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Cinnamaldehyde Derivatives Inhibit Coxsackievirus B3-Induced Viral Myocarditis

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Abstract

The chemical property of cinnamaldehyde is unstable *in vivo*, although early experiments have shown its obvious therapeutic effects on viral myocarditis (VMC). To overcome this problem, we used cinnamaldehyde as a leading compound to synthesize derivatives. Five derivatives of cinnamaldehyde were synthesized: 4-methylcinnamaldehyde (1), 4-chlorocinnamaldehyde (2), 4-methoxycinnamaldehyde (3), α-bromo-4-methylcinnamaldehyde (4), and α-bromo-4-chlorocinnamaldehyde (5). Neonatal rat cardiomyocytes and HeLa cells infected by coxsackievirus B3 (CVB3) were used to evaluate their antiviral and cytotoxic effects. *In vivo* BALB/c mice were infected with CVB3 for establishing VMC models. Among the derivatives, compound 4 and 5 inhibited the CVB3 in HeLa cells with the half-maximal inhibitory concentrations values of 11.38 ± 2.22 μM and 2.12 ± 0.37 μM, respectively. The 50% toxic concentrations of compound 4 and 5-treated cells were 39-fold and 87-fold higher than in the cinnamaldehyde group. Compound 4 and 5 effectively reduced the viral titers and cardiac pathological changes in a dose-dependent manner. In addition, compound 4 and 5 significantly inhibited the secretion, mRNA and protein expressions of inflammatory cytokines TNF-α, IL-1β and IL-6 in CVB3-infected cardiomyocytes, indicating that brominated cinnamaldehyde not only improved the anti-vital activities for VMC, but also had potent anti-inflammatory effects in cardiomyocytes induced by CVB3.

Key Words: Anti-inflammatory, Cinnamaldehyde, Coxsackievirus B3, Myocarditis

INTRODUCTION

Viral myocarditis (VMC), an inflammatory disease of heart muscle secondary to viral infection (Cooper, 2009), is an important cause of dilated cardiomyopathy worldwide (Schultz *et al*., 2009). A recent study showed that myocarditis was the cause of sudden cardiac death in 8.6% of cases (Fabre and Sheppard, 2006). Enteroviruses, particularly coxsackievirus B (CVB), as the most common viruses resulting in viral myocarditis, are reportedly contribute to at least 50% cases of infection-caused heart diseases (Seong *et al*., 2001).

Treatment of VMC is dependant on the clinical presentation and severity of disease (Swedberg *et al*., 2005; Jessup *et al*., 2009; Schultz *et al*., 2009). Seeing that the most common inducement of myocarditis is virus infection, it would be credible that antiviral vaccines or antiviral medications could be valuable in the treatment of viral myocarditis (Blauwet and Cooper, 2010). The effects of some antiviral substances in the treatment of acute myocarditis have been evaluated in animal models and a few clinical cases. The role of antiviral therapy for more chronic myocarditis associated with persistent viral genomes has still being investigated (Feldman and McNamara, 2000; Schultz *et al*., 2009). Although interferon alpha and ribavirin can improve the survival rate of mice with acute myocarditis (Matsumori *et al*., 1987, 1988), optimal virus-specific preventive procedures against myocarditis have not been clinically evaluated and no standard exists for their use in clinical practice. Looking for potent antiviral agents without affecting host cells' normal physiological function is the primary task in designing new effective therapeutic drugs.

Previous studies have shown that cinnamaldehyde (3-phenyl-2-propenal), a major component of the essential oil of cinnamon bark isolated from *Cinnamomum* trees, possesses multiple biological activities including anti-platelet aggrega-

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1: R=CH_3
2: R=Cl
3: R=CH_3O
Cinnamaldehyde: R=H

4: R=CH_3
5: R=Cl

Fig. 1. Chemical structures of the derivatives of cinnamaldehyde (1-5).

tion, anti-bacterial, anti-viral, and anti-inflammation properties (Beige *et al*., 1996; Youn *et al*., 2008). Our recent study showed that cinnamaldehyde reduced the viral titer and improved the survival rate of VMC mice. Furthermore, cinnamaldehyde reduced plasma nitric oxide (NO) content, NF-κB expression and decreased inflammatory cell infiltrate in the myocardium of VMC mice (Ding *et al*., 2010). Despite these potential advantages, cinnamaldehyde, as an aldehyde with reducing property, can be oxidized to cinnamic acid rapidly in blood. The therapeutic effect of cinnamic acid on viral myocarditis was not observed *in vivo*. In order to overcome the instability of cinnamaldehyde *in vivo*, we synthesized a series of derivatives using cinnamaldehyde as the leading compound (structure shown in Fig. 1). The evaluation of the antiviral and cytotoxic effects of these derivatives on HeLa cells infected by CVB3 and neonatal rat cardiomyocytes are described in this report. We present a series of derivatives to determine if added substituents raise antiviral activity.

MATERIALS AND METHODS

Synthesis of (E)-3-p-tolylacrylaldehyde (1), (E)-3-(4-chlorophenyl) acrylaldehyde (2), and (E)-3-(4-methoxyphenyl)acrylaldehyde (3)

0.25 mol substituted-benzaldehyde (4-methylbenzaldehyde, 30.0375 g; 4-chlorobenzaldehyde, 35.1425 g; 4-methoxybenzaldehyde, 34.0370 g) and KOH (1.60 g) in ethanol (50 ml) were stirred at 5-10°C and protected by nitrogen. Acetaldehyde (8.81 g, 0.20 mol) was added in a dropwise manner within 15 min. The mixture was stirred at 5°C for 10 h, adjust pH to 5 with acetic acid. After pouring this whole mixture into water, the oily material was extracted with diethyl ether and then the organic layer was washed with saturated aqueous NaCl solution and water consecutively. The solvent was removed in vacuo, recrystallized with 50% ethanol, and the products were yellow crystals 1 (4.38 g, 12%), 2 (6.25 g, 15%) and 3 (9.32 g, 23%). 1: Mp: 40-42°C; IR (solid): 2822, 2744, 1684, 1626, 1604, 1508, 1448, 1324, 1210, 1129, 1109, 1008, 808 cm^{-1}; NMR (400 MHz; $CDCl_3$): δ 9.68 (1H, d, J=8.0), 7.46 (2H, d, J=8.0), 7.45 (1H, d, J=16.0), 7.23 (2H, d, J=8.0), 6.68 (1H, dd, J=8.0 16.0), 2.38 (3H, s). 2: Mp: 59-61°C; IR (solid): 2992, 2853, 1687, 1626, 1590, 1490, 1411, 1299, 1247, 1121, 1085, 1012, 977, 807 cm^{-1}; NMR (400 MHz; $CDCl_3$): δ 9.69 (1H, d, J=8.0), 7.80 (1H, d, J=16.0), 7.54 (2H, d, J=8.0), 7.48 (2H, d, J=8.0), 6.89 (1H, dd, J=8.0 16.0). 3: Mp: 56-57°C; IR (solid): 2939, 2843, 2765, 1666, 1626, 1602, 1570, 1511, 1264, 1249, 1177, 1131, 1008, 978, 826, 808 cm^{-1}; NMR (400 MHz; $CDCl_3$): δ 9.65 (1H, d, J=8.0), 7.52 (2H, dd, J=4.8 6.4), 7.42 (1H, d, J=16), 6.94 (2H, dd, 4.8 6.8), 6.61 (1H, dd, 8.0 15.6), 3.86 (3H, s).

Synthesis of (Z)-2-bromo-3-p-tolylacrylaldehyde (4)

Compound 1 (3.67 g, 0.025 mol) and acetic acid (10 ml) were added and the solution was stirred in an ice bank to drop the temperature below 5°C. Dropwise liquid bromine (4.15 g) was slowly added in a dropwise fashion and this mixture was stirred in the ice bank for 30 min. Then, K_2CO_3 (2.76 g, 0.02 mol) was added to the mixture until no bubbles were present. This solution was stirred at 80°C for 1.5 h. After cooling the mixture to 30°C, 25 ml water was added into it to precipitate. The yellow crystals were recrystallized with 80% ethanol, and to create a yellow compound 4 (6.95 g, 81.26%). Mp: 64-66°C; IR (solid): 2851, 1933, 1693, 1595, 1560, 1284, 1106, 1082, 895, 817 cm^{-1}; NMR (400 MHz; $CDCl_3$): δ 9.31 (1H, s), 7.92 (2H, d, J=8.0), 7.85 (1H, s), 7.29 (2H, d, J=8.0), 2.41 (3H, s).

Synthesis of (Z)-2-bromo-3-(4-chlorophenyl) acrylaldehyde (5)

Compound 2 (4.17 g, 0.025 mol) and acetic acid (10 ml) were added and the solution was stirred in an ice bank to drop the temperature below 5°C. Liquid bromine (4.10 g) was slowly added in a dropwise manner and stirred in the ice bank for 30 min. Then, K_2CO_3 (2.76 g, 0.02 mol) was added to the mixture until no bubbles were present and the solution was stirred at 80°C for 1.5 h. After cooling the mixture to 30°C, 25 ml water was added into it to precipitate. The yellow crystals were recrystallized with ethanol to create a yellow compound 4 (4.45 g, 72.48%). Mp: 91-92°C; IR (solid): 2832, 2359, 1687, 1601, 1586, 1489, 1408, 1282, 1116, 1091, 1016, 895, 822 cm^{-1}; NMR (400 MHz; $CDCl_3$): δ 9.34 (1H, s), 7.95 (2H, d, J=8.0), 7.85 (1H, s), 7.46 (2H, d, J=8.0).

Cardiomyocytes culture and cytotoxicity assay

Cardiomyocytes culture and cytotoxicity assay were performed according to a previously described procedure (Zhang *et al*., 2012). Briefly, rat cardiomyocytes were isolated from the ventricles of one to three-day-old Sprague-Dawley rats. The hearts were excised from neonatal rats under ethylether anesthesia, minced with scissors and dispersed with PBS. The cells were digested with 0.05% collagenase type II and 0.05% trypsin in PBS for 10 min at 37°C. The supernatants were transferred to DMEM containing 10% FBS, and the digestion was repeated four times. The resulting cell suspension was centrifuged, and the pellet was re-suspended in DMEM. The cells were plated in culture flasks for 1.5 h to remove nonmyocytes. Cardiomyocytes were cultured on 96-well plates maintaining in DMEM (containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 3 days. The cells were incubated with 0.01-1000 μM compounds 1-5 or cinnamaldehyde for 72 h. Dimethyl sulfoxide (DMSO) was used to dissolve compounds 1-5 and cinnamicaldehyde. The solutions were diluted with the medium before use to ensure all assays' final DMSO concentration didn't exceed 0.05%. Colorimetric MTT assay was used to analyze cell proliferation. 20 μl of MTT (5 mg/ml) was added into each well of the plate which was then maintained at 37°C for 4 h. After aspirating the supernatant, 150 μl of DMSO was added into each well. A 96-well microplate reader (Bio-Rad, Tokyo, Japan) was used to measure the absorbance at 570 nm. The following formula was used to

determine the cytotoxicity: percent of inhibition (%)=100-(absorbance value of test compound-absorbance value of blank)/(absorbance value of control-absorbance value of blank)×100. The 50% toxic concentration (TC_{50}) was determined by the compound's concentration inhibiting 50% of the viability of untreated cell cultures. At least three tests of the mean dose-response curve was used to calculate the TC_{50}.

Virus and anti-viral activity assay

The CVB3 (Nancy strain), purchased from the Microbiology Department of Fourth Military Medical University (Xi'an, China), was maintained by passage through human cervical carcinoma HeLa cells (ATCC CCL-2). Viral titers were evaluated by cytopathic effect in HeLa cells. Virus stocks were titrated on HeLa monolayer cells in 96-well plates by making tenfold dilutions (eight wells per dilution). Plates were incubated at 37°C in a CO_2 incubator for 5 d and the results were read under the light microscope. Titres were expressed as 50% tissue culture infective dose ($TCID_{50}$) values, calculated according to Reed and Muench (1938).

The antiviral activity of test compounds against CVB1 was evaluated by the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino) carbonyl-2H-tetrazolium hydroxide]) method as previously described (Chiang *et al*., 2002). Briefly, cardiomyocytes were cultured to confluency on 96-well plates (final cell density: ≈1×10^4 cell /well) and then infected with CVB3 (100TCID50, 20 μl per well) for 2 h in six replicates. After that, the cells were washed with phosphate-buffered saline (PBS) for three times, followed by incubation with serial dilutions of compound 1-5 and cinnamaldehyde. After 72 h, 50 μl of mixed solution of 0.1 ml phenazine methosulfate and XTT (5 mg/5 ml) were added to each well and then incubated for 2 h to producing XTT formazan. A 96-well microplate reader (Bio-Rad, Tokyo, Japan) was utilized to detect the optical densities with the test wavelength being 450 nm and the reference wavelength being 690 nm. The effect of viral inhibition was determined by the following formula: viral inhibition rate=(ODtv-ODcv)/(ODcd-ODcv)×100%. ODtv, ODcv and ODcd denote the absorbance of virus infected cells treated with test compound, the virus control and the cell control, respectively. The half-maximal inhibitory concentrations (IC_{50}) and the therapeutic index (TI, TC_{50}/IC_{50}) of compound 1-5 and cinnamicaldehyde were calculated.

Treatment of CVB3 infected mice

Four-week-old male BALB/c mice were purchased from the Center of Experimental Animals of the Fourth Military Medical University and housed under pathogen-free condition. These animals were randomly divided into fourteen groups (n=10) as follows: normal group, vehicle group (0.5% carboxymethylcellulose (CMC)-saline), model group, cinnamaldehyde group (40 mg /kg), interferon alpha (10 mg /kg) and compound 2, 4, 5 groups (20, 40 and 60 mg/kg). To establish VMC models, animals were inoculated intraperitoneally (i.p.) with 0.1 ml RPMI 1640 medium that contains 100×$TCID_{50}$ of CVB3. The vehicle group was treated with only 0.1 ml RPMI 1640 medium. From the 2nd day the interferon alpha group was intraperitoneally administered interferon once per day. The animals in the compound 2, 4, 5, vehicle and model groups were administered compound 2, 4, 5 or 0.5% CMC-saline interferon by oral gavage at the same time. At day 7, animals were euthanized by an overdose of sodium pentobarbital. To assess the severity of acute CVB3-induced myocarditis, ventricular tissue samples of isolated hearts were prefixed in buffered formalin, embedded in paraffin and finally sectioned into 4-μm slices. Sections were subsequently stained with H&E and examined by light microscopy. The impairment degree of CVB3-induced myocarditis was evaluated by the ratio of the heart section with inflammation to the entire heart section using a microscope eyepiece grid with magnification ×200. The score system was as follow: no lesion (grade 0); <25% of the heart section involved (grade 1); 25-50% involved (grade 2); 50-75% involved (grade 3); >75% involved (grade 4) (Nishio *et al*., 1999). The animal experiments were performed in accordance with the 'Guidelines for Animal Experimentation' of the Fourth Military Medical University.

Virus titration from heart homogenate

Part of the heart was weighed, homogenized in 10% (w/v) PBS and then centrifuged at 5000 rpm for 10 min. The supernatant was collected for $TCID_{50}$ assay to determine virus titer. Briefly, 96-well plates was seeded with HeLa cell monolayers and then serial 10-fold dilutions (100 μl) of supernatant added into the wells. After 5 days after infection, virus titers were calculated according to the last dilution leading to cells in 50% of wells showing a cytopathic effect. Results were presented as Log $TCID_{50}$/mg of tissue.

TNF-α, IL-1β and IL-6 expression

After infected with CVB3, cardiomyocytes were incubated with cinnamaldehyde (10 μM) and compound 2, 4 and 5 (10 μM). After 12 h, the TNF-α, IL-1β and IL-6 mRNA expression in cardiomyocytes was assayed using real-time PCR (Chen *et al*., 2005). The supernatants were collected after 72 h, and the levels of TNF-α, IL-1β and IL-6 in the culture supernatant were measured with ELISA, using commercially available ELISA kits (Rapidbio, West Hills, CA, USA).

Western blot analysis

The cardiomyocytes infected with CVB3 were incubated with cinnamaldehyde (10 μM) and compound 2, 4 and 5 (10 μM). After 24 h, whole-cell lysates were prepared according to the procedure previously described (Cao *et al*., 2011). Samples containing equal amounts of protein were separated by 15% SDS-PAGE and transferred onto nitrocellulose membranes. After washing, the membranes were blocked with 5% skimmed milk at room temperature for 2 h, and then incubated overnight at 4°C with the TNF-α, IL-1β, IL-6 and GAPDH antibodies (R&D Systems, Minneapolis, MN, USA). The membranes were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were visualized using Supersignal chemiluminescence substrate (Pierce, Rockford, IL, USA). Band densities were determined using Quantity One Software (BioRad, Hercules, CA, USA). The intensity of the resulting bands was expressed as the ratio between proteins of interest and GAPDH.

Statistics

All data were expressed as mean ± SD. Significant differences between two groups were determined by Student's t-test and one-way ANOVA was used for multiple comparisons. $p<0.05$ was accepted as statistically significant for all tests

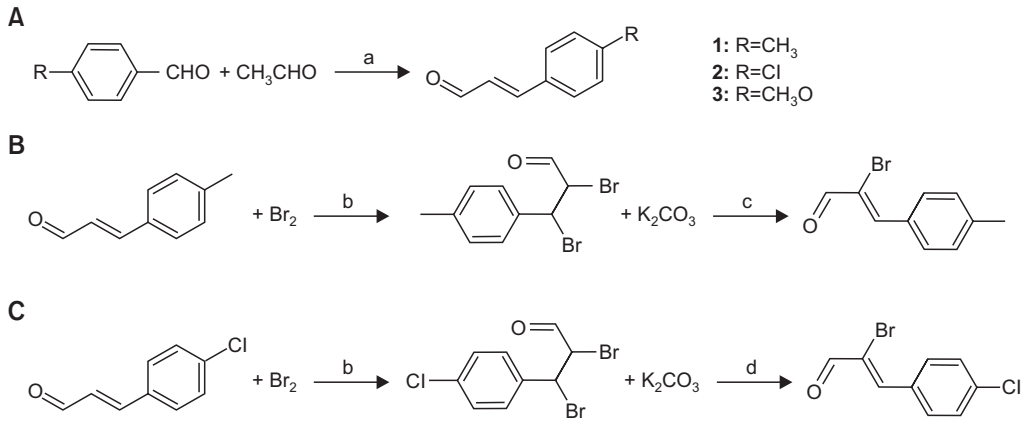

Fig. 2. Reagents and conditions. (A) KOH, EtOH, 5-10°C, 3 h; (B) CH_3COOH, 0-5°C, 30 min. (C) 80°C, 1.5 h; (D) 80°C, 1 h.

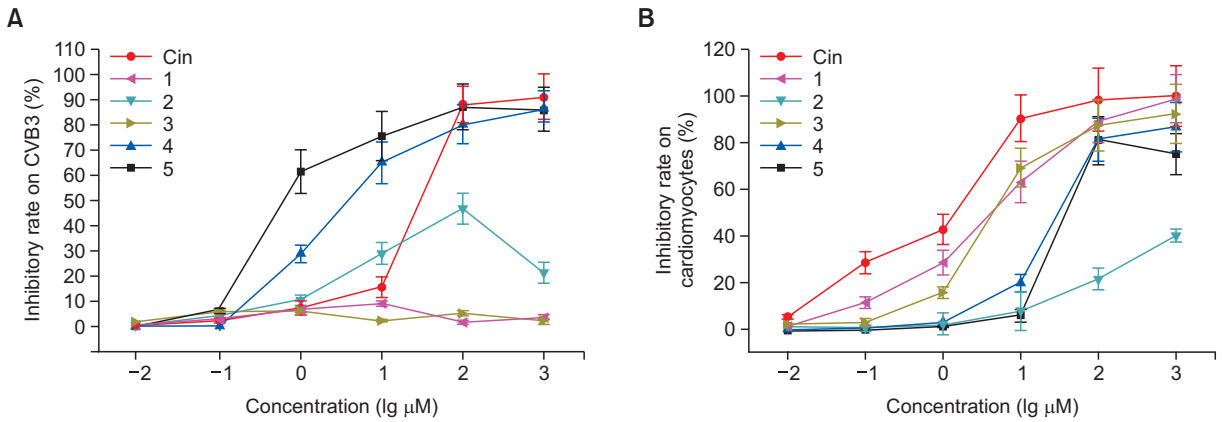

Fig. 3. The toxicity and anti-viral activity. (A) Anti-viral activity. Cardiomyocytes were infected with CVB3 for 1 h, and incubated with compound 1-5 or cinnamaldehyde (0.01-1000 μM). After 72 h, viral titers were determined using $TCID_{50}$ assay. (B) Cytotoxicity of compound 1-5 and cinnamaldehyde (Cin) on cardiomyocytes. Cardiomyocytes were treated with 0.01-1000 μM compound 1-5 or cinnamaldehyde for 72 h. Cell proliferation was analyzed by MTT assay. Values are expressed as mean ± SD.

RESULTS

Preparation of compounds

There are several published synthetic routes for cinnamaldehyde derivatives (Battistuzzi *et al*., 2003; Nordqvist *et al*., 2011). We modified and built synthetic schemes to yield compound 1-5 (Fig. 2). The substituted-benzaldehydes were combined with acetaldehyde to yield substituted- cinnamaldehydes (compound 1-3). Compound 1 and 2 were combined with liquid bromine to yield 2,3-dibromo-3-p-tolylpropana or 2,3-dibromo-3-(4-chlorophenyl) propanal, respectively. After dehydrobromination, yellow crystals (compound 4, yield was 81.26%; compound 5, yield was 72.48%) were obtained.

Cytotoxic and anti-viral activities of cinnamaldehyde derivatives on cardiomyocytes

Cardiomyocytes were infected with CVB3 and treated with compound 1-5 and cinnamaldehyde at concentrations in the range of 0.01-1000 μM for 72 h and then evaluated by the MTT assay. IC_{50} of compound 1-5 were calculated (De Logu *et al*., 2000). In addition, the cytotoxic effects of compound 1-5 were evaluated in the neonatal rat cardiomyocytes using MTT assay. In our experiments, compound 2, 4, 5 and cinnamaldehyde inhibited the replication of CBV3 in the cardiomyocytes in concentration-dependent manners (Fig. 3A). Compound 4 and 5 showed strong inhibitory effects with IC_{50} values of 11.38 ± 2.22 μM and 2.12 ± 0.37 μM, respectively, compound 2 showed a weak inhibitory effect with IC_{50} value of 2109.08 ± 157.69 μM, while the other compounds exhibited no activities (Table 1). Fig. 3B showed the cytotoxic effects of compound

Table 1. Inhibitory effects of compounds 1-5 on infected cardiomyocytes

Compound	CC_{50} (μM)	IC_{50} (μM)	TI
Cinnamaldehyde	0.98 ± 0.12	24.18 ± 4.77	0.04
1	3.96 ± 0.29*	-	-
2	6,972.47 ± 734.08*	2,109.08± 157.69*	3.31
3	7.88 ± 0.82*	-	-
4	38.61 ± 4.89*	11.38 ± 2.22*	3.39
5	85.69 ± 7.63*	2.12 ± 0.37*	40.42

Values are expressed as mean ± SD.
Significant differences between two groups were determined by Student's *t*-test. *p<0.05 vs. cinnamaldehyde group.

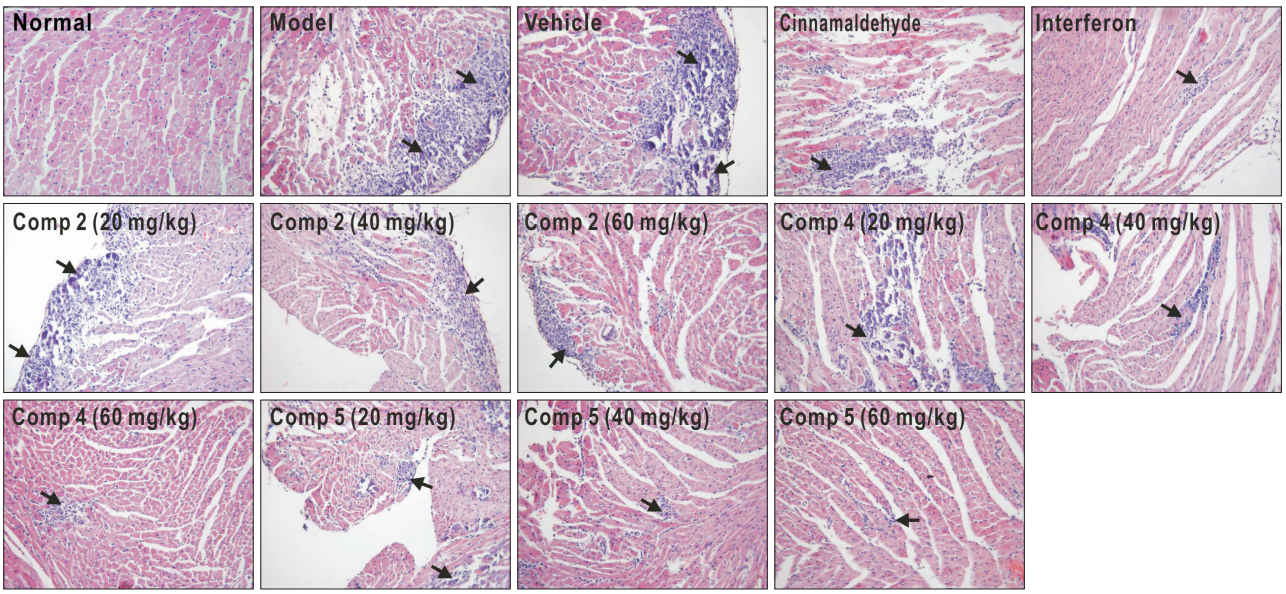

Fig. 4. Histology images of myocardial tissue. Seven days after CVB3 challenges, paraffin sections of heart tissues were stained with hematoxylin-eosin and viewed by light microscopy (original magnification×200). The representative photographs of heart tissue from normal, CVB3 control, vehicle control, cinnamaldehyde (40 mg/kg), interferon (10 mg/kg) and compound 2, 4, 5 (20, 40 and 60 mg/kg) groups. Mononuclear cell inflammation and necrotic cardiomyocyte site were indicated by a small black arrow.

Table 2. Therapeutic effects of compound 2, 4 and 5 on viral myocarditis *in vivo* (n=10)

Group	Dose (mg/kg/day)	Pathological score (grade)	Viral titers (Log $TCID_{50}$/mg)
Normal	-	0 ± 0	0 ± 0
Model	-	2.95 ± 0.60	3.40 ± 0.70
Vehicle	-	2.90 ± 0.60	3.30 ± 0.82
Cinnamaldehyde	40	2.40 ± 0.50*	2.80 ± 0.63
Interferon	10	1.30 ± 0.50*	1.50 ± 0.71*
Compound 2	20	2.85 ± 0.70	3.30 ± 0.48
Compound 2	40	2.85 ± 0.70	3.20 ± 0.63
Compound 2	60	2.75 ± 0.60	3.10 ± 0.57
Compound 4	20	2.80 ± 0.70	3.10 ± 0.74
Compound 4	40	2.10 ± 0.60*	2.90 ± 0.57
Compound 4	60	1.90 ± 0.60*	2.40 ± 0.67*
Compound 5	20	1.95 ± 0.40*	2.80 ± 0.42
Compound 5	40	1.75 ± 0.60*	2.40 ± 0.52*
Compound 5	60	1.30 ± 0.50*	1.70 ± 0.67*

Values are expressed as mean ± SD.
Significant differences between two groups were determined by Student's *t*-test. *p<0.05 vs. model group.

1-5 and cinnamaldehyde on cardiomyocytes. The CC_{50} values of compound 1, 3 and cinnamaldehyde were 3.96 ± 0.29 μM, 7.88 ± 0.82 μM and 0.98 ± 0.12 μM, respectively (Table 1). Compound 4 and 5 showed weak cytotoxic effects with CC_{50} values of 38.61 ± 4.89 μM and 85.69 ± 7.63 μM, while compound 2 exhibited no obvious cytotoxicity. The TI values of compound 2, 4, 5 and cinnamaldehyde were 3.31, 3.39, 40.42 and 0.04, respectively. So in the next study, we chose compound 2, 4, 5 for further study and compared their therapeutic effects on VMC *in vivo*.

Therapeutic effects of cinnamaldehyde derivatives on mouse model of VMC

The morphometrical analysis was used to assess the severity of myocarditis (Fig. 4, Table 2). In the infected control group, histopathological investigations showed large infiltrates of lymphocytes associated with severe myocardial necrosis. The cardiac pathological score was 2.95 ± 0.60. Compared with the infected control group, the myocardial damage was significantly reduced by compound 4 and 5 in dose-dependent manners. However, there was no significant difference between the compound 2 group and model group.

Furthermore, viral titers in the infected mice were signifi-

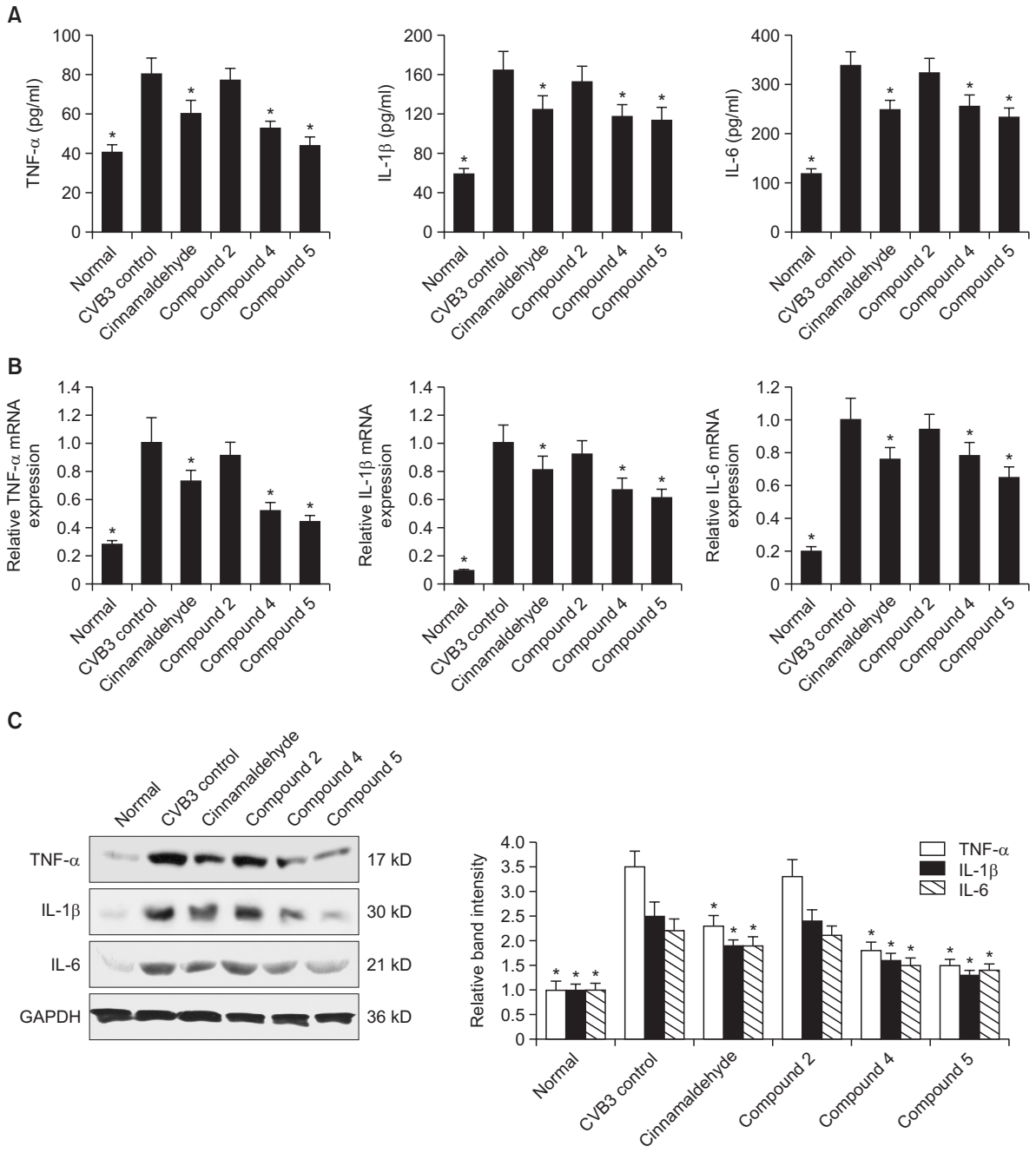

Fig. 5. The inhibitory effects of compound 2, 4, 5 on TNF-α, IL-1β and IL-6. Cardiomyocytes were exposed to CVB3 for 1 h, washed with PBS three times, and incubated with compound 2, 4, 5, or cinnamaldehyde (10 μM). After 72-hours of culturing, the supernatants were collected, and the productions of TNF-α, IL-1β and IL-6 were detected with ELISA (n=6) (A). The TNF-α, IL-1β and IL-6 mRNA expression in cardiomyocytes were assayed using real-time PCR after culturing for 12 h (B). (C) Western blot depicting TNF-α, IL-1β and IL-6 expression in the cardiomyocytes. Typical results are depicted in left panel along with the statistical analysis of TNF-α, IL-1β or IL-6 to GAPDH expression in right panel (n=3). Values are expressed as mean ± SD. Significant differences between two groups were determined by one-way ANOVA. **p*<0.05 vs. CVB3 control.

cantly decreased by the treatment of compound 4, 5 and interferon. The cardiac CVB3 titer in the model group was 3.40 ± 0.70 Log $TCID_{50}$/mg. Compound 4 and 5 inhibited viral titers in dose-dependent manners. The level of CBV3 titers in compound 4 (60 mg/kg) group and compound 5 (40 and 60 mg/kg) groups were significantly decreased, compared with those in the model group (p<0.05). Results showed that compound 5 had highest efficiency on 11 VMC than other cinnamaldehyde derivatives, being consistent with *in vitro* results.

Anti-inflammatory activities of cinnamaldehyde derivatives

Neonatal rat cardiomyocytes were infected with CVB3 and

then incubated with cinnamaldehyde (10 μM) and compound 2, 4 and 5 (10 μM) to examine their effects on TNF-α, IL-1β and IL-6 levels. After 12 h, the TNF-α, IL-1β and IL-6 mRNA expressions in cardiomyocytes were assayed using real-time PCR (Chen *et al*., 2005). The culture supernatants were collected after 72 h, and their TNF-α, IL-1β and IL-6 levels were measured with ELISA. According to our data, the levels of TNF-α, IL-1β and IL-6 in viral-infected cardiomyocytes were significantly inhibited and reached 43.60 ± 4.77, 112.72 ± 13.60 pg/ml and 232.80 ± 19.62 pg/ml, respectively, after application of compound 5. These values are lower than those of the viral control group (80.12 ± 8.40, 163.64 ± 19.75 and 337.46 ± 29.22 pg/ml, respectively) (Fig. 5A). Compound 4 and 5 also inhibited the expressions of TNF-α, IL-1β and IL-6 mRNA (Fig. 5B). We further evaluated the effect of cinnamaldehyde, compound 2, 4 and 5 on the expression of TNF-α, IL-1β and IL-6 by Western blot analysis (Fig. 5C). The TNF-α, IL-1β and IL-6 expression in viral-infected cardiomyocytes was markedly increased (3.52 ± 0.31, 2.53 ± 0.28 and 2.27 ± 0.24, respectively) compared with normal cells. Notably, compound 4 and 5 markedly diminished CVB3-mediated TNF-α, IL-1β and IL-6 expression. These results indicate that the two compounds could suppress CVB3-induced TNF-α, IL-1β and IL-6 expressions at the transcription and translation levels.

DISCUSSION

One of the major causes of acute myocarditis is CVB3 which can lead to tissue remodeling and even result in the disease itself (Kashimura *et al*., 2004; Dennert *et al*., 2008). In this study, we compared the anti-CVB3 activities of compound 1-5 and cinnamaldehyde on CVB3-induced viral myocarditis. Among the derivatives, compound 2, 4 and 5 inhibited the CVB3 in HeLa cells with IC_{50} values of 2109.08 ± 157.69, 11.38 ± 2.22 and 2.12 ± 0.37 μM, respectively. CC_{50} in compound 2, 4 and 5-treated cardiomyocytes were 6972.47 ± 734.08, 38.61 ± 4.89 and 85.69 ± 7.63 μM, which were 7114-fold, 39-fold and 87-fold higher than in the cinnamaldehyde group, suggesting that the toxicities of compound 2, 4 and 5 were much lower than that of cinnamaldehyde. In addition, the present studies demonstrated that compound 4 and 5 had effectively protective effect on CVB3-infected mice. They could reduce the viral titers and cardiac pathological changes in dose-dependent manners. Furthermore, compound 4 and 5 significantly inhibited the secretion and mRNA expressions of inflammatory cytokines TNF-α and IL-β in CVB3-infected cardiomyocytes, indicating that brominated cinnamaldehyde not only improved the anti-vital activity, but also had potent anti-inflammatory effects in cardiomyocytes induced by CVB3.

At present, the treatment of viral myocarditis is conventional supportive therapy. Specific therapies are lacking and in great demand (Swedberg *et al*., 2005; Schultz *et al*., 2009; Blauwet and Cooper, 2010). It is a primary task to look for potent antiviral agents without affecting host cells' normal physiological function in designing new effective therapeutic drugs. Cinnamaldehyde, α, β-unsaturated carbonyl derivative with a monosubstituted benzene ring, has demonstrated to suppress the growth of influenza A/PR/8 virus and CVB3 (Hayashi *et al*., 2007). It has been reported that cinnamaldehyde is unstable *in vivo* and can be oxidized to cinnamic acid rapidly in circulating blood. Resulted from β-oxidation of cinnamic acid, glycine or glucuronic acid conjugates of benzoic acid can be formed as the primary urinary metabolites of cinnamaldehyde (Yuan *et al*., 1992). Our previous studies showed that 10 to 1000 μM cinnamic acid displayed significant anti-CVB3 activity in primary cultured myocardial cells. Cinnamaldehyde possessed similar antiviral activity to cinnamic acid when being administered intraperitoneally (Ding *et al*., 2010). However, cinnamaldehyde could reduce plasma nitric oxide (NO) content, NF-κB, inducible nitric oxide synthase and TLR4 expression in myocardium from VMC mice 7 days after viral inoculation, while not observed in the mice treated with cinnamic acid. These results suggested that cinnamaldehyde might be a new lead compound for treating VMC.

Cinnamaldehydes are commonly synthesized in one or more steps by the Wittig reaction, Horner-Wadsworth-Emmons reaction, Peterson reaction, oxidation of primary allylic alcohols or crossed aldol condensation (van Staden *et al*., 2002; Nordqvist *et al*., 2011; Gu and Tian, 2012). We used substituted-benzaldehyde and acetaldehyde in one step to obtain cinnamaldehyde derivatives. This synthesis was readily available, low toxic, and easy to handle. Then, we compared their VMC therapeutic effects and cytotoxicity with cinnamaldehyde at a concentration range of 0.01-1000 μM. Among them, compound 4 and 5 had promising therapeutic effects and less toxicity than cinnamaldehyde *in vitro*.

Acute VMC has three phases including an original viral infection,autoimmune response and reshaping of cardiac structure and function (Dennert *et al*., 2008). In the incipient stage of CVB3 infection, the direct attack of the virus on the cardiac muscle cells is the main pathogenic process (Liu and Mason, 2001; Wang *et al*., 2007). We used the CVB3-inoculated male BABL/C mice for duplicating the VMC model. On the 3rd day, the mice exhibited signs of illness, including lethargy, progressive weight loss, and death. The structure of normal myocardium was clear, and the cell nucleus was clear. We found severe inflammation infiltration, necrosis, and fibrosis in infected murine hearts. With the activation of host immunologic function, the viral titer gradually dropped in the target organs. But in cardiomyocytes, non-renewable cells, viral titer might be long-standing, even being achieved at 30 days (Mall *et al*., 1991). So to detect VMC infected with CVB3, the activities of biochemical markers, virus titers of the organs and the histology changes of the heart are the most particular characteristics (Wang *et al*., 2009). After the mice were given compound 4 and 5 for 6 days, the damage of cardiomyocytes was reduced and necrosis and infiltration were significantly decreased, compared with the infected control group. Furthermore, the cardiac viral titers in the mice administrated with compound 4 and 5 were significantly decreased compared with the CVB3 control. These results suggested that compound 4 and 5 might improve the cardiac injury in the VMC animal models.

Although virus in the evolution of acute VMC is very important, it can attack on myocardial cells directly. The main cause of myocyte injury is an excessively activated immune response, which is triggered by the virus (Dennert *et al*., 2008). Previous studies showed that the expression of many pro-inflammatory cytokines in the heart of mice with viral myocarditis were too much. Among these cytokines, TNF-α, IL-1β and IL-6 conduce to the CBV3 pathological changes in myocardial disease (Shen *et al*., 2009; Gui *et al*., 2012). So, in this study,we discussed the effects of compound 2, 4 and 5 on the

TNF-α, IL-1β and IL-6 production and mRNA and protein expression in CVB3 infected neonatal rat cardiomyocytes. Our experiments showed that compound 4 and 5 could inhibit not only the production, but also the mRNA and protein expressions of TNF-α, IL-1β and IL-6 in CVB3 infected cardiomyocytes. These results suggested that compound 4 and 5 had potent anti-inflammatory activities. In addition, we observed that compound 5 inhibited CVB3-induced NF-κB activation, IκB-α degradation and phosphorylation by dose-dependent method, and decreased hearts' Toll like receptor (TLR) 4 protein level (Zhang *et al*., 2012). However, further studies involving structure-activity relationship are needed.

Furthermore, cinnamaldehyde is unstable in rat blood with a half-life of 4 min (Yuan *et al*., 1992). Our previous study showed that cinnamaldehyde maintained a very low concentration in plasma by the oral route (Zhao *et al*., 2014). In this study, we showed the antivital activities were markedly increased when cinnamaldehyde di-substituted with bromine and chlorine/methyl. We assumed that these substituents might raise the stability of cinnamaldehyde and further increases anti-viral activity. To evaluate the pharmacokinetic behavior of compound 2, 4 and 5 in rats, we recently developed a high performance liquid chromatographic method with UV detection for the determination of them in rats after a single oral administration at a dose of 20 mg/kg (unpublished data). A period of 24 h stability was tested by analyzing compounds in blood at room temperature and indicated that compound 2, 4 and 5 were stable for 24 h. In addition, the preliminary data showed that the peak concentrations of compound 2, 4 and 5 were achieved about 1.2 h, 3.3 h and 2.1 h after oral administration. From above, it seems likely that the substituted cinnamaldehyde exhibited more satisfactory biological effects and pharmacokinetic properties. However, the precise details of pharmacokinetics and the tissue distribution of these compounds need more elucidation so that the molecular mechanisms involved could be better understood.

In conclusion, our results demonstrated that chlorinated and brominated cinnamaldehyde can inhibit CVB3 replication and CVB3-induced cardiac injury potently both *in vitro* and *in vivo*. Compound 4 and 5, particularly compound 5, showed improved anti-vital and anti-inflammatory activity, low toxicity relative to cinnamaldehyde.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Battistuzzi, G., Cacchi, S. and Fabrizi, G. (2003) An efficient palladium-catalyzed synthesis of cinnamaldehydes from acrolein diethyl acetal and aryl iodides and bromides. *Org. Lett.* **5**, 777-780.

Beige, J., Weber, A., Engeli, S., Offermann, G., Distler, A. and Sharma, A. M. (1996) Angiotensinogen-M235T genotype and post-transplant hypertension. *Nephrol. Dial. Transplant.* **11**, 1538-1541.

Blauwet, L. A. and Cooper, L. T. (2010) Myocarditis. *Prog. Cardiovasc. Dis.* **52**, 274-288.

Cao, W., Xie, Y. H., Li, X. Q., Zhang, X. K., Chen, Y. T., Kang, R., Chen, X., Miao, S. and Wang, S. W. (2011) Burn-induced apoptosis of cardiomyocytes is survivin dependent and regulated by PI3K/Akt, p38 MAPK and ERK pathways. *Basic Res. Cardiol.* **106**, 1207-1220.

Chen, R., Zhou, H., Beltran, J., Malellari, L. and Chang, S. L. (2005) Differential expression of cytokines in the brain and serum during endotoxin tolerance. *J. Neuroimmunol.* **163**, 53-72.

Chiang, L. C., Chiang, W., Chang, M. Y., Ng, L. T. and Lin, C. C. (2002) Antiviral activity of Plantago major extracts and related compounds *in vitro*. *Antiviral Res.* **55**, 53-62.

Cooper, L. T., Jr. (2009) Myocarditis. *N. Engl. J. Med.* **360**, 1526-1538.

De Logu, A., Loy, G., Pellerano, M. L., Bonsignore, L. and Schivo, M. L. (2000) Inactivation of HSV-1 and HSV-2 and prevention of cell-to-cell virus spread by Santolina insularis essential oil. *Antiviral Res.* **48**, 177-185.

Dennert, R., Crijns, H. J. and Heymans, S. (2008) Acute viral myocarditis. *Eur. Heart J.* **29**, 2073-2082.

Ding, Y., Qiu, L., Zhao, G., Xu, J. and Wang, S. (2010) Influence of cinnamaldehyde on viral myocarditis in mice. *Am. J. Med. Sci*. **340**, 114-120.

Fabre, A. and Sheppard, M. N. (2006) Sudden adult death syndrome and other non-ischaemic causes of sudden cardiac death. *Heart* **92**, 316-320.

Feldman, A. M. and McNamara, D. (2000) Myocarditis. *N. Engl. J. Med.* **343**, 1388-1398.

Gu, Y. and Tian, S. K. (2012) Olefination reactions of phosphorus-stabilized carbon nucleophiles. *Top. Curr. Chem.* **327**, 197-238.

Gui, J., Yue, Y., Chen, R., Xu, W. and Xiong, S. (2012) A20 (TNFAIP3) alleviates CVB3-induced myocarditis via inhibiting NF-κB signaling. *PLoS ONE* **7**, e46515.

Hayashi, K., Imanishi, N., Kashiwayama, Y., Kawano, A., Terasawa, K., Shimada, Y. and Ochiai, H. (2007) Inhibitory effect of cinnamaldehyde, derived from Cinnamomi cortex, on the growth of influenza A/PR/8 virus *in vitro* and *in vivo*. *Antiviral Res.* **74**, 1-8.

Jessup, M., Abraham, W. T., Casey, D. E., Feldman, A. M., Francis, G. S., Ganiats, T. G., Konstam, M. A., Mancini, D. M., Rahko, P. S., Silver, M. A., Stevenson, L. W. and Yancy, C. W. (2009) 2009 focused update: ACCF/AHA guidelines for the diagnosis and management of heart failure in adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines: developed in collaboration with the International Society for Heart and Lung Transplantation. *Circulation* **119**, 1977-2016.

Kashimura, T., Kodama, M., Hotta, Y., Hosoya, J., Yoshida, K., Ozawa, T., Watanabe, R., Okura, Y., Kato, K., Hanawa, H., Kuwano, R. and Aizawa, Y. (2004) Spatiotemporal changes of coxsackievirus and adenovirus receptor in rat hearts during postnatal development and in cultured cardiomyocytes of neonatal rat. *Virchows Arch*. **444**, 283-292.

Liu, P. P. and Mason, J. W. (2001) Advances in the understanding of myocarditis. *Circulation* **104**, 1076-1082.

Mall, G., Klingel, K., Albrecht, M., Seemann, M., Rieger, P. and Kandolf, R. (1991) Natural history of Coxsackievirus B3-induced myocarditis in ACA/Sn mice: viral persistence demonstrated by quantitative in situ hybridization histochemistry. *Eur. Heart J*. **12 Suppl D**, 121-123.

Matsumori, A., Crumpacker, C. S. and Abelmann, W. H. (1987) Prevention of viral myocarditis with recombinant human leukocyte interferon alpha A/D in a murine model. *J. Am. Coll. Cardiol.* **9**, 1320-1325.

Matsumori, A., Tomioka, N. and Kawai, C. (1988) Protective effect of recombinant alpha interferon on coxsackievirus B3 myocarditis in mice. *Am. Heart J*. **115**, 1229-1232.

Nishio, R., Matsumori, A., Shioi, T., Ishida, H. and Sasayama, S. (1999) Treatment of experimental viral myocarditis with interleukin-10. *Circulation* **100**, 1102-1108.

Nordqvist, A., Bjorkelid, C., Andaloussi, M., Jansson, A. M., Mowbray, S. L., Karlen, A. and Larhed, M. (2011) Synthesis of functionalized

cinnamaldehyde derivatives by an oxidative Heck reaction and their use as starting materials for preparation of Mycobacterium tuberculosis 1-deoxy-D-xylulose-5-phosphate reductoisomerase inhibitors. *J. Org. Chem.* **76**, 8986-8998.

Reed, L. J. and Muench, H. (1938) A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol.* **27**, 493-497.

Schultz, J. C., Hilliard, A. A., Cooper, L. T., Jr. and Rihal, C. S. (2009) Diagnosis and treatment of viral myocarditis. *Mayo Clin. Proc.* **84**, 1001-1009.

Seong, I. W., Choe, S. C. and Jeon, E. S. (2001) Fulminant coxsackieviral myocarditis. *N. Engl. J. Med.* **345**, 379.

Shen, Y., Kan, Q. C., Xu, W., Chu, Y. W. and Xiong, S. D. (2009) Coxsackievirus B3 infection induced viral myocarditis by regulating the expression pattern of chemokines in cardiac myocytes. *Iran. J. Allergy Asthma Immunol.* **8**, 1-9.

Swedberg, K., Cleland, J., Dargie, H., Drexler, H., Follath, F., Komajda, M., Tavazzi, L., Smiseth, O. A., Gavazzi, A., Haverich, A., Hoes, A., Jaarsma, T., Korewicki, J., Levy, S., Linde, C., Lopez-Sendon, J. L., Nieminen, M. S., Pierard, L. and Remme, W. J.; Task Force for the Diagnosis and Treatment of Chronic Heart Failure of the European Society of Cardiology (2005) Guidelines for the diagnosis and treatment of chronic heart failure: executive summary (update 2005): The task force for the diagnosis and treatment of chronic heart failure of the European Society of Cardiology. *Eur. Heart J.* **26**, 1115-1140.

van Staden, L. F., Gravestock, D. and Ager, D. J. (2002) New developments in the Peterson olefination reaction. *Chem. Soc. Rev.* **31**, 195-200.

Wang, Y. F., Wang, X. Y., Ren, Z., Qian, C. W., Li, Y. C., Kaio, K., Wang, Q. D., Zhang, Y., Zheng, L. Y., Jiang, J. H., Yang, C. R., Liu, Q., Zhang, Y. J. and Wang, Y. F. (2009) Phyllaemblicin B inhibits Coxsackie virus B3 induced apoptosis and myocarditis. *Antiviral Res.* **84**, 150-158.

Wang, Y. X., da Cunha, V., Vincelette, J., White, K., Velichko, S., Xu, Y., Gross, C., Fitch, R. M., Halks-Miller, M., Larsen, B. R., Yajima, T., Knowlton, K. U., Vergona, R., Sullivan, M. E. and Croze, E. (2007) Antiviral and myocyte protective effects of murine interferon-β and -α_2 in coxsackievirus B3-induced myocarditis and epicarditis in Balb/c mice. *Am. J. Physiol. Heart Circ. Physiol.* **293**, H69-H76.

Youn, H. S., Lee, J. K., Choi, Y. J., Saitoh, S. I., Miyake, K., Hwang, D. H. and Lee, J. Y. (2008) Cinnamaldehyde suppresses toll-like receptor 4 activation mediated through the inhibition of receptor oligomerization. *Biochem. Pharmacol.* **75**, 494-502.

Yuan, J., Bucher, J. R., Goehl, T. J., Dieter, M. P. and Jameson, C. W. (1992) Quantitation of cinnamaldehyde and cinnamic acid in blood by HPLC. *J. Anal. Toxicol.* **16**, 359-362.

Zhang, Y., Cao, W., Xie, Y. H., Yang, Q., Li, X. Q., Liu, X. X. and Wang, S. W. (2012) The comparison of α-bromo-4-chlorocinnamaldehyde and cinnamaldehyde on coxsackie virus B3-induced myocarditis and their mechanisms. *Int. Immunopharmacol.* **14**, 107-113.

Zhao, H., Xie, Y., Yang, Q., Cao, Y., Tu, H., Cao, W. and Wang, S. (2014) Pharmacokinetic study of cinnamaldehyde in rats by GC-MS after oral and intravenous administration. *J. Pharm. Biomed. Anal.* **89**, 150-157.