Antiviral Activity of Coxsackievirus B3 3C Protease Inhibitor in Experimental Murine Myocarditis

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Background. We investigated the efficacy of a 3C protease inhibitor (3CPI) in a murine coxsackievirus B3 (CVB3) myocarditis model. CVB3 is a primary cause of viral myocarditis. The CVB3 genome encodes a single polyprotein that undergoes a series of proteolytic events to produce several viral proteins. Most of this proteolysis is catalyzed by the 3C protease (3CP).

Methods and Results. By way of a micro-osmotic pump, each mouse received 3CPI in 100 mM 3CPI in 100 µL of 100% dimethyl sulfoxide (DMSO) during a 72-hour period. On the day of pump implantation, mice (n = 40) were infected intraperitoneally with 10⁶ plaque-forming units of CVB3. For the infected controls (n = 50), the pump was filled with 100% DMSO without 3CPI. The 3-week survival rate of 3CPI-treated mice was significantly higher than that of controls (90% vs 22%; P < .01). Myocardial inflammation, viral titers, and viral RNA levels were also reduced significantly in the 3CPI-treated group compared with these measures in the controls.

Conclusions. The protein-based drug 3CPI inhibited the activity of 3CP of CVB3, significantly inhibited viral proliferation, and attenuated myocardial inflammations, subsequent fibrosis, and CVB3-induced mortality in vivo. Thus, this CVB3 3CPI has the potential to be a novel therapeutic agent for the treatment of acute viral myocarditis during the viremic phase.

Coxsackievirus B3 (CVB3) is an enterovirus of the picornavirus family and is the most common cause of human viral myocarditis, which can progress to chronic myocarditis and dilated cardiomyopathy [1, 2]. CVB3 has a 7.4-kb positive-stranded RNA genome that is translated as a monocistronic polyprotein. After the expression of 4 capsid viral proteins (VP1–VP4) and 7 nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) during translation, 2 viral proteases, 2A and 3C, cleave the viral polyprotein cotranslationally into mature peptides [3, 4]. These enzymes are attractive targets for the development of therapeutic antiviral agents, because effective therapies for prevention and treatment of the diseases caused by CVB3 infection are not currently available [5].

A previous study reported the antiviral activity and cytotoxicity of a novel inhibitor of the human rhinovirus (HRV) 3C protease (3CP) AG7088 [6]. Because the CVB3 3CP is highly homologous with HRV 3CP in amino acid sequence (approximately 64%), we developed a potent CVB3 3CPI using the backbone protein structure of AG7088. This completely prevented viral proliferation in HeLa cells in vitro at 50 µM [7]. In this study, we administered CVB3 3CPI dissolved in 100% dimethyl sulfoxide (DMSO) to mice using subcutaneously implanted micro-osmotic pumps and evaluated the antiviral activity of the 3CPI against CVB3-induced viral myocarditis. This protein-based 3CPI inhibited the proliferation of CVB3 and reduced myocardial inflammations and deaths from viral myocarditis.
METHODS

Synthesis of the 3CPI
We generated a model for the structure of CVB3 3CP based on information from the structure of HRV 3CP [8, 9] in a complex with the inhibitor AG7088 (Protein Data Bank accession code 1CQQ) [6]. Synthesis of the inhibitor was as described in [7].

Antiviral Activity of 3CPI in Serially Collected Sera From Mice After Micro-Osmotic Pump Implantation
The protocols used in this study conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 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 International and abides by the Institute of Laboratory Animal Resources guide. We anesthetized 6-week-old male BALB/c mice by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). We loaded 50 mM 3CPI in 100 μL of 100% DMSO into micro-osmotic pumps (DURECT Corp), which were designed to deliver 100 μL of solution at 1.0 μL/h for 72 hours. The pumps were implanted subcutaneously in the backs of mice (n = 5). Serum was collected from the retro-orbital venous plexus at 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, and 144 hours. We measured the serum-expression levels of 3CPI using an antiviral assay and by quantifying viral RNA production in vitro. In brief, HeLa cells were infected with CVB-H3 (multiplicity of infection [MOI] = 100). After preincubation for 1 hour, cells were treated with the collected sera at 1% (v/v) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum. After 24 hours of incubation, 15 μL of the cell proliferation detection reagent supplied with the Cell Counting Kit 8 (Dojindo Laboratories) was added and the cells were incubated for a further 2 hours. Light absorbance was measured at 450 nm wavelengths, using a microplate reader (VersaMax, Molecular Devices). We used noninfected HeLa cells as controls and arbitrarily set absorbance to 100. Data are presented as mean plus or minus the standard error of the mean from 3 independent experiments.

Antiviral Activity of 3CPI in Serially Collected Sera: Viral RNA Amplification by Real-Time Polymerase Chain Reaction
To quantify viral RNA (VP1) expression, we performed real-time polymerase chain reaction (PCR) using 5 ng DNA as previously described [10]. HeLa cells were infected with CVB3-H3 virus as previously described [11], CVB-H3 (derived from the infectious cDNA copy of the cardiotropic H3 strain), at an MOI of 100. After preincubation for 1 hours, cells were treated with the collected sera at 1% (v/v) in DMEM supplemented with 5% fetal bovine serum. After 6 hours of incubation, RNA was purified from virus-infected cells with the collected sera (easy-spin total RNA extraction kits, iNtRON Biotechnology). For RNA quantification, we synthesized complementary DNA (cDNA) using 1 μg RNA through a reverse transcription reaction using antisense VP1 primer. Real-time PCR quantitative RNA or DNA analyses were performed in an ABI Prism 7000 Sequence Detection System using the SYBR green fluorescence quantification system (Applied Biosystems) to quantify amplicons. The standard PCR conditions were 95°C for 10 minutes, then 40 cycles at 95°C (30 seconds), 55°C (30 seconds), and 72°C (30 seconds), followed by a standard denaturation curve. Primer sequences were designed using Primer Express software (Applied Biosystems) with nucleotide sequences found in the GenBank database. The primer sequences were as follows: (1) for the internal control gene, glyceraldehyde phosphate dehydrogenase (GAPDH): 5’-GCCAA GGATATCCATGACAAC-T3’ and 5’-CTGCTGGTGTCAGCCA CAGA-3’; (2) for VP1: 5’-CACGCGATCTCGTGATGATTT-3’ and 5’-GTCAGCATGCGTGTACC-TTA-3’.

PCR conditions were optimized with regard to primer concentration, absence of primer—dimer formation, and the efficiency of amplification of target genes and the housekeeping gene control. We used SYBR Green PCR Master Mix (Applied Biosystems), 200 nm specific primers, and 2.5 ng cDNA in each reaction. We based the threshold for positive output on negative controls. For RNA analysis, we used the calculations for determining the relative level of gene expression according to the instructions from the user’s bulletin (P/N 4303859) from Applied Biosystems, by reference to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the sample, using the cycle threshold (Ct) method. The mean Ct values from triplicate measurements were used to calculate the expression of the target gene, with normalization to the GAPDH housekeeping gene (internal control) using the 2−ΔΔCt formula, also according to the user’s bulletin. The levels of negative controls without RNA or DNA and without reverse transcriptase were also calculated.

Murine Viral Myocarditis Model
CVB3 was derived from the infectious cDNA copy of the cardiotropic H3 strain (CVB3-H3). We used an acute viral myocarditis model in mice that represents cell-mediated direct viral toxicity and T-cell-mediated myocardial injury rather than persistent or acute immune response. The mean 3-week survival rate of mice in this model was <30% [11, 12]. We anesthetized 6-week-old male BALB/c mice by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). Micro-osmotic pumps (DURECT) containing either 3CPI in DMSO or DMSO alone (control) were implanted subcutaneously into the backs of BALB/c mice (CVB3+3CPI group, n = 40; CVB3+solvent control group, n = 50). The 3CPI was administered at 50 μM/h. Both groups were infected on day 0 by intraperitoneal injection with 106 plaque-forming units (PFU) of CVB3-H3. Mice were euthanized via cervical dislocation, and
sera and organs were collected on days 3, 7, 14, and 21. These euthanized mice (CVB3+solvent control group, n = 4; CVB3+3CPI group, n = 5 for each day) were excluded from the survival analysis. To exclude the toxic effect of 100% DMSO, we implanted micro-osmotic pumps filled with 100% DMSO alone in the control mice (solvent group; n = 15) and recorded any skin changes and mortality that occurred without CVB3 infection.

Histopathology and Organ Viral Titers
We homogenized the basal parts of the hearts and half of the pancreas in DMEM with 5% fetal bovine serum and determined the supernatant viral titers by PFU assay [11, 12]. The apical parts of the hearts and half of the pancreas were fixed in 10% formalin, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin–eosin or picrosirius red. Sections were graded for inflammation by 2 different investigators using a semiquantitative scale of 0–3 (0 = no myocarditis; 3 = widespread and confluent inflammation). We evaluated the percentage area of myocardial fibrosis by computer-assisted analysis of areas stained with picrosirius red (Image-Pro Plus, Media Cybernetics).

Detection of Positive- and Negative-Strand Viral RNA in the Heart and Pancreas
To quantify positive- and negative-strand viral RNA production, we purified RNA from virally infected hearts and pancreas. For RNA quantification, we synthesized cDNA by putting 1 μg RNA through a reverse transcription reaction using an antisense VP1 primer (for positive-strand RNA) and a sense VP1 primer (for negative-strand RNA). Real-time PCR quantitative RNA or DNA analyses were performed in an ABI Prism 7000 Sequence Detection System using the SYBR green fluorescence quantification system (Applied Biosystems) to quantify amplicons.

Statistics
Data are presented as mean plus or minus the standard error of the mean. Differences in measured parameters between control and target groups were examined using the Mann–Whitney nonparametric U test (SPSS 12.0 for Windows, IBM SPSS). Survival rates were analyzed using the Kaplan–Meier method, and P < .05 was considered statistically significant.

RESULTS
Antiviral Activity of 3CPI in Serial Serum Samples After Micro-Osmotic Pump Implantation
We used in vitro antiviral assay and real-time PCR to evaluate antiviral activity of 3CPI in the serial sera from mice after micro-osmotic pump implantation. In a cell viability test, antiviral activity was detected in the sera 2 hours after micro-osmotic pump implantation, peaked at 72 hours, and lasted for 6 days (Figure 1A). The viral RNA level in infected HeLa cells was significantly reduced by the sera from 2 hours to 6 days after micro-osmotic pump implantation (Figure 1B). No skin necrosis or infection was observed in the implanted site ≥7 days after micro-osmotic pump implantation. There were no deaths, and we found no pathology in the hearts of these mice at autopsy. These data indicate that 3CPI was continuously released without adverse effect for 7 days after micro-osmotic pump implantation.

In Vivo Effect of 3CPI in the Murine Myocarditis Model
Only 22% of the CVB3+solvent–treated control mice survived to 3 weeks versus 90% of the CVB3+3CPI group (P < .0001; Figure 2). In the solvent-only treatment control group, all the mice survived up to the time of euthanasia and had no evidence of skin necrosis. Thus, 3CPI had a protective effect against the mortality caused by viral myocarditis.

Figure 1. Antiviral effect of coxsackievirus B3 (CVB3) 3C protease inhibitor (3CPI) in the serum by in vitro antiviral assay (A) and real-time polymerase chain reaction (B). After micro-osmotic pumps containing 3CPI in 100% dimethyl sulfoxide were implanted subcutaneously in the backs of BALB/c mice without CVB3 infection, serum was collected from the retro-orbital venous plexus serially. a, Antiviral activity was detected in the sera at 2 h after micro-osmotic pump implantation, peaked at 72 h, and lasted for 6 d. b, Viral RNA production was significantly inhibited in the sera from 2 h to 6 d after micro-osmotic pump implantation. Noninfected HeLa-UVM cells were used as controls, and absorbance was set arbitrarily to 100. Data are presented as the mean plus or minus the standard error of the mean from 3 independent experiments.
Histopathology of Inflammation and Fibrosis
Myocardial inflammation in the CVB3+3CPI group on day 7 was markedly decreased compared with that in the CVB3+solvent controls (mean scores of 1.8 ± 0.45 vs. 3.0 ± 0.0; P < .05; Figure 3a). The area of myocardial fibrosis was significantly decreased in the CVB3+3CPI group compared with that of the controls (3.9% ± 1.8% vs. 8.5% ± 1.3%; P < .05). The relative area of myocardial fibrosis on day 14 was significantly less than on day 21 in both groups, but that of the CVB3+3CPI group was significantly less than that of the control group (2.3% ± 0.7% vs. 3.7% ± 0.4%; P < .05; Figure 3b). The viral titers in hearts at day 3 were not significantly different between groups. However, on day 7, viral titers in the hearts were significantly lower in the CVB3+3CPI group than in controls (3.2 ± 0.0 log_{10} PFU/mg vs. 4.1 ± 0.1 log_{10} PFU/mg heart, P < .05; Figure 3a). In the pancreas, viral titers in the CVB3+3CPI group were significantly lower than titers in the controls on days 3 and 7 (day 3, 8.4 ± 0.0 log_{10} PFU/mg vs. 9.7 ± 0.1 log_{10} PFU/mg; day 7, 3.9 ± 0.0 log_{10} PFU/mg vs. 4.6 ± 0.0 log_{10} PFU/mg; P < .05; Figure 4b). Regarding pancreatic pathology, we observed massive pancreatic exocrine gland destruction and inflammatory cell infiltrations in both groups on day 7. These pathologic changes were recovered relatively faster in the CVB3+3CPI group than in controls on day 21 (Supplementary Figure 1). Both positive- and negative-strand viral RNA levels in the hearts and pancreas were significantly reduced in the CVB3 3CPI group on days 3 and 7 (Figure 4b). Viral titers and viral RNA production in the heart and pancreas were too low to be detected on days 14 and 21.

DISCUSSION
We found that 3CPI was released effectively through subcutaneous micro-osmotic pumps implanted in the backs of mice. Importantly, CVB3 3CPI, which is able to inhibit the proliferation of CVB3 in cell cultures, exhibited a therapeutic effect against murine myocarditis. The CVB3 3CPI released in the sera of mice inhibited viral proliferation in the heart and pancreas, decreased myocardial inflammation, prevented subsequent fibrosis, and improved the most important biological consequence, the survival rate.

During virus infection, viral RNA is translated into monocistronic polyproteins using the host’s translational machinery. These polyproteins are processed subsequently mainly by viral proteases 2A and 3C into individual mature structural and nonstructural proteins, which are essential for viral replication and assembly. Thus, viral proteases 2A and 3C play critical roles in the viral life cycle. The proteases of picornaviruses are also actively involved in viral pathogenesis by cleavage of host proteins, such as translation and transcription initiation factors [13–15], signaling molecules, and structural proteins [16–18]. In particular, viral protease 3C reportedly cleaves and inactivates numerous transcription factors, including the cyclic adenosine monophosphate response element–binding protein, the octamer-binding transcription factor, the cellular TATA-binding protein, and the histone protein H3, leading to profound shutdown of the host-cell transcriptional and translational machinery [19–21]. A previous study suggested that viral protease 3C induces apoptosis through the upregulation of the Bax protein and the cleavage of the Bid protein, resulting in the release of cytochrome c from mitochondria into the cytoplasm of infected cells [22]. Therefore, 3CPI might effectively improve survival rates and reduce inflammation because it inhibits the 3CP function that induces apoptosis.

Because 3CPI inhibits posttranslational viral activity, it did not block the entrance of the virus, which uses the coxsackie and adenovirus receptor and the decay-accelerating factor. Although antiviral activity was shown in sera at 2 hours after 3CPI administration, it was difficult to establish whether 3CPI could block virus proliferation in the organs (Figure 4), because 3CPI did not show any organ preference: In contrast, CVB3-H3 is highly cardiotoxic [11, 12]. Because 3CPI is soluble only in DMSO, we could not attain higher serum levels of 3CPI because of the risk of solvent toxicity. Therefore, if 3CPI could be modified to a water-soluble form by chemical engineering, the proliferation of CVB3 in the heart and pancreas might be inhibited completely.

We have tested the antiviral activity of 3CPI against other enteroviruses, especially coxsackievirus A24 (CVA24). CVA24 is responsible for most cases of acute hemorrhagic conjunctivitis, which is characterized by conjunctivitis, keratitis, foreign body sensation, and pain, but respiratory symptoms and severe neurological symptoms similar to those caused by poliovirus (acute flaccid paralysis) also have been reported [23]. We also found that 3CPI significantly prevented the proliferation of CVA24 by an in vitro antiviral activity assay (data not shown). Thus, CVB3 3CPI has potential as a therapeutic agent against...
Figure 3. Histopathology of inflammation and fibrosis. A, 3C protease inhibitor (3CPI)–attenuated myocardial inflammation and fibrosis. Myocardial inflammation (i–iv) and fibrosis (v–vi) were significantly reduced in the treatment group. B, The percent area of myocardial fibrosis on day 14 was significantly less than on day 21 in all groups, but that in the coxsackievirus B3 plus -3CPI group was still significantly less than that in the control group. i–iv, hematoxylin-eosin staining, magnification ×40 (i, ii), ×400 (iii, iv); v and vi, picrosirius red staining, magnification ×200 (*P < .05).
Enteroviruses other than CVB3 in that members of the genus Enterovirus, which also includes poliovirus and echovirus, have a highly conserved region in the 3CP sequence.

The current management of human viral myocarditis depends on supportive therapy to prevent systolic dysfunction [1]. Inhibition of viral infections, which are major contributors to early direct myocardial damage [16], may be very effective during the viremic phase as another therapeutic option [24]. Because of the rapid deteriorating and fatal nature of human viral myocarditis, blocking any viral infection should be started as soon as possible. We have shown here that CVB3 3CPI, released effectively into mice using a micro-osmotic pump, could prevent the proliferation of coxsackievirus and reduce mortality from viral myocarditis.

Figure 4. Viral titers and viral clearance in the organs. A, 3C protease inhibitor (3CPI)–attenuated heart and pancreas viral titers. In the hearts, the viral titers on day 3 were not significantly different between groups. On day 7, viral titers were significantly lower in the coxsackievirus B3 (CVB3) plus 3CPI group than in controls. In the pancreas, viral titers in the CVB3+3CPI group were significantly lower than in the controls both on days 3 and 7.

B, Viral RNA productions by real-time polymerase chain reaction. Both positive- and negative-strand viral RNA in the heart and pancreas were significantly reduced in the CVB3+3CPI group. Data are presented as mean plus or minus the standard error of the mean from 3 independent experiments (*P < .05). Abbreviation: PFU indicates plaque-forming units.
In conclusion, CVB3 3CPI could be used as a potential novel therapeutic agent for the treatment of acute human viral myocarditis during the viremic phase. However, further experiments are needed to determine more accurately the concentration of 3CPI released in sera and to modify the structure of 3CPI with the aim of attaining higher sera levels without producing solvent toxicity.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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