

Fig. 2. Time-of-addition experiments (a–c) and the effect of SERMs on extracellular and intracellular HCV RNA in simultaneous infection (d) and in persistent infection (e). Huh 7.5.1 cells were treated with tamoxifen (TMX) (10 μ M, a), raloxifene (RLX) (10 μ M, b), or ICI 182,780 (ICI) (30 μ M, c) during the following time periods: 0–2 h, 2–48 h, or 24–48 h after JFH-1 infection (moi 0.1). Forty-eight hours after infection, the culture supernatant was harvested, and HCV RNA was extracted and subjected to quantitative real-time RT-PCR to determine the number of copies of the JFH-1 genome. The data are the averages of three independent experiments and the standard deviation. d) Effect of treatment with SERMs for 3 days on extra- and intra-cellular HCV RNA levels. Huh 7.5.1 cells were infected with JFH-1 (moi 0.1) just after addition of the SERMs. Three days later, RNA was extracted from the cells and from the culture supernatant. The amount of HCV RNA was measured by quantitative real-time RT-PCR. Brefeldin A (BFA) was used as a positive control. e) Huh 7.5.1 cells were infected at a moi of 0.01, 3 days before addition of drugs. The infected cells were treated with SERMs for 48 h. RNA was subsequently extracted from the cells and the culture supernatant to determine the viral genome copy number. The results are presented as the percentage of control cells without drug. The data are the averages of triplicates and the error bars represent standard deviation. **P*-value < 0.05. One representative experiment of two independent experiments is shown.

Next we treated this (#4-1, genotype 2a) and another replicon (#5-15, genotype 1b) [5] with the SERMs for 3 days and examined the effect of the compounds on the HCV NS5A protein levels by western blotting. As shown in Fig. 3b, the SERMs except ICI 182,780 reduced the level of NS5A in

accordance with the results in Fig. 3a. ICI 182,780 seemed to slightly reduce NS5A protein in #5-15 replicon cell. The SERMs did not reduce the protein levels of GAPDH in the subgenomic replicon cells (Fig. 3b). These results indicated that SERMs, at least tamoxifen, raloxifene and clomifene,

control cell growth are indicated by solid lines and dotted lines, respectively. b) Effect of the following ER α antagonists: ICI 182,780 (closed triangles), ZK164015 (closed rectangles), and MPP (open rectangles). c) Effect of the following ER α agonists: 17 β -estradiol (closed triangles), diethylstilbestrol (open rectangles), and PPT (closed circles). The results are presented as percentages of the control cells that were not treated with drugs. Values are the averages of triplicates, and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown. d) Huh 7.5.1 cells were infected (moi 0.01) in the presence of tamoxifen (TMX), clomifene (CLM), raloxifene (RLX), or ICI 182,780 (ICI) and incubated for 5 days. Cell lysates were blotted with anti-core and anti-GAPDH antibodies as described in the Section Materials and methods.

were effective not only against HCV genotype 2a but also HCV genotype 1b and that the compounds inhibited a HCV replication step. The growth of the replicon cells was suppressed by treatment with 10 μM of clomifene. Clomifene at concentrations less than 10 μM and tamoxifen, raloxifene and ICI 182,780 at 10 μM concentration or lower did not inhibit cell growth (Fig. 3c).

3.4. SERMs inhibited entry of HCVpp but not VSVpp

To further examine the inhibition of early viral processes by the SERMs, we used infectious HCV pseudo-particles (HCVpp). Because HCVpp enter into cell dependent on HCV envelope protein but replicate dependent on retroviral system in the cell, we can exclude other effects of the drug except effect on HCV entry system. Pseudo-particles with the viral envelope glycoprotein mimic the entry of the parental virus, and this system has been used for investigation of HCV entry [7,8,18,20,21]. The infectious titer is determined by luciferase activity. We added tamoxifen to HCVpp- or VSVpp-

containing medium and incubated Huh 7.5.1 cells with this medium for 3 h. After washing the cells, fresh medium was added, and the cells were incubated for 3 days. Treatment with tamoxifen reduced the luciferase activity of the cells that were infected with HCVpp in a dose-dependent fashion. In contrast, the luciferase activity caused by VSVpp was not reduced by the same concentrations of tamoxifen (Fig. 4a). We also examined the effect of other SERMs, such as clomifene, raloxifene, ICI 182,780, ZK164015, and MPP, on HCVpp infection. All of these SERMs inhibited the luciferase activity caused by HCVpp but not the activity caused by VSVpp (Fig. 4b). ICI 182,780 showed a weaker effect compared to tamoxifen, clomifene and raloxifene. Next, we examined the effects of these drugs on various genotypes of HCVpp. Although the extent of inhibition was varied, the compounds inhibited all of the genotypes that were examined (Fig. 4c). At a concentration of 10 μM , ICI 182,780 inhibited all of the genotypes of HCVpp other than genotype 2a. These results suggested that the SERMs inhibit entry of all genotypes of HCV.

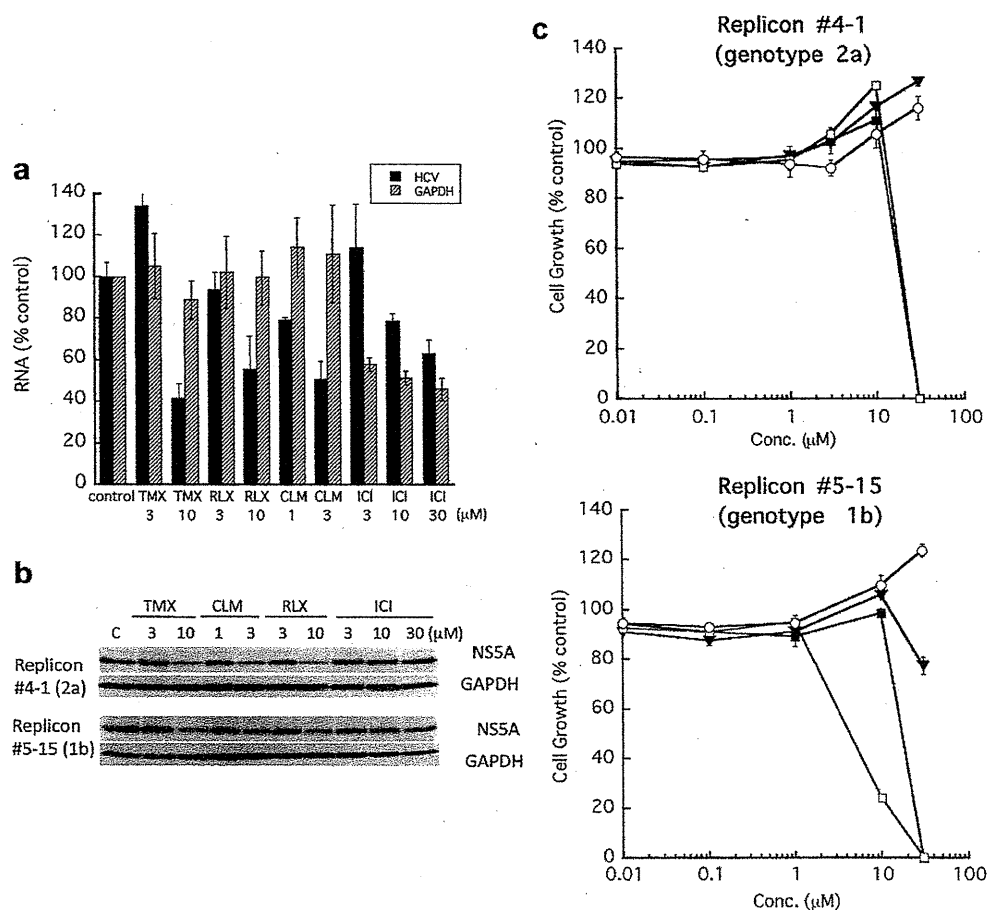


Fig. 3. The effect of SERMs on cells that harbored a subgenomic replicon. A subgenomic-replicon-harboring cell line clone #4-1 (genotype 2a) was treated with SERMs for 48 h. The total RNA was extracted from the cells, and amount of HCV RNA genome was measured. As an internal control, relative amount of GAPDH RNA was measured and indicated as percentage of control cells without drug (a). Another subgenomic-replicon-harboring cell line, clone #5-15 (genotype 1b) was treated with SERMs for 3 days. Cell lysates were subjected to western blotting with an anti-NS5A antibody or an anti-GAPDH antibody (b). Cells that were grown for 3 days in the presence of tamoxifen (closed rectangles), clomifene (open rectangles), raloxifene (closed triangles), or ICI 182,780 (open circles) were measured using the MTT assay. Cell growth is expressed as a percentage of control cells without drug (c). The values are the average of triplicate and the error bars represent the standard deviation of the mean. One representative experiment of two independent experiments is shown.

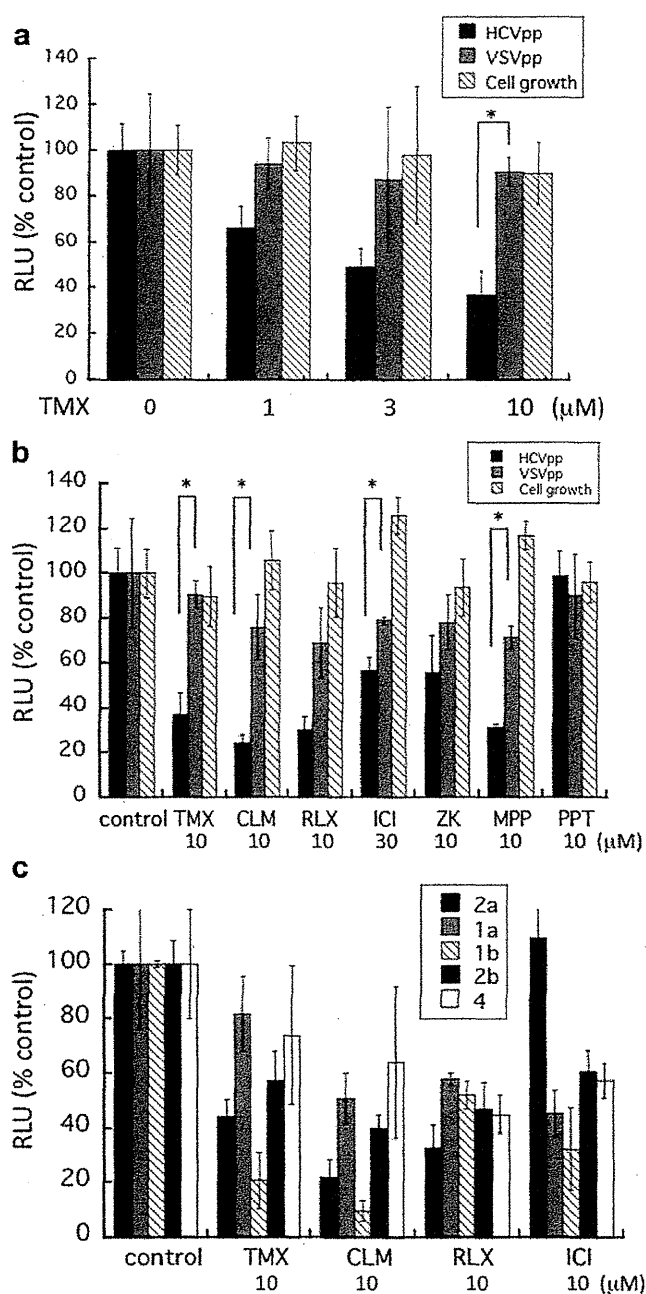


Fig. 4. Effect of SERMs on HCV pseudo-particle (HCVpp) infection. a) Huh 7.5.1 cells were incubated with pseudo-particles (HCVpp or VSVpp) in the presence or absence of tamoxifen for 3 h. The supernatants were removed, medium was added back to the cell cultures, and the cells were incubated for another 3 days. The VSVpp preparation was diluted 600 times so it was infected at similar RLU activity levels compared to HCVpp (approximately 5000 RLU). A parallel culture without pseudo-particles was analyzed using the MTT assay to evaluate the effect of the drugs on cell growth. b) Effects of various SERMs on HCVpp and VSVpp infection. c) Effects of SERMs on the various genotypes of HCVpp infection. The control luciferase activities were approximately 5000 RLU (genotype 2a), 3000 RLU (genotype 1a), 2400 RLU (genotype 1b), 3900 RLU (genotype 2b), and 860 RLU (genotype 4). The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars are the standard deviation of the mean. **P*-value < 0.05. One representative experiment of three independent experiments is shown.

3.5. Effect of tamoxifen on the attachment and entry steps

To better understand how tamoxifen blocks HCV entry, we performed an experiment to discriminate between the inhibition of HCV attachment to cells and the inhibition of post-binding entry events. HCV attaches to several cellular receptors via its E1 and E2 envelope proteins and enters via clathrin-mediated endocytosis [14–16]. We used HCVpp because infection with HCVpp is thought to simulate HCV entry [7,17,18] and the entry is independent of HCV replication. HCVpp binding to the cellular receptors was performed at 4 °C for 1.5 h. Under these conditions, HCVpp bind to the cells but entry is not efficient. The inoculum was removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. In protocol I, the drug was administered during the binding step at 4 °C. After the shift to 37 °C, treatment with the drug was performed during first hour (protocol II) or after 1 h at 37 °C (protocol III) to distinguish between the inhibition of early and late post-binding events (Fig. 5a). The inoculum was removed after treatment, and fresh medium was added to the cells. We used chloroquine, a lysosome-tropic agent, as a control inhibitor for early entry (protocol II) [19]. We also used an anti-CD81 antibody that specifically inhibits HCV entry through the inhibition of the HCV cellular receptor protein CD81 at early entry [20,21]. As expected, chloroquine inhibited luciferase activity when the cells were treated during the early post-binding step (protocol II). This result suggested that endocytosis occurred primarily during the first post-binding period (protocol II). Anti-CD81 markedly inhibited luciferase activity during protocol II as reported [18,19]. Tamoxifen treatment did not result in clear differences between the protocols and the compound displayed similar activity regardless of the treatment period (Fig. 5b left). As a control, the same experiment was performed using VSVpp. Chloroquine inhibited the early entry step of VSVpp, but anti-CD81 and tamoxifen did not show any inhibition (Fig. 5b right).

Tamoxifen is a lipophilic weak base and inhibits acidification intracellularly [22]. Therefore, we examined whether the inhibition of the endocytosis of HCVpp by tamoxifen was dependent on its function as a weak base. Chloroquine is a weak base and inhibits endosome acidification. The pH sensitivity is considered a good indication of clathrin-dependent endocytosis. Previous reports have indicated that chloroquine inhibited HCVcc and HCVpp infection [14,19]. We adjusted the medium to pH 5.5 and incubated the cells in this acidic medium in the presence or absence of tamoxifen for 2 h post-binding. The acidification of the medium did not affect either the entry of HCVpp or the cell growth (Fig. 5c). Treatment with tamoxifen in the medium with a normal pH (pH 7.1) reduced HCVpp entry, and treatment with the drug in the acidic medium also reduced entry to a similar extent. In contrast, chloroquine treatment in regular medium reduced HCVpp entry, but entry was restored in the acidic medium (Fig. 5c). These results indicate that the inhibitory effect of tamoxifen was not dependent on the function of this compound as a base, unlike the effects of chloroquine.

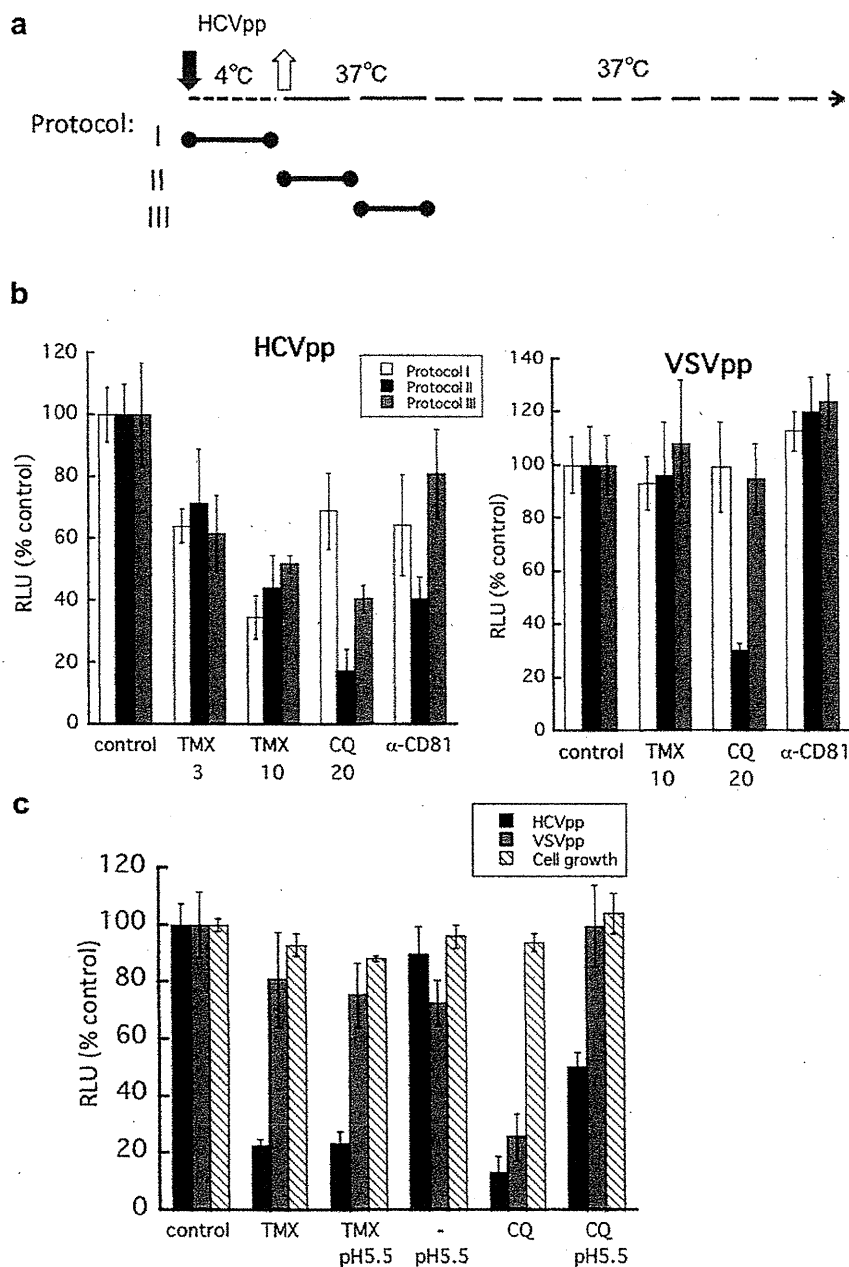


Fig. 5. Effect of tamoxifen on the attachment and endocytosis of HCVpp. a) Experimental design. HCVpp attachment to cells was performed at 4 °C for 1.5 h in the presence or absence of drug. Under these conditions, HCVpp bind to the cells but do not efficiently enter the cells. The inoculum was then removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. The cells were treated with drug during the binding period at 4 °C (protocol I), during first hour after the shift to 37 °C (protocol II), or 1 h after the shift to 37 °C (protocol III). The drug-containing medium was removed for every treatment, and new medium was supplied to the cells. b) Effects of tamoxifen (TMX) (3 and 10 μM), chloroquine (CQ) (20 μM) and anti-CD81 antibody (20 μg/ml) on HCVpp attachment (protocol I) and post-binding events (protocol II and III) (left). Effects of tamoxifen, chloroquine and anti-CD81 on VSVpp (right). c) Effects of exposure to low pH on the inhibition of HCVpp entry by tamoxifen and chloroquine. The cells were incubated with HCVpp at 4 °C for 1.5 h in the absence of drug. After removing the inoculum, regular (pH 7.1) or acidic medium that was adjusted with HCl to pH 5.5, either with or without drug (tamoxifen, 10 μM, chloroquine, 20 μM), was added to the cells. The cells were subsequently incubated at 37 °C. The drug-containing medium was removed after 2 h of incubation, and the cells were incubated for an additional 3 days with fresh, regular medium. The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown.

4. Discussion

We screened for HCV inhibitors using the JFH-1-Huh 7.5.1 cell culture system and found that tamoxifen and ERα antagonists, but not ERα agonists, inhibited HCV JFH-1

infection. Although there are some reports about the HCV inhibitory effects of tamoxifen and other SERMs, we presented further information about the inhibitory effects of these substances. The time-of-addition experiments (Fig. 2a–c) suggested that these SERMs inhibit the entry and replication

steps in the HCV life cycle. These SERMs, except ICI 182,780, reduced level of HCV genome (genotype 2a) and NS5A (genotypes 1b and 2a) in the subgenomic replicon cells (Fig. 3), which supports the hypothesis that the inhibitory effect of the SERMs occurred during the replication steps. Further we observed that SERMs preferentially reduced extracellular HCV RNA compare to intracellular HCV RNA in the newly (Fig. 2d) and persistently infected cells (Fig. 2e). It suggests that the SERMs also target post replication step(s) in the viral life cycle, such as assembly and release. A low concentration of tamoxifen (0.1 μ M) accumulated intracellular HCV RNA (Fig. 2d), which suggests that SERMs target post replication step(s) more efficiently than replication steps. Additionally, these compounds inhibited HCVpp infection (Fig. 4), which supports an inhibitory effect during the entry step. The inhibition of entry was mediated through the inhibition of viral binding to cells and through the inhibition of a post-binding event (Fig. 5b). Taken together, SERMs seemed to target multiple steps of the HCV viral life cycle.

Among the SERMs, ICI 182,780 did not exhibit distinct inhibition of replication in the replicon cells (Fig. 3a and b), although the compound seemed to inhibit entry and replication steps according to the result of time-of-addition experiment (Fig. 2c). The replicon cells are derived from Huh 7 cell. Although viral sensitivity to the SERMs might be dependent on the cell that HCV infect, it remains unclear whether ICI 182,780 inhibits replication step or not. However, the compound affected post replication step in a similar manner to other SERMs (Fig. 2d and e). It is unlikely that ICI 182,780 is an inhibitor with different mechanisms.

The inhibitory effect of tamoxifen on HCV replication has been previously reported by Watashi et al. [23]. They also demonstrated that ICI 182,780 inhibited HCV replication. This effect was discovered using a cell line that harbored a subgenomic replicon (genotype 1b). Additionally, these researchers reported that RNA interference-mediated knock-down of ER α , not ER β , reduced HCV replication, but the reduction was not related to ERE-mediated transactivation activity. They suggested that ER α interacts with the HCV viral polymerase NS5B and that ER α promotes the participation of NS5B with the HCV replication complex. Using the Huh 7.5.1-JFH-1 screening system, Gastaminza et al. and Chockalingam et al. found that toremifene and raloxifene, respectively, function as HCV inhibitors. Gastaminza et al. [24] reported that toremifene inhibited HCV infection by inhibiting both the entry and release steps of the viral life cycle. Chockalingam et al. [25] determined that raloxifene inhibited the entry and replication steps, as we also observed. Our results are in accordance with these previous reports and other information about the inhibitory effects of SERMs.

Pseudo-particle experiments confirmed that SERMs affected the entry step of HCV viral life cycle (Fig. 4b), although the inhibitory effects were lower compare to those against HCVcc (Fig. 2a–c). The reason for the difference in sensitivity may account for some difference in the two entry systems. Otherwise, in the treatment with the drugs for the first 2 h of HCVcc infection, some amount of the drugs might enter

the cell and remain affecting the other steps. The SERMs affected not only genotype 2a but also other genotypes of HCVpp that were examined, suggests that these chemicals have effects on various genotypes of HCV. Although the SERMs appeared to inhibit multiple steps of the HCV life cycle, the primary target step in the viral life cycle might be the entry step. In the time-of-addition experiments, treatment with tamoxifen or raloxifene during the first 2 h was more effective than treatment during the subsequent 2–48 h (Fig. 2a). These SERMs are thought to primarily prevent viral entry and to inhibit post replication step and replication at higher concentrations.

As shown in Fig. 5, an experiment that could discriminate an effect on viral attachment from an effect at the post-binding processes indicated that tamoxifen inhibited both steps. The inhibition of endocytosis by tamoxifen was not rescued by exposure to a low pH. This suggests that the observed inhibition is the result of a mechanism that is independent of the compound's function as a base. HCV entry is a highly complicated process that involves numerous viral and cellular factors. Tamoxifen is thought to target multiple steps that are involved in the attachment and entry steps of the HCV life cycle, which results in high levels of inhibition.

At present, the mechanism of the entry inhibition by SERMs is not clear. It is possible that tamoxifen targets viral molecules, but we have no evidence to support this hypothesis. ER α might be a target molecule because all of the antagonists of ER α that were examined had an inhibitory effect. Watashi et al. indicated that ER α is involved in HCV replication [23]. ER α is thought to be present in the cytoplasm, which is where HCV replicates. However, it is doubtful that ER α is present on the cell surface where viral entry occurs. The addition of 17 β -estradiol with tamoxifen did not prevent the inhibitory effect of tamoxifen in the HCVpp experiment (data not shown). This result suggests that tamoxifen does not compete with 17 β -estradiol for the target molecules involved in HCV entry. Additionally, a pure ER α antagonist, ICI 182,780, was a less effective inhibitor of the entry step. Based on these results, it is thought that the molecule responsible for HCV entry that is targeted by SERMs is not ER α .

Tamoxifen has various targets other than ER α , such as P-glycoprotein (GPR30), calmodulin, and protein kinase C [26]. GPR30 (G protein-coupled receptor protein 30) is a membrane-associated estrogen receptor that is distinct from the classical ER [27]. Tamoxifen and ICI 182,780 are agonists of GPR30 [28]. We examined the effect of a specific GPR30 agonist, G-1, and a GPR30 antagonist, G-15, on HCVpp infection [29]. G-1 and G-15 did not inhibit HCVpp infection. Conversely, HCVpp infection was observed to increase upon addition of these compounds (data not shown). This result suggested that GPR30 is not involved in the inhibition of HCV entry.

We previously reported that a typical PKC inhibitor, bisindolylmaleimide I (BIM I), inhibited HCV replication [2]. BIM I (10 μ M) inhibited both HCVpp and VSVpp infection in a similar manner by approximately 50% (data not shown). This suggests that BIM I has a different mechanism for the

inhibition of entry compared to tamoxifen. PKC is not thought to be involved in the HCV-specific inhibition of entry by SERMs.

There were few reports of HCV entry inhibitors until the development of the cell-culture JFH-1 infection system. It has recently been reported that fluphenazine, trifluoperazine and related chemicals exhibit a strong, dose-dependent inhibition of HCV entry without significantly affecting the entry of VSVpp [24,25]. These compounds are structurally similar to chlorpromazine, which is an inhibitor of the clathrin-coated pit formation that is required for HCV entry [14]. Interestingly, these compounds and the SERMs have a common structural characteristic: planar, multiple aromatic rings with a tertiary amine side chain. Tamoxifen, raloxifene and ER α antagonists all have this structure, but the ER α agonists do not have these structures. Fluphenazine and related chemicals may inhibit HCV entry through a mechanism that is similar to tamoxifen.

In summary, we observed a significant HCV inhibitory effect of various SERMs using the Huh 7.5.1 cell-JFH-1 infection system. Additionally, we demonstrated that SERMs could be useful for the treatment of HCV. Because it takes a great deal of time and money to develop a new drug from a novel chemical compound, it may be easier to use previously developed drugs that can be used for new applications. Tamoxifen, toremifene, and raloxifene are all drugs that have been in use for an extended period of time. In our present *in vitro* study, the effective concentrations for the HCV inhibitory effects of the SERMs were approximately 0.1–10 μ M. In the case of tamoxifen, 20 mg per day, administered for 8 weeks resulted in plasma concentrations of approximately 0.5 μ M. These concentrations could be sufficient to exert an anti-HCV effect. SERMs should be investigated to determine their efficacy for treating HCV clinically. Further examination of the mechanism of the entry inhibition mediated by SERMs would produce significant new data relevant to the understanding of HCV entry.

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Antiviral Activity of Glycyrrhizin against Hepatitis C Virus *In Vitro*

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Abstract

Glycyrrhizin (GL) has been used in Japan to treat patients with chronic viral hepatitis, as an anti-inflammatory drug to reduce serum alanine aminotransferase levels. GL is also known to exhibit various biological activities, including anti-viral effects, but the anti-hepatitis C virus (HCV) effect of GL remains to be clarified. In this study, we demonstrated that GL treatment of HCV-infected Huh7 cells caused a reduction of infectious HCV production using cell culture-produced HCV (HCVcc). To determine the target step in the HCV lifecycle of GL, we used HCV pseudoparticles (HCVpp), replicon, and HCVcc systems. Significant suppressions of viral entry and replication steps were not observed. Interestingly, extracellular infectivity was decreased, and intracellular infectivity was increased. By immunofluorescence and electron microscopic analysis of GL treated cells, HCV core antigens and electron-dense particles had accumulated on endoplasmic reticulum attached to lipid droplet (LD), respectively, which is thought to act as platforms for HCV assembly. Furthermore, the amount of HCV core antigen in LD fraction increased. Taken together, these results suggest that GL inhibits release of infectious HCV particles. GL is known to have an inhibitory effect on phospholipase A2 (PLA2). We found that group 1B PLA2 (PLA2G1B) inhibitor also decreased HCV release, suggesting that suppression of virus release by GL treatment may be due to its inhibitory effect on PLA2G1B. Finally, we demonstrated that combination treatment with GL augmented IFN-induced reduction of virus in the HCVcc system. GL is identified as a novel anti-HCV agent that targets infectious virus particle release.

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Introduction

Hepatitis C virus (HCV) infection is a major public health problem since most cases cause chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma. Current treatment of chronic hepatitis C is based on the combination of pegylated interferon-alpha (IFN- α) and ribavirin. However, approximately 50% of treated patients infected with genotype 1 do not respond, or show only a partial or transient response, and therapy causes significant side effects [1]. In Japan, glycyrrhizin (GL) preparations (stronger neo-minophagen C

(SNMC)) have been used for more than 20 years as a treatment for chronic hepatitis patients who do not respond to IFN therapy.

GL is the major component of licorice root extract, and is composed of glycyrrhetic acid. GL has been shown to possess several beneficial pharmacological activities, including anti-inflammatory activity [2], anti-tumor activity [3], anti-allergic activities [4], and anti-viral activities [5]. Several mechanisms of the GL-induced anti-inflammatory effect are reported, such as inhibition of thrombin-induced platelet aggregation [6], inhibition

of prostaglandin E2 production [7] and inhibition of phospholipase A2 (PLA2) [8].

Many anti-viral effects of GL have been reported previously, for example, against herpes simplex type 1 (HSV-1) [9], varicella-zoster virus (VZV) [10], hepatitis A (HAV) [11] and B virus (HBV) [12], human immunodeficiency virus (HIV) [13], severe acute respiratory syndrome (SARS) and coronavirus [14], Epstein-Barr virus (EBV) [15], human cytomegalovirus [16] and influenza virus [17]. GL has been considered as a potential treatment for patients with chronic hepatitis C, and long term administration of GL to patients is effective in suppressing serum alanine aminotransferase (ALT) levels and histological change [18]. However, a direct anti-viral effect of GL against HCV has never been reported.

In this study, we evaluated the anti-HCV effects of GL, and demonstrated that GL targeted the release step of infectious HCV particles from infected cells. We found that the suppression of virus release by GL may be derived from its inhibitory effect on group 1B PLA2 (PLA2G1B). These findings suggest possible novel roles for GL in the treatment of patients with chronic hepatitis C.

Materials and Methods

Cell culture and reagents

The human hepatoma cell line, Huh7, and its derivative cell line, Huh7.5.1, provided by Francis Chisari (Scripps Research Institute, La Jolla, CA), were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) [19]. Huh7 cells harboring the subgenomic replicon [20] [21] were maintained in complete DMEM supplemented with 0.5 mg/ml G418 (Geneticin, Life Technologies Japan Ltd., Tokyo, Japan). GL (20 β -carboxyl-11-oxo-3 α -norolean-12-en-3 β -yl-2-O- β -D-glucopyranuronosyl- β -D-glucopyranosiduronic acid) and IFN- α were kindly provided by the Minophagen Pharmaceutical Co., Ltd., (Tokyo, Japan) and MSD K.K., (Tokyo, Japan) respectively. Oleyloxyethyl phosphorylcholine (OPC) (Cayman Chemical Company, Ann Arbor, MI), sPLA2IIA inhibitor I (MERCK, Darmstadt, Germany), anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Human CD81 (BD Pharmingen, San Jose, CA) antibodies were purchased. The solvents were distilled water (GL), ethanol (OPC), and DMSO (sPLA2IIA inhibitor).

Quantification of HCV core antigen and cell viability

The production of cell culture-produced HCV (HCVcc) has been previously reported [22]. Purification of LD has been previously reported [23]. The concentration of HCV core antigen in filtered culture medium, in cell lysates and in LD fraction of infected cells was determined using the Lumipulse Ortho HCV antigen kit (Ortho Clinical Diagnostics, Tokyo, Japan). Cell viability was analyzed by using Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturers' protocol.

Electroporation of HCV RNA lacking E1 and E2

In vitro synthesis of HCV RNA JFH1 lacking E1 and E2 (JFH1delE1E2), and electroporation were performed as described previously [22].

HCV pseudoparticle (HCVpp) assay

HCVpp harboring E1 and E2 glycoproteins of the JFH-1 clone (genotype 2a) (HCVpp2a) and the TH clone (genotype 1b) (HCVpp1b) were produced as previously described [24]. Pseudotype virus with VSV G glycoprotein (VSVpp) were also generated [24]. Huh7 or Huh7.5.1 cells were seeded into 48-well plates, incubated overnight at 37°C, and then infected with the HCVpp in the presence of various concentration of GL. Several hours post-infection, medium was replaced with DMEM with 10% FBS, and the cells were harvested 48 hours later to determine intracellular luciferase activity (Luciferase Assay System, Promega).

HCV subgenomic replicon assay

The assay for the genotype 1b and 2a subgenomic reporter replicon has been previously reported [20] [21]. After 72 hours of treatment with GL, the replicon-transfected cells were harvested for either measurement of luciferase activity (Promega) or HCV RNA titer, as described previously [25]. The replication efficiency of HCV in each preparation was calculated as the percentage of luciferase activity or HCV RNA titer compared with that of cells subjected to the control treatment.

Extra- and intracellular infectivity

To determine extracellular HCV infectivity, naïve Huh7 cells were inoculated with cell culture supernatant medium containing HCVcc. After 3 hours of incubation, the medium was replaced with DMEM containing 10% FBS, and the cells were cultured for an additional 72 hours. The infectious HCV titer in the culture medium was determined by quantification using the Lumipulse Ortho HCV antigen kit or by immunostaining of the HCV core antigen. Using an immunoassay that also provided results indicative of HCV infectivity [26], we confirmed a good correlation between the levels of core antigen and infectious titers (data not shown). To estimate intracellular infectivity, cells in the culture plates filled with DMEM containing 10% FBS were subjected to four cycles of freezing and thawing, using dry ice and a 37°C water bath. Cells in the culture plates were centrifuged at 1,200 rpm for 5 min at 4°C to remove cell debris, and the supernatants were collected to evaluate infectivity as above.

RNA interference

The siRNA targeted to PLA2G1B, 5'-GCUGGACAGCUGUAAAUUUTT-3', and scramble negative control siRNA to PLA2G1B were purchased from Sigma (Tokyo, Japan). Cells in a 24-well plate were transfected with siRNA using HiPerFect transfection reagent (Qiagen, Tokyo, Japan) following the manufacturer's instructions.

Quantification of triglyceride

Triglyceride (TG) was measured with a Triglyceride kit (Wako, Tokyo, Japan) according to the manufacturer's instructions.

Indirect immunofluorescence assay

The inoculated cells were fixed with methanol and immunostained with a mouse monoclonal anti-core antibody and a rabbit polyclonal anti-NS5A antibody [22], followed by an Alexa Fluor 555-conjugated anti-mouse secondary antibody (Life Technologies Japan Ltd.).

Transmission electron microscopy (EM)

Cells were fixed with 1.5% glutaraldehyde in 1.0% cacodylate buffer, pH 7.4, for 5 min, and then post-fixed with 2% OsO₄ in phosphate buffer, pH 7.4, for 1 hour. The cells were dehydrated in ethanol and embedded in Epon. Ultrathin sections were double stained and examined at an accelerating voltage of 80 keV. Immuno-EM (IEM) were performed by using the labeled-(strept) avidin-biotin (LAB) kit according to the manufacturer's instructions (Zymed laboratories, San Francisco, CA) as described previously [27].

Statistical Analysis

Assays were performed at least four independent experiments. Data are expressed as the mean \pm SD. Statistical analysis was performed using Student's *t* test.

Results

Anti-HCV effects of GL

To assess the anti-HCV effects of GL, HCVcc-infected cells were treated with various concentrations of GL for 72 hours, and then the levels of HCV core antigen and infectivity of the medium were determined. HCV core antigen levels were reduced by 29% with 500 μ M GL (Figure S1). As shown in Figure 1A, infectivity of supernatant following GL treatment at 3, 30, or 500 μ M was reduced by 12, 62, or 71% of the control levels, respectively. The calculated 50% effective concentration (EC₅₀) was 16.5 μ M. There was no effect on cell viability after these treatments (Figure 1B). These results suggest that GL effectively inhibited the production of infectious HCV.

HCV propagates in hepatocytes throughout its lifecycle, including the stages of attachment, entry, uncoating, translation, genome replication, assembly, budding, and release. To investigate which step of the HCV lifecycle GL inhibited, we used the HCVpp system for evaluating attachment and entry, and the HCV replicon system for translation and genome replication. Treatment of HCVpp2a with GL resulted in a moderate reduction of luciferase activity in the cells infected with HCVpp, with an EC₅₀ value of 728 μ M (Figure 1C). On the other hand, there was no significant reduction of luciferase activity in the cells infected with HCVpp1b (Figure 1D) and VSVpp (Figure 1E). No cytotoxic effects of GL were observed (data not shown).

Huh7 cells harboring the type-2a subgenomic replicon were treated with various concentrations of GL for 72 hours. Relative luciferase activities of GL-treated cells were inhibited in a dose-dependent manner with an EC₅₀ value of 738 μ M (Figure 1F). A similar result was obtained by using the type-1b subgenomic replicon (data not shown). We also transfected HCV RNA lacking E1E2 (JFH1delE1E2) and monitored the effect of GL

on HCV replication to avoid reinfection of Huh7 cells. There was no significant reduction of HCV RNA titers in the cells (Figure 1G). There was no significant cytotoxicity seen following these treatments (data not shown).

To investigate the effect of GL on entry, HCV particles were treated with increasing concentrations (0 to 1500 μ M) of GL. The viral samples were then used to inoculate Huh7 cells cultured in GL-containing medium. Several hours post-infection, medium was replaced with DMEM without GL. The levels of HCV core antigen in the medium were determined at 72 h postinfection (p.i.). There was no significant reduction of HCV production (Figure 1H). These results indicated that GL did not inhibit HCV entry and replication significantly.

Effects of GL on infectious HCV particle release

To further assess whether GL treatment affects other steps of the viral lifecycle, we analyzed infectious HCV particle assembly and release following GL treatment. Supernatant or crude cell lysates of HCVcc-infected cells treated with GL were used to inoculate naïve Huh7 cells to determine extra- and intracellular specific infectivity, respectively. Specific infectivity was determined as the ratio of infectious virus titer to HCV core antigen level, as described previously [28]. As shown in Figure 2A, the extracellular specific infectivity titer was inhibited by 57% by GL at a concentration of 500 μ M, on the other hand, the intracellular specific infectivity titer was increased 3.8-fold over that of controls at the same concentration of GL (Figure 2B). There was no significant cytotoxicity following these treatments (data not shown).

It has been previously reported that virus assembly takes place around lipid droplets (LDs) [29]. By immunofluorescence staining, we examined the subcellular co-localization of HCV core (Figure 2C) or NS5A (Figure 2D) with LDs in HCVcc-infected cells with or without GL treatment. Un-infected cells were shown in Figure 2E. We observed HCV proteins colocalized with LDs (Figure 2C and 2D). Intensity profiles along the line segments, shown on the bottom of the images, demonstrated that core proteins were tightly colocalized with LD in the HCVcc-infected cells treated with GL, when compared with untreated cells (Figure 2C lower panel). We quantified the size of LDs in HCV-infected cells (Figure 2D) and un-infected cells (Figure 2E) with GL-treatment. We found that GL did not affect the size of LDs in un-infected cells (Figure 2F right panel). On the other hand, the size of LDs increased in HCV-infected cells with GL-treatment (Figure 2F left panel).

HCVcc-infected cells (Figure 2G) and un-infected cells (Figure 2H), treated with GL, were prepared for EM analysis. In the cytoplasm of HCV-infected cells, we observed increased numbers of LDs in close proximity to endoplasmic reticulum (ER) and the electron-dense signals on ER attached to LD (Figure 2G upper panel), which are thought to act as platforms for the assembly of viral components [29]. Interestingly, in the cytoplasm of HCV-infected cells after treatment with GL, accumulated electron-dense particles were observed on ER attached to LD (Figure 2G lower panel). IEM experiments showed that anti-core antibody stained the membrane around LDs (Figure 2I lower panel). In naïve Huh7 cells, the close association of LDs with ER was rarely observed (Figure 2H).

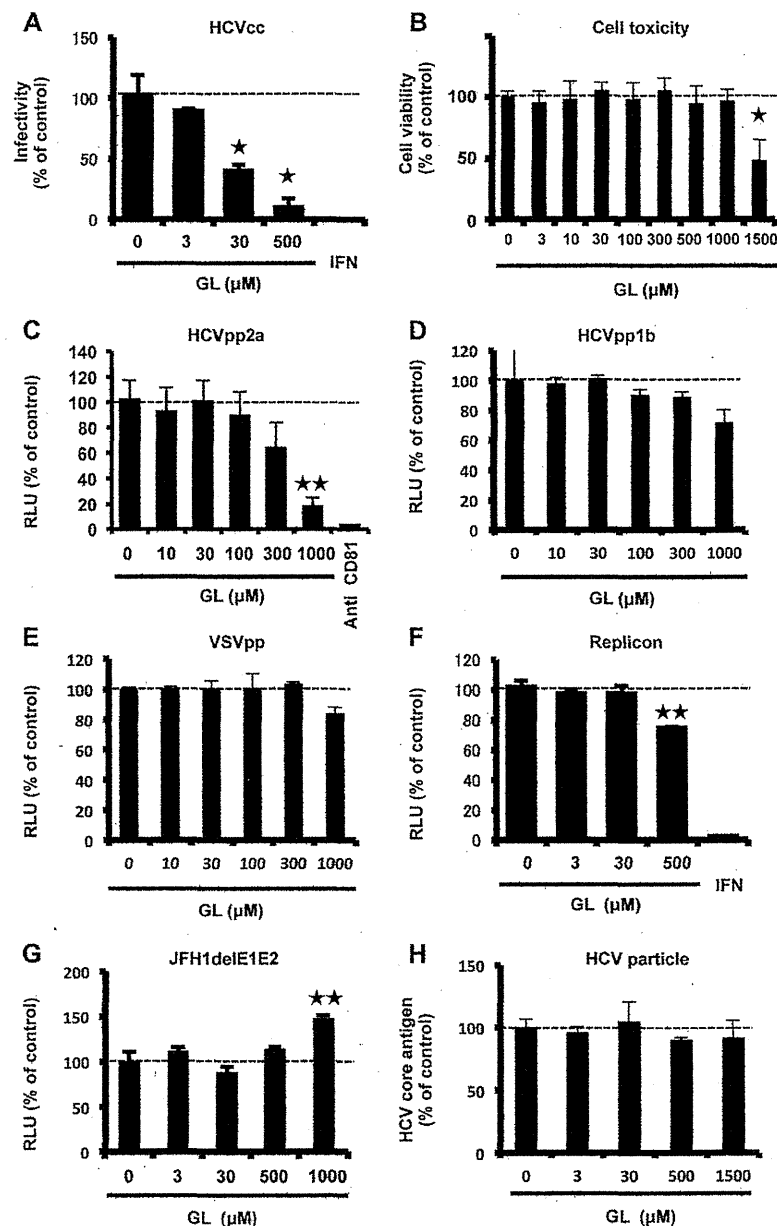


Figure 1. Anti-HCV effects of GL. (A) HCVcc-infected cells were treated with various concentrations of GL for 72 hours. Naïve Huh7 cells were inoculated with supernatant and cultured for 72 hours. Infectivity was determined by immunostaining. (B) Cell viability was assessed using Cell Titer-Glo Luminescent Cell Viability Assay. Huh7 cells were infected with HCVpp2a (C), HCVpp1b (D), and VSVpp (E) in various concentrations of GL for 24 hours, and then medium was replaced. Effects of GL on entry of HCVpp and VSVpp were determined by measuring the luciferase activity at 72 hours post-transfection. (F) Huh7 cells harboring the type-2a subgenomic replicon were treated with various concentrations of GL for 72 hours. Replication efficiency of the replicon was estimated by measuring the luciferase activity. (G) The effects of GL on HCV replication were tested by electroporation of HCV RNA lacking E1E2 (JFH1delE1E2). (H) HCV particles were treated with increasing concentrations (0 to 1500 μM) of GL. The viral samples were then used to inoculate Huh7 cells with GL-containing medium. Several hours post-infection, medium was replaced with DMEM without GL. The levels of HCV core antigen of the medium were determined at 72 h postinfection (p.i.). IFN (300 IU/ml) was used as a positive control for reduced HCV replication. Anti-human CD81 antibody (10 μg/ml) was used as a positive control for reduced HCV entry to the cells. Results are expressed as the mean ± SD of the percent of the control from four independent experiments. *P < 0.05, **P < 0.005 versus control (0 μM treatment).

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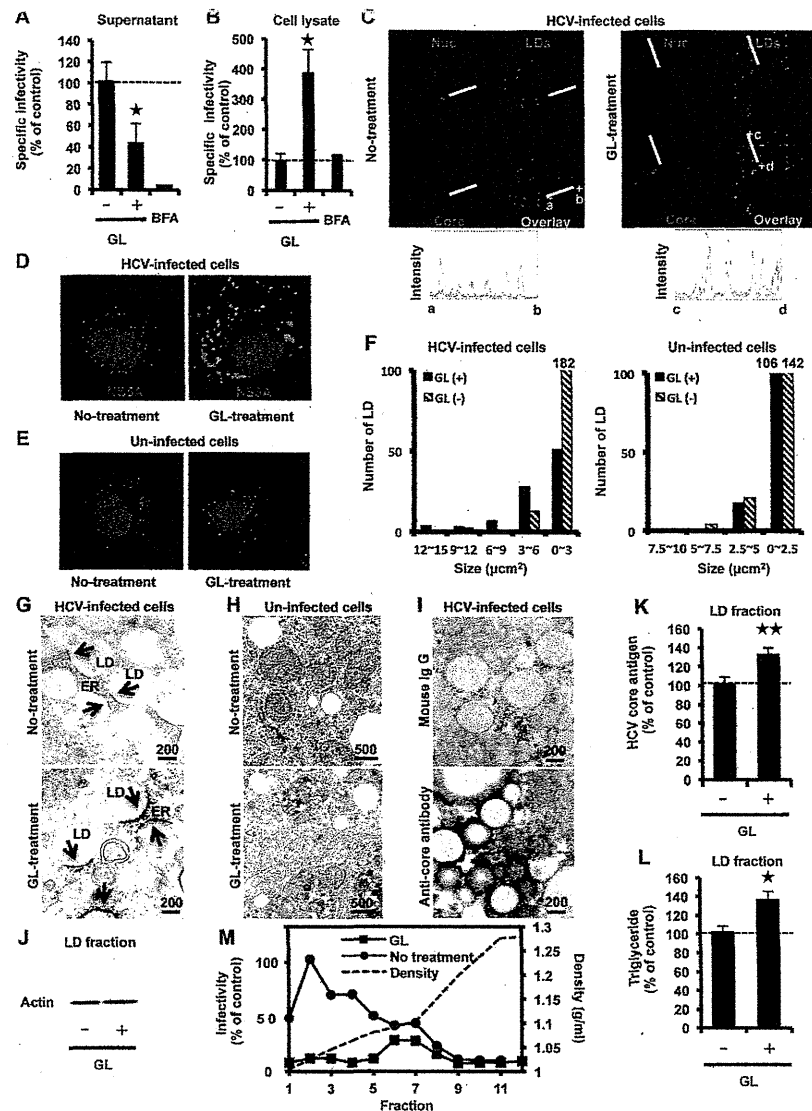


Figure 2. Effects of GL on release of infectious HCV particles. HCVcc-infected cells were treated with GL at a concentration of 500 μM for 72 hours. Untreated cells were used as controls. Extra- (A) and intracellular specific infectivity (B) were determined. Subcellular co-localization of HCV core (C) or NS5A (D) with LDs in HCVcc-infected cells with or without GL treatment. (E) Uninfected cells. LDs and nuclei were stained with BODYPI 493/503 (green) and DAPI (blue), respectively. (C) Points a and b, as well as c and d, define two line segments that each cross several structures. Intensity profiles along the line segments shown on the bottom of the images. (F) The size of LDs in uninfected cells (right panel) and HCV-infected cells (left panel) were quantified. Transmission EM of LDs in infected cells (G) and uninfected cells (H) treated with GL at 500 μM . Arrows indicate electron-dense signals (G upper panel) and particles (G lower panel). (I) IEM using the LAB method of LDs in infected cells treated with GL at 500 μM . Mouse IgG (upper panel) or anti-core monoclonal antibody (lower panel) was used for primary antibody. (J) Immunoblotting with anti-actin antibody in the LD fraction. Quantification of HCV core antigen (K) and TG (L) in the LD fraction. The LD fraction was collected from cell lysates. The ratio of HCV core antigen level in the LD fraction to that in total cell lysate was determined. (M) HCVcc-infected cells were treated with GL at 500 μM for 72 hours. Untreated cells were used as controls. Supernatant was ultracentrifuged through a 10-60% sucrose gradient and the infectivity of each fraction was determined. Infectivity of fraction 2 of untreated cells was assigned the arbitrary value of 100%. The density of each fraction was measured by refractive index measurement. Brefeldin A (1 μM for 24 hours) was used as a positive control for reduced HCV release. Results are expressed as the mean \pm SD of the percent of the control from four independent experiments. * $P < 0.05$, ** $P < 0.005$ versus control (0 μM treatment). Scale bars, 200 and 500 nm.

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To confirm the accumulation of core antigen around LD, we purified the LD [23], and quantified HCV core antigen and TG in the LD fraction, followed by immunoblotting with anti-actin antibody (Figure 2J). Analysis of the levels of HCV core antigen and TG in the LD fraction of the total cell lysate showed that the amount in GL-treated cells was increased by 31% and 35% compared with controls, respectively (Figure 2K and 2L). Taken together, these results suggested that GL inhibits release, but not assembly and budding, of infectious HCV particles in cells.

To characterize the infectivity of HCV particles released from HCVcc-infected cells treated with GL, supernatant from cell cultures treated or not treated with GL was subjected to continuous 10–60% (w/v) sucrose density gradient centrifugation, and the infectivity titer of each fraction was measured. A reduction in infectivity by GL-treatment was observed in fractions 1–7 (Figure 2M). These results suggest that GL may decrease the amount of HCV infectious particles in the supernatant.

Role of PLA2 in HCV lifecycle

GL is known to have an inhibitory effect on PLA2 [8]. PLA2 is classified into several groups and their biological functions are not the same. It is unknown which group of PLA2 is targeted by GL. We analyzed the effect of GL on PLA2G1B and PLA2G2A, which were major groups of PLA2 family. To confirm the effects of GL on expression of PLA2G1B, cells, transfected with an expression plasmid for PLA2G1B, were treated with GL and OPC, which is a specific inhibitor for PLA2G1B. Treatment with GL effectively decreased the cellular level of PLA2G1B (Figure S2). To verify whether PLA2 has a role in viral entry and replication, we tested the effect of PLA2 inhibitors on HCVpp infection and the replicon system, respectively. OPC has no significant effect on virus entry and replication (Figure 3A and 3B). On the other hand, sPLA2IIA inhibitor I, which is a specific inhibitor for PLA2G2A, inhibited both HCVpp entry (Figure 3A) and subgenomic replicon replication (Figure 3B). There was no significant cytotoxicity seen after these treatments (data not shown).

To evaluate the effects of PLA2 inhibitors on HCVcc infectivity, infected cells were treated with PLA2 inhibitors and extra- and intracellular specific infectivity were measured (Figure 3C and 3D). OPC slightly decreased specific infectivity of virus in the supernatant and significantly increased specific infectivity of virus in the cell lysate. On the other hand, sPLA2IIA inhibitor I significantly decreased the specific infectivity of virus in both the supernatant and cell lysate. To confirm the importance of PLA2G1B in HCV release, we silenced PLA2G1B with its specific siRNA and monitored its effect on HCV release. PLA2G1B siRNA decreased the cellular level of PLA2G1B (Figure S3). Suppression of PLA2G1B reduced core protein level in the medium (Figure 3E left panel) and increased specific infectivity in the cells (Figure 3E right panel). We performed GL treatment with or without OPC and showed that GL and OPC had no additive effect when applied together (Figure 3F). There was no significant cytotoxicity seen after these treatments (data not shown). Taken together, these results suggest that the suppression of virus release by GL may be derived from its inhibitory effect on PLA2G1B. These

results also suggested that PLA2G1B has a role in virus release.

Antiviral effects of IFN along with GL

We have demonstrated that the target causing the anti-HCV effect of GL differs from that of IFN. To analyze the antiviral effect of IFN combined with GL, HCVcc-infected cells were treated with 0.1 and 1.0 IU/ml of IFN in combination with various concentrations of GL. HCV core level in culture medium (Figure 4A) and in the cell (Figure 4B), specific infectivity in culture medium (Figure 4C) and in the cells (Figure 4D) were measured. Regardless of the IFN concentration, HCV core level and specific infectivity of the supernatant decreased in response to GL treatment in a dose dependent manner (Figure 4A and 4C). On the other hand, HCV core level and specific infectivity of the cell increased (Figure 4B and 4D), suggesting that GL inhibited HCV release. The results indicated that a combination therapy of IFN with GL could be an effective treatment for HCV.

Effect of GL on IFN induction and secretion proteins

The IFN-inducing ability of GL has also been previously reported [30]. We evaluated IFN stimulated gene induction by GL, but no effects were observed (Figure S4). PLA2 is known to be associated with various intracellular trafficking events and secretion of very low-density lipoprotein (VLDL) [31]. HCV particles are known to be secreted using the host membrane trafficking system [32]. There is now increasing evidence that VLDL participates in HCV assembly and release [33]. Therefore, we analyzed the level of albumin, an abundantly secreted protein from hepatocytes, and apolipoprotein E (ApoE), a component of lipoproteins, in the culture supernatants of Huh7 cells and found that they were not influenced by GL treatment (Figure S5).

Discussion

Recently, Ashfaq et al. found the inhibitory effect of GL on HCV production in patient serum infected Huh7 cells [34]. Their cell culture system does not produce HCV efficiently. Thus, it does not permit analysis of the complete viral life cycle. In this study, we observed distinct suppression of HCV release by GL, using the HCVcc system (Figure 1A). Anti-viral effects of GL on early steps in the viral lifecycle have been reported previously, for example the inhibition of endocytosis of influenza A virus (IAV), the direct fusion of HIV-1 [35], the penetration of the plasma membrane of HAV [11] and EBV [15], the virus entry of SARS [14], and infection by pseudorabies virus [36]. GL effectively inhibits the replication of VZV [10], HSV-1 [9], EBV [15] and HIV [13]. This is the first report that GL can suppress virus release, however, the detailed mechanisms of these remain elusive. It has also been reported that GL had a membrane stabilizing effect [37] and a reduction of membrane fluidity [35], [38]. HCV uses cellular membrane structure in its lifecycle [39], [40]. Thus, it is conceivable that membrane alterations may play a negative role in the HCV lifecycle.

We found core protein accumulation on LDs in GL-treated cell (Figure 2C, 2I and 2K). This inverse correlation between

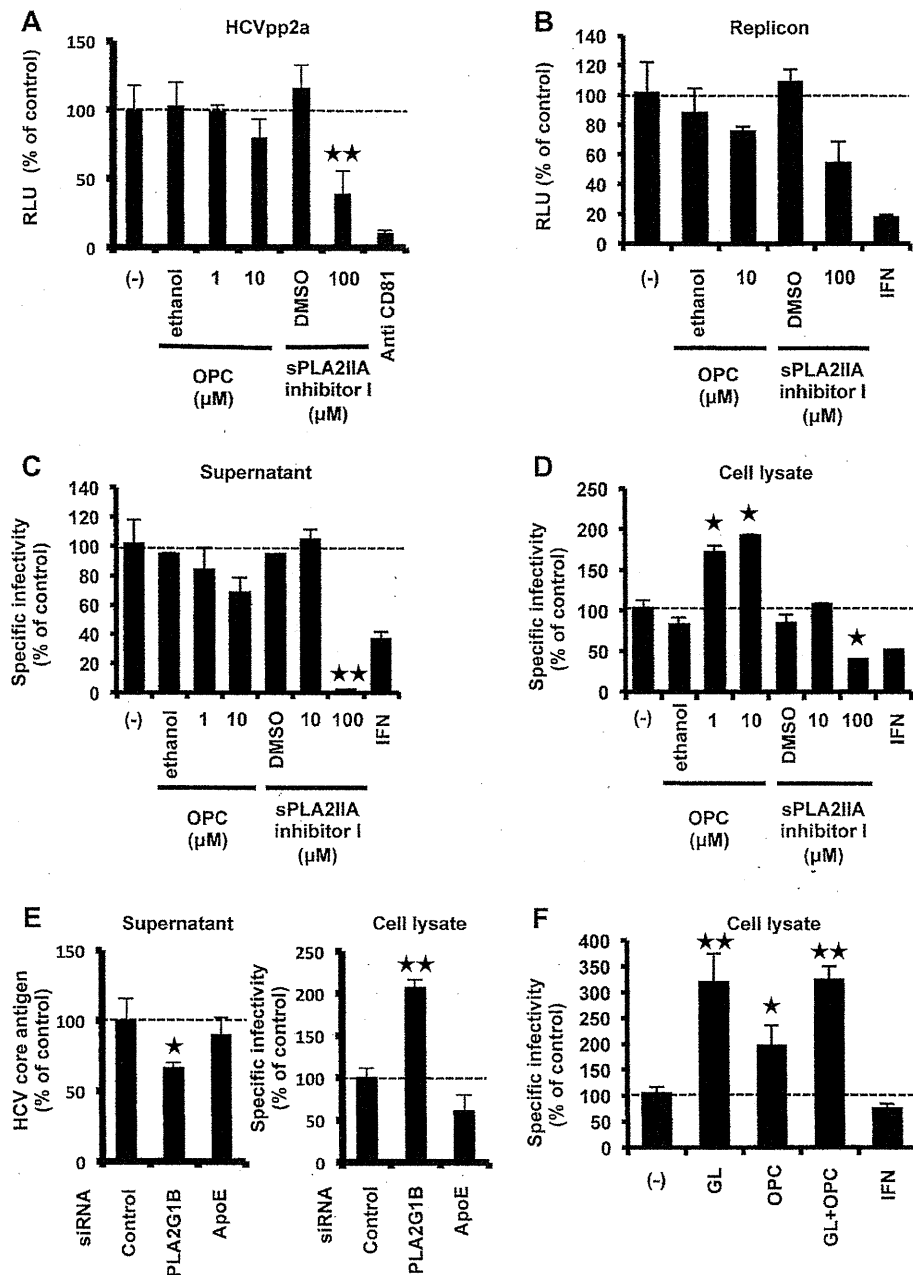


Figure 3. A role of PLA2 in HCV lifecycle. (A) Huh7 cells were infected with HCVpp in the presence and absence of OPC or sPLA2IIA inhibitor for 2 hours, then medium was replaced. Effects of PLA2 inhibitor on the entry of HCVpp were determined by measuring the luciferase activity at 72 hours post-infection. Anti-human CD81 antibody (10 μg/ml) was used as a positive control for reducing HCV entry to the cells. (B) Huh7 cells harboring the type-2a subgenomic replicon were treated with OPC or sPLA2IIA inhibitor for 72 hours. Replication efficiency of the replicon was estimated by measuring HCV RNA titer. HCVcc-infected cells were treated with PLA2 inhibitor for 72 hours. Specific infectivity of the supernatant (C) and cell lysate (D) were evaluated by quantifying the HCV core antigen in cells at 72 hours post-infection. (E) Effects of siRNA against PLA2G1B on core level in the medium (left panel) and specific infectivity in HCV-infected cells (right panel). ApoE siRNA was used as a positive control for reduced HCV infectivity. (F) HCVcc-infected cells were treated with GL (500 μM) with or without OPC (10 μM), and intracellular specific infectivity was measured. IFN (10 IU/ml) was used as a positive control. Results are expressed as the mean ± SD of the percent of the control from four independent experiments. *P < 0.05, **P < 0.005 versus control (0 μM treatment).

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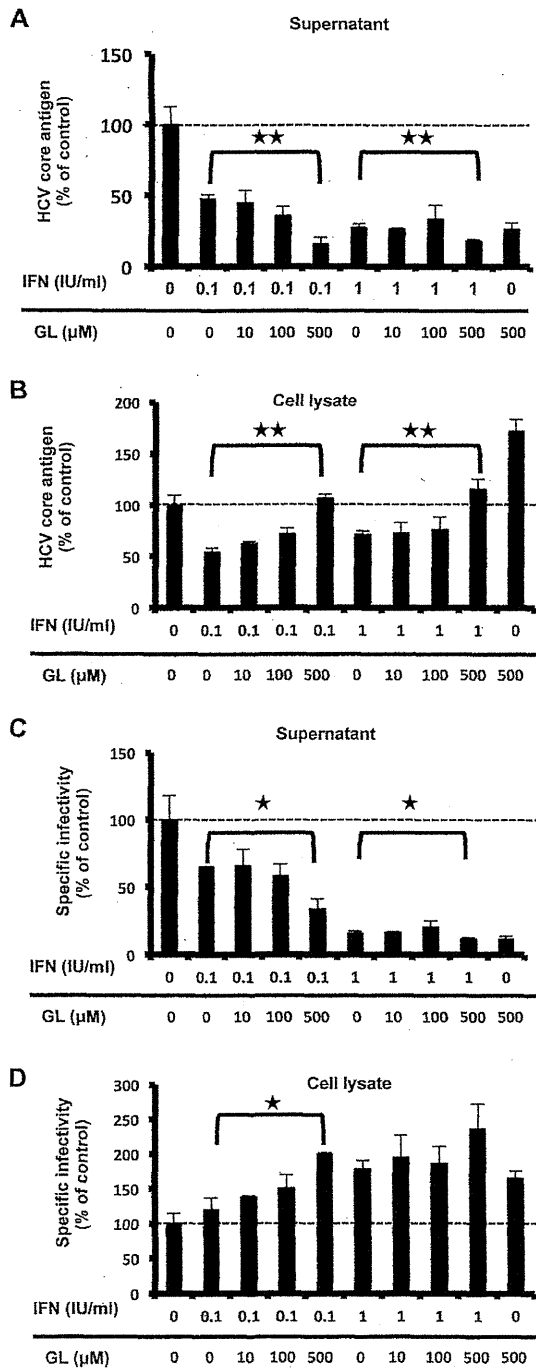


Figure 4. Anti-HCV effects IFN in combination with GL. HCVcc-infected cells were treated with IFN alone, or IFN with GL for 72 hours. HCV production was assessed by measuring the HCV core antigen in culture medium (A) and cell (B). Specific infectivity in culture medium (C) and cell (D) were measured. Results are expressed as the mean \pm SD of the percent of the control from four independent experiments. * $P < 0.05$, ** $P < 0.005$ versus IFN mono-therapy.

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the efficiency of virus production and core protein accumulation on LDs was also observed that colocalization of HCV protein with LDs was low in cases of the chimera Jc1, supporting up to 1,000-fold higher infectivity titers compared with JFH1 [41], [29]. In this study, we demonstrated that GL did not affect the size of LDs in un-infected cells (Figure 2F right panel). On the other hand, the size of LDs increased in HCV-infected cells with GL-treatment (Figure 2F left panel), probably because accumulated-HCV enhanced the formation of LDs [29].

We demonstrated the importance of PLA2G1B in HCV release by PLA2G1B inhibitor and siRNA against PLA2G1B (Figure 3). The overexpression of PLA2G1B did not have any effect on HCV release (data not shown), probably because enough PLA2G1B existed in the cells. This result is generally observed in other host factors that involved in HCV lifecycle. For example, overexpression of the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein (hVAP-33), which has a critical role in the formation of HCV replication complex, did not increase HCV replication [42]. PLA2 family proteins have been known as lipid-signaling molecules, inducing inflammation [43]. On the basis of the nucleotide sequence, the superfamily of PLA2 enzymes consists of 15 groups, comprising 4 main types: cytosolic PLA2 (cPLA2), calcium-independent PLA2, platelet activating factor acetyl hydrolase/oxidized lipid lipoprotein associated PLA2, and the secretory PLA2 (sPLA2) including PLA2G1B, 2A, and 4A [44]. In this study, we showed that GL, PLA2G1B inhibitor, and PLA2G1B siRNA inhibited HCV release and that GL and OPC had no additive effect when applied together, suggesting that suppression of HCV release by GL may be derived from its inhibitory effect on PLA2G1B. The role of PLA2G1B in the HCV lifecycle has not been reported. In this study, we also demonstrated that PLA2G2A inhibitor decreased entry, replication, and assembly of infectious HCV particles in cells (Figures 3A, 3B, 3C, and 3D). The role of PLA2G2A in the HCV lifecycle has not been reported. PLA2G2A is known to affect the secretion of VLDL (30). Therefore, PLA2G2A may contribute to HCV assembly. In the case of PLA2G4A, Menzel et al. showed that inhibition of PLA2G4A produces aberrant HCV particles [45]. These observations suggest that PLA2 has a role in several steps of the HCV lifecycle.

In this study, we showed that the EC_{50} of GL treatment for intracellular infectivity was 16.5 μ M (Figure 1A). It has been reported that the maximum peripheral concentration of GL in normal patients is 145 μ M [46]. The placebo-controlled phase I/II trial revealed no significant effect on viral titer [47]. In vivo, accumulated HCV in GL treated cells may cause lysis and apoptosis of the cells, leading to the release of infectious particles in the circulation. This may be a major limitation to use GL mono-therapy against HCV infection in patients. On the other hand, combination treatment with GL augmented the IFN-induced reduction in HCV core antigen levels (Figure 4A).

Although a number of natural compounds with anti-HCV activities were identified in recent years (Silymarin, EGCG, Ladanein, Naringenin, Quercetin, Luteolin, Honokiol, 3-hydroxy caruillignan C, and other things) [48], many aspects concerning their mechanisms of action remain unknown. In this study, GL is identified as a novel anti-HCV agent that targets the release

steps of infectious HCV particles. We found that the suppression of viral release by GL may be due to an inhibitory effect of PLA2G1B. These observations provide a basis for development of an improved IFN-based combination therapy against chronic hepatitis C.

Supporting Information

Figure S1. Anti-HCV effect of GL. HCVcc-infected cells were treated with various concentrations of GL for 72 hours. HCV production was assessed by measuring the level of HCV core antigen in culture medium. Results are expressed as the mean \pm SD of the percent of the control from four independent experiments. IFN (10 IU/ml) was used as a positive control. * $P < 0.05$, ** $P < 0.005$ versus control (0 μ M treatment). (TIF)

Figure S2. Effect of GL on expression of PLA2G1B. A human PLA2G1B cDNA was inserted into the EcoRI site of pCAGGS, yielding pCAGPLA2G1B. Since there was no effective antibody to detect endogenous expression of PLA2G1B, 293T cells transfected with the pCAGPLA2G1B plasmid were treated with GL (500 μ M) for 72 hours and lysed in lysis buffer, followed by immunoblotting with anti-PLA2G1B and anti-actin antibodies. OPC (10 μ M) was used as a positive control to reduce PLA2G1B protein in the cells. (TIF)

Figure S3. Effect of PLA2G1B siRNA on expression of PLA2G1B. HCVcc infected-Huh7 cells in a 24-well plate were transfected with siRNAs targeted to PLA2G1B and scramble negative control siRNA, followed by immunoblotting with anti-PLA2G1B and anti-actin antibodies. (TIF)

Figure S4. Effect of GL on IFN induction. The pSRE-Luc vector contains the firefly luciferase reporter gene, downstream

of the IFN-Stimulated Response Element (ISRE) cis-acting enhancer element. The pRL-TK vector contains the renilla luciferase reporter downstream of the herpes simplex virus thymidine kinase (HSV-TK promoter), and was used as an internal control. Huh7 cells transfected with the pSRE-Luc vector and the pRL-TK vector were treated with various concentrations of GL for 72 hours, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System. IFN (300 U/ml) was used as a positive control. Results are expressed as the mean \pm SD percent of the controls (treatment with IFN). (TIF)

Figure S5. Effect of GL on secretion of lipoprotein and the host proteins. Huh7 cells were treated or untreated with GL at 500 μ M for 72 hours. ApoE and albumin in the culture supernatants were measured by immunoblotting and ELISA, respectively. Results are expressed as the mean \pm SD of the percent of the control from four independent experiments. (TIF)

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Author Contributions

Conceived and designed the experiments: YM NW RS SI TS T. Miyamura T. Matsuura TW SH K. Wake K. Watashi. Performed the experiments: YM H. Aoyagi H. Aizaki. Analyzed the data: YM H. Aoyagi H. Aizaki. Contributed reagents/materials/analysis tools: MM TD. Wrote the manuscript: YM H. Aoyagi.

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Replication of Hepatitis C Virus Genotype 3a in Cultured Cells

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See Covering the Cover synopsis on page 1;
see editorial on page 13.

Hepatitis C virus (HCV) genotype 3a is widespread worldwide, but no replication system exists for its study. We describe a subgenomic replicon system for HCV genotype 3a. We determined the consensus sequence of an HCV genome isolated from a patient, and constructed a subgenomic replicon using this clone. The replicon was transfected into HuH-7 cells and RNA replication was confirmed. We identified cell culture-adaptive mutations that increased colony formation multiple-fold. We have therefore established a genotype 3a replicon system that can be used to study this HCV genotype.

Keywords: Virology; Experimental Model; HCVGT3; In Vitro Culture System.

Hepatitis C virus (HCV) infection leads to chronic infection and advanced liver diseases in most infected adults.¹ Of the 6 major HCV genotypes, genotypes 1 and 2 are the most prevalent in North America, Europe, and Japan,^{2,3} and are the most highly studied. However, other genotypes display specific characteristics. For example, genotype 3a infection can result in hepatic steatosis⁴ and telaprevir and boceprevir are less effective against genotype 3a.⁵ Therefore, the pathogenesis and inhibitor sensitivity of all HCV genotypes should be studied. Although HCV subgenomic replicons are useful for understanding viral/host factors involved in HCV replication and inhibitor sensitivity, only HCV replicons for genotypes 1a, 1b, and 2a have been established.⁶⁻⁹ Here, we report on the robust genotype 3a replication system.

An almost complete HCV genome was recovered from the serum of a patient with post-transplantation recurrent HCV infection. This serum exhibited higher infectivity than other tested sera toward primary human hepatocytes (Supplementary Figure 1A). The isolate, named S310, contained the following structural elements: a 5'UTR (nt 1-339), an open reading frame encoding 3021 aa (nt 340-9402), and a 3'UTR (nt 9403-9654). Only the last 44 nt of the X-region (nt 9611-9654) could not be recovered. Two major virus populations were found; S310/A contained Ala, Thr, Thr, and Ile, and S310/B

contained Thr, Ala, Ala, and Thr, at the 7th, 151st, 431st, and 472nd aa of the NS3 protein, respectively. S310 was clustered into genotype 3a by phylogenetic analysis (Supplementary Figure 1B). The complexity of the virus quasi-species in the serum was analyzed by sequencing the hypervariable region. Identical amino acid sequences in all 10 hypervariable region clones indicated a very low degree of diversity. The hypervariable region sequence of the JFH-1 strain also exhibited monoclonality,¹⁰ which can be important for efficient replication in cultured cells.

Subgenomic replicons SGR-S310/A and SGR-S310/B were constructed and their replication efficiency was evaluated by G418-resistant colony-formation assay. After 3 weeks, a small number of colonies were visible for both replicons (Figure 1A). Because more colonies were observed in SGR-S310/A than in SGR-S310/B, we focused on SGR-S310/A (henceforth called SGR-S310). Ten cell colonies of SGR-S310 were isolated and analyzed for HCV replication. The mean RNA titer was $9.1 \times 10^7 \pm 4.6 \times 10^7$ copies/ μ g total RNA (Figure 1B). HCV RNA (approximately 8 kb) was detected by Northern blotting (Supplementary Figure 2A). Viral proteins in the replicon cells were detected by immunofluorescence and Western blotting (Supplementary Figure 2B and 2C). To determine whether the G418 resistance of the cells was transmissible by cellular RNA transfection, we electroporated total cellular RNA isolated from 4 replicon clones into naïve HuH-7 cells. Multiple G418-resistant colonies appeared after transfection of the RNA isolated from the replicon clones (Supplementary Figure 3A), but not from the naïve HuH-7 cells. These results indicate that the replicon RNA in the parental colonies could replicate in naïve cells. Thus, the G418-resistant colonies that were isolated from cells electroporated with SGR-S310 synthetic RNA contained replicating viral RNA.

Replicating genomes have been shown to accumulate cell culture adaptive mutations, which increase their replication potential. To examine whether SGR-S310 acquired mutations, the complete HCV sequences from 10 replicon clones were sequenced. At least one nonsynonymous mutation was detected in the NS3-NSSB region of each replicon clone (Figure 1B). The following mutations were identified: T1286I in the NS3 helicase (6 of 10

Abbreviation used in this paper: HCV, hepatitis C virus.

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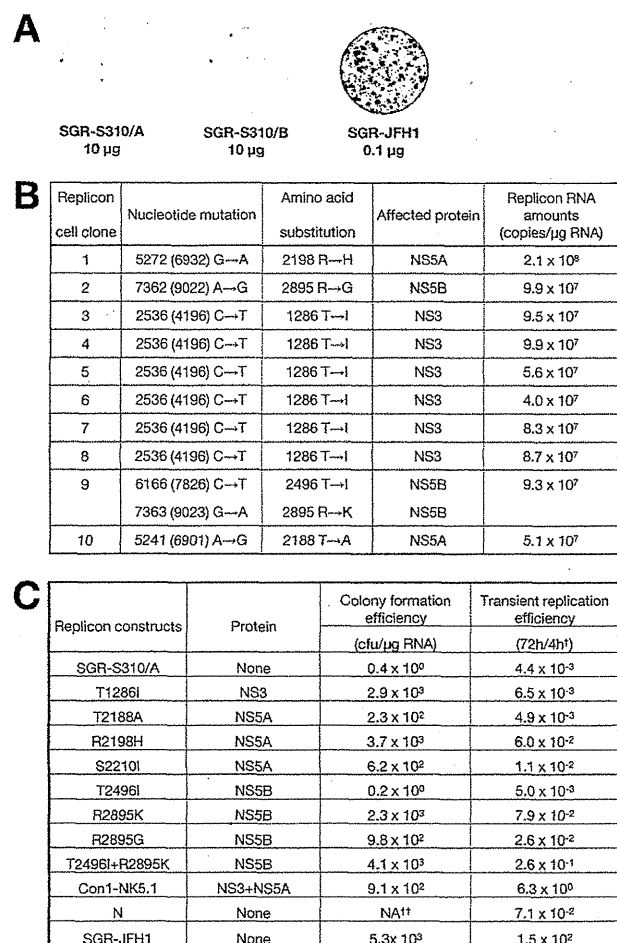


Figure 1. S310 subgenomic replicon analysis. (A) Three million HuH-7 cells were electroporated with 10 µg RNA from SGR-S310/A or SGR-S310/B or 0.1 µg RNA from SGR-JFH1. G418-selected colonies were fixed and stained after 3 weeks. (B) Non-synonymous mutations identified in the replicon genomes and HCV RNA titers in the replicon clones. Nucleotide positions within the S310 subgenomic replicon and within the full-length S310 genome (in parentheses) are given. (C) Replication potential of the adaptive mutants as determined by the colony-formation assay using Neo-replicons and by the transient replication assay using Fluc-replicons. †72 h/4 h, transient replication efficiency was determined as a ratio of luciferase activity in the transfected cells between 72 h and 4 h post transfection. ††NA, not available.

clones); T2188A or R2198H in NS5A (2 clones); an R2895G substitution in NS5B (1 clone); and T2496I in NS5A plus R2895K in NS5B (1 clone). These mutations and the S2210I mutation (corresponding to S2204I in genotype 1 replicon)^{7,8} were introduced, individually or in combination, into the parental SGR-S310 and the colony-formation efficiencies of the mutant replicons were tested. All mutations, except T2496I, increased the colony formation, indicating an adaptive phenotype (Figure 1C, Supplementary Figure 3B). Transient replication efficiency was also tested using firefly luciferase reporter replicons. SGR-S310/Luc did not replicate in Huh-7.5.1 cells, whereas the adaptive mutants displayed varying degrees of replication (Figure 1C, Supplementary Figure 3C). Adaptive mutations T2496I and R2895K, when combined to-

gether, most efficiently enhanced the colony formation as well as transient replication (Figure 1C). Interestingly, T1286I and R2895G found in our study correspond to the Con1 adaptive mutations T1280I and R2884G, respectively.^{11,12} T2188A or R2198H in NS5A were identified in 2 replicon clones and are located close to S2210I. Indeed, S2210I also enhanced SGR-S310 replication, suggesting that this region might be important for HCV replication. S310 replicons with adaptive mutations were compared with genotype 1b (Con1 and N) and 2a (JFH-1) replicons. Colony-formation efficiencies of most S310 adaptive replicons were at levels comparable with Con1 and JFH-1 (Figure 1C, Supplementary Figure 3B). In contrast, S310 adaptive replicons replicated less efficiently than Con1-NK5.1 and JFH-1 replicons in transient replication assays. However, genotype 1b N replicon replicated at a level similar to some S310 adaptive replicons (Figure 1C, Supplementary Figure 3C). Future studies will dissect the detailed mechanisms that underlie the effects of these mutations.

Successful generation of a genotype 3a replicon provided a unique opportunity to compare the susceptibility of genotype 3a (SGR-S310), 1b (Con1¹³), and 2a (JFH-1/4-1¹³) replicons to HCV inhibitors. Interferon-alfa dose-dependently decreased the replication of all tested genotypes (Figure 2A), whereas a protease inhibitor, BILN-2061, was more effective against replicons from genotypes 1b and 2a than 3a (Figure 2B). The non-nucleoside polymerase inhibitor JTK-109 was more potent against genotype 1b and 3a (Figure 2C). However, the nucleoside polymerase inhibitor, PSI-6130, equally inhibited all genotypes (Figure 2D).

In conclusion, we established a subgenomic replicon for genotype 3a, which should be useful for understanding the specific characteristics of this genotype and for the screening of antiviral chemicals that are effective against this genotype. Construction of a full-length infectious S310 clone is in progress.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2012.09.017>.

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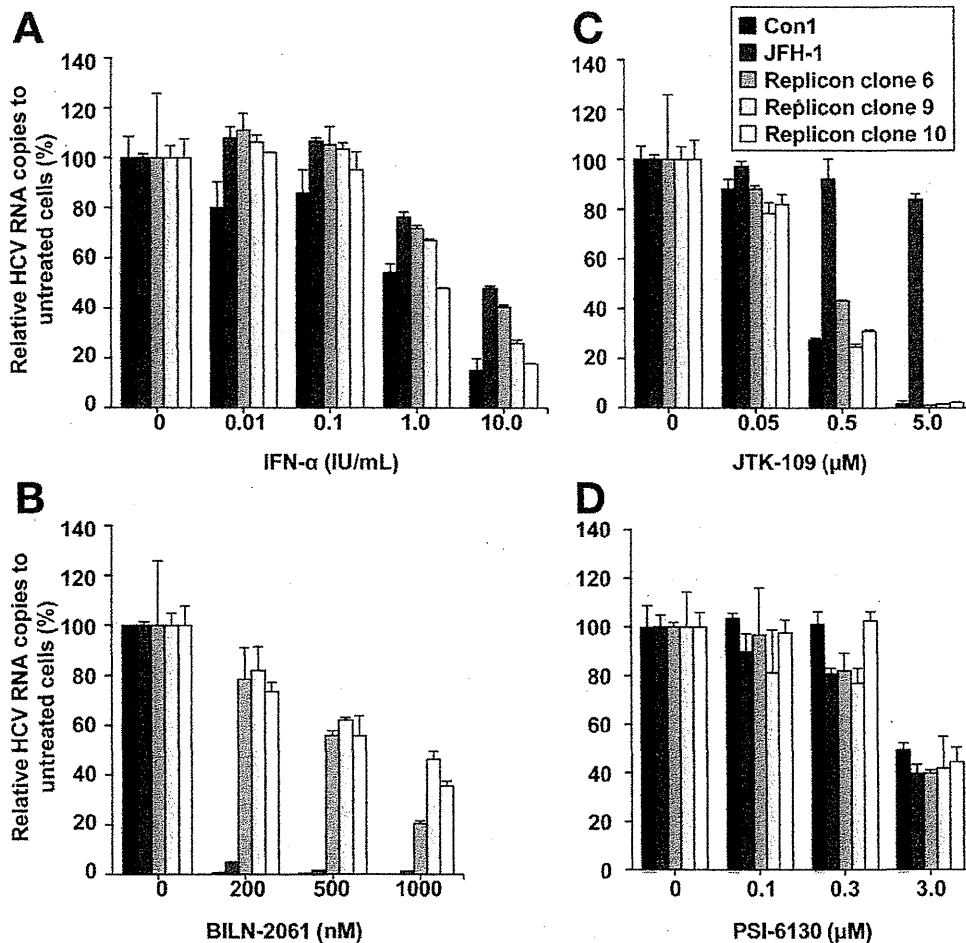


Figure 2. Effects of antiviral inhibitors on the replication of S310 subgenomic replicons. Three cell clones (clone 6, 9 and 10) carrying genotype 3a S310 replicons and one cell clone each harboring genotype 1b Con1 and genotype 2a JFH-1 replicons were treated with the indicated concentrations of (A) interferon alpha, (B) HCV protease inhibitor BILN-2061, (C) the non-nucleoside polymerase inhibitor JTK-109, and (D) the nucleoside polymerase inhibitor PSI 6130 for 72 hours and replication levels were measured by quantifying intracellular HCV RNA. Results are means \pm standard deviations of 3 replicates.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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