Protective Effects of 20(S)-Protopanaxtriol on Viral Myocarditis Infected by Coxsackievirus B3

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Key Words

Myocarditis • 20(S)-Protopanaxtriol • Coxsackievirus B3

Abstract

Objective: Coxsackievirus B3 (CVB3) is a dominant causative agent for viral myocarditis. So far, effective therapies for the treatment of the disease are not available. 20(S)-Protopanaxtriol is a major component of *Panax pseudoginseng* and has been clinically used for the treatment of heart diseases. However, it is not known whether 20(S)-protopanaxtriol exerts any anti-viral effects. Thus, the aim of this study was to investigate the therapeutic effects of 20(S)-protopanaxtriol against CVB3 in vivo and in vitro. **Methods:** The antiviral effects of 20(S)-protopanaxtriol in vitro were evaluated in HeLa cells infected by CVB3. Then, we examined the protective effects of 20(S)-protopanaxtriol on CVB3-induced myocarditis in BALB/c mice. These mice were treated with 20(S)-protopanaxtriol at doses of 100–400 mg·kg⁻¹·day⁻¹ for 7 days and compared with the controls. **Results:** We found that 20(S)-protopanaxtriol possessed potent antiviral effects on CVB3 in vitro. Compared with control mice, virus titers and pathological changes in the hearts were significantly decreased in the 20(S)-protopanaxtriol-treated group. Furthermore, biochemical markers of myocardial injury such as plasma lactate dehydrogenase and creatine kinase were decreased to nor-

mal levels. **Conclusions:** These data provide the possibility that 20(S)-protopanaxtriol can be used as a potential therapeutic means for treatment of viral myocarditis.

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Introduction

Viral myocarditis has been recognized as a cause of congestive heart failure for more than 50 years, but it is still a challenging disease for diagnosis and treatment [1, 2]. The history and clinical features are often nonspecific, and practical serological markers are not available during the acute phase of the disease. Even after proper diagnosis, no clinically proven treatment exists to prevent the development of subsequent dilated cardiomyopathy and death. Many viruses have been implicated as possible causes of myocarditis. Coxsackievirus B3 (CVB3) is one of them. Recently, it has been reported that infection of the cardiac myocyte is required for the induction of cardiac dysfunction and inflammation when mice were systemically infected with CVB3 [3]. This demonstrates a crucial role of CVB3 infection of cardiac myocytes in the heart during the development of CVB3-mediated myocarditis [4].

Although much work on the development of vaccines and therapeutic agents for the treatment of myocarditis

Fig. 1. Structure of 20(S)-protopanaxatriol.

has been conducted, no antiviral agents are available for clinical use. In this study, we investigated the antiviral effects of a natural extract from a traditional Chinese medicinal plant.

Panax notoginseng (Burk.) F.H. Chen is a well-known traditional Chinese medicinal plant, with the first recorded usage dating from the 16th century. It can help improve blood flow through the coronary arteries, suggesting that it can be useful for treatment of arteriosclerosis, high blood pressure and angina [5]. The roots of *P. notoginseng* are considered to contain analgesic, anti-inflammatory, antiphlogistic, antiseptic, astringent, cardiogenic, discutient, diuretic, hemostatic, hypoglycemic and styptic components [6]. 20(S)-Protopanaxatriol is one of the most important triterpene extracted from this herb. Its biological effects have been widely studied. For example, it improves cardiac function and protects myocardial cells from reperfusion injury. However, little information is available in the literature about 20(S)-protopanaxatriol effects on viral myocarditis induced by CVB3 infection. Therefore, the objective of the present study was to investigate the possible antiviral effects of 20(S)-protopanaxatriol against CVB3 in vitro and in vivo.

Materials and Methods

Compounds

20(S)-Protopanaxatriol (>97% pure) was obtained from the Laboratory of Phytochemistry, Kunming Institute of Botany, Chinese Academy of Sciences (China) [7]. The chemical structure is shown in figure 1.

Viruses and Cells

The CVB3 Nancy strain and the HeLa cell line were generously provided by the Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China. HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml

streptomycin, at 37°C in a humid atmosphere with 5% CO₂. The media used for the cytotoxic assay as well as for antiviral assays contained 2% of fetal calf serum.

Animals

Male BALB/c mice (4 weeks old, 14–16 g) were purchased and maintained at the Experimental Animal Center, Zhengzhou University (clean grade, certificate number 0009706). The animals were housed under constant conditions at a temperature of 23 ± 1°C, with 40 ± 5% humidity, and on a 12-hour light/12-hour dark cycle. They had free access to pellet food and water.

Determination of Cytotoxicity

To determine the 50% toxic concentration (TC₅₀) of 20(S)-protopanaxatriol, confluent HeLa cell monolayers grown in 96-well plates were incubated with serial 2-fold dilutions of compound in 100 µl test medium. The plates were incubated at 37°C and checked daily by phase contrast microscopy. After 48 h of incubation, 20 µl MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] (0.5 mg/ml) was added to each well and allowed to react for 4 h. After removal of the supernatant, 100 µl of DMSO was added to each well. Plates were incubated at room temperature for 30 min and the optical density was measured at the dual wavelengths of 570 and 630 nm. TC₅₀ was defined as the concentration of compound required to reduce the viability of untreated cell cultures by 50%. The mean dose-response curve of at least three tests was used to calculate the TC₅₀.

Antiviral Activity

HeLa cell monolayers were grown in 96-well flat-bottomed microtiter plates. After removal of culture medium, 50 µl of drug solution and a constant amount of 100 50% tissue culture infective dose (TCID₅₀) CVB3 in a volume of 50 µl was added to the cells. After 24 h, 20 µl MTT (0.5 mg/ml) was added to each well and allowed to react for 4 h. After removal of the supernatant, 100 µl of DMSO was added to each well. Plates were incubated at room temperature for 30 min and the optical density was measured at the dual wavelengths of 570 and 630 nm. IC₅₀ is the concentration of the drug required to inhibit 50% of the viral cytopathic effect. The mean dose-response curve of at least three tests was used to calculate the IC₅₀.

In vivo Antiviral Activity Experiment – Experiment Design and Antiviral Therapy

In this study, 90 mice were inoculated intraperitoneally with CVB3 (100 TCID₅₀). Forty-five of the inoculated mice received 20(S)-protopanaxatriol orally at doses of 100 (n = 15), 200 (n = 15) and 400 mg·kg⁻¹·day⁻¹ (n = 15) for 7 days. The doses were set based on our observation that three doses of 20(S)-protopanaxatriol (100, 200 and 400 mg/kg/day) had no toxicity in the mouse (data not shown). Oral administration with 150 mg·kg⁻¹·day⁻¹ (n = 15) of ribavirin (RBV) was used as a positive control. CVB3-inoculated mice (n = 15) were treated with a 0.9% NaCl solution daily and used as infected controls. Uninoculated mice (n = 15) were treated with an oral dose of 0.9% saline solution daily and used as normal control. Mice were sacrificed on the 7th day. Blood was collected retro-orbitally and the serum was separated to measure the activities of lactate dehydrogenase (LDH) and creatine kinase (CK) by using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) to analyze myocardial injury. The heart

Fig. 2. 20(S)-Protopanaxatriol decreases LDH and CK levels in CVB3-infected mice. Mice were treated with RBV (as positive control) and different doses of 20(S)-protopanaxatriol after CVB3 infection. Serum levels of LDH and CK were measured at day 7. $n = 15$ for each group. ** $p < 0.01$ versus the CVB3-infected group.

Table 1. Cytotoxic effects of 20(S)-protopanaxatriol on HeLa cells and antiviral activity against CVB3

Drug	TC ₅₀ , $\mu\text{g/ml}$	IC ₅₀ , $\mu\text{g/ml}$	TI
20(S)-Protopanaxatriol	28.77 $\pm$ 3.61	2.74 $\pm$ 0.33	10.56 $\pm$ 1.59
Ribavirin	722 $\pm$ 66.2	190 $\pm$ 21.66	3.81 $\pm$ 0.09

TI = Therapeutic index = TC₅₀/IC₅₀.

was divided into two parts: one part was homogenized in DMEM to determine virus titers by TCID₅₀ assays, and the other part was fixed for histological and electron microscopy analysis.

Virus Titers of the Heart

Mice were sacrificed 8 days after infection. The hearts were removed and washed with physiological saline, then homogenized in DMEM containing 4% fetal calf serum. The cellular debris was removed by centrifugation at 2,000 g for 10 min at 4°C. Virus titers were measured in 10-fold serial dilutions of the supernatant in DMEM by TCID₅₀ assays in HeLa cells.

Fig. 3. Virus titers of the hearts of each group. Mice were sacrificed at day 7, and the hearts were homogenized and virus titers were determined as described in Materials and Methods. $n = 15$ for each group. * $p < 0.05$, ** $p < 0.01$ versus the CVB3-infected group.

Histology

Histology was performed to examine the state of myocardial injury. The hearts were dissected from the mice, fixed in 10% formalin solution, embedded in paraffin, sectioned serially to a thickness of 3 μm , and stained with hematoxylin-eosin (HE). HE-stained sections were blindly and independently examined under a light microscope for evaluation of myocarditis.

Electron Microscopy

Myocardial injury was further examined by transmission electron microscopy. Heart samples were fixed in 4% formaldehyde and 1% glutaraldehyde. After dehydration, embedding, sectioning and staining, these sections were observed under a JEOL 200CX transmission electron microscope (Tokyo).

Statistical Analyses

Data are expressed as the mean $\pm$ SD. One-way analysis of variance with unpaired Student's t test was used to determine group differences. For all data, $p < 0.05$ was considered statistically significant.

Results

20(S)-Protopanaxatriol Has Potent Anti-CVB3 Activity in vitro

The cytotoxicity and antiviral activity of 20(S)-protopanaxatriol against CVB3 were examined in HeLa cells. As shown in table 1, TC₅₀ of RBV and 20(S)-protopanaxatriol on HeLa cells were 722 and 29 $\mu\text{g/ml}$, respectively. Both 20(S)-protopanaxatriol and RBV inhibited the replication of CVB3 in HeLa cells. The IC₅₀ for inhibition of CVB3 replication in HeLa cells was 190 $\mu\text{g/ml}$ for RBV

Fig. 4. Histological features of CVB3-induced myocarditis. Representative micrographs (HE staining) from each group are shown. The arrow indicates the foci of mononuclear cell infiltration, and the arrowhead indicates myocardial necrosis. $\times 400$.

Fig. 5. Ultrastructural features of CVB3-induced myocarditis. In the CVB3-infected group, the cardiac muscles were in disarray, and sarcomere fragmentation was observed (arrow). In addition, mitochondria had shrunk (arrowheads). $\times 10,000$.

and $2.74 \mu\text{g/ml}$ for 20(S)-protopanaxtriol, respectively, indicating that the inhibitory effects of 20(S)-protopanaxtriol against CVB3 were stronger than that of RBV.

20(S)-Protopanaxtriol Decreases LDH and CK Levels in CVB3-Infected Mice

Infection of CVB3 resulted in myocarditis in mice as indicated by increased plasma levels of LDH and CK (fig. 2). However, treatment with 20(S)-protopanaxtriol led to an obvious decrease in the activities of LDH and CK in infected mouse serum compared with control groups.

20(S)-Protopanaxtriol Inhibits CVB3 Replication and CVB3-Induced Myocarditis in Mice

Virus titers of the hearts in the infected mice were clearly decreased after treatment with 20(S)-protopanaxtriol at doses of 200 and $400 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (fig. 3). Histological examination revealed that CVB3 infection induced focal myocardial necrosis accompanied by mononuclear cell infiltration, which is compatible with the histological features of myocarditis (fig. 4). Treatment with either RBV or 20(S)-protopanaxtriol or apparently

reduced mononuclear cell infiltration and the myocardium damage was relieved.

Ultrastructural changes in the myocardium were further examined by transmission electron microscopy. In the CVB3-infected group, the cardiac muscle was in disarray, and sarcomere fragmentation was observed. In addition, mitochondria were shrunken (fig. 5). Treatment with the 20(S)-protopanaxtriol group led to the reduction of these changes, similarly to the treatment of RBV.

Discussion

Although the exact molecular mechanism responsible for viral myocarditis is not completely clarified, the cardiac damage caused by CVB3 is considered to be mediated by the following mechanisms, including autoimmune reaction [8], direct cytotoxicities [9] and virus-induced apoptosis [10–12]. In the clinical sites, immunosuppressive agents (azathioprine, prednisone and cyclosporine), intravenous immunoglobulin and antiviral agents (interferon, pleconaril and acyclovir) are often

used for treatment of the disease [13]. However, most of these treatments are for improving symptoms. Recently, effective ingredients isolated from traditional Chinese herbs have been shown to have antiviral activities including CVB3 [14, 15]. 20(S)-Protopanaxatriol is the major component isolated from the roots of *P. notoginseng*, yet its biological effects on myocarditis have not been fully studied. In the present study, we found that 20(S)-protopanaxatriol has potent anti-CVB3 activities both in vitro and in vivo and can significantly improve CVB3-induced myocarditis in mice.

Of note, the therapeutic index of 20(S)-protopanaxatriol on CVB3-infected HeLa cells was higher than that of RBV. In addition, our study showed that 20(S)-protopanaxatriol, when administered to a murine model of viral myocarditis, not only markedly decreased the virus titers in serum, but also protected against CVB3-induced cardiac injury and inflammatory cell infiltration. Throughout the in vitro experiment, the cytotoxicity of 20(S)-protopanaxatriol seemed to be higher than that of RBV, and even high doses by oral administration are not only effective but also safe in vivo [16]. The finding of reduced cardiac necrosis and mononuclear cell infiltration after treatment with 20(S)-protopanaxatriol suggests that 20(S)-protopanaxatriol may have several therapeutic functions against CVB3-induced myocarditis in vivo. Several mechanisms may be operative for anti-viral effects of 20(S)-protopanaxatriol. For example, 20(S)-protopanax-

atriol may be directly involved in an anti-inflammatory process caused by CVB3 infection, or may protect myocardiocytes from cell death (apoptosis). In our preliminary study, treatment with 20(S)-protopanaxatriol for 24 h significantly prevents from HeLa cell apoptosis induced by CVB3 infection (data not shown). This notion is further supported by the observation that biochemical markers including CK and LDH used to identify myocardial injury were normalized in 20(S)-protopanaxatriol-treated mice. It is possible that 20(S)-protopanaxatriol protected myocardial cells from the harmful effects of CVB3 by decreasing the leakage of the enzymes from the myocardial cells into the blood stream. In spite of this, the molecular mechanisms for 20(S)-protopanaxatriol anti-viral effects remain to be disclosed in future.

In conclusion, our study showed that 20(S)-protopanaxatriol had antiviral effects on CVB3 virus-induced myocarditis in mice. These findings suggest that 20(S)-protopanaxatriol may be potentially useful for the treatment of patients suffering from viral myocarditis.

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