ORI2 Inhibits Coxsackievirus Replication and Myocardial Inflammation in Experimental Murine Myocarditis

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We purified ORI2 [3-(3,4-dihydroxyphenyl)acrylic acid 1-(3,4-dihydroxyphenyl)-2-methoxycarbonylethyl ester] from an extract of the plant Isodon excisus. We tested the antiviral effect of ORI2 in a coxsackievirus-induced myocarditis model. Coxsackievirus B3 (CVB3) is a common cause of myocarditis and dilated cardiomyopathy. Activation of extracellular signal-regulated kinase (ERK) and Akt signaling in virus-infected cells is essential for CVB3 replication. Antiviral compounds were screened by HeLa cell survival assay. Several purified natural compounds were added to HeLa cells cultured in 96-well plates for 30 min after 1 multiplicity of infection (m.o.i) CVB3 infection. ORI2 significantly improved HeLa cell survival in a dose-dependent manner. For in vivo studies, BALB/c mice (n=20) were infected with CVB3, then 10 of the mice were treated by daily intraperitoneal injections of ORI2 (100 mM) for 3 consecutive days. ORI2 treatment significantly improved early survival in the treated mice compared to untreated mice (85% vs. 50%, respectively). Organ virus titers and myocardial damage were significantly lower in the ORI2-treated mice than in untreated mice. These results demonstrate that ORI2, delivered by intraperitoneal injection after CVB3 infection, has a significant antiviral effect by markedly inhibiting virus replication, resulting in a decrease in organ virus titer and myocardial damage. ORI2 may be developed as a potential therapeutic agent for the treatment of CVB3 infections.

Key words coxsackievirus B3; myocarditis; Isodon excisus; antiviral effect

Coxsackievirus B3 (CVB3) is a cardiotropic virus that binds to the coxsackie-adenovirus receptor to infect host cells. CVB3 is a member of the family Picornaviridae, and along with polioviruses, it belongs to the Enterovirus genus. Although most enterovirus infections are subclinical, acute myocardial inflammation triggered by these infections can induce severe arrhythmias and sudden cardiac death, or may lead to the development of chronic myocarditis and dilated cardiomyopathy.2–8 We previously reported several antiviral drugs developed from small chemical compounds. An inhibitor of enterovirus protease 3C (3CPI) showed a particularly strong antiviral effect in a murine viral myocarditis model. It significantly reduced viral replication in the heart and myocardial damage. Moreover, survival of CVB3-infected mice was dramatically increased by 3CPI treatment.9 These results suggested that inhibition of virus replication could be an effective method for anti-enteroviral therapy. To discover new anti-enterovirus agents, we have screened natural compounds for their ability to inhibit enterovirus replication and to regulate cell survival signaling molecules such as Akt. We found that ORI2 [3-(3,4-dihydroxyphenyl)acrylic acid 1-(3,4-dihydroxyphenyl)-2-methoxycarbonylethyl ester], isolated from Isodon excisus, inhibits the effects of CVB3 on HeLa cells and has a particularly strong antiviral effect in a HeLa cell survival assay. I. excisus is widespread in Korea and has been used in traditional folk medicine for detoxification and gastrointestinal disorders.10 However, the biological effects of ORI2 are poorly characterized.

ORI2 significantly inhibited virus replication with inhibition of Akt signaling. In the CVB3 myocarditis model, virus titer in the heart and pancreas, and myocardium damage were dramtically decreased in the CVB3+ORI2 treated group compared to the CVB3 group. These findings suggest that ORI2 has as strong potential to be developed as a drug for anti-enterovirus therapy.

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Virus and Cell Lines
Coxsackievirus B3 was derived from an infectious cDNA copy of the cardiotropic H3 variant of CVB3. The virus titer was determined by the plaque-forming assay in HeLa cells as we described previously. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. HeLa-UVM cells were obtained from Dr. E. S. Jeon (Samsung Medical Center, Seoul, Korea). 11)

Purification of a Various Natural Compounds
More than 100 natural compounds were purified from plant as followed previous report.12) In brief, dried whole I. excisus plants (2.5 kg) were percolated with MeOH for 2 weeks. The residue obtained after removal of the solvent (45 g) was diluted with H2O (1 L) and extracted three times with 1 L of EtOAc. Final purification was achieved by HPLC on a C18 column; fractions were eluted with a gradient of acetonitrile in H2O, yielding pure compound (5.8 mg). On the basis of the results, 1H-NMR, and 13C-NMR spectral data, the purified compound was identified as [3-(3,4-dihydroxyphenyl) acrylic acid 1-(3,4-dihydroxyphenyl)-2-methoxycarbonylethyl ester] (C19H18O8; molecular weight (MW) 374) (Fig. 1B).

In Vitro Cell Survival Assay
We screened more than 100 compounds using the antiviral activity of various natural compounds by an in vitro cell survival assay. In brief, HeLa cells were infected with 10^4 plaque-forming unit (PFU)/mL CVB3. After pre-incubation for 30 min, cells were treated with natural compounds serially diluted in DMEM supplemented with 5% fetal bovine serum. After 18 h of incubation, 10 μL of the cell proliferation detection reagent supplied with the Cell Counting Kit 8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was added and the cells were incubated for a further 2 h. Light absorbance was measured at 450 nm using a microplate reader (VersaMax; Molecular Devices, CA, U.S.A.). 9) The compound observed more than 70% survival rate compare to without virus infection that was selected for second round experiment.

Western Blot Analysis
Cells were lysed in radio immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 8.0, 0.1% sodium dodecyl sulfate (SDS), 1% NP40, 150 mM NaCl, 0.5% sodium deoxycholate). Aliquots (10 μg) of total cell extracts were loaded onto 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Following gel electrophoresis, proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked in 5% non-fat dry milk solution in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and probed with anti-enterovirus VP1 (1:1000, mouse monoclonal antibody; Novocasta), anti-phospho Akt (Ser473) and total Akt, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (1:1000, rabbit polyclonal antibodies; Cell Signaling). 13,14)

Murine Viral Myocarditis Model
All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Samsung Biomedical Research Institute (IACUC, SBRI-20121227001). SBRI is accredited by the As-
Antiviral Effect of ORI2 in the Myocarditis Model

Either ORI2 (100 µM) or saline alone (control) was injected for 3 consecutive days from day 0 p.i. (CVB3+ORI2 group, n=10; CVB3 control group, n=10). Mice were euthanized at days 3, 7, and 14 p.i. and the heart and pancreas were collected for organ virus titer measurement and histology. Mouse survival was recorded upon 14d the termination of experiment.

Histopathology and Organ Viral Titers The heart and pancreas were collected, and the virus titers were measured on days 3, 7, and 14 p.i. The basal parts of the hearts were homogenized in DMEM medium with 5% fetal bovine serum, and viral titers in the supernatants 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).

Statistics The data are presented as the mean±S.E.M. Differences in measured parameters between control and target groups were examined using the Mann–Whitney non-parametric t-test (GraphPad Prism 3.0 for Windows; GraphPad Software, La Jolla, CA, U.S.A.). Survival rates were analyzed using the Kaplan–Meier method. A p<0.05 was considered significant.

RESULTS

In Vitro Antiviral Effect of ORI2 To find antiviral compound in extracts from plants, we have screened more than 100 compounds using a HeLa cell survival assay. Survival of HeLa cells infected with CVB3 was measured after 2h CCK-8 treatment. In the first screening, ORI2 was found to have an antiviral effect. In particular, ORI2 significantly increased HeLa cell survival in a dose-dependent manner (Fig. 1A). Treatment with 1 mM ORI2 resulted in HeLa cell survival of >90%.

ORI2 Inhibit CVB3 Replication and AKT Signal Activity in HeLa Cells A monolayer of HeLa cells was infected with CVB3 at 1 m.o.i. for 30 min and then treatment with ORI2 at concentrations ranging from 1 µM to 100 µM. After 18h, total protein was extracted and subjected to Western blot analysis. As shown in Fig. 2A, the expression of CVB3 capsid protein VP1 was dramatically reduced by ORI2 treatment in a dose-dependent manner. This result suggests that ORI2 ef-
effectively inhibited CVB3 replication in infected HeLa cells. In a previous report, CVB3 replication was found to be regulated by host cell signaling involving AKT.17) AKT phosphorylation (Ser473) was significantly reduced by ORI2 treatment in a high dose (Fig. 2B), suggesting that this may be the mechanism underlying inhibition of CVB3 replication in HeLa cells by ORI2. But ERK signaling activity was not decreased by ORI2 treatment with CVB3 infection.

Survival Curves and Organ Virus Titer in Myocarditis Model Next we examined the antiviral effect of ORI2 in a mouse myocarditis model. The 2-week survival rate was significantly higher in the CVB3+ORI2 group than in the CVB3-infected control group (85% vs. 50%, respectively; p<0.05) (Fig. 3A). The virus titers in the heart and pancreas were significantly lower in the CVB3+ORI2 group than in the control group at day 3 p.i. (heart, 6.8±0.1 vs. 8.7±0.1 log PFU/mg, p<0.05; pancreas, 7.7±0.1 vs. 9.0±0.1 log PFU/mg, p<0.05) (Fig. 3B).

Histopathology Finding Myocardial inflammation and damage were evaluated by H&E staining on days 3 and 7 p.i. On day 7, inflammation and myocardium damage were markedly lower in CVB3+ORI2 mice than in control mice (Figs. 4a–d). In contrast, pancreatic cell inflammation was similar in ORI2-treated mice and control mice (Figs. 4e–h). Thus, ORI2 may be used as a therapeutic drug for enteroviral myocarditis.

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