Soluble Coxsackievirus B3 3C Protease Inhibitor Prevents Cardiomyopathy in an Experimental Chronic Myocarditis Murine Model

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ABSTRACT

Background: Coxsackievirus B3 (CVB3) is a common cause of myocarditis and dilated cardiomyopathy. CVB3 3C protease (3CP) cleaves the viral polyprotein during replication. We tested whether a water soluble 3CP inhibitor (3CPI) had antiviral effects in a chronic myocarditis model.

Methods: Chronic myocarditis was established using DBA/2 strain mice. Starting on post-infection (p.i) day 3, CVB3-infected mice (n = 41) were treated with 3CPI by daily intraperitoneal (i.p.) injection at a concentration of 50 μM (1.7 mg/kg/day) per day for 3 consecutive days. Additional mice (n = 49) were injected with PBS as a control.

Results: The 5-week survival rate was significantly higher with 3CPI treatment (82.3% versus 47.9%; P < 0.05). Organ virus titers at day 3 and 7 and myocardial damage were significantly lower in 3CPI-treated mice. Echocardiography at day 31 indicated strong protection of heart function by 3CPI (FS, 51.2 ± 1.5 versus 26.1 ± 1.5%; P < 0.001). Hemodynamic measurements indicated that 3CPI treatment markedly reduced CVB3-induced LV dysfunction on day 31 (dP/dTmax, 5302 ± 352 versus 4103 ± 408 mmHg/s, P < 0.05; dP/dTmin, −3798 ± 212 versus −2814 ± 206 mmHg/s, P < 0.01).

Conclusions: Water soluble 3CPI was delivered through i.p. injection after CVB3 infection. This agent preserved heart function and decreased organ viral titers and myocardial damage. Soluble 3CPI may be beneficial in the treatment of cardiomyopathy associated with enterovirus infection.

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1. Introduction

Enteroviruses of the Picornaviridae family, particularly Coxsackievirus B3 (CVB3), are considered among the most common etiological agents of myocarditis. Although most enterovirus infections are subclinical, acute myocardial inflammation can induce severe arrhythmias and sudden cardiac death, and may lead to the development of a chronic myocarditis and dilated cardiomyopathy (DCM) (Cooper, 2002). We reported previously on the antiviral effects of several possible drugs in an experimental model of CVB3-induced acute myocarditis in Balb/C mice. IL-1 receptor antagonist protein (IRAP), Coxsackie and Adenovirus receptor (CAR) antagonist protein gene therapy, and 3C protease inhibitor (3CPI) were tested in a model of acute severe myocarditis in the Balb/C strain (Lim et al., 2002, 2006; Yun et al., 2012). We also reported on the antiviral activity of water-insoluble CVB3 3CPI dissolved in 100% dimethyl sulfoxide (DMSO), which was delivered to the subcutaneous region by an implanted micro-osmotic pump in Balb/C mice. This formulation of 3CPI effectively suppressed CVB3-induced myocardial damage and reduced mortality (Yun et al., 2012), but we encountered limitations in the delivery of water-insoluble 3CPI and the timing of drug administration after CVB3 infection.
Experimental models of chronic CVB3-induced myocarditis in various mouse strains have been used to explore the pathogenesis of human myocarditis (Herskowitz et al., 1985; Huber and Lodge, 1986; Khatib et al., 1987; Koide et al., 1992). Among these, the DBA/2 strain offers a specific model of human chronic myocarditis characterized by delayed virus replication and propagation in the heart, because susceptibility to viral infection is lower than that of Balb/C. The correlation between viral replication and myocardial damage has been studied at the onset of chronic murine CVB3-induced myocardial infection (Kandolf et al., 1993). The Balb/C model has a very short viremic phase, making it impossible to deliver the drug at the appropriate time (Lim et al., 2002). To overcome the limitations of the acute Balb/C mouse model and water-insoluble 3CPI for delivery, we re-established a chronic CVB3 myocarditis model using DBA/2 mice and produced water-soluble 3CPI (LDD1588) by modifying the 3CPI-9b chemical structure (Yun et al., 2012). In this report, we show that the water-soluble 3CPI, which was injected intraperitoneally for 3 days from post-infection (p.i.) day 3, had strong antiviral effects. 3CPI markedly inhibited virus replication, which was reflected in decreased virus titers in various organs, and less myocardial damage, and subsequently reduced mortality. 3CPI treatment also prevented the progression from post-myocarditis remodeling to DCM.

2. Materials and methods

2.1. Virus

CVB3 was derived from the infectious cDNA copy of the cardiotropic H3 variant of CVB3 (CVB3-H3). Viral titer was determined by plaque-forming assay in HeLa cells as described previously (Knowlton et al., 1996; Lim et al., 2012).

2.2. Development of water-soluble 3CPI

We previously reported a strong anti-enteroviral effect of a 3CPI (Yun et al., 2012) that had to be dissolved in DMSO. To improve solubility, we modified the R-configuration of a previously water-insoluble 3CPI (9b structure) (Fig. 1A) (Kim et al., 2012).

2.3. Screening water soluble 3CPIs

We confirmed the solubility of water-soluble 3CPIs and antiviral efficiency in an in vitro cell culture system. Briefly, $5 \times 10^5$ HeLa cells were infected with CVB3-H3 at an m.o.i. of 1 (multiplicity of infection = 1). CVB3-H3 and the modified 3CPIs were added. After 24 h incubation, 15 mL of the cell proliferation reagent supplied with the Cell Counting Kit 8 (Dojindo Labs, Tokyo, Japan) was added, and the cells were incubated for an additional 2 h. Absorbance at 450 nm was then measured on a microplate reader. Uninfected HeLa cells were used as a control and the values for the controls were set arbitrarily to 100. The data are presented as mean ± SEM from three independent experiments (Yun et al., 2012). The 3CPI (S2) chemical was selected as the best candidate for the in-vivo experiments and named LDD1588 (Fig. 1B).

2.4. Murine chronic viral myocarditis model

The animal experimental protocols used in this study conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Samsung Biomedical Research Institute (SBRI). SBRI is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.
Fig. 2. Chronic myocarditis model in DBA/2 mice. (A) Changes of organ viral titers by PFU assay. Organ viral titers were highest at day 7 and infectious virus was detected on p.i. day 21. (B) Organ injuries estimated by TnI and AST/ALT using pooled sera at p.i. days 3, 7, 14 and 31. Serum TnI level was highest at day 7 and remained elevated during the experimental time period up to p.i. day 31. AST/ALT levels were elevated to day 21 in a pattern that was delayed compared to TnI. (C) Cardiac inflammation was observed by HE stain (a–d), and myocyte membrane disruption and death was confirmed by von Kossa (e–h) and Evans blue stain (i–l). Virus replication was represented by pancreatitis (m–p). Scale bars indicate 100 μm (a–h and m–p) or 200 μm (i–l). Data are presented as the mean plus or minus the standard error of the mean from three independent experiments.

International and abides by the Institute of Laboratory Animal Resources guide.

In acute myocarditis model as in Balb/C mice, infectious virus is detected by day 10 post-infection. The mortality by 3 weeks is over 90%, and mice usually died before developing the heart failure phenotype. To develop post infectious dilated cardiomyopathy phenotype, we used the DBA/2 strain instead of the Balb/C strain. Five-week-old male DBA/2 mice were inoculated on day 0...
by intraperitoneal injection of $2 \times 10^3$ plaque-forming units (PFUs) of CVB3-H3. Mice were euthanized via cervical dislocation after anesthesia by ketamine injection (100 mg/kg) and the serum and various organs (heart, liver, spleen, and pancreas) were collected on p.i. days 3, 7, 14, 21, and survived mice were leaved to observe the heart failure development at days 31. To evaluate myocardial damage, mice were injected with Evans blue dye for 8 h before euthanasia. Viral titer was measured by a plaque-forming assay in samples from the heart and pancreas. Cardiac damage and liver damage were assessed by measuring the levels of troponin I (Tnl), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in the serum using the Fujifilm Dri-Chem 3000 system according to the manufacturer’s instructions (Fujifilm, Seoul, Korea).

2.5. Histopathology and organ viral titers

The heart and pancreas were collected and viral titers measured on days 3, 7, 14, and 21 after intraperitoneal (i.p) CVB3-H3 infection. The basal parts of the hearts were homogenized in DMEM medium with 5% fetal bovine serum, and supernatant viral titers were measured by plaque-forming assay. The apical parts of the hearts were fixed in 10% formalin, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin–eosin (H&E) for inflammation detection or Picrosirius red and von Kossa staining for fibrosis and myocardium disruption. The extent of inflammation was graded in the tissue sections, and the percent area of myocardial fibrosis was evaluated using the NIH image quantification method (Lim et al., 2008).

2.6. Antiviral effect of 3CPI in the DBA/2 myocarditis model

To test the antiviral effects of water-soluble 3CPI (LDD-1588), either 3CPI at 50 μM (1.7 mg/kg/day) or saline alone (control) was injected i.p. for 3 consecutive days from p.i. day 3 (CVB3 + 3CPI group, $n=41$; CVB3 group, $n=49$). Mice were euthanized via cervical dislocation after anesthesia by ketamine injection (100 mg/kg), and the serum, heart, liver, spleen, and pancreas were collected as described above. These mice (CVB3 group, $n=4$; CVB3 + 3CPI group, $n=5$ for each day) were excluded from the survival analysis. Mice were monitored two times a day.

2.7. Mouse echocardiography

M-mode echocardiograms were performed in CVB3-H3 infected mice treated with or without 3CPI. Adult mice were anesthetized with 1% isoflurane on p.i. day 31 and then subjected to echocardiography as described previously (Lim et al., 2008; Xiong et al., 2007).

2.8. Hemodynamic measurements

At 31 days after CVB3 infection with or without water-soluble 3CPI, hemodynamic measurements were made using a microtip
pressure–volume (PV) catheter (SPR-838, Millar Instruments; Houston, TX, USA). Briefly, mice were anesthetized using a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). The animals were intubated with a blunt 21-gauge needle using the tracheotomy method and ventilated with a custom-designed constant-pressure ventilator at 75 breaths/min using room air. An anterior thoracotomy was performed, and a small apical stab was made with a 27-gauge needle to expose the left ventricular (LV) apex. The microtip catheter was inserted retrogradely into the LV cavity along the cardiac longitudinal axis until stable PV loops were obtained. Mice were euthanized via cervical dislocation after recording. Loops were acquired after 20 min of stabilization with the ventilator turned off for 5–10 s. The sampling rate was 1000/s using the ARIAX P–V conductance system (Millar Instruments) coupled to a PowerLab 16/30A/D converter (AD Instruments, Mountain View, CA, USA).

2.9. Statistics

The data are presented as the mean ± SEM. Differences in measured parameters between control and target groups were examined using the Mann–Whitney nonparametric t-test (GraphPad Prism3.0 for Windows, GraphPad Software, La Jolla, CA, USA). Survival rates were analyzed using the Kaplan–Meier method. A P-value < 0.05 was considered significant.

3. Results

3.1. Phenotype of chronic viral myocarditis in DBA/2 mice

We re-established a CVB3-induced chronic viral myocarditis model in the DBA/2 strain. Viral titers peaked in the heart and pancreas on p.i. day 7 and decreased quickly (Fig. 2A), suggesting that virus proliferation was delayed in the DBA/2 strain compared with the Balb/C strain, in which virus titers peak on p.i. day 3. Changes in serum TnI level were similar to those of the heart viral titers up to p.i. day 21, but serum TnI level remained elevated to the end of the experiment on p.i. day 31. AST/ALT levels were elevated to p.i. day 21, but the pattern was delayed compared to TnI (Fig. 2B), possibly reflecting ongoing organ damage or remodeling after viral injury in the heart and liver. Histological myocardial damage as assessed by Evans blue stain (Fig. 2C, i–l) and necrotic lesions with calcium (Ca²⁺) uptake continued expanding through p.i. day 21 (as assessed by H&E stain (Fig. 2C, a–d) and von Kossa stain (Fig. 2C, e–h)). Taken together, these findings demonstrate that pathological changes in the hearts of CVB3-infected DBA/2 mice progressed to DCM through a remodeling process.

3.2. Survival curves and echocardiography in a DBA/2 myocarditis model

The survival rate of CVB3-H3 infected DBA/2 mice on p.i. day 31 was 52% (9/20). Mice died between p.i. days 10 and 20 (Fig. 3A). We performed echocardiography to evaluate heart size and function on p.i. day 31. The LV internal dimension in systole (LVIDs), diastole (LVIDd), and fractional shortening (FS) were measured in CVB3-infected DBA/2 mice and control mice. CVB3-infected DBA/2 mice had larger LVIDs and LVIDd and smaller FS compared to control mice (LVIDs, 1.68 ± 0.15 versus 2.65 ± 0.12, LVIDd, 3.15 ± 0.07 versus 3.59 ± 0.14, FS, 47.16 ± 3.78 versus 26.32 ± 1.1 in CVB3-infected and control mice, respectively (n = 5)) (Fig. 3B).
3.3. Antiviral effect of 3CPI in the chronic myocarditis model

The 5-week survival rate of the CVB3-infected control group was 47.9%. The survival rate was significantly higher in the CVB3 + 3CPI group than in the CVB3 group (82.3% versus 47.9%; *P* < 0.05) (Fig. 4A). In this DBA/2 myocarditis mouse model, viral titers in the heart and pancreas were highest on p.i. day 7, and the organ viral titers were significantly lower in CVB3 + 3CPI group than in the untreated controls (heart, 3.8 ± 0.1 versus 8.7 ± 0.1 log PFU/mg; *P* < 0.05; pancreas, 5.7 ± 0.1 versus 9.0 ± 0.1 log PFU/mg; *P* < 0.05) (Fig. 4B).

3.4. Histopathology

Myocardial inflammation and damage were evaluated by H&E and von Kossa staining on p.i. days 7 and 21. On day 7, inflammation and myocardial Ca^{2+} uptake were markedly lower in CVB3 + 3CPI mice than in CVB3 mice (Fig. 5A, a–d). On p.i. day 21, the area of Ca^{2+} deposition was also significantly lower in the CVB3 + 3CPI group (Fig. 5A, e–h and B), but there were no differences in the pancreas (Fig. 5A, i and j). In CVB3 infected mice, the left ventricle was progressively dilated, reflecting post-myocarditic LV remodeling causing the typical phenotype of DCM. This post-myocarditic LV remodeling was inhibited by 3CPI treatments (Fig. 5A, k and l), providing strong evidence that the new water-soluble 3CPI decreased myocardial injury by inhibiting viral proliferation and consequently prevented LV remodeling from progressing to LV dilatation.

3.5. Preservation of heart function by 3CPI after CVB3 infection

Echocardiography on p.i. day 31 also showed a strong preservation of heart function by 3CPI treatment in CVB3-H3 infected. LV function was significantly preserved (FS, 51.2 ± 1.5% versus 26.1 ± 1.5%; *P* < 0.001; *n* = 4 each group) (Fig. 6A). Ninety percent of CVB3-infected mice not treated with 3CPI showed LV dilatation. Hemodynamic measurements using a microtip PV catheter demonstrated that 3CPI treatment markedly reduced CVB3-induced LV dysfunction compared with controls on p.i. day 31 (dP/dTmax, 5302 ± 352 versus 4103 ± 408 mmHg/s; *P* < 0.05; dP/dTmax, −3798 ± 212 versus −2814 ± 206 mmHg/s; *P* < 0.01) (Fig. 6B).

4. Discussion

In this work, we successfully re-established a model of chronic viral myocarditis that can be used to study antiviral drugs for the subacute treatment of viral myocarditis and may be applicable to the clinical course of the condition in humans. In our study, a water-soluble 3CPI, LDD1588, that was injected intraperitoneally during the subacute viremic phase significantly decreased virus proliferation and prevented progression from post-myocarditic LV remodeling to DCM.

Many antiviral drugs have been tested against CVB3-induced myocarditis in Balb/C mice, and the results have suggested that acute virus proliferation induces the cellular immune response, which leads to direct cardiac injury. Although cellular immunity
**Fig. 6.** 3CPI preserved left ventricular function. (A) Echocardiography on p.i. day 31 showed the strong anti-remodeling effect of 3CPI, n = 4 each group (FS, 51.2 ± 1.5% versus 26.1 ± 1.5%; P < 0.001; control, virus non-infected). (B) Hemodynamic measurement was performed by microtip PV catheter. 3CPI administration significantly preserved heart function (dP/dTmax, 5302 ± 352 versus 4103 ± 408 mmHg/s, P < 0.05; dP/dTmin, −3798 ± 212 versus −2814 ± 206 mmHg/s, P < 0.01). Cardiac output displayed normal levels. Data are presented as the mean plus or minus the standard error of the mean from 3 independent experiments. LVIDd, left ventricular diastolic dimension; LVIDs, left ventricular systolic dimension; FS, fractional shortening, NS P > 0.05, *P < 0.05, ***P < 0.001.

initiates acute myocarditis in Balb/C mice, myocyte-reactive antibodies are detected in the serum of infected animals during periods of inflammation and cardiac necrosis (Yun et al., 2012). We previously reported that early inflammatory cytokine receptor blockers and the virus receptor have an antiviral effect (Kim et al., 2006; Lim et al., 2002, 2006). Pinkert et al. (2009) also showed antiviral effects using an adenovirus system that expressed soluble CAR-Fc, but the use of a viral vector system has not been fully established for clinical applications.

We reported previously that CVB3 3CPIs have an antiviral effect against CVB3 and several enteroviruses (Yun et al., 2012). We found that CVB3 3CPI inhibited enterovirus 3C protease activity in vitro, suppressed the proliferation of CVB3 in cell culture, and had a therapeutic effect against acute murine myocarditis. At the same time, we encountered some limitations in transferring the results to clinical applications. First, because of its chemical composition, the initial 3CPI was not water soluble and so could not be injected directly into the body. Second, because Balb/C mice are highly...
susceptible to CVB3 and become too ill to manipulate from p.i. day 3, the antiviral drug needed to be administered at the same time as the virus infection, on day 0. In most clinical situations, an antiviral drug would be administered only after the patient becomes ill. A model of chronic myocarditis with a longer time course was needed to overcome the limitations of this previous model.

To do so, we re-established a chronic myocarditis model using the DBA/2 strain of mice. Susceptibility to Coxsackievirus-induced myocarditis differs considerably between strains of mice (Wolfram et al., 1986). Most strains develop severe acute myocarditis, and only a few special strains of mice develop chronic myocarditis that mimics human viral myocarditis and DCM (Wolfram et al., 1985). Chronic CVB3-induced myocarditis has been described previously in DBA/2 strains with histopathological features similar to those observed in the heart in humans. In our CVB3-infected DBA/2 mouse model, replication of viral particles correlated strongly with pathological changes associated with chronic myocarditis on p.i. day 31. In previous studies, the viral titers in the myocardium were significantly lower in the chronic period than in the acute period, and the myocardium showed persistent CVB3 infection during the chronic period (Klingel et al., 1992; Klingel and Kandolf, 1993; Sato et al., 1994). Our results are consistent with these earlier experimental data (Figs. 2 and 3). In our experiments, about 52% of DBA/2 mice survived until p.i. 31 days. Echocardiography showed that the LV chamber was dilated, as seen typically in DCM as a result of post-myocarditic remodeling. The serum TNF level peaked at the same time as the organ viral titers on p.i. day 7. TNF level increased again on p.i. day 31, indicating persistent myocardial damage. These findings strongly suggest that CVB3-induced myocarditis had progressed from post-myocarditic remodeling to DCM in this DBA/2 mouse model.

Administration of the water-soluble 3CPI (LDD1588) in this DBA/2 mouse model of chronic myocarditis prevented CVB3 proliferation and post-myocarditic remodeling, and preserved heart function. These findings suggest that the water-soluble 3CPI, LDD1588, has potential in clinical antiviral treatment. 3CPI can be delivered intraperitoneally on p.i. day 3 and significantly inhibits virus proliferation in the heart and pancreas. Reducing myocardial damage by limiting viral proliferation in the heart might protect against progression from post-myocarditic LV remodeling to DCM.

In summary, we re-established the chronic CVB3 myocarditis model in DBA/2 mice and produced water-soluble 3CPI (LDD1588). The water-soluble 3CPI was injected intraperitoneally for 3 days from p.i. day 3 and had strong antiviral effects by markedly inhibited virus replication. This agent also decreased virus titers in various organs, myocardial damage, and mouse mortality. In addition, LDD1588 treatment prevented the progression from post-myocarditic remodeling to DCM. These findings strongly suggest that soluble 3CPI may be beneficial in the treatment of cardiomyopathy associated with enterovirus infection.

Conflict of interest

None.

Acknowledgments

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