Anti-Influenza Activity of Betulinic Acid from Zizyphus jujuba on Influenza A/PR/8 Virus

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Abstract
Betulinic acid, a pentacyclic triterpene isolated from Jujube tree (Zizyphus jujuba Mill), has been known for a wide range of biological and medicinal properties such as antibacterial, antimalarial, anti-inflammatory, antihelminthic, antinoceptive, and anticancer activities. In the study, we investigated the antiviral activity on influenza A/PR/8 virus infected A549 human lung adenocarcinoma epithelial cell line and C57BL/6 mice. Betulinic acid showed the anti-influenza viral activity at a concentration of 50 μM without a significant cytoxicity in influenza A/PR/8 virus infected A549 cells. Also, betulinic acid significantly attenuated pulmonary pathology including increased necrosis, numbers of inflammatory cells and pulmonary edema induced by influenza A/PR/8 virus infection compared with vehicle- or oseltamivir-treated mice in vivo model. The down-regulation of IFN-γ level, which is critical for innate and adaptive immunity in viral infection, after treating of betulinic acid in mouse lung. Based on the obtained results, it is suggested that betulinic acid can be the potential therapeutic agent for virus infection via anti-inflammatory activity.

Key Words: Betulinic acid, Zizyphus jujuba, Influenza A/PR/8, A549, Inflammation

INTRODUCTION
Influenza virus is (-)-strand RNA virus containing viral genome consists of eight segments of single-strand RNA, and is a common cause of respiratory infection known as “the flu”. Influenza virus is included in Orthomyxoviridae family and known to have 3 different serotypes including A, B, and C. Among them, serotype B and C were known to infect only human, but serotype A shows broad-spectrum in infection in mammals and even in poultry (Siemons et al., 1974; Webster et al., 1992).

Influenza A virus have two surface proteins, hemagglutinin (HA) and neuraminidase (NA), and sub-classified by the antigenicity of HA and NA. HA was known to help virus to attach cells and NA is glycoside hydrolase enzyme that cleave the glycosidic linkages of neuraminic acids. Recently, several inhibitors targeting NA were introduced as anti-influenza drug, and known to efficiently prevent the spreading of virus, which includes oseltamivir and zanamivir. However, recently the occurrence of resistant virus against NA inhibitors was reported, and which make us to find other antiviral candidates against influenza virus (Ward et al., 2005).

Zizyphus jujuba Mill, (Jujube tree) is indigenous to China over 4000 years and is widely distributed in Europe, eastern Asia, and Australia (Huang et al., 2008). Dry fruits of Z. jujuba have been utilized as poplar food and tea additives or favor for a long time (Li et al., 2007). The extract of Z. jujuba has been traditionally recognized as an outstanding source of anorexia, fatigue, and loose stools (Guo et al., 2010b). To date, it has been revealed that Z. jujuba contains the wide range of constituents including flavonoids (Pawłowska et al., 2009), triterpenic acids (Guo et al., 2010a), phenolic acids and amino acids (Choi et al., 2011).

Betulinic acid (BeA) is pentacyclic lupane-type triterpene that are widely distributed throughout the higher plant (Rastogi et al., 2015). The jujube tree (Zizyphus spp.) is known as one of the most extensively stated sources of BeA produced in considerable quantity (Dubey and Goel, 2013). In recent...
years, BeA has been reported to show a wide range of biological and medicinal properties such as of antibacterial, antimalarial, anti-inflammatory, anthelmintic, anticoagulant, and anticancer activities of BeA (Yogeesswari and Sriram, 2005; Rastogi et al., 2015). Especially, its derivatives are a promising new therapeutic agent for the treatment of HIV infection (Dang et al., 2013). The interest in BeA with antiviral activities on a few viruses led to examine it against influenza A/PR/8 virus infected in A549 cells and C57BL/6 mice. The aim of the present study was compare the anti-influenza virus activity of BeA in vitro and in vivo models.

MATERIALS AND METHODS

Isolation of BeA from Z. jujuba

The pulverized dried roots (14.5 kg) of Z. jujuba were macerated with MeOH (2×60 L) for each one week at room temperature. The MeOH extract was concentrated in vacuo to give a crude extract (0.5 kg). The concentrated extract was suspended in H₂O and acidified with 1N HCl to pH 3. The acidic solution was extracted with EtOAc to yield 186.2 g of EtOAc fraction. The aqueous residue was basified with NaOH to pH 9 and extracted with CHCl₃ to provide an alkaloid fraction (1.7 g) which was not applied in this study. The EtOAc fraction was subjected to a normal silica gel CC with a mixture of CHCl₃ and MeOH (1:1 to 1:10). Among them, the EA-3 fraction (50.1 g) was fractionated by a normal silica gel CC with a mixture of CHCl₃ and MeOH (100:1 to 5:1), giving seven fractions. Fraction 3 and 4 yielded BeA (betulinic acid, 5.4 g) by the re-crystallization with 100% MeOH.

Betulinic acid (BeA): whitis, amorphous powder, ¹H NMR (400 MHz, CD₃OD) : δ2.95 (1H, t, J=11.4, 5.0 Hz), 3.46 (1H, t, J=7.8 Hz, H-3), 2.74 (1H, td, like H-13), 2.68 (1H, m, H-11), 2.63 (1H, dt, J=12.7, 3.0 Hz, H-16a), 2.26 (1H, m, H-22a), 2.24 (1H, m, H-21a), 1.93 (1H, m, H-12), 1.90 (1H, m, H-15a), 1.87 (1H, m, H-2), 1.79 (3H, s, H-30), 1.76 (1H, t, J=11.4, H-18), 1.66 (2H, dt, J=13.0 Hz, 3.0, H-1), 1.59 (1H, m, H-22b), 1.56 (2H, m, H-6a and H-16b), 1.52 (1H, m, H-21b), 1.42 (1H, m, H-7a), 1.41 (1H, m, H-6b), 1.40 (1H, m, H-7b), 1.39 (1H, m, H-9), 1.28 (1H, m, H-15b), 1.23 (3H, s, H-23), 1.07 (3H, s, H-27), 1.06 (3H, s, H-24), 1.01 (3H, s, H-26), 0.83 (3H, s, H-25), 0.82 (1H, m, H-5) and ¹³C NMR (100 MHz, CD₃OD) : δ178.9 (C-28), 151.3 (C-20), 109.9 (C-29), 72.3 (C-3), 56.6 (C-17), 55.9 (C-5), 51.0 (C-9), 49.8 (C-18), 47.8 (C-19), 42.9 (C-14), 41.1 (C-8), 39.5 (C-1), 39.3 (C-4), 38.6 (C-13), 37.6 (C-22), 37.5 (C-10), 34.8 (C-7), 32.9 (C-16), 31.2 (C-21), 30.3 (C-15), 28.7 (C-23), 28.3 (C-2), 26.1 (C-12), 21.2 (C-11), 19.5 (C-30), 18.8 (C-6), 16.4 (C-24 and C-25), 16.3 (C-26), 14.9 (C-27).

Virus, cells, and reagents

Influenza A/PR/8 virus was obtained from by ATCC (American Type Culture Collection, Manassas, VA, USA). A549 cells were purchased from ATCC (Rockville, MD, USA) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimyotic solution. Antibiotic-antimyotic solution, FBS, and DMEM were supplied by Gibco BRL (Invitrogen Life Technologies, Karlsruhe, Germany). TPCK-Trypsin was purchased from Pierce (Thermo Fisher Scientific, Rockford, IL, USA). Both sulforhodamine B (SRB) and oseltamivir were purchased from Sigma-Aldrich (St. Louis, MO, USA). The tissue culture plates were purchased from Falcon (BD Biosciences, San Jose, CA, USA). All other chemicals were of reagent grade.

In vitro antiviral activity assay

Antiviral activity was evaluated by the SRB method using cytopathic effect (CPE) reduction, as previously reported (Song et al., 2014). A549 cells were seeded in a 96-well plate at a concentration of 2×10⁴ cells per well and were incubated for 24 h. Next day, the diluted virus suspension containing TPCK-trypsin of 1 μg/mL was placed in each well and were added the selected concentration of BeA. Virus-infected non-compound-treated cells were used as viral controls, while non-infected non-compound-treated cells were used as cell controls. After incubation for 2 days, A549 cells were washed with PBS, and ice-cold 70% acetone was added and incubated for 30 min at -20°C. After removing the acetone, 96-well plates were dried in a dry oven for 30 min, after which we added 0.4% (w/v) SRB in 1% acetic acid solution to each well for 30 min at room temperature. SRB was then removed, and the plates were washed with 1% acetic acid before oven-drying. After drying for 1 day, SRB was then solubilized with 10 mM un-buffered Tris-based solution, and the absorbance was then read at 540 nm using a VERSAmax microplate reader ( Molecular Devices, Palo Alto, CA, USA) with a reference absorbance at 620 nm. The antiviral activity of each test compound in influenza A/PR/8 virus-infected cells was calculated as a percentage of the corresponding untreated control.

Mice and virus infection

C57BL/6 mice between 6 and 7 weeks of age were purchased from SPL laboratory animal company (KOATCH Bio, Pyeongtaek, Korea). Mice were infected intranasally with 5×10⁴ pfu/30 μl of influenza A/PR/8 virus. Mice were maintained in animal facility at the Kangwon National University. All experiments were approved by the Institutional Animal Care and Use Committees of the Kangwon National University.

Histology and scoring

Lung tissue was washed with PBS containing and fixed in 4% formaldehyde for 1 hour at 4°C. The tissues were dehydrated by gradually soaking them in alcohol and xylene and then embedded in paraffin. The paraffin-embedded specimens were cut into 10 μm sections, stained with hematoxylin and eosin (H&E) and viewed with a digital light microscope (Olympus, Tokyo, Japan). As previously described (Shim et al., 2007), we used a scoring system to evaluate the level of lung tissue destruction, epithelial cell layer damage, polymorphonuclear cell infiltration into the site, and alveolitis.

Cytokine analysis

The levels of interferon gamma (IFN-γ), interleukin-1b (IL-1b), and tumor necrosis factor-α (TNF-α) were measured by mouse ELISA Ready-SET-GO kit (eBioscience), according to the manufacturer’s instructions.

Statistics

The Kaplan-Meier method was used to determine the statistical significance of differences in survival time. We performed the Log-Rank test (Mantel-Cox), using SPSS 12.0K.
for Windows. To compare the differences between two groups, Student’s t-test was used. To compare multiple groups, we carried out one-way ANOVA, followed by the Tukey-HSD post hoc test.

RESULTS

Isolation and Determination of BeA from Z. jujuba

BeA were isolated from the methanolic extract of Z. jujuba using a series of column chromatography followed by the recrystallization. The structure of BeA was determined on the basis of NMR spectroscopic data (Fig. 1A). In $^{13}$C NMR spectrum, the pattern of 30 carbon signals including the characteristic peaks at $\delta_C$ 151.3 (C-20) and 109.9 (C-29) exhibited the presence of the lupane-type triterpene having an olefinic bond. Additionally, the signals at $\delta_C$ 178.9 (C-28) and 78.1 (C-3) showed that this compound bear a carboxylic and hydroxyl moieties, respectively. After confirming the location of characteristic groups by HMBC correlation, it was determined as betulinic acid (BeA) (Yili et al., 2009).

Antiviral activity of BeA against influenza A/PR/8/34 virus

The antiviral activities of BeA against influenza A/PR/8/34 were assessed using the SRB method, which monitors the alteration of CPE induced by virus infection. The antiviral assays demonstrated that BeA possessed strong antiviral activity of about 98% against influenza A/PR/8/34 virus at the concentration of 50 $\mu$M and antiviral activity of about 30% at the same virus at the concentration 10 $\mu$M (Fig. 1B, 1C). BeA was not
toxic to A549 cells with cell viability of about 100% at the concentration of 50 μM (Data not shown).

**Antiviral activity of BeA against influenza A/PR/8 virus infected mice**

To confirm the anti-influenza activity of BeA in vivo, BeA or oseltamivir were administered to mice as follows: BeA (10 mg/kg/dose) or oseltamivir (30 mg/kg/dose), dissolved in PBS, was intraperitoneally administered daily for 7 days after influenza virus infection. The mice were infected intranasally with 30 μL of influenza A/PR/8 virus suspension 5×10^3 plaque forming unit (PFU).

We monitored daily the body weight of influenza A/PR/8 virus-infected mice for all experiments to compare anti-influenza virus effect of BeA with oseltamivir treatment. However, BeA and oseltamivir did not attenuated body weight loss induced by infection of influenza A/PR/8 virus (Fig. 2A). Further evidence of the anti-influenza effects of BeA on influenza A/PR/8 virus-infected mice lung tissue was provided viral gene expression by real-time PCR analysis. However, BeA did not influence on influenza A/PR/8 virus replication (Fig. 2B). Interestingly, however, influenza infected mice treated with BeA significantly attenuated pulmonary pathology including increased necrosis, numbers of inflammatory cells and pulmonary edema induced by influenza A/PR/8 virus infection, assessed at 7 days post infection, as compared with vehicle- or oseltamivir-treated mice (Fig. 3A). In addition, there are significant differences in scoring system to evaluate the level of lung tissue destruction, epithelial cell layer damage, polymorphonuclear cell infiltration into the site, and alveolitis as compared with influenza A/PR/8 virus infected group (Fig. 3B).

**Alteration of pulmonary cytokine signature in PR8-infected mice after the treatment of BeA and oseltamivir**

Virus-induced cytokines conduct a major role in recruiting leukocytes to the site of infection and activating innate immune responses to induce inflammation. Despite their protective roles, severe inflammation induced by cytokine storm was known to be associated with influenza-induced pulmonary pathology. To evaluate cytokines production at protein levels, mice were infected and treated by the same scheme as before and 6 hrs after final administration of BeA and oseltamivir, lungs from mice were obtained. The levels of cytokines including IFN-γ, IL-1β and TNF-α in lungs were measured with ELISA. The intranasal infection of influenza A/PR/8 virus increased the levels of IFN-γ, IL-1β and TNF-α at day 7 post infection as compared with PBS-treated control mice. Oseltamivir did not induce significant changes in the IFN-γ, IL-1β and TNF-α levels over vehicle treatment in influenza A/PR/8 virus-infected mice. Interestingly, at day 7 post infection, although BeA did not reduce the level of IL-1β and TNF-α, it significantly decreased the levels of IFN-γ as compared with that of oseltamivir treatment (Fig. 4).

**DISCUSSION**

The present study demonstrated that BeA inhibits the proliferation of influenza A/PR/8 virus with a dose dependent manner (0.4-50 μM) in A549 cells without significant cytotoxicity. After confirming antiviral activity of BeA in vitro model, we further studied whether BeA exerts sufficient therapeutic efficacy in influenza virus infected mouse model. Although BeA did not restore the body weight loss after the infection of influenza A/PR/8 virus similar to oseltamivir used as the positive control, the histochemical staining result showed BeA significantly reduced the inflammation and pulmonary edema induced by influenza virus.

Also, the change of IFN-γ level after treating of BeA in mouse lung supported that BeA can be the potential therapeutic agent for the inflammation by virus infection. IFN-γ has been known to have critical for innate and adaptive immunity in some viral, bacterial and protozoal infections. Collectively, these results suggested that BeA decrease inflammatory cytokine levels, especially IFN-γ, and help influenza A/PR/8 virus-
Infected mice to rapidly recover from severe pulmonary inflammation. Although further studies are necessary to clarify the detailed anti-influenza mechanisms, it is suggested that BeA can be the potential therapeutic agent for treating influenza viral infection via anti-inflammatory.

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