °C prior to use.

2.4. Animals

Sixty 7-week-old female BALB/c mice were purchased from the Animal Centre, Institute of Medicine, Hubei province, China. Mouse studies were performed according to the guidelines of this institution (No. 00020502). All animal studies were complied with the Hubei Provincial Animal Care and Use Committee and the animal experiment guidelines of the Animal Experimentation Ethics Committee of Huazhong Agricultural University.

2.5. qPCR standard curve preparation

The concentration of purified PCV-2 positive clone plasmid was measured (0.2 μg/μL) and then converted to copy number-based value, that is, 1×10^{10} copies/μL. The purified plasmid of known copy numbers was prepared in 10-fold serial dilutions for amplification. The slope, correlation coefficient and intercept of the standard curve were −3.967890, 0.996291, and 55.906414, respectively (Fig. 1). The linear equation was $y = -3.967890x + 55.906414$, where y is the cycle threshold, and x is the copy number of plasmid template (Fig. 1).

2.6. Cytotoxicity test of ACT against PK-15

The cytotoxicity test of ACT against PK-15 was performed as previously described with some modifications (Pan et al., 2008). The ACT was diluted in 2-fold-series from 1.0 mg/mL (4% DMSO) to 500, 250, 125, 62.5, 31.3 and 15.6 μg/mL (DMSO 2%, 1%, 0.5%, 0.25%, 0.125% and 0.0625%, respectively). ACT at each concentration was each added to a 48 well plate (250 μL/well) in triplicate. Freshly trypsinized PK-15 cells were suspended and adjusted to 2×10^5 cell/mL, and then added to a 48 well plate (250 μL/well) to mix with ACT at different concentrations. DMSO control and cell control were prepared in the same way. The cells were incubated under 5% CO_2 at 37 °C for 48 h. Thereafter, the medium was removed and 20 μL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution (Promega, USA) was added to each well with a final MTT concentration of 5 mg/mL. After additional 4 h incubation, the cell medium was replaced with 150 μL DMSO. The plate was shaken for 10 min, and absorbance was measured at 570 nm to determine the viability relative to the untreated control.

2.7. Dose effect on PCV2 proliferation

ACT was diluted from the maximum non-toxic level to five different concentrations (62.5, 31.3, 15.6, 7.81 and 3.91 μg/mL) using cell growth medium, and then added to a 48 well plate (250 μL/well), with triplicate wells for each drug level. PK-15 cells (2×10^5 cell/mL) and PCV-2 (1 MOI) were added to each well and then incubated under 5% CO_2 at 37 °C for 24 h. The supernatant was removed and cell cultures were treated with D-glucosamine (Sigma, America) (C.M. Liu et al., 2006; J. Liu et al., 2006). The maintenance broth was replaced for 48 h continuous culture. After 3 freeze-thaw cycles, cells were collected from all wells. The viral load was measured by qPCR assays in order to examine the dose effect of ACT on PCV2 proliferation.

2.8. Dosing schedule effect on PCV-2 proliferation in vitro

The virus was cultured using the synchronous inoculation method and divided into two groups according to the MOI (1 MOI and 10 MOI). Each group was further divided into three subgroups based on the dosing schedule, i.e., 0, 4 or 8 h after inoculation. The ACT was added to the selected drug levels that showed an optimal inhibitory effect in triplicate. The culture was treated with 300 mM D-glucosamine 24 h after inoculation, and the maintenance medium was replaced for an additional 48 h continuous incubation. After 3 freeze-thaw cycles, cells were harvested from all wells. The viral load was measured via qPCR assays to examine the dosing schedule effect of ACT on PCV2 proliferation.

2.9. Testing antiviral activity of ACT in vivo

Sixty female BALB/c mice were randomly divided into 4 groups, i.e., ACT dose 1 (200 μg/mL), ACT dose 2 (20 μg/mL), positive control (ribavirin, 1000 μg/mL), and negative control (saline). Each group was inoculated with PCV2 by intraperitoneal injection of 0.2 mL per mouse as well as intranasal administration of 0.02 mL per mouse. ACT or ribavirin was administered by intraperitoneal injection of 0.2 mL 24 h after inoculation and was repeated everyday for 5 consecutive days. The negative control was injected with saline. At 7, 14 and 21 days after inoculation, five mice were randomly selected from each group and sacrificed. The inguinal lymph nodes, spleen and lungs were collected for measurement of viral load using qPCR assay.

2.10. Determination of PCV2 viral load by quantitative PCR

DNA extraction from the samples was performed using the E.Z.N.A.™ Viral DNA Kit (OMEGA, USA) according to the manufacturer's instructions. The DNA was used to quantify the PCV2 genomic DNA copy numbers by real-time PCR. GenBank entry FJ598044 was used for the primer and probe design. The Cap gene region (corresponding to nucleotides 1033–1734 bp of the whole PCV2 genome) (Cheung, 2003) was chosen for the primer and probe design because it has a lower nucleotide homology with *porcine circovirus* type 1 (PCV1) than ORF1 (~65%) (Liu et al., 2001). The forward (5′-CCAGGAGGGCGTTCTGACT-