Supporting Materials and Method

Instrumentations

The high-performance countercurrent chromatography (HPCCC) instrument was Dynamic Extractions Spectrum HPCCC (Dynamic Extractions, Berkshire, UK). The HPCCC instrument was coupled with an IOTA S 300 pumps (ECOM, Czech Republic), a Sapphire 600 UV-VIS variable wavelength detector (ECOM, Czech Republic), and a Gilson FC 203B fraction collector (Middleton, WI, USA). Identification of isolates was carried out by an AVANCE 500 spectrometer (Bruker, Karlsruhe, Germany) for ^{1}H NMR and ^{13}C NMR, and a 6460 Q-TOF mass spectrometer (Agilent Technologies, CA, USA).

Reagents and plant materials

Organic solvents for HPCCC separation and HPLC analyses were analytical grade and purchased from Daejung-Chemical and Metals Co. Ltd. (Kyunggi-Do, Korea). Ultra purified water was prepared by Millipore Milli-Q water purification system (Millipore, MA, USA). Roots of *Vitisvinifera* were purchased from local herbal drugs market in Seoul, Korea. An NS5B polymerase inhibitor, (sofosbuvir), E2 inhibitor (Bush et al., 2014), and two NS3/4A protease inhibitors (telaprevir and boceprevir) were purchased from PharmaBlock (China, Nanjing).

Antiviral activity in the JFH-1-GFP infectious HCV cell culture system

Antiviral activity assay was performed in 384-well plates in the presence or absence of inhibitors. In brief, Huh-7.5 cells were seeded in 384-well plates at 2.5 x 10^3 cells/well and incubated for 2 h at 37℃ with 3-fold serially diluted test compounds. Afterwards, Huh-7.5 cells were inoculated with HCVcc expressing an NS5A-GFP fusion protein (Hwang et al., 2013). After 3 days of incubation at 37℃cultured cells were fixed with 2% paraformaldehyde in PBS containing10 μg/mL Hoechst 33326 (Sigma) for 30 min at room temperature. HCV RNA replication was analyzed by determining the number of GFP-positive cells using fully automated confocal microscopy (Image press Ultra/Molecular Devices) [200X]. In addition, the total cell numbers was used as marker for cytotoxicity.

Western blot analysis

1.5 X 10^5 cells (Huh7.5-J6/JFH1 or Bart79I) were plated onto a 6-well plate (Costar 3610) and supplemented with DMSO or vitisin B at the indicated concentrations. At 3 days after incubation, whole-cell extracts were prepared in RIPA buffer (150 mMNaCl, 1 % Triton X-100, 1 % deoxycholic acid sodium salt, 0.1 % sodium dodecyl sulfate, 50 mMTris-HCl, pH 7.5, 2 mM EDTA; genDEPOT) containing a cocktail of protease inhibitors (Complete, Roche Diagnostic at a final concentration of 1 tablet per 50 ml RIPA buffer) and quantitated by the Bradford assay (Bio-Rad). Equal amounts of protein were electrophoresed on an SDS–polyacrylamide gel, subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford), and probed with mouse anti-NS3, anti-NS5A, or anti-NS5B antibodies (1:1000, Virostat). A time-response curve was also determined by performing similar western blot analyses at 24, 48, and 72 h after treating cells with 0.5 μM of vitisin B.

Analysis of vitisin B-resistant HCV variants

Huh7.5-Bart79I cells stably harboring a subgenomic GT1b HCV replicon were

cultured in 10cc plate with standard Huh7.5 media containing 1 μM of vitisin B and 375 μg/mL of G418 for 4 weeks. Colonies were picked and propagated until growth had exceeded a 24 well plate flask at approximately 1-2 weeks. Total cellular RNA was extracted using the RNeasy® mini kit (Qiagen) in accordance with the manufacturer's instructions. HCV replicon RNA was reverse-transcribed and amplified with Emerald-Amp GT PCR master mix (Takara), using 10 sets of primers covering the entire non-structural regions of Bart79I. The PCR products were sequenced, and compared to wild-type Bart79I sequence.

Expression and purification of recombinant NS3 protein

Recombinant HCV genotype 1b NS3 helicase was expressed and purified following the reference protocol with some modifications (Hanson, Hernandez, Shadrick & Frick, 2012). The expression plasmid (pET28a/His-NS3) containing helicase of HCV NS3gene from HCV genotype 1b was transformed in *E. coli* BL21 DE3 pLysS and expression induction by 0.5mM IPTG overnight at 22℃. Bacteria were harvested and resuspended in buffer A [50 mM HEPES (pH 7.5), 300mMNaCl, 20mM imidazole and 5% glycerol]. After sonication, the lysate was centrifuged and supernatant was filtered at 4℃. NS3 protein was purified from the supernatant by chromatography through a His-tag purification resin (Roche) and washed with 20 mL of buffer A. Column bound NS3 protein was eluted with buffer B containing 0.5 M imidazole. Fractions containing NS3 were dialyzed in protein storage buffer [25mM HEPES(pH 7.5), 150mMNaCl and 50% glycerol], and verified by SDS-PAGE gelstained with Coomassie Brilliant Blue.

Recombinant HCV GT 1b NS3 helicase which was used in DARTS assay was expressed and purified following the reference protocol with some modifications (Tani et al., 2009). The expression plasmid (pET21a/His-NS3) containing full-length of HCV NS3 gene from HCV GT1b was transformed in E. coli BL21 DE3 pLysS and expression induction by 0.5 mM IPTG overnight at 22℃. Bacteria were harvested and resuspended in buffer A [50 mM HEPES (pH 7.5), 300 mMNaCl, 20 mM imidazole and 5% glycerol]. After sonication, the lysate was centrifuged and supernatant was filtered at 4℃. NS3 protein was purified from the supernatant by chromatography through a HP Ni-NTA column (GE Healthcare) and washed with 10 mL of buffer A. Column bound NS3 protein was eluted with buffer B containing 1 M imidazole. Fractions containing NS3 were dialyzed in protein storage buffer [25 mM HEPES (pH 7.5), 150 mMNaCl and 50% glycerol], and verified by SDS-PAGE gel stained with coomassie brilliant blue. Protein concentration of homogeneous HCV NS3 was determined spectrophotometrically using the Bradford assay.

Recombinant HCV genotype 1b NS3/4A protease which was used in an NS3/4A protease assay was expressed and purified following Du et al. with minor modifications(Du, Hou, Guan, Tong & Wang, 2002). The expression plasmid (pET28a/His-NS3/4A) containing the full-length of HCV NS3/4A gene from HCV genotype 1b was transformed in E. coli Rosetta2 DE3 pLysSand induction was preceded with 0.1mM IPTG overnight at 30℃. The cells were harvested and resuspended in buffer A [50 mM HEPES (pH 7.5), 300 mMNaCl, 20 mM imidazole, and 5% glycerol]. After sonication, the lysate was centrifuged and supernatant was filtered at 4℃. The NS3/4A protein was purified from the supernatant by chromatography through a HP Ni-NTA column (GE Healthcare), washed with 10 mL buffer A and subsequently, column bounded NS3/4A protein was eluted with Buffer B[50 mM HEPES (pH 7.5), 300 mMNaCl, 1M imidazole and 5% glycerol]. Fractions containing NS3/4A were dialyzed in protein storage buffer [25 mM HEPES (pH 7.5), 150 mMNaCl, and 50% glycerol], and NS3/4Avisualized by coomassie brilliant blue staining pattern of SDS-PAGE

gel. The protein concentration of homogeneous HCV NS3/4A was determined spectrophotometrically using the Bradford assay.

DARTS assay

1 μg of recombinant NS3 protein was incubated at 25℃ with either 2 or 4 μM of vitisin B for 1 h on the rocker and pronase was treated for 30 seconds per each sample, respectively (The volume of total reaction solution must maintain below 100 μL). Next, SDS sample buffer was mixed with the reaction solution before boiling samples at 100℃ for 5 min. Samples were electrophoresed on an SDS–polyacrylamide gel, subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford), and probed with a mouse anti-NS3 (1:1000, Virostat).

Fluorometricin vitro NS3/4A protease assay

HCV NS3/4A protease activity was determined fluorometrically with a commercial SensoLyte 520 HCV protease assay kit (ANASPEC, #71145) using a fluorescence energy transfer peptide according to the manufacturer's instructions with some modifications.Boceprevir (Chemical Book #394730-60-0) was used as a positive control. The protease assay was performed in 1x assay buffer in 40 μL reaction volume at 25℃. The enzyme (15 ng) was pre-incubated with compounds without substrate for 15 min at 25℃. The cleavage reaction was started by the addition of 1x substrate and carried out at 25℃ for 10 min. After incubation, fluorescence intensity was measured by a spectrophotometer (SpectraMax M5/Molecular Devices) at 490 nm excitation and 520 nm emission.

Pharmacokinetic study of vitisin B

To assess vitisin B is able to reach its target organ, liver, at a sufficient concentrations, tissue distribution study of vitisin B was carried out. The Vitisin B(dissolved in dimethylsulfoxide: PEG200: normal saline = 2:48:50, v/v) was intraperitoneally injected at a dose of 10 mg/kg to rats. At 60 min (n = 5) and 480 min (n = 5) later, blood from each rat was collected from the carotid artery and then, each rat was euthanized with CO_2. After centrifugation of the blood sample, two 50-μL aliquots of plasma sample were stored at –80℃ until further use in the LC-MS/MS analysis. Following complete systemic perfusion with 0.9% injectable NaCl solution, approximately 1 g of each brain, fat, heart, kidney, liver, lung and spleen was excised, washed with 0.9% injectable NaCl solution and blotted dry with paper tissue. Each tissue was homogenized with four volumes of 0.9% injectable NaCl solution using a tissue homogenizer (Ultra-Turrax, T25, Janke and Kunkel, IKA-Labortechnik, Staufen, Germany). After centrifugation at 9,000 g for 10 min, two 50-μL aliquots of the supernatant were stored in a –80^{o}C freezer until LC-MS/MS. The plasma or tissue concentrations of vitisin B were determined by a previously reported LC-MS/MS method (Choi WK, 2015).

Cytokine assay

Serum from a vitisin B-treated rat (10 mg/kg) after 1 hour (n=4) and 8 hours (n=4) was collected. Serum concentration of TNF and IL-6 were measured using ELISA kits (ELISA MAX^{TM} Deluxe Sets for rat TNF and LEGEND MAX^{TM} for rat IL-6, Biolegend, US) according to the manufacturer's instructions.

HCVpp entry assay

The plasmid pNL4-1 Fluc Re expressing all human immunodeficiency virus proteins except Gag and Pol was described previously (Bartosch, Dubuisson & Cosset, 2003). The plasmid pCon1 E1E2 expresses HCV E1 and E2 glycoproteins. 293T cells were seeded into 10cc plates at a density of cells 5 X 10^6 1 day before transfection. Cells were transfected with 8 μg HIV gag-pol, 8 μg pNL4-1 Fluc Re and 8 μg pCon1 E1E2 plasmid using lipofectamine 2000 (Invitrogen). After transfection (48 h), culture supernatants of transfected cells were collected and centrifuge at 3000rpm for 15min with a 0.45 mm filter (Sartorius stedim). For the HCVpp transduction assay, Huh7.5 cells were seeded into 96-well plates at a density of 1.8 X 10^4 cells per well in 100 μl DMEM containing 10% FBS. 24 h later, HCVppDMEM was replaced with 100 μl with 1mg/mlHexadimethrine-bromide (sigma). After 24 h, HCVpp containing medium was discarded and 100 μl DMEM containing 10% FBS was added to well. At 48 h after incubation, cells were incubated for 3 h at 37 ℃ in the presence of EZ-CYTOX (10 % tetrazolium salt; Dogen) reagent to assess the cytotoxicity. Firefly luciferase activities were measured by using a luciferase reagent (Luciferase assay system; Promega).

HCV replication, assembly, and particle production assay

Huh7.5-J6/JFH-1 cells were seeded into 6-well plates at a density of 3 X 10^5cells per well with DMEM containing 10% FBS. After 24 hr, they are incubated with either DMSO, or increasing concentrations of vitisin B for 3, 6, 12, 24, 48, and 72 hrs. Total RNAs were extracted from treated cells and virus-containing media were harvested at each time point. Naïve Huh7.5 cells were infected with these virus-containing media after concentration with centricon. Total RNAs were extracted from Huh7.5 cells infected with virus-containing media. qRT-PCR analysis was performed to measure effect of vitisin B on HCV replication and virus particle production.

References for supporting information

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