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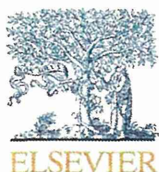
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Specific inhibition of hepatitis C virus entry into host hepatocytes by fungi-derived sulochrin and its derivatives

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ABSTRACT

Hepatitis C virus (HCV) is a major causative agent of hepatocellular carcinoma. Although various classes of anti-HCV agents have been under clinical development, most of these agents target RNA replication in the HCV life cycle. To achieve a more effective multidrug treatment, the development of new, less expensive anti-HCV agents that target a different step in the HCV life cycle is needed. We prepared an in-house natural product library consisting of compounds derived from fungal strains isolated from seaweeds, mosses, and other plants. A cell-based functional screening of the library identified sulochrin as a compound that decreased HCV infectivity in a multi-round HCV infection assay. Sulochrin inhibited HCV infection in a dose-dependent manner without any apparent cytotoxicity up to 50 μM. HCV pseudoparticle and trans-complemented particle assays suggested that this compound inhibited the entry step in the HCV life cycle. Sulochrin showed anti-HCV activities to multiple HCV genotypes 1a, 1b, and 2a. Co-treatment of sulochrin with interferon or a protease inhibitor telaprevir synergistically augmented their anti-HCV effects. Derivative analysis revealed anti-HCV compounds with higher potencies ($IC_{50} < 5$ μM). This is the first report showing an antiviral activity of methoxybenzoate derivatives. Thus, sulochrin derivatives are anti-HCV lead compounds with a new mode of action.

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1. Introduction

Hepatitis C virus (HCV) infection is a major causative agent of chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma [1]. The standard anti-HCV therapy has been a co-treatment with pegylated-interferon (IFN)α and ribavirin, but this therapy is limited by less efficacy to certain HCV genotypes, poor tolerability, serious side effects, and high cost [2,3]. In addition to the newly approved protease inhibitors, telaprevir and boceprevir, a variety of anti-HCV candidates are under clinical development. Although these drugs improve the virological response rate, the emergence of drug-resistant virus is expected to be a significant problem. Moreover, these compounds are expensive due to their complex structure and the many steps required for their total synthesis. To overcome the drug-resistant virus and achieve a long-term antiviral effect, multidrug treatment is essential. Thus, the development of drugs targeting a different step in the HCV life cycle and presumably requiring low cost is urgently needed.

HCV propagates in hepatocytes through its viral life cycle including: attachment and entry (defined as the early step in this study); translation, polyprotein processing, and RNA replication (the middle step); and assembly, trafficking, budding, and release (the late step) (Supplementary Fig. S1). The middle step has been extensively analysed, especially after the establishment of the HCV replicon system [4]. The early step can be analysed with HCV pseudoparticle (HCVpp) [5,6], which is a murine leukemia virus- or human immunodeficiency virus-based pseudovirus carrying HCV E1 and E2 as envelope proteins. The HCV-producing cell culture system (HCVcc) is used for analyzing the whole life cycle [7–9]. In addition, the HCV trans-complemented particle (HCVtcp) system carrying an HCV subgenomic replicon RNA packaged in HCV E1 and E2-containing particles can evaluate the life cycle from the early to the middle step [10]. The majority of anti-HCV agents currently under clinical development, such as inhibitors of protease, polymerase, NS5A, and cellular cyclophilin, inhibit polyprotein processing and/or RNA replication. A desirable approach

Abbreviations: HCV, hepatitis C virus; IFN, interferon; HCVpp, HCV pseudoparticle; HCVcc, HCV derived from cell culture; HCVtcp, HCV trans-complemented particle; MOI, multiplicity of infection; HBs, HBV envelope protein; CsA, cyclosporin A; VSV, vesicular stomatitis virus.

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to achieving efficient multidrug therapy is to identify new antiviral drugs targeting different steps in the viral life cycle. A combination of drugs with different targets can greatly decrease the emergence of drug-resistant virus.

Natural products generally contain more characteristics of high chemical diversity than combinatorial chemical collections, and therefore have a wider range of physiological activities [11,12]. They offer major opportunities for finding novel lead structures that are active in a biological assay. Moreover, biologically active natural products are generally small molecules with drug-like properties, and thus development costs of producing orally active agents tend to be lower than that derived from combinatorial chemistry [13]. In addition, there is a wide variety of natural compounds reported to possess antiviral activity [14,15]. In the present study, we have taken advantage of the potential of natural products by screening a natural product library derived from fungal extracts with a cell-based assay that supports the whole life cycle of HCV.

2. Materials and methods

2.1. Cell culture

Huh-7.5.1 [8] and HepaRG cells [16] were cultured as described previously.

2.2. Natural product library and reagents

Natural products were extracted essentially as previously described [17]. Culture broths of fungal strains isolated from seaweeds, mosses, and other plants were extracted with CH_2Cl_2. The crude extracts were separated by silica gel column chromatography to purify compounds. The chemical structure of each compound was determined by NMR and mass spectrometry analyses. Thus, we prepared an in-house natural product library consisting of approximately 300 isolated compounds.

Cyclosporin A was purchased from Sigma. Bafilomycin A1 and chlorpromazine were purchased from Wako. Heparin was obtained from Mochida Pharmaceutical. IFNα was purchased from Schering-Plough.

2.3. Compound screening

Huh-7.5.1 cells were treated with HCV J6/JFH1 at a multiplicity of infection (MOI) of 0.15 for 4 h. The cells were washed and then cultured with growth medium treated with 10 μM of each compound for 72 h. The infectivity of HCV in the medium was quantified. Cell viability at 72 h post-treatment was simultaneously measured. Compounds that decreased the cell viability to less than 50% of that without treatment were eliminated for further evaluations. Normalised infectivity was calculated as HCV infectivity divided by cell viability. Compounds reducing the normalised infectivity to less than 40% were selected as initial hits. The initial hits were further evaluated for data reproduction and dose-dependency.

2.4. HCVcc assay

HCVcc was recovered from the medium of Huh-7.5.1 cells transfected with HCV J6/JFH-1 RNA as described [7]. HCVcc was infected into Huh-7.5.1 cells at 0.15 MOI for 4 h. After washing out the inoculated virus, the cells were cultured with normal growth medium in the presence or absence of compounds for 72 h. The infectivity of HCV and the amount of HCV core protein in the medium were quantified by infectious focus formation assay and chemiluminescent enzyme immunoassay (Lumipulse II HCV core assay, ortho clinical diagnostics), respectively [7,18].

2.5. Immunoblot analysis

Immunoblot analysis was performed as described previously [19]. The anti-HCV core antibody (2H9) was used as a primary antibody with 1:1000 dilution [7].

2.6. MTT assay

The viability of cells was quantified by using a Cell Proliferation Kit II XTT (Roche Diagnostics) as described previously [20].

2.7. HCV replicon assay

Huh-7.5.1 cells were transfected with an HCV subgenome replicon RNA (SGR-JFH1/Luc) for 4 h and then incubated with or without compounds for 48 h [21]. The cells were lysed with 1xPLB (Promega), and the luciferase activity was determined with a luciferase assay system (Promega) according to the manufacturer's protocol [22].

2.8. HCVpp assay

HCVpp was recovered from the medium of 293T cells transfected with expression plasmids for HCV JFH-1 E1E2, MLV Gag-Pol, and luciferase, which were kindly provided from Dr. Francois-Loic Cosset at Universite de Lyon [5]. Vesicular stomatitis virus pseudoparticles (VSVpp) was similarly recovered with transfection by replacing HCV E1E2 with VSV G.

Huh-7.5.1 cells were preincubated with compounds for 3 h and were then infected with HCVpp in the presence of compounds for 4 h. After washing out virus and compounds, cells were incubated for an additional 72 h before recovering the cell lysates and quantifying the luciferase activity.

2.9. HCVtcp assay

The HCVtcp assay was essentially performed as described [10]. Briefly, Huh-7 cells were transfected with expression plasmids for the HCV subgenomic replicon carrying the luciferase gene and for HCV core-NS2 based on genotype 1a (RMT) (kindly provided by Dr. Michinori Kohara at Tokyo Metropolitan Institute of Medical Science), 1b (Con1), and 2a (JFH-1) [4,10,23] to recover HCVtcp. HCVtcp can reproduce RNA replication as well as HCV-mediated entry into the cells [10].

2.10. Synergy analysis

To determine whether the effect of the drug combination was synergistic, additive, or antagonistic, MacSynergy (kindly provided by Mark Prichard), a mathematical model based on the Bliss independence theory, was used to analyse the experimental data shown in Fig. 3A. In this model, a theoretical additive effect with any given concentrations can be calculated by $Z = X + Y(1-X)$, where X and Y represent the inhibition produced by each drug alone, and Z represents the effect produced by the combination of two compounds if they were additive. The theoretical additive effects were compared to the actual experimental effects at various concentrations of the two compounds and were plotted as a three-dimensional differential surface that would appear as a horizontal plane at 0 if the combination were additive. Any peak above this plane (positive values) indicates synergy, whereas any depression below the plane (negative values) indicates antagonism. The 95% confidence interval of the experimental dose–response was considered to reveal only effects that were statistically significant.

3. Results

3.1. Screening of natural products possessing anti-HCV activity

We extracted culture broths of fungal strains isolated from seaweeds, mosses, and other plants and purified compounds as described in the Section 2 [17]. The chemical structure of each compound was determined by NMR and mass spectrometry analyses. Thus, we prepared an in-house natural product library consisting of approximately 300 isolated compounds. As shown in the Section 2, compounds reducing the normalised HCV infectivity to less than 40% as compared with DMSO were selected as primary hits. The primary hits were then validated by examining the reproducibility, dose-dependency, and cell viability in the HCVcc system. Sulochrin [methyl 2-(2,6-dihydroxy-4-methylbenzoyl)-5-hydroxy-3-methoxybenzoate] (Fig. 1A) was one of the compounds showing the highest anti-HCV activity, and the following analyses focus mainly on this compound.

3.2. Sulochrin decreased HCV infectivity in HCV cell culture assay

To characterise the anti-HCV activity of the compounds, Huh-7.5.1 cells were infected with HCV J6/JFH1 at an MOI of 0.15 and then cultured for 72 h in the presence or absence of compounds. In this system, infectious HCV is secreted into the medium and then re-infects into uninfected cells to support the spread of HCV during a 72 h period (Section 2). Cell cultures were treated with sulochrin or cyclosporin A (CsA) as a positive control in this mul-

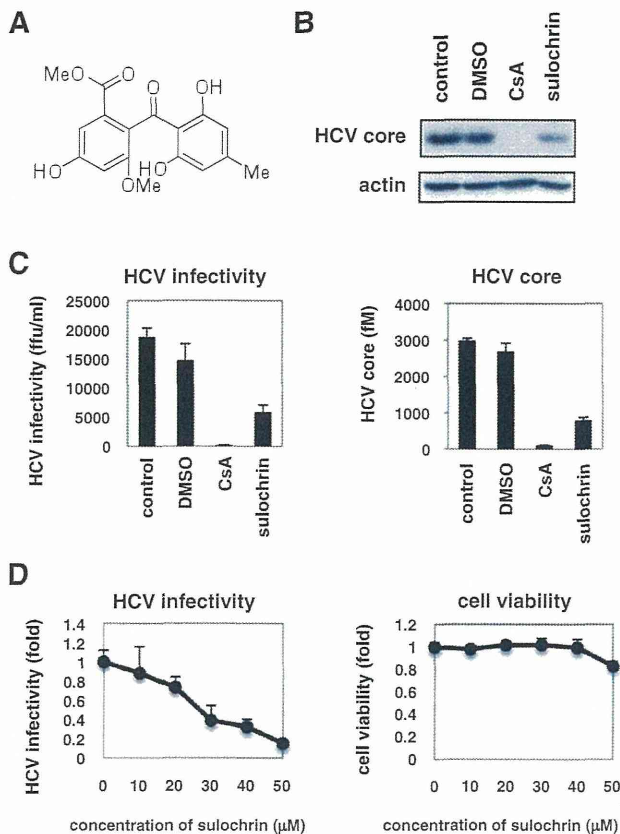


Fig. 1. Sulochrin decreased HCV production in a multi-round HCV infection assay. (A) Chemical structure of sulochrin. (B) Huh-7.5.1 cells were infected with HCV J6/JFH-1 at an MOI of 0.15 for 4 h and then incubated with or without 0.3% DMSO, 2 μM cyclosporin A (CsA), or 30 μM sulochrin for 72 h. The resultant medium was inoculated into naïve Huh-7.5.1 cells to detect intracellular HCV core and actin protein at 48 h postinoculation by immunoblot. (C) HCV infectivity (left) and HCV core protein (right) in the medium as prepared in (B) were quantified as shown in the Section 2. (D) HCV infectivity (left) determined as shown in (C) with varying concentrations (0–50 μM) of sulochrin. Cell viability was examined by MTT assay (right).

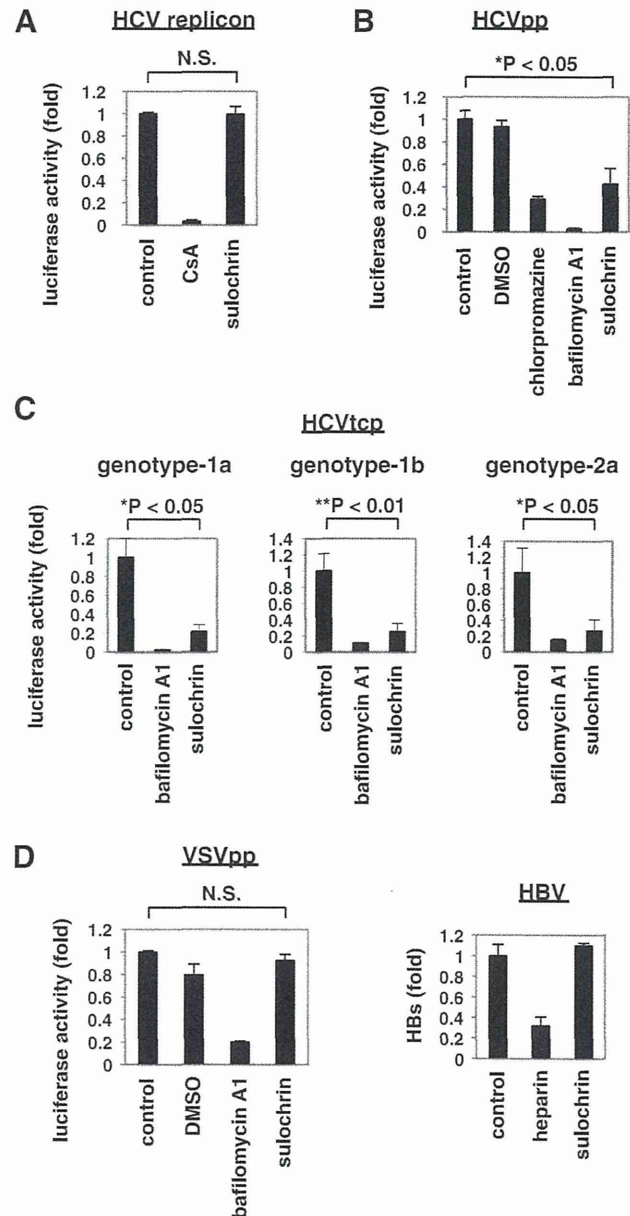


Fig. 2. Sulochrin blocked HCV entry. (A) Replicon assay. Huh-7.5.1 cells were transfected with an HCV subgenomic replicon RNA for 4 h followed by treatment with or without the indicated compounds for 48 h. Luciferase activity driven by the replication of the subgenomic replicon was quantified. (B and C) HCV pseudoparticle (HCVpp) and trans-complemented particle (HCVtcp) assay. Huh-7.5.1 cells were pretreated with the indicated compounds for 3 h and then infected with HCVpp (B) or HCVtcp (C) for 4 h. After washing out virus and compounds, cells were further incubated for 72 h and harvested for measuring luciferase activity driven by the infection of HCVpp or HCVtcp. HCVtcp assay was performed with HCV E1 and E2 derived from genotypes 1a (RMT), 1b (Con1), and 2a (JFH1). (D) Left, the pseudoparticle assay was performed as shown in (B) with VSV G instead of HCV E1 and E2. Right, HBV infection assay. HepaRG cells were pretreated with the indicated compounds for 3 h and then infected with HBV for 16 h. After washing out virus and compounds, cells were incubated for an additional 12 days. HBV infection was evaluated by measuring HBs secretion from the infected cells. Heparin was used as a positive control that inhibits HBV entry.

ti-round infection system. To examine the level of infectious HCV particles produced from the cells, the resultant medium was inoculated into naive Huh-7.5.1 cells to detect HCV core protein in the cells. As shown in Fig. 1B, intracellular production of HCV core but not that of actin was reduced in the cells inoculated with sulochrin- and CsA-treated medium (Fig. 1B). Quantitative analysis showed that sulochrin decreased HCV infectivity and HCV core protein in the medium to 1/3–1/4 of the untreated levels (Fig. 1C). Reduction of HCV infectivity by sulochrin was dose-dependent without serious cytotoxicity up to 50 μM (Fig. 1D).

3.3. Sulochrin blocked HCV entry

We investigated the step in the HCV life cycle that was inhibited by sulochrin. The middle step of the life cycle including translation and RNA replication was evaluated with the transient replication assay by using the HCV subgenomic replicon. Sulochrin had little effect on the replicon activity at doses up to 50 μM (Fig. 2A). In the HCVpp system, which reproduced the early step of HCV infection including entry, sulochrin significantly inhibited HCVpp infection (Fig. 2B). Sulochrin also inhibited the infection of HCVtcp, which reproduced both the viral entry and RNA replication, further supporting that this compound targeted the entry step (Fig. 2C). In contrast, VSV G-mediated viral entry efficiency was not altered by sulochrin treatment (Fig. 2D). Additionally, HBV entry was not inhibited by the presence of sulochrin (Fig. 2D). These data suggest that the inhibitory activity of sulochrin on viral entry is specific to HCV. The anti-HCV entry activity of sulochrin was conserved among different HCV genotypes, 1a (RMT), 1b (Con1), and 2a (JFH-1) [4,10,23] (Fig. 2C).

3.4. Synergistic effect of cotreatment of sulochrin with IFNα or telaprevir

We examined the anti-HCV activity of sulochrin co-administered with clinically available anti-HCV agents, IFNα and a prote-

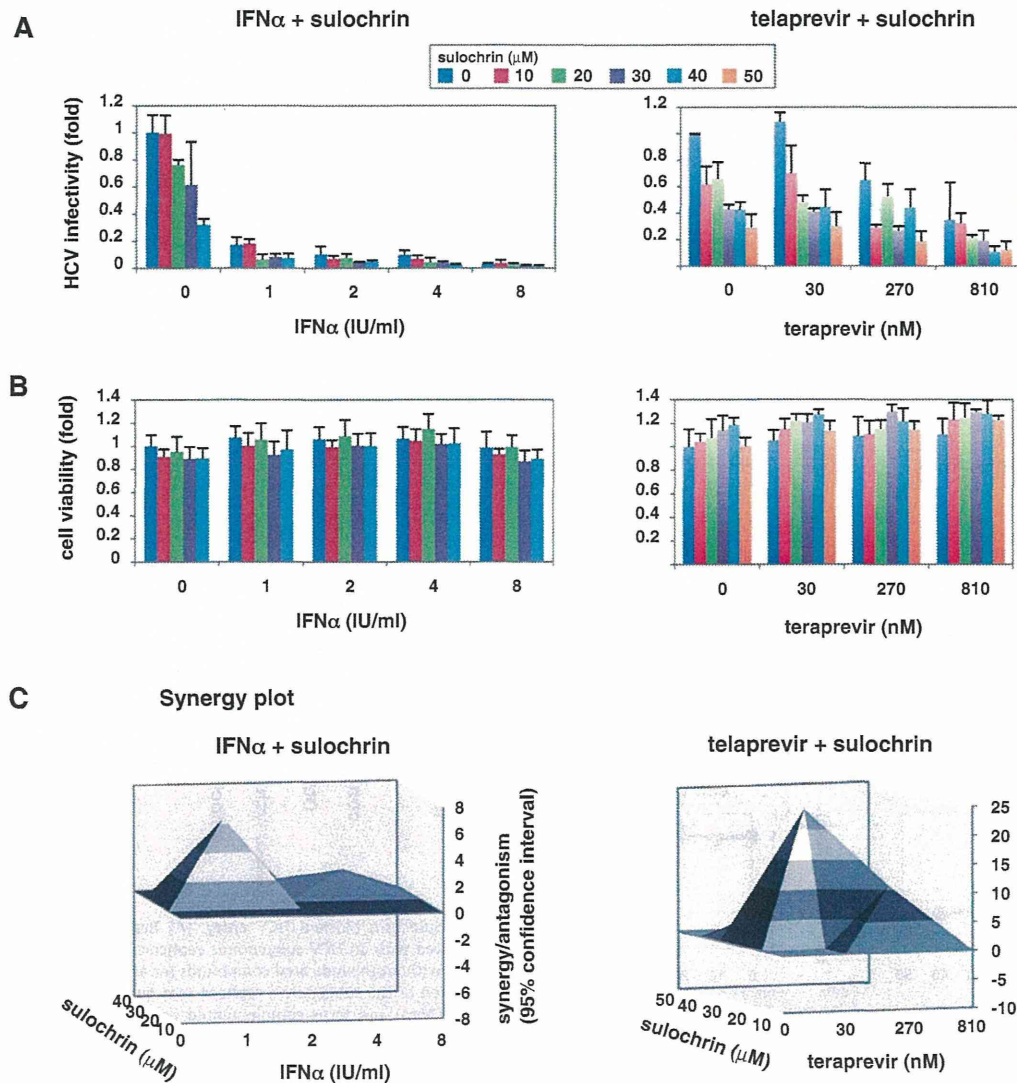


Fig. 3. Cotreatment of sulochrin with IFNα or telaprevir. (A, B) Huh-7.5.1 cells infected with HCV were treated with the indicated concentrations of sulochrin with IFNα (left) or telaprevir (right) to determine HCV infectivity in the medium (A) as shown in Fig. 1C. Cell viability was also quantified (B). (C) Synergy analysis. The results of the combinations shown in (A) were analysed with a mathematical model, MacSynergy, as described in the Section 2. The three-dimensional surface plot represents the difference between actual experimental effects and theoretical additive effects of the combination treatment (95% confidence interval). The theoretical additive effects are shown as the zero plane (dark gray) across the z-axis. A positive value in the z-axis as a peak above the plane indicates synergy, and a negative value with a valley below the plane indicates antagonism. Sulochrin in combination with IFNα (left) or telaprevir (right) produced synergistic antiviral effects that were greater than the theoretical additive effects.

ase inhibitor telaprevir. As shown in Fig. 3, addition of sulochrin with IFNα or telaprevir led to a further decrease in HCV infectivity (Fig. 3A) without significantly enhancing cytotoxicity (Fig. 3B) at any given concentrations. Thus, the combination of sulochrin and IFNα or telaprevir always resulted in a greater reduction in HCV infectivity as compared with that achieved by either agent alone. Synergy/antagonism analysis with the Bliss independence model showed that the experimental anti-HCV activity in combination with sulochrin and IFNα or telaprevir showed a peak above the zero plane in the z-axis, which shows the calculated theoretical additive effect (Fig. 3C). Any peak above the zero plane indicates more than an additive effect, namely, synergy (Section 2). The data clearly indicate that sulochrin had a synergistic anti-HCV effect with both IFNα and telaprevir.

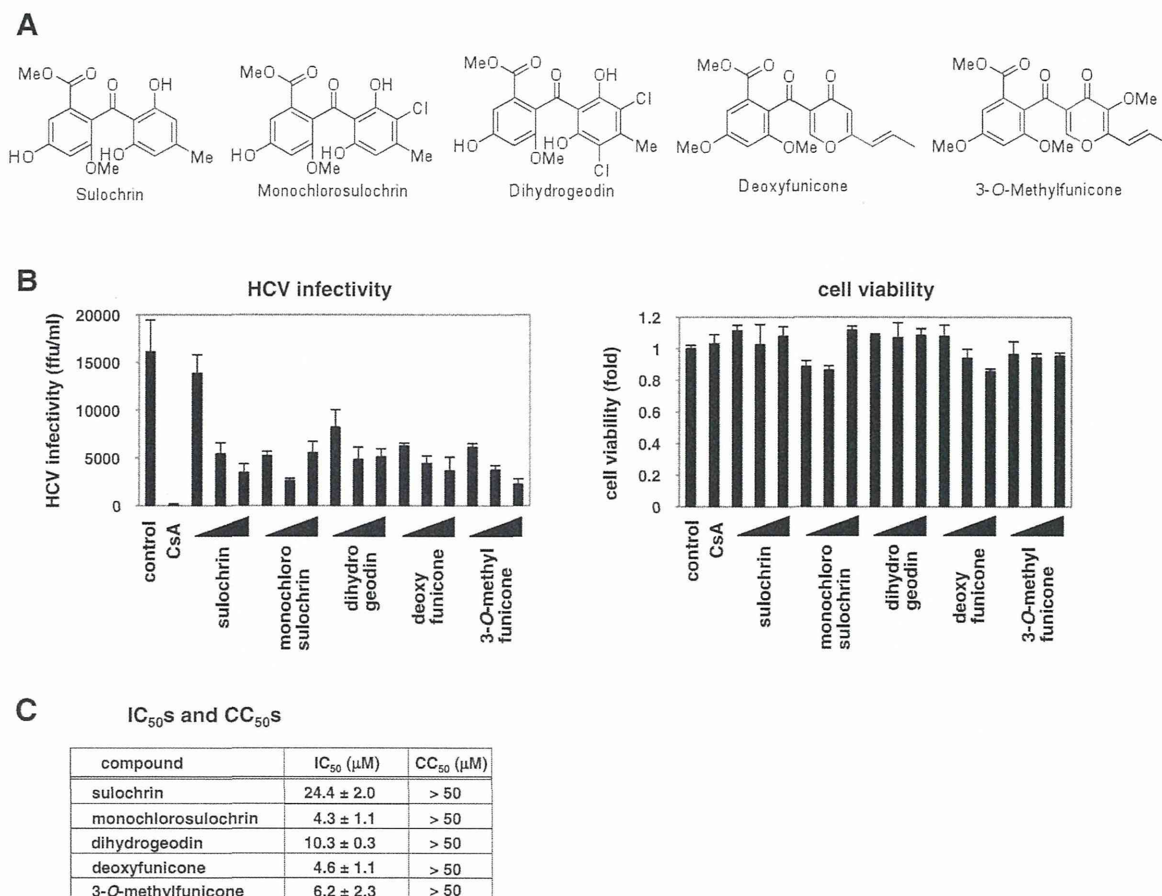
3.5. Derivative analysis of sulochrin

We examined the anti-HCV activity of a series of sulochrin derivatives (Fig. 4A) in the HCVcc system. Monochlorosulochrin and dihydrogeodin, mono- or dichloro-substituted derivatives of sulochrin, possessed even higher anti-HCV activity than sulochrin (Fig. 4B and C). Deoxyfunicone, of which one aromatic ring was replaced by a 4-pyrone ring, had approximately 5-fold greater HCV inhibitory activity as compared with sulochrin (Fig. 4B and C). An additional compound, 3-O-methylfunicone, also possessed anti-HCV activity (Fig. 4B and C). These data suggest that the 1,3-dihydroxy-5-methylbenzene moiety of sulochrin is important for anti-HCV activity. Furthermore, funicone derivatives as well as sulochrin derivatives are likely to be lead compounds for a new class of anti-HCV agents.

4. Discussion

In the present study, we prepared a natural product library consisting of approximately 300 isolated compounds derived from fungi extract [17]. Among these compounds, we focused on sulochrin, which reduced HCV infectivity in the HCVcc system. Sulochrin suppressed the viral entry efficiencies both in the HCVpp and the HCVtcp systems, suggesting that this compound blocked HCV envelope-mediated entry. HCV was reported to enter host cells through clathrin-dependent endocytosis after engagement to host receptors [24–27]. Sulochrin is not likely to be a general inhibitor of clathrin-dependent endocytosis, but rather is specific for HCV entry, as it did not affect the entry of other viruses such as VSVpp and HBV, which were reported to enter by clathrin-dependent manners [28,29].

Sulochrin inhibits eosinophil degranulation, activation, and chemotaxis [30,31]. It also inhibits VEGF-induced tube formation of human umbilical vein endothelial cells [32]. In addition, 3-O-methylfunicone, a sulochrin derivative possessing anti-HCV activity, has an anti-tumor activity [33]. It is unknown if these activities of the compounds are related to their anti-HCV activity. The establishment of drug-resistant virus and the identification of the target molecule are in progress to reveal the mechanism of action of sulochrin and its derivatives. However, the present study is the



compound	IC_{50} (μM)	CC_{50} (μM)
sulochrin	24.4 ± 2.0	> 50
monochlorosulochrin	4.3 ± 1.1	> 50
dihydrogeodin	10.3 ± 0.3	> 50
deoxyfunicone	4.6 ± 1.1	> 50
3-O-methylfunicone	6.2 ± 2.3	> 50

Fig. 4. Derivative analysis of sulochrin. (A) Chemical structures of sulochrin derivatives examined in this study, monochlorosulochrin, dihydrogeodin, deoxyfunicone, 3-O-methyfunicone, as well as sulochrin. (B) Anti-HCV effects of the sulochrin derivatives (10, 30, and 50 μM) were investigated as shown in Fig. 1C. (C) The IC_{50} and CC_{50} values of the sulochrin derivatives are shown.

first report to demonstrate the antiviral activity of these compounds. It is important to note that sulochrin inhibited the entry of HCV genotype 1a and b, which are the dominant genotypes in North America, Europe, and East Asia, indicating that this compound has potential clinical applications. Promising applications of entry inhibitors include the prevention of HCV recurrence in patients after liver transplantation. In patients with HCV-related end-stage liver diseases undergoing liver transplantation, re-infection of the graft is universal and characterised by accelerated progression of liver diseases. Entry inhibitors may be effective especially in these conditions under robust re-infection of HCV into hepatocytes. In the present study, we showed that co-treatment of sulochrin with IFNα and a protease inhibitor, teleprevir, synergistically augmented the anti-HCV effects of these approved drugs. These results suggest the possibility that co-treatment with sulochrin and probably its effective derivatives helps to inhibit the spread of HCV infection. We also identified the chemical structure and the derivatives of sulochrin as lead compounds for anti-HCV agents. Further derivatives analysis may identify more preferable anti-HCV agents.

In conclusion, our results demonstrate that sulochrin and its derivatives are potent and selective inhibitors of HCV infection in cell culture. Although further studies including an analysis of mode of action and pharmacological properties *in vivo* are required, this class of compounds should be pursued for its clinical potential in the treatment of HCV infection.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.100.

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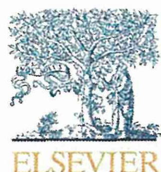

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A class II phosphoinositide 3-kinase plays an indispensable role in hepatitis C virus replication

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ABSTRACT

Phosphoinositides function as fundamental signaling molecules and play roles in diverse cellular processes. Certain types of viruses may employ host cell phosphoinositide signaling systems to facilitate their replication cycles. Here we demonstrate that the β isoform of class II PI3K (PI3K-C2β) plays an indispensable role in hepatitis C virus (HCV) propagation in human hepatocellular carcinoma cells. Knockdown of PI3K-C2β abrogated HCV propagation in the cell. Using an HCV replicon system, we found that knockdown of PI3K-C2β substantially repressed the full-genome replication, while showing relatively small reductions in sub-genome replication, in which structural proteins including core protein were deleted. We also found that HCV core protein showed the binding activity towards D4-phosphorylated phosphoinositides and overlapped localization with phosphatidylinositol 3,4-bisphosphate in the cell. These results suggest that the phosphoinositide generated by PI3K-C2β plays an indispensable role in the HCV replication cycle through the binding to HCV core protein.

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1. Introduction

Phosphoinositides (PIs) are lipid molecules that play crucial roles in diverse cellular functions [1]. In most cases, PIs generated in response to specific stimuli recruit their binding proteins to certain intracellular sites where these proteins exert their functions [2]. Accumulating evidence has identified a number of PI-binding proteins and their functions in fundamental cellular activities, such as cell proliferation, cell growth, vesicle trafficking, and cytoskeletal reorganization [1,2]. PI species are interconverted through phosphorylation and dephosphorylation by a set of specific PI kinases and PI phosphatases. For example, phosphatidylinositol 4-phosphate (PtdIns(4)*P*) is phosphorylated by phosphatidylinositol phosphate 5-kinases to produce phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), which is further phosphorylated by class I PI 3-kinases (PI3Ks) to produce phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). This PtdIns(4)*P*–PtdIns(4,5)P_2–PtdIns(3,4,5)P_3 axis is well characterized in its regulation and physiological functions. PtdIns(4)*P* binds to the pleckstrin homology (PH) domain of certain proteins to regulate the intracellular trafficking of proteins and lipids. PtdIns(4,5)P_2 plays a role in regulating cytoskeletal reorganization through the binding to several actin-binding proteins, while PtdIns(3,4,5)P_3 governs cell proliferation and survival through the binding to a set of PH-domain proteins, such as the protein kinase AKT.

Hepatitis C virus (HCV) is an RNA virus that propagates in human hepatic cells and becomes a causative agent for several hepatic disorders, such as steatosis, fibrosis, and hepatocellular carcinomas [3]. HCV is an enveloped virus, of which the viral nucleocapsid is comprised of one type of protein, the core protein. An accumulating body of evidence suggests that the core protein affects diverse host cell function, including proliferation, apoptosis, and metabolism [4], although underlying molecular mechanism of how the core protein acts on these cellular processes remains largely undefined. HCV enters into cells via clathrin-mediated endocytosis, replicates on certain endomembrane structures, and exits from cells using the lipoprotein secretion system [5–7]. These observations clearly show that HCV employs fundamental host cell functions, including cytoskeletal reorganization and intracellular trafficking systems, to facilitate its replication, thereby suggesting that multiple PIs may participate in the HCV replication cycle. Indeed, several studies have demonstrated that type III phosphatidylinositol 4-kinases (PI4K-IIIα and PI4K-IIIβ), which produce PtdIns(4)*P*, are required for HCV replication in HuH-7 hepatocellular carcinoma cells [8–12]. It has been also reported that PI4K-IIIα binds to the HCV nonstructural NS5A protein and participates in the replication complex formation [13–15]. In addition, NS5A

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protein is known to activate class I PI3K and increase survival signals [16,17]. However, participation of other PIs and their metabolizing enzymes in the HCV replication cycle remains largely unknown.

In this study, we show that the β isoform of class II PI3K (PI3K-C2β), a potential phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2)- and phosphatidylinositol 3-phosphate (PtdIns(3)P)-producing enzyme, is required for the HCV genome replication process. We also demonstrate that PtdIns(3,4)P_2 directly binds to HCV core protein *in vitro* and, in HCV-replicating cells, localizes at endomembrane structures with the core protein. Our study is the first report to demonstrate the requirement of PI3K-C2β for the HCV replication cycle.

2. Materials and methods

2.1. HCV replication assay

Replicon RNAs (SGR, SGR-GND, FGR, and FGR-GND) were prepared from pSGR-JFH1/luc, pSGR-JFH1(GND)/luc, pFGR-JFH1/luc, and pFGR-JFH1(GND)/luc as described previously [18,19]. HuH-7.5.1-8 cells on 48-well plates were transfected with one of the replicon RNAs as described above and then cultured for 22–72 h. Luciferase activity was determined using the Luciferase Assay System (Promega) according to the manufacturer's protocol.

2.2. Lipid binding assay

In order to prepare liposomes, egg phosphatidylcholine (500 μg, Avanti Polar Lipids) and *sn*-1-palmitoy-2-oleoylphosphatidylethanolamine (125 μg, Sigma–Aldrich) along with 3 μg each of PtdIns(3)P, PtdIns(4)P, phosphatidylinositol 5-phosphate, PtdIns(3,4)P_2, PtdIns(4,5)P_2, or PtdIns(3,4,5)P_3 were dried under vacuum. All PIs used in this study were in the dipalmitoylated form and obtained from Cayman. The dried lipids were resuspended in 100 μl of resuspension buffer (50 mM 3-morpholinopropanesulfonic acid–NaOH [pH 7.2], 100 mM NaCl, and 1 mM DTT) and incubated at 65 °C for 1 h. The liposomes were then frozen in liquid nitrogen and thawed at 37 °C for three cycles. The liposome binding assay was performed in a solution containing 100 μl of liposome solution and 2 μl of fraction-17 (of Sephacryl S-300 column chromatography) containing recombinant core protein. After incubation at 4 °C for 1 h, liposomes were collected by centrifugation at 20,000*g* for 20 min, and core protein precipitated with liposomes

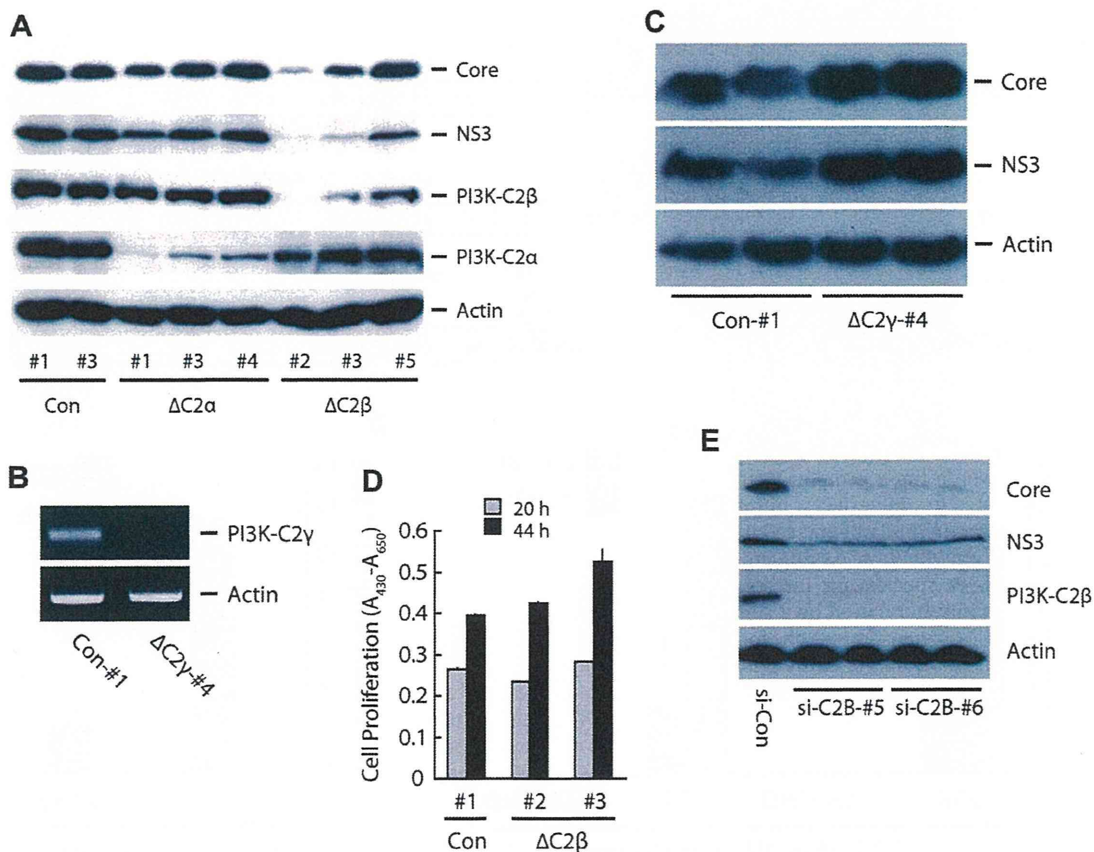

Fig. 1. PI3K-C2β is indispensable for HCV propagation in HuH-7.5.1-8 cells. (A) Control (Con-#1 and #3), PI3K-C2α-knockdown (ΔC2α-#1, #3, and #4), and PI3K-C2β-knockdown (ΔC2β-#2, #3, and #5) cells were infected with HCV. After a 5-day culture, viral proteins (core and NS3) accumulated in cells were detected by immunoblotting. Expression of PI3K-C2α and PI3K-C2β along with actin (as a loading control) was also represented. (B) RNA fractions from Control (Con-#1) and PI3K-C2γ-knockdown (ΔC2γ-#4) cells were subjected to RT-PCR analysis to detect PI3K-C2γ and actin mRNAs. RNA extraction and RT-PCR were conducted as described under Section 2 (HCV entry assay section). (C) Control (Con-#1) and PI3K-C2γ-knockdown (ΔC2γ-#4) cells were infected with HCV. After a 5-day culture, viral proteins (core and NS3) accumulated in the cells were detected by immunoblotting. (D) In order to determine the proliferation of control (Con-#1) and PI3K-C2β-knockdown (ΔC2β-#2 and #3) cells, 2.5 × 10^3 cells were plated on 96-well plates and cultured for the indicated times. Proliferation was determined as described under Section 2. Data are represented as the mean ± SD from quadruplicated experiments. (E) HuH-7.5.1-8 cells were transfected with indicated siRNAs (si-Con, si-C2B-#5, and si-C2B-#6) twice with a 3-day interval. Cells were then infected with HCV. After a 5-day culture, viral proteins (core and NS3) accumulated in the cells were detected by immunoblotting. Expression of PI3K-C2β along with actin (as a loading control) was also represented. Typical data from three independent experiments are presented (A, C, E).

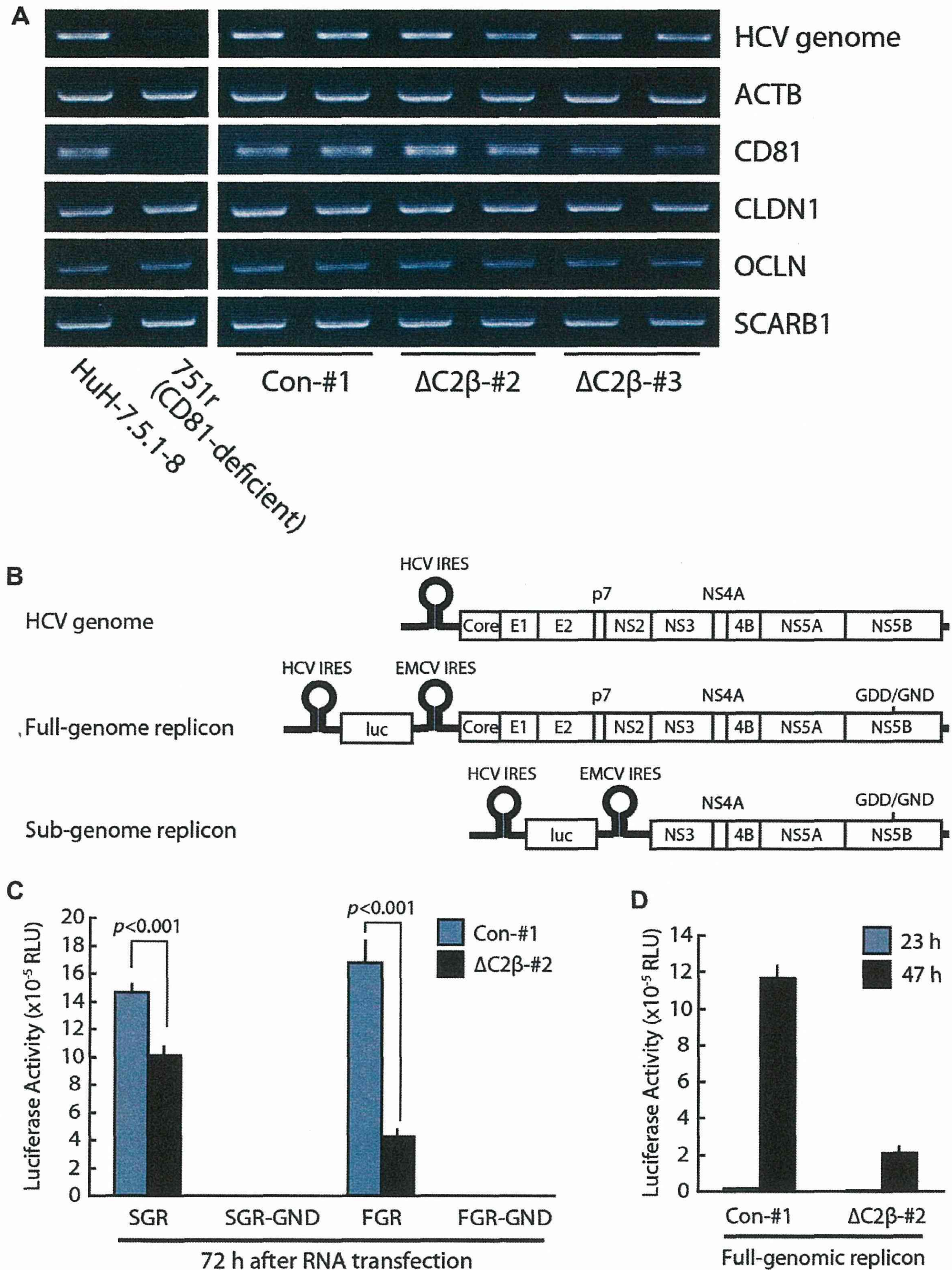

Fig. 2. PI3K-C2β knockdown represses HCV replication. (A) PI3K-C2β knockdown does not affect HCV entry. Control (Con-#1) and PI3K-C2β-knockdown (ΔC2β-#2 and #3) cells as well as HuH-7.5.1-8 (as a positive control) and CD81-deficient 751r (as a negative control) cells were subjected to HCV entry assay described under Section 2. Experiments were performed in duplicate (except positive and negative controls) and typical images from repeated experiments are presented. (B) Schematic diagram of HCV genomic and replicon RNA. (C) Control (Con-#1, gray column) and PI3K-C2β-knockdown (ΔC2β-#2, filled column) cells were transfected with RNA from either the sub-genomic replicon (SGR), replication-defective SGR (SGR-GND), full-genomic replicon (FGR), or replication-defective FGR (FGR-GND). After a 72-h culture, the luciferase activity of each sample was determined as described in Section 2. Data are represented as the mean ± SD from triplicated experiments. An unpaired Student's *t*-test was used to calculate statistical significance. (D) Control (Con-#1) and PI3K-C2β-knockdown (ΔC2β-#2) cells were transfected with the full-genomic replicon RNA. After the indicated time of culture, the luciferase activity of each sample was determined and represented as described above.

was analyzed by immunoblot analysis. The relative intensities of immunoreactive core protein bands were measured using the ImageJ Java applet.

2.3. HCV entry assay

Cells (1.4×10^5) were plated onto a well of a 12-well plate and cultured for 1 day. After removal of the medium, 0.35 ml of HCV-containing culture medium were added onto the cells and incubated for 2 h at 37 °C. Then, the medium was changed to normal growth medium supplemented by 0.1% Pluronic F-68 (Invitrogen). After the incubation at 37 °C for 2 h, the cells were rinsed with PBS containing 0.1% Pluronic F-68, followed by the incubation in 0.5 ml of AccuMax (Innovative Cell Technologies) for 5 min at room temperature in order to detach cells from the vessel and remove bound viruses but not internalized viruses [20]. The cells were then collected by centrifugation, and RNA was extracted using TRIzol Reagent (Invitrogen). The RNA fractions were transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Takara), followed by PCR analyses. In order to analyze the level of HCV genome RNA and transcripts for host cell factors, PCR was conducted in a 10-μl reaction mixture using 20 ng of cDNA as a template and Platinum PCR Super Mix (Invitrogen) according to the manufacturer's protocol. Primers used in this study were listed in Supplementary Table S1.

2.4. Supplementary methods

For cell culture, transfection, DNA construction, recombinant core protein preparation, immunoblot analysis, immunofluorescent assay and HCV infection, please consult Supplementary Data.

3. Results and discussion

3.1. PI3K-C2β is indispensable for HCV propagation in cells

Class II PI3Ks, that are relatively insensitive towards wortmannin, catalyze phosphorylation of PtdIns and PtdIns(4)*P* to produce PtdIns(3)*P* and PtdIns(3,4)P_2, respectively. Biological processes potentially controlled by these PIs (PtdIns(3)*P* for endocytosis; PtdIns(3,4)P_2 for endocytosis and proliferation) may affect HCV propagation [1,21]. In the human genome, there are three isoforms of class II PI3K, PI3K-C2α, PI3K-C2β, and PI3K-C2γ. The expression of all isoforms in HuH-7.5.1-8 cell was detected by immunoblot analysis (see Fig. 1A) and reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 1B and data not shown). Thus, we performed short hairpin RNA (shRNA)-mediated gene silencing of PI3K-C2α, PI3K-C2β, and PI3K-C2γ in HuH-7.5.1-8 cell to test their contribution to HCV propagation in the cell. We established multiple stable clones (ΔC2α-#1, #3, and #4; ΔC2β-#2, #3, and #5; ΔC2γ-#4) in which the expression of PI3K-C2α, PI3K-C2β, or PI3K-C2γ was reduced to various extents (see Fig. 1A and B) as well as stable clones harboring control constructs (Con-#1 and #3). These cells were infected with cell-cultured HCV (JFH1 strain) and incubated for 5 days to allow the virus propagation. As shown in Fig. 1A, while HCV core and NS3 proteins were accumulated in control cells, the accumulation of these viral proteins was greatly reduced in PI3K-C2β-knockdown cells. Quite severe reduction in HCV protein accumulation was observed in ΔC2β-#2, in which PI3K-C2β expression was almost undetectable; while ΔC2β-#3 and ΔC2β-#5, in which PI3K-C2β expression was modestly inhibited, showed partial reduction in HCV protein accumulation (Fig. 1A). This result suggests a dose-dependent requirement of PI3K-C2β in HCV propagation in the cell. In contrast, the effect of PI3K-C2α knockdown on viral protein accumulation was limited, although PI3K-C2α expression was almost undetectable in the cell (see ΔC2α-#1, Fig. 1A). In addition, the depletion of PI3K-C2γ (ΔC2γ-#4), as estimated by RT-PCR (Fig. 1B), did not affect viral protein accumulation (Fig. 1C). Thus we focused on PI3K-C2β functions in HCV propagation using ΔC2β-#2 and ΔC2β-#3 cells for further analyses, because these cell lines showed significant decreases in viral protein accumulation at 5 days after the infection (Fig. 1A). It should be noted that these PI3K-C2α-, PI3K-C2β-, and PI3K-C2γ-knockdown cells did not show decreased proliferation (Fig. 1D and data not shown). Further the introduction of PI3K-C2β-directed small interfering RNAs (siRNAs) (si-C2B-#5 and si-C2B-#6) into HuH-7.5.1-8 cells induced significant reduction of PI3K-C2β expression and resulted in decreases in viral protein accumulation at 5 days after the infection when compared to control cell (Fig. 1E). These non-coding small RNAs (si-C2B-#5, si-C2B-#6, and PIK3C2B-SH) target distinct sequences of PI3K-C2β mRNA, excluding the possibility that the inhibitory effect of PI3K-C2β knockdown on HCV propagation was due to the off-target effect. In addition, as shown in Fig. S1, the expression of mouse PI3K-C2β restored HCV propagation, that was repressed in PI3K-C2β-knockdown cell. We also tested virus release from PI3K-C2β-depleted cells and found that, as reflecting the reduction of HCV protein accumulation in cells (Fig. 1A), culture supernatant from HCV-infected ΔC2β-#2 and ΔC2β-#3 cells showed reduced infectivity towards naïve HuH-7.5.1-8 cells (Fig. S2). These observations taken together suggest that PI3K-C2β plays an indispensable role in HCV propagation in cells.

3.2. PI3K-C2β plays a role in HCV genome replication process

Both of PI3K-C2β-produced PIs, PtdIns(3)*P* and PtdIns(3,4)P_2, are implicated in endocytosis, the cellular process which HCV

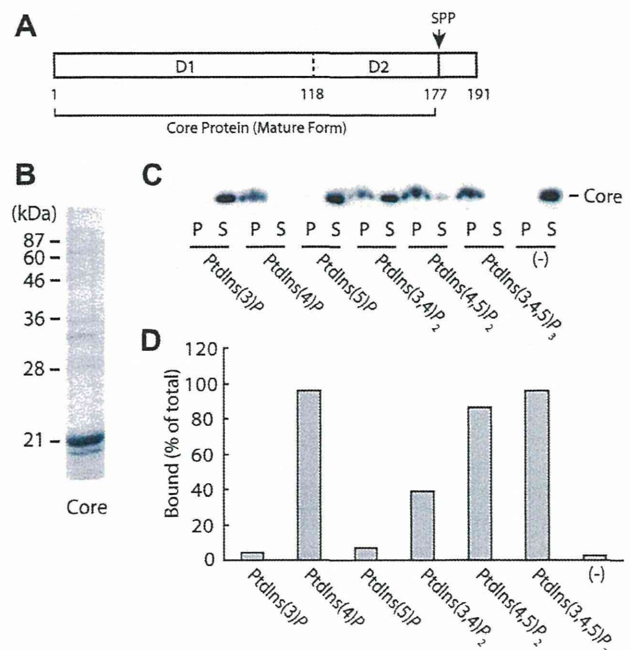

Fig. 3. HCV core protein binds to phosphoinositides *in vitro*. (A) Schematic diagram of HCV core protein. SPP, signal peptide peptidase. (B) Coomassie brilliant blue staining of recombinant core protein prepared by Sephacryl S-300 column chromatography (fraction-17). (C) Recombinant core protein bound to liposomes containing indicated PIs (P) and left unbound (S) were detected by immunoblot analysis. (D) Quantification of the bound core protein against the total amount shown in (C). Typical data from two independent experiments are presented.