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Antiviral Activity of Chrysin Derivatives against Coxsackievirus B3 *in vitro* and *in vivo*

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Abstract

Chrysin is a 5,7-dihydroxyflavone and was recently shown to potently inhibit enterovirus 71 (EV71) by suppressing viral 3C protease ($3C^{pro}$) activity. In the current study, we investigated whether chrysin also shows antiviral activity against coxsackievirus B3 (CVB3), which belongs to the same genus (*Enterovirus*) as EV71, and assessed its ability to prevent the resulting acute pancreatitis and myocarditis. We found that chrysin showed antiviral activity against CVB3 at 10 μM, but exhibited mild cellular cytotoxicity at 50 μM, prompting us to synthesize derivatives of chrysin to increase the antiviral activity and reduce its cytotoxicity. Among four 4-substituted benzyl derivatives derived from C(5) benzyl-protected derivatives 7, 9-11 had significant antiviral activity and showed the most potent activity against CVB3 with low cytotoxicity in Vero cells. Intraperitoneal injection of CVB3 in BALB/c mice with 1×10^6 $TCID_{50}$ (50% tissue culture infective dose) of CVB3 induced acute pancreatitis with ablation of acinar cells and increased serum CXCL1 levels, whereas the daily administration of 9 for 5 days significantly alleviated the pancreatic inflammation and reduced the elevation in serum CXCL1 levels. Collectively, we assessed the anti-CVB3 activities of chrysin and its derivatives, and found that among 4-substituted benzyl derivatives, 9 exhibited the highest activity against CVB3 *in vivo*, and protected mice from CVB3-induced pancreatic damage, simultaneously lowering serum CXCL1 levels.

Key Words: Antiviral activity, Coxsackievirus B3, Chrysin, Flavonoid, Pancreatitis

INTRODUCTION

Coxsackievirus B3 (CVB3) belongs to the genus *Enterovirus*, together with enterovirus, poliovirus, and echovirus, and is a causative agent of human diseases including viral myocarditis, which may result in acute heart failure and dilated cardiomyopathy (Yajima and Knowlton, 2009). To date, no effective antiviral therapies have been approved for either the prevention or treatment of diseases caused by CVB3. Although the main symptoms of mice infected with CVB3 are acute and chronic myocarditis, acute pancreatic inflammation was also reported, which is in accordance with human disease manifestations after CVB3 infection (Ramsingh, 1997; Tracy *et al*., 2000). Thus, mice with CVB3-induced pancreatitis could act as murine models of acute human viral pancreatitis. Interestingly, it was previously suggested that CVB3-induced pancreatitis could be a predisposing factor for the development of myocarditis, and that CVB3-induced myocarditis only occurs following pancreatitis (Tracy *et al*., 2000). Thus, the ability to prevent CVB3-induced pancreatitis might be crucial for the effectiveness of antiviral drug candidates against CVB3 infection.

Chrysin (5,7-dihydroxyflavone) is a natural flavonoid present in plants. It is found in large quantities in honey and propolis (Schnitzler *et al*., 2010). Flavonoids exhibit a wide range of pharmacological and biological activities, including antitumor, antibiotic, antifungal, and antiviral activities (Kumar and Pandey, 2013). Recently, Yang's group reported that chrysin and its 7-diisopropyl phosphate analogue showed a significant anti-enterovirus 71 (EV71) effect through strong inhibition of

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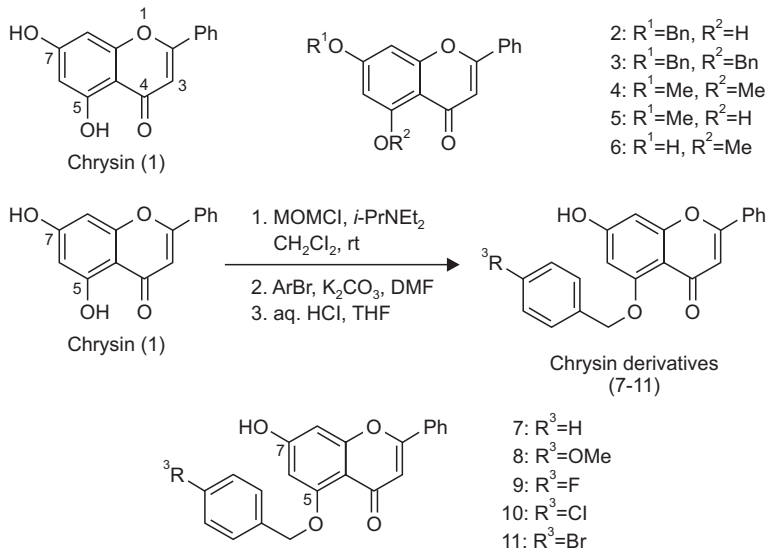

Fig. 1. Synthesis of chrysin derivatives.

EV71 replication (Wang *et al*., 2014). However, no studies on the anti-CVB3 activity of chrysin and its derivatives have been reported to date. Continuing our efforts towards the synthesis and biological evaluation of flavonoid-based natural products (Kim *et al*., 2014; Lee *et al*., 2014a; Lee *et al*., 2014b; Baek *et al*., 2015), we embarked on the synthesis of chrysin derivatives and studied the antiviral effect of chrysin and its derivatives against CVB3. Herein, we report the antiviral activity of chrysin-derivatives against CVB3 *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines and viruses

The CVB3 virus (ATCC VR-30, Manassas, VA, USA) was obtained from the division of vaccine research of the Korea Center Disease Control and prevention, and was propagated at 37°C in Vero cells (ATCC, Manassas, VA, USA), which are kidney epithelial cells that originated from an African green monkey. Vero cells were used for the infection of CVB3 since these cells have intrinsic genetic deletion of a type I locus resulting in the lack of type I IFN production, making them suitable targets for CVB3 infection. Vero cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic solution. Antibiotic-antimycotic solution, trypsin-EDTA, FBS, and MEM were purchased from Gibco BRL (Invitrogen Life Technologies, Karlsruhe, Germany). Tissue culture plates were purchased from Falcon (BD Biosciences, San Jose, CA, USA).

Preparation of chrysin derivatives

Known chrysin derivatives 2-6 were prepared from chrysin (1) according to the procedure previously described in the literature (Kim *et al*., 2014; Lee *et al*., 2014a; Lee *et al*., 2014b; Baek *et al*., 2015). C(5) benzyl- and 4-substituted benzyl-protected chrysin derivatives 7-11 were prepared using conventional synthetic methods in three steps (MOM protection of chrysin, benzylation, and deprotection of MOM group) as described in Fig. 1.

Animal model

Wild-type inbred BALB/c mice were purchased from Charles River Laboratories (Orient Bio Inc., Sungnam, Korea). Mice were maintained under specific pathogen-free conditions in the experimental facility at Kangwon National University. The experiments were approved by the Institutional Animal Care and Use committees of Kangwon National University. The female 4-week-old BALB/c mice weighing 13 g to 15 g were divided equally into four groups, and intraperitoneally inoculated with 1×10^6 $TCID_{50}$ of CVB3 in 200 μl Vero cell lysate. Mice inoculated with Vero cell lysate only without CVB3 infection were used as control. After being anesthetized with ether, all mice were sacrificed on day 5 post-infection for histological analysis. Sera were taken at day 5 post-infection.

Sulforhodamine B (SRB) assays

Assays of antiviral activity and cytotoxicity were evaluated by the sulforhodamine (SRB) method using the cytopathic effect (CPE) induced by viral infection as recently reported (Song *et al*., 2014). The infectivity of each virus was determined by measuring intact cells attached to plates after addition of $CCID_{50}$ (50% cell culture infective dose) of CVB3 virus. The antiviral drug candidates were added to each well at the indicated time points and the cytopathic effect was monitored at 48 h post-infection. Cell morphology was also assessed after SRB assay under a microscope at 4×10 magnification (AXIOVERT10, ZEISS, Germany) and images were acquired. The absorbance of SRB in each well was read at 540 nm using a VERSAmax microplate reader (Molecular Devices, Palo Alto, CA, USA) and a reference absorbance of 620 nm. The antiviral activity of each test compound in CVB3-infected cells was calculated as a percentage of the corresponding maximum survival of non-infected cells after normalization with untreated infected control cells.

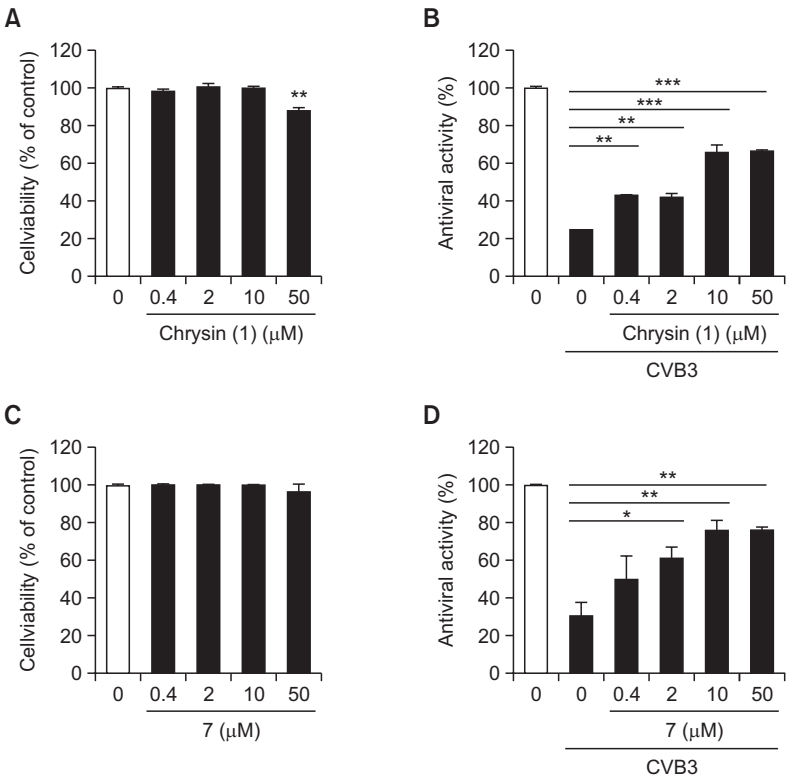

Fig. 2. The antiviral activity of chrysin against CVB3. (A) Cytotoxicity and (B) antiviral activity of chrysin and (C) cytotoxicity and (D) antiviral activity of 7 against coxsackievirus B3 (CVB3) in Vero cells. Vero cells were infected with CVB3, after which they were treated with the indicated concentrations (0.4, 2, 10, and 50 μM) of 7 for 48 h. Antiviral activity was investigated using a cytopathic effect (CPE) reduction assay. Data are presented as means ± S.D. from three independent experiments each carried out in triplicate.

Histology

The pancreata of mice were removed and washed in phosphate-buffered saline before being fixed with 4% formaldehyde. The tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E). The number of acini was counted by a pathologist using a blind test.

Cytokine measurement

The levels of chemokine (C-X-C motif) ligand 1 (CXCL1) were measured by a DuoSet Mouse ELISA Kit (R&D Systems, Minneapolis, MN, USA) and ELISA MAX standard sets (Biolegend, Inc., San Diego, CA, USA). All experiments were performed according to each manufacturer's instructions.

Statistical analysis

To compare multiple groups, we carried out one-way analysis of variance (ANOVA) followed by the Tukey post hoc test using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). Values of $p<0.05$ were considered significant at a 95% confidence interval.

RESULTS

Construction of derivatives of chrysin

Chrysin was shown to demonstrate potent antiviral activity against EV71 by suppressing viral 3C protease ($3C^{pro}$) activity (Wang *et al*., 2014). Since CVB3 belongs to the same genus, *Enterovirus*, as EV71, we assessed whether it showed antiviral activity against CVB3 also. To this end, chrysin was added to Vero cells at a cell culture infectious dose 50% ($CCID_{50}$) (concentrations ranging from 0.4 to 50 μM), and the cells surviving after CVB3 infection were assessed by SRB assay. We found that chrysin showed antiviral activity against CVB3 at 10 μM, but also induced mild cellular cytotoxicity at 50 μM (Fig. 2A, B). Thus, we decided to synthesize derivatives of chrysin (1) to increase the antiviral activity and reduce its cytotoxicity.

Initially, we prepared C(5)/C(7) methyl- or benzyl-protected derivatives to identify the effect of C(5) and C(7) hydroxyl on the antiviral activity against CVB3 (Fig. 1). Among the tested seven derivatives, C(5) benzyl-protected derivative 7 showed the most potent antiviral effect on CVB3 with low cytotoxicity (Fig. 2C, D). Based on the basic structure of 7, we decided to further prepare various 4-substituted benzyl derivatives of 7 including 8-11 (Fig. 1), and tested their antiviral effects on CVB3 in an effort to discover more potent derivatives.

The chrysin derivatives 9-11 have antiviral activity against CVB3 infection *in vitro*

Interestingly, among the four 4-substituted benzyl derivatives, 9-11 had significant antiviral activity at 50 μM. Especially, 9 showed significant antiviral activity even at 5 μM, without inducing cytotoxicity (Fig. 3A, B). In addition, we also checked the morphology of Vero cells infected with CVB3 after treatment with 9-11 using the SRB method. In the absence of infection of Vero cells with CVB3, cells treated with the ve-

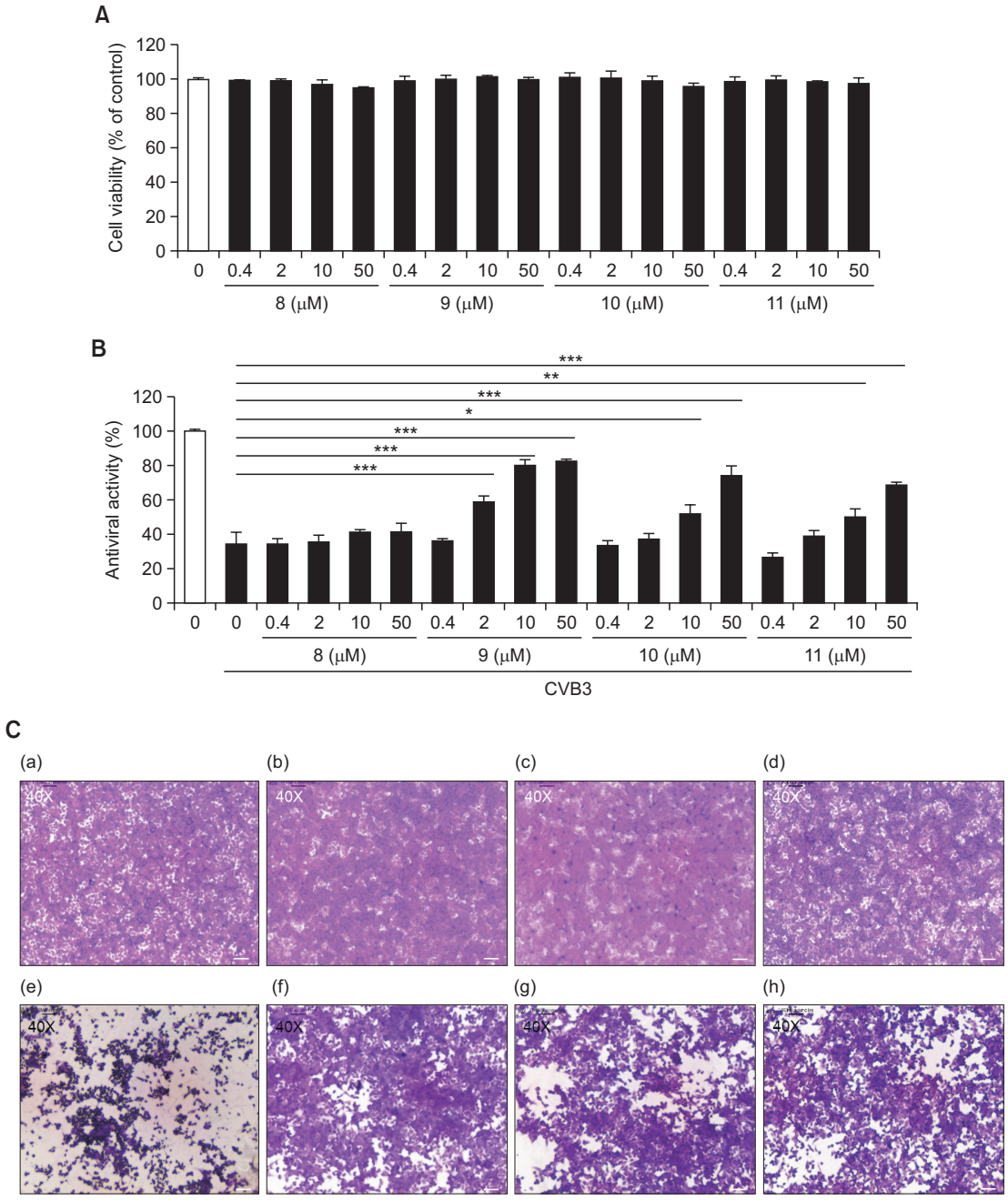

Fig. 3. The antiviral activity of 4-substituted benzyl chrysin derivatives 8-11 against CVB3. (A) Cytotoxicity and (B) antiviral activity of 8-11 against coxsackievirus B3 (CVB3) in Vero cells. Vero cells were infected with CVB3, after which they were treated with the indicated concentrations (0.4, 2, 10, and 50 μM) of 8-11 for 48 h. Antiviral activity was investigated using a cytopathic effect (CPE) reduction assay. Data are presented as means ± S.D. from three independent experiments each carried out in triplicate. (C) Morphological assessment of CVB3-infected Vero cells following treatment with 4-substituted benzyl derivatives of 7. (a) Non-infected cells; (b) non-infected cells treated with 9; (c) non-infected cells treated with 10; (d) non-infected cells treated with 11; (e) CVB3-infected cells; (f) CVB3-infected cells treated with 9; (g) CVB3-infected cells treated with 10; (h) CVB3-infected cells treated with 11.

hicle or 10 μM of each compound showed typical spread-out shapes with normal morphology (Fig. 3C). Infection with CVB3 in the absence of drug treatment resulted in a severe CPE (Fig. 3C). On the contrary, the addition of 9, 10, and 11 to the Vero cells infected with CVB3 inhibited the formation of a visible CPE (Fig. 3C). Collectively, these results suggest that the 9-11 flavonoid derivatives have significant antiviral activity against CVB3 without inducing cytotoxicity in Vero cells.

The chrysin derivative 9 showed significant reduction in serum CXCL1 levels after CVB3 infection

To ascertain the antiviral effect of 9 and 10 *in vivo*, BALB/c

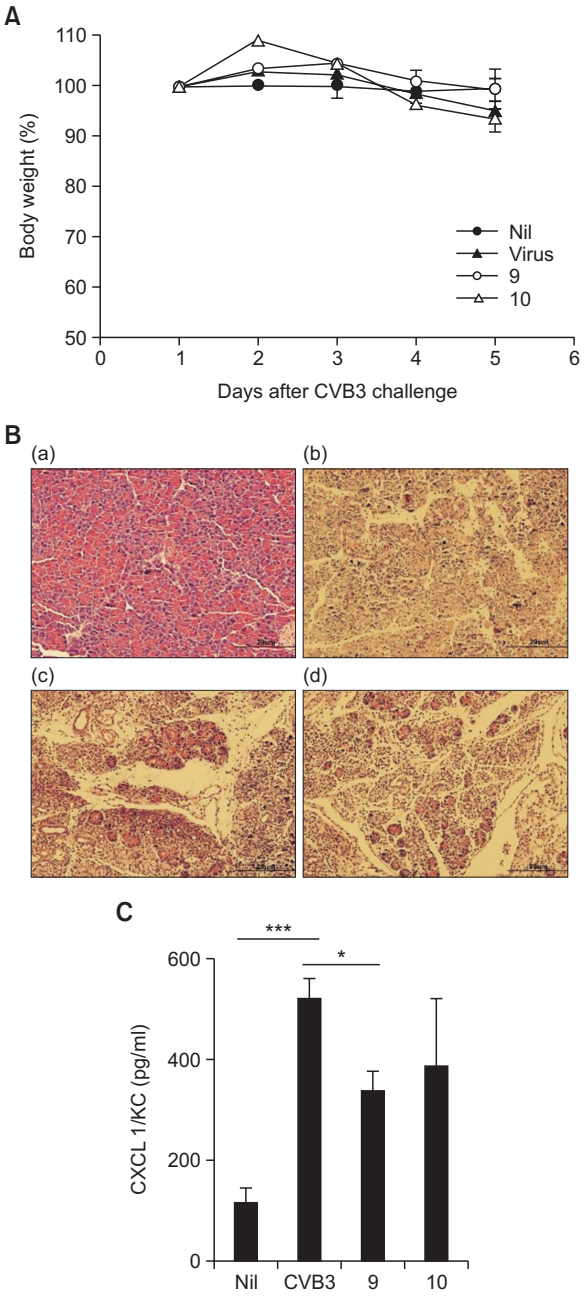

Fig. 4. Administration of 9 mitigate damage to the pancreas and reduce chemokine levels. (A) 9, 10, and control groups of BALB/c mice were infected with a 1×10^6 $TCID_{50}$ (50% tissue culture infective dose) dose of coxsackievirus B3 (CVB3) and then assessed by body weight and survival. (B) Representative hematoxylin and eosin staining (H&E) of pancreas section of (a) uninfected, (b) infected CVB3 and treated (c) 9 and (d) 10 (Scale bar=20 μm). (C) Serum chemokine levels in mice treated with 9 and 10. The sera were taken at day 5 post-infection. Levels of CXCL1 at 5 days were determined in the sera after intraperitoneal infection by the DuoSet Mouse ELISA Kit.

mice were intraperitoneally injected with CVB3 at 1×10^6 tissue culture infectious dose 50% (TCID50). Initially, we monitored the change in body weight of CVB3-infected mice after treatment with 9 and 10. There was a slight weight loss in CVB3-infected mice, but the treatment with 9 and 10 could not significantly prevent the body weight loss after CVB3 infection (Fig. 4A). Next, we assessed the induction of CVB3-associated pancreatitis in mice, since it was well-known that the pancreata of mice are one of the major target organs of CVB3 (Kemball *et al*., 2010). For the pathological analysis, histology sections were obtained from the pancreata of infected mice. Uninfected pancreata of mice were histologically normal, while after five days of CVB3 infection, they showed almost complete ablation of acinar cells, as well as infiltration of inflammatory cells. However, in CVB3-infected mice, we could find partially intact acinar cells, albeit major parts of the pancreata were also destroyed by CVB3 infection (Fig. 4B).

Finally, we obtained sera from mice at five days post-infection, and measured the serum levels of CXCL1. In the absence of CVB3 infection, the serum levels of CXCL1 were undetectable; CVB3 infection significantly increased serum CXCL1 levels at five days post-infection. Interestingly, daily administration of 9 for five consecutive days significantly reduced the CXCL1 levels. Thus, it is possible that 9 has antiviral activity against CVB3 and consequently resulted in the lowering of CXCL1 levels in the serum of infected mice.

DISCUSSION

CVB3 infection in humans is known to cause heart-muscle infection, whereas in mice, it results in acute pancreatitis and viral myocarditis. To date, no effective antiviral therapies have been approved for either the prevention, or the treatment of diseases caused by CVB3. Recently, it was also reported that the development of some cases of type 1 diabetes was associated with CVB3 infections, highlighting the physiological significance of CVB3 infection in the pancreas (Jaeckel *et al*., 2002; Park *et al*., 2009; Alirezaei *et al*., 2012).

Based on a recent study demonstrating the antiviral activity of chrysin against EV71 (Wang *et al*., 2014), we started to assess the antiviral activity of chrysin against CVB3. Previously, it was also reported that total flavonoid extracts from *Selaginella moellendorffii* Hieron containing amentoflavone showed significant inhibitory effects on CVB3 *in vitro* and exhibited significant antiviral activity against CVB3 infection *in vivo*. The study showed that the mice infected with CVB3 had defects in their blood circulation, and viral replication was found in their heart and kidneys (Yin *et al*., 2014). Although the anti-CVB3 activity of amentoflavone was also suggested to be mediated by the inhibition of fatty acid synthase expression (Wilsky *et al*., 2012), amentoflavone possessed higher cytotoxicity with relatively weaker antiviral activity than the total flavonoid extracts from *Selaginella moellendorffii* Hieron (Yin *et al*., 2014). Thus, it seems that there may be a synergistic effect exerted by flavonoids, or other antiviral compounds may be present in the total flavonoid extracts from *Selaginella moellendorffii* Hieron. The antiviral activity of calycosin-7-O-beta-D-glucopyranoside (CCGR) against CVB3 has also been demonstrated. CCGR is one of the main isoflavonoids isolated from Astragalus membranaceus var. Mongholicu (BGE.) Hsiao, and was found to suppress CVB3-induced cytotoxicity with an IC_{50} value of 25 μg/mL, while alleviating CVB3-induced acute myocarditis in mice and improving survival (Zhu *et al*., 2009).

It was reported that levels of some serum cytokines and chemokines are elevated during CVB3 infection (Lundgren *et*

al., 2009); among them, we found CXCL1 levels to be dramatically increased in CVB3-infected mice. The transcription of the CXCL1 gene is regulated by nuclear factor kappa B (NF-κB)-binding elements within the promoter region (Wood *et al*., 1995), and thus the expression of CXCL1 could be NF-kB-dependent as previously suggested (Lee *et al*., 2012; Burke *et al*., 2014). Interestingly, a recent study suggested that the viral protease $3C^{pro}$ of CVB3 cleaves inhibitor of kappa B (IκBα), and it was sufficient to cause apoptosis of infected cells (Zaragoza *et al*., 2006). Although it is still unknown whether the activation of NF-κB could have antiviral effects or not, the inhibition of CVB3 $3C^{pro}$ by chrysin may inhibit the cleavage of IκBα and attenuate NF-κB-mediated CXCL1 transcription as well as inhibit the cleavage of viral proteins. Thus, the reduced level of serum CXCL1 could be the result of reduced viral replication and inhibition of NF-κB translocation into the nucleus, which is in accordance with the alleviation of apoptotic cell death of pancreatic acinar cells by chrysin after CVB3 infection in mice. Alternatively, we propose that the increased levels of CXCL1 could be caused by increased levels of NF-κB signaling, which, in turn, may be due to increased endoplasmic reticulum (ER) stress caused by CVB3 infection. In this regard, the elevation of ER stress in cells infected with CVB3 was reported, (Zhang *et al*., 2010) but further studies will be needed to elucidate the role of chrysin and chrysin derivatives in the regulation of ER stress response.

In the current study, we assessed the anti-CVB3 activities of chrysin and its derivatives, and found that among 4-substituted benzyl derivatives, 9 exhibited the highest activity against CVB3 *in vivo*, protected mice from CVB3-induced pancreatic damage, as well as attenuated serum CXCL1 levels in CVB3-infected mice.

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