

**Fig. 5.** Activation effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) on hepatitis C virus (HCV) RNA polymerase of genotypes 1a, 1b, and 2a. The polymerase activation effects of CyPA and CyPB on HCV 1a (H77 and RMT), 1b (HCR6, NN, and Con1), and 2a (J6CF and JFH1) were examined. HCV RdRp (100 nM) was incubated with 50× CyPA and 5× CyPB. The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements.

different from the physiological concentrations in cells [4,37]. However, under the optimal HCV transcription condition, HCV RdRp activation was observed by CyPA and CyPB (Fig. 1).

The amount of CyPA varies by cell type [59]. In some cells, CyPB may also contribute to HCV 1b replication because it localizes in the endoplasmic reticulum and plasma membranes [60,61], which form a membrane web in which an HCV replication complex exists [1].

PPI activity of CyPs is essential for HCV replicon activation [32,53]. CyP inhibitors (DEBIO-025, NIM811, and SCY-635) inhibit PPI activity. The PPI activity of CyPA contributed to HCV RdRp activation and CyP-NS5A binding [36]. The PPI activity of CyPA partly contributed to the activation of HCR6 (1b) RdRpwt in vitro (Fig. 3A,  $p < 0.01$ ). The PPI activity of CyPB may not be essential for RdRp activation because the activation ratio was not large between CyPB and CyPBΔPPI, although the experiment showed a statistically significant difference (Fig. 3B). There may be differences in the RdRp activation mechanisms of CyPA with and without PPI activity. This finding will help with the development of new CyPA inhibitors that target domains other than PPI.

The mechanism of HCV RdRp activation by CyPs is not clear. In the least, CyPA and CyPB enhanced the early stage of HCV transcription, including the template RNA binding of HCV RdRp (Fig. 4) [29,41,45]. The productive template-polymerase binding is the late-limiting step of transcription initiation by HCV RdRp in vitro, and a small fraction of HCV RdRp was active in vitro [62,63]. CyP may enhance this step on many HCV RdRp molecules to show apparent activation of RdRp in vitro.

Considering the controversial reports on CyP and HCV replication [29,33,35,41,43,44], it can be concluded that CyPA is the major factor of HCV genome replication and that the activation of HCV RdRp may require other factors such as NS5A to condense CyPA around the HCV RdRp. Although many HCV treatment approaches have been applied in addition to Peg-IFN, ribavirin, and NS3/NS4a protease inhibitor [64–67], more effort has to be made to ensure an HCV cure. This

study and that of Heck et al. [54] demonstrated similar activation kinetics and genotype specificity of CyPB activation (Figs. 2 and 5). CyPB also has the potential to activate HCV 1b genome replication in a limited condition, and it should also be included as the target of inhibitor development because HCV 1b is the genotype that is most resistant to treatment [13].

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagen.2012.08.017>.

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# Japanese Reference Panel of Blood Specimens for Evaluation of Hepatitis C Virus RNA and Core Antigen Quantitative Assays

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**An accurate and reliable quantitative assay for hepatitis C virus (HCV) is essential for measuring viral propagation and the efficacy of antiviral therapy. There is a growing need for domestic reference panels for evaluation of clinical assay kits because the performance of these kits may vary with region-specific genotypes or polymorphisms. In this study, we established a reference panel by selecting 80 donated blood specimens in Japan that tested positive for HCV. Using this panel, we quantified HCV viral loads using two HCV RNA kits and five core antigen (Ag) kits currently available in Japan. The data from the two HCV RNA assay kits showed excellent correlation. All RNA titers were distributed evenly across a range from 3 to 7 log IU/ml. Although the data from the five core Ag kits also correlated with RNA titers, the sensitivities of individual kits were not sufficient to quantify viral load in all samples. As calculated by the correlation with RNA titers, the theoretical lower limits of detection by these core Ag assays were higher than those for the detection of RNA. Moreover, in several samples in our panel, core Ag levels were underestimated compared to RNA titers. Sequence analysis in the HCV core region suggested that polymorphisms at amino acids 47 to 49 of the core Ag were responsible for this underestimation. The panel established in this study will be useful for estimating the quality of currently available and upcoming HCV assay kits; such quality control is essential for clinical usage of these kits.**

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide (15). There is no protective vaccine against this virus, and once an individual is infected, HCV often establishes persistent infection and leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (9). The most widely used therapy for HCV infection is the combined administration of pegylated alpha interferon and ribavirin (29). However, this treatment is problematic, as it has limited efficacy, high cost, and severe adverse effects (8, 25). To estimate the outcome of antiviral therapy, and to understand the state of viral propagation, it is important to determine the HCV viral load in chronic hepatitis C patients by the use of accurate and reliable HCV quantitative assays (9, 14). For this purpose, several commercial assay kits for HCV RNA and core antigen (Ag) quantification are currently used in Japan. For quantification of HCV RNA levels, two real-time quantitative reverse transcription-PCR (qRT-PCR)-based assay kits are available, including the COBAS AmpliPrep/COBAS TaqMan HCV test (CAP/CTM-RNA; Roche Diagnostics, Tokyo, Japan) and the Abbott RealTime HCV test (ART-RNA; Abbott Japan, Tokyo, Japan). These assays are known to have high sensitivity and a wide dynamic range, but they require technical skill and attention to maintaining the specified conditions (4–6, 16, 24, 33–35). Alternatively, HCV viremia can be quantified by assessment of HCV core Ag level (1–3, 7, 10, 12, 13, 17–22, 27, 30–32). Five HCV core Ag assay kits are commercially available in Japan, including Architect HCV Ag (Architect-Ag; Abbott Japan), Lumipulse Ortho HCV Ag (Lumipulse-Ag; Fujirebio, Tokyo, Japan), Lumispot Eiken HCV Ag (Lumispot-Ag; Eiken Chemical, Tokyo, Japan), the Ortho HCV Ag ELISA test (ELISA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan), and the Ortho HCV Ag IRMA test (IRMA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan). These assays have some disadvantages compared to those measuring HCV RNA (notably, low sensitivity and narrow range of quantification) but also have some advantages (including ease of use, reduced risk of

contamination, reduced cost, and reliability even with samples stored at room temperature for extended periods of time [1, 32]). Although core Ag levels are thought to be related closely to HCV RNA titers, the correlation and linearity of core Ag levels have not yet been fully evaluated. In addition, these quantitative parameters are known to be affected by nucleotide and amino acid sequences at the target regions of the assays (5, 6, 28, 34), and this sequence variation depends on genotypes or predominant strains in specific geographical regions.

In this study, we established a Japanese reference panel of samples for evaluation of HCV RNA and core Ag levels by collecting donated blood specimens that tested positive for HCV RNA and anti-HCV antibodies. Using this reference panel, we evaluated the HCV loads in these specimens with two HCV RNA assay kits and five core Ag assay kits and assessed correlations among the data generated by these kits.

## MATERIALS AND METHODS

**Preparation of reference panel.** To establish a reference panel for HCV quantitative assays, a total of 80 donated plasma samples were selected. All of these specimens, supplied by the Japanese Red Cross Blood Centers, tested positive for the presence of HCV RNA and anti-HCV antibodies. These samples, collected in Japan from May to September of 2007, were obtained from Japanese blood donor volunteers in various regions of

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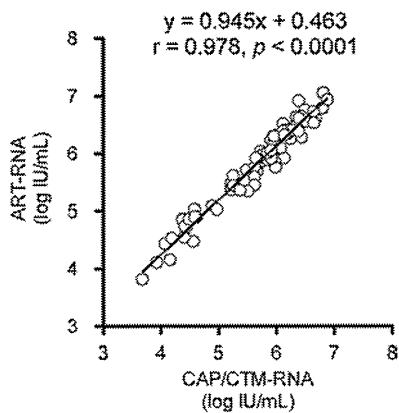


FIG 1 Correlation of HCV RNA titers as quantified by two commercial kits.

Japan. The samples were divided into 1-ml aliquots and stored at  $-80^{\circ}\text{C}$  until use.

**Quantification of HCV RNA and core Ag.** The HCV RNA titer was measured with two real-time qRT-PCR kits, CAP/CTM-RNA (detection range,  $1.5 \times 10^1$  to  $6.9 \times 10^7$  IU/ml) and ART-RNA (detection range,  $1.2 \times 10^1$  to  $1.0 \times 10^8$  IU/ml). Additionally, samples were assessed using five HCV core Ag assay kits, including Architect-Ag (detection range, 3 to 20,000 fmol/liter), Lumipulse-Ag (detection range, 50 to 50,000 fmol/liter), Lumispot-Ag (detection range, 20 to 400,000 fmol/liter), ELISA-Ag (detection range, 44.4 to 3,600 fmol/liter), and IRMA-Ag (detection range, 20 to 20,000 fmol/liter). All assays were performed by the respective manufacturers at their research laboratories.

**Sequencing and genotyping of HCV in reference panel samples.** Viral RNA was extracted with the QIAamp viral RNA kit (Qiagen, Valencia, CA) from 140  $\mu\text{l}$  of each plasma sample. HCV RNA was amplified by RT-PCR with primers corresponding to the 5' untranslated region (UTR) (43S-IH, 5'-CCTGTGAGGAAGTACTGTCTTC-3'; c/s17-ssp, 5'-CCGG GAGAGCCATAGTGGTCTGCG-3') and the E1 region (1323R-IH, 5'-G GCGACCAGTTCATCATCAT-3'); the amplified products were sequenced directly. HCV genotypes of the isolated strains were assigned by phylogenetic analysis using an alignment with a representative strain of each genotype.

**Statistical analysis.** The correlations of obtained quantitative data were assessed by Pearson's correlation coefficient analysis, and values for  $r$  and  $P$  were calculated. A  $P$  value of  $<0.05$  was considered to indicate statistical significance. Analysis was performed using Prism 5 software (GraphPad Software, Inc., La Jolla, CA).

**Nucleotide sequence accession numbers.** The accession numbers of C-01 to C-80 are AB705312 to AB705391, respectively.

## RESULTS

**Quantification of HCV RNA levels.** The reference panel established in this work was used to measure HCV RNA levels with the CAP/CTM-RNA and ART-RNA kits. The correlation of the data obtained with the two kits is shown in Fig. 1. The RNA titers of these samples were distributed evenly, and all values were within the dynamic ranges of both assays. The HCV titers ranged from 3.68 to 6.88 and 3.82 to 7.08 log IU/ml in CAP/CTM-RNA and ART-RNA, respectively, and the correlation was significant ( $r = 0.978$ ;  $P < 0.0001$ ).

**Quantification of HCV core Ag levels.** HCV core Ag levels were measured using Architect-Ag, Lumipulse-Ag, Lumispot-Ag, ELISA-Ag, and IRMA-Ag kits. Among the 80 specimens in the reference panel, core Ag levels could be measured in all samples using Architect-Ag and ELISA-Ag kits, whereas core Ag levels

were below the detection limit in 4, 2, and 1 samples using Lumipulse-Ag, Lumispot-Ag, and IRMA-Ag kits, respectively (Fig. 2; also, see Fig. S1 in the supplemental material). Significant correlations were observed between assays of HCV core Ag and HCV RNA ( $r = 0.9065$  to  $0.9666$  and  $P < 0.0001$  compared with CAP/CTM-RNA data [Fig. 2]);  $r = 0.8877$  to  $0.9552$  and  $P < 0.0001$  compared with ART-RNA data [see Fig. S1 in the supplemental material]). The theoretical lower limits of detection of these assays were calculated by use of these correlation formulas and were 3.2 and 3.4 log IU/ml for Architect-Ag, 4.2 and 4.2 log IU/ml for Lumipulse-Ag, 3.7 and 3.9 log IU/ml for Lumispot-Ag, 3.6 and 3.8 log IU/ml for ELISA-Ag, and 3.6 and 3.8 log IU/ml for IRMA-Ag (compared to CAP/CTM-RNA and ART-RNA, respectively). These calculated detection limits were substantially higher than those for the RNA quantitative assays (1.18 and 1.08 log IU/ml for CAP/CTM-RNA and ART-RNA, respectively).

In addition, we found that several samples showed considerable deviation from the linear regression (Fig. 2; also, see Fig. S1 in the supplemental material). To identify the deviating samples, we used Bland-Altman plot analysis (Fig. 3; also, see Fig. S2 in the supplemental material). This plot shows the difference between the titer values of HCV RNA and core Ag as a function of the average of these two values. Several samples demonstrated discordance between the measured HCV RNA and core Ag levels. Among these samples, we focused on samples with discordant results in multiple core Ag assays compared to both RNA quantitative assays. For sample C-01, core Ag levels were underestimated when measured with Architect-Ag, Lumipulse-Ag, and Lumispot-Ag in comparison with CAP/CTM-RNA (Fig. 3) and when measured with Architect-Ag, Lumipulse-Ag, Lumispot-Ag, and IRMA-Ag in comparison with ART-RNA (see Fig. S2 in the supplemental material). Likewise, for sample C-73, core Ag levels were underestimated when measured with Architect-Ag, Lumipulse-Ag, and IRMA-Ag in comparison with CAP/CTM-RNA (Fig. 3) and when measured with Architect-Ag and Lumipulse-Ag in comparison with ART-RNA (see Fig. S2 in the supplemental material). Thus, sample-specific underestimation was observed in several HCV core Ag kits.

**Nucleotide sequences in core region of reference panel samples.** To clarify the sources of these underestimates of HCV core Ag levels, HCV RNA was extracted from each of the samples in the reference panel, and the nucleotide sequences of core regions were determined. Phylogenetic analysis with these sequences permitted classification of the individual strains by genotype. Of 80 samples in the reference panel, 1 (1.3%) was genotype 1a, 35 (43.8%) were genotype 1b, 26 (32.5%) were genotype 2a, and 18 (22.5%) were genotype 2b (Table 1; also, see Fig. S3 in the supplemental material). These strains were distributed evenly among reference strains of each genotype and cover the sequence diversity of strains isolated in Japan (see Fig. S3 in the supplemental material). The genotypes of samples associated with underestimated core Ag values (samples C-01 and C-73) were both classified as genotype 2a.

Predicted amino acid sequences of HCV core protein were aligned with the consensus core protein sequence for the genotype 1b strains obtained in this study (see Fig. S4 in the supplemental material). Excluding the genotype-specific sequence variations, a specific amino acid polymorphism was identified at amino acid (aa) residue 48 (Ala to Thr) in samples C-01 and C-73. Sample C-01, which yielded underestimated values in most core Ag assays, also possessed an additional polymorphism in the same region,

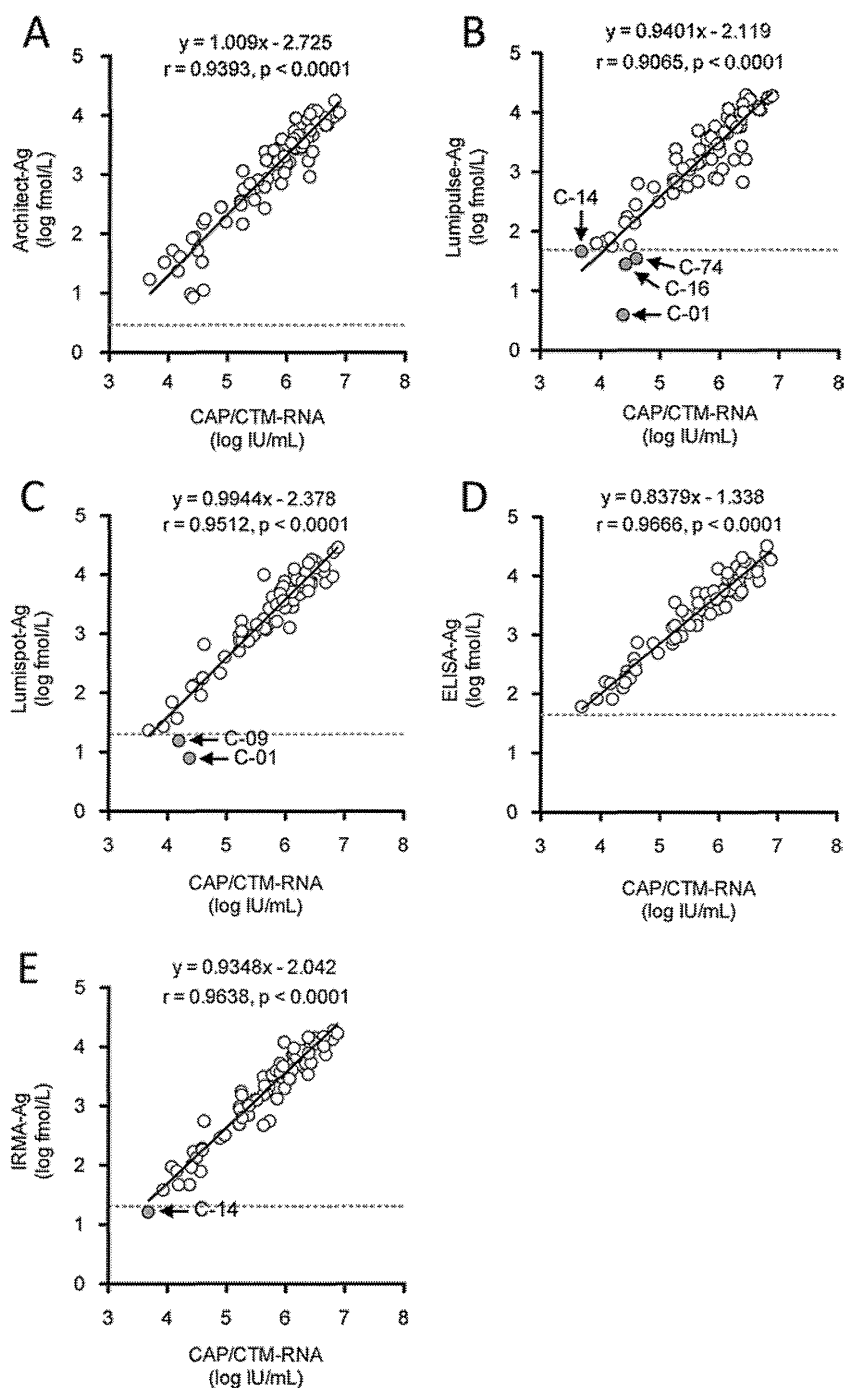


FIG 2 Correlation between CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. Data for core Ag levels were converted to log fmol/liter prior to analysis. In each plot, the lower limit of detection of the respective core Ag assay is indicated by a dotted line. Data for samples below the lower detection limit of each assay are indicated by shaded circles labeled with the respective sample designations.

specifically an Arg-to-Gly substitution at aa 47. We suspected that these polymorphisms altered the antigenicity of the core protein, thereby reducing detected core Ag levels and leading to underestimation of values by the core Ag quantification kits. To assess the correlation of these polymorphisms with the underestimation of core Ag values, strains containing polymorphisms in this region (at aa 47 to 49 [Fig. 4]) were identified in Bland-Altman plots of HCV RNA and core Ag (Fig. 3; also, see Fig. S2 in the supplemental

material). A total of 12 strains exhibited polymorphisms at these positions, including 2 strains of genotype 1b, 8 of genotype 2a, and 2 of genotype 2b (Table 1). In the Bland-Altman plot of CAP/CTM-RNA and Architect-Ag, 4 of 12 values (for samples C-01, C-16, C-73, and C-74) were located under the line of the lower 95% limit of agreement (Fig. 3A). Likewise, in the plot of CAP/CTM-RNA and Lumipulse-Ag, 3 of 12 values (those for samples C-01, C-67, and C-73) were located under the line of the lower

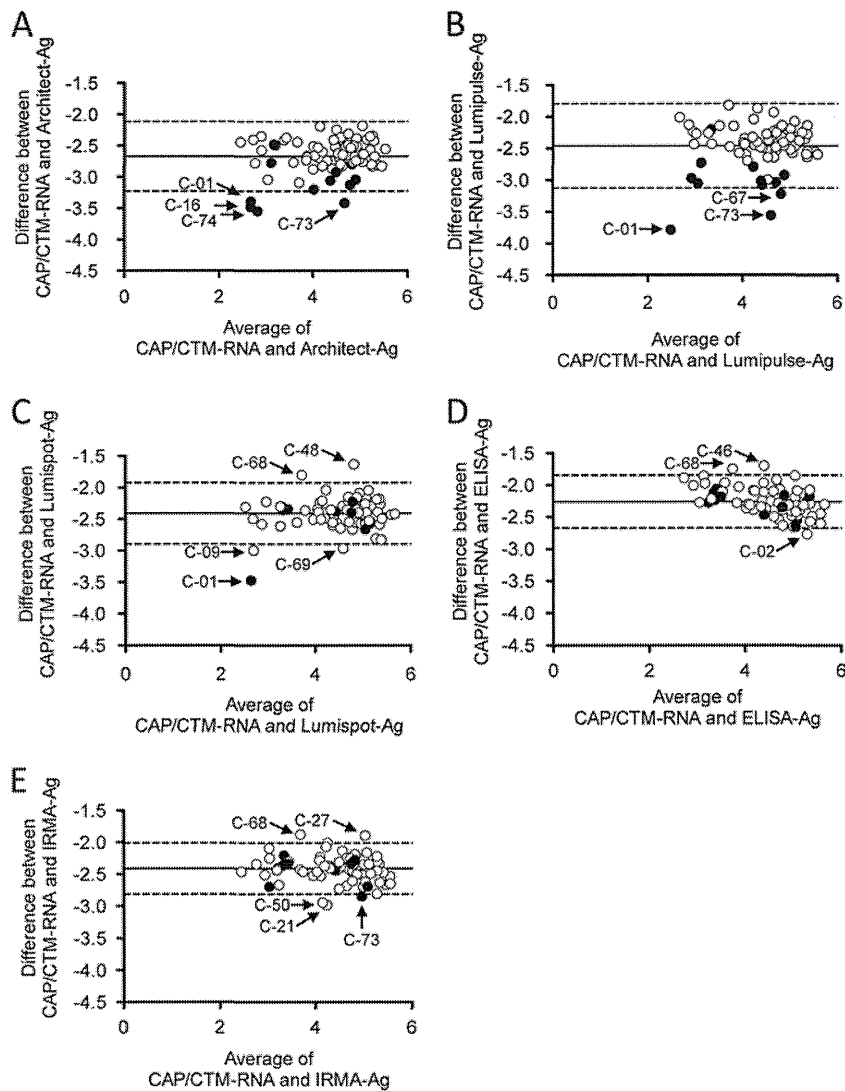


FIG 3 Bland-Altman plot analysis of CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. These plots show the difference between the values of HCV RNA and core Ag as a function of the average of these two values. Data for core Ag levels were converted to log fmol/liter prior to analysis. The bias and 95% limits of agreements are indicated by solid and dashed lines, respectively. Data for samples with polymorphisms at amino acid residues 47 to 49 are indicated by solid circles. Data points outside the 95% limits are indicated by arrows labeled with the sample designations.

95% limit of agreement (Fig. 3B). In these plots, underestimation for samples that lacked these polymorphisms (at aa 47 to 49) was not detected. In the plot of CAP/CTM-RNA and Lumispot-Ag, only 1 sample (C-01) was located under the line of the lower 95% limit of agreement, but this sample exhibited the most discordant

value (Fig. 3C). In the plot of CAP/CTM-RNA and ELISA-Ag, no correlation between polymorphisms at these positions and underestimation was observed (Fig. 3D). In the plot of CAP/CTM-RNA and IRMA-Ag, sample C-73 was located under the line of the lower 95% limit of agreement, as were other samples that lacked polymorphisms at aa 47 to 49 (Fig. 3E). Similar trends were observed in comparison with ART-RNA levels (see Fig. S2 in the supplemental material). Based on these results, the levels of HCV core Ag measured with Architect-Ag and Lumipulse-Ag seem to be more strongly affected by single polymorphisms at these positions. In the case of Lumispot-Ag, underestimation may be limited to specimens with multiple polymorphisms at these positions.

TABLE 1 Number of reference panel strains with polymorphisms at amino acid residues 47 to 49 of the HCV core region

Genotype	No. (%) of strains	
	Total	With polymorphisms
1a	1	0
1b	35	2 (5.7)
2a	26	8 (30.8)
2b	18	2 (11.8)
Total	80	12 (15.0)

### DISCUSSION

The quantification of HCV viral load is essential for selecting an appropriate antiviral strategy and for monitoring the efficacy of treatment. Since HCV is known to be highly variable and rapidly

aa	1		60
1b-cons.	MSTNPKPQRKTKRNTNRRPQDVKFFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRG		
C-16 (1b)	.....		P.....
C-53 (1b)	.....		P.....
C-01 (2a)	.....		GT.....
C-03 (2a)	.....	L.....	T.....
C-12 (2a)	.....	T.....	T.....
C-65 (2a)	.....	T.....	A.....
C-67 (2a)	.....		T.....
C-71 (2a)	.....		T.....
C-73 (2a)	.....		T.....
C-76 (2a)	.....		A.....
C-08 (2b)	.....		P.....
C-74 (2b)	.....		P.....

FIG 4 Alignment of the first 60 amino acids of the HCV core region of strains with polymorphisms at amino acid residues 47 to 49. The position numbers are given at the top. Dots indicate identical amino acids. The consensus sequence of 1b strains (1b cons.) isolated in this study was determined and used as a reference sequence. Genotypes of strains are given in parentheses. Positions of polymorphisms are indicated by inverted triangles above the sequence alignment.

evolving (23, 26), the assays for quantifying this virus should be unaffected by sequence polymorphisms. In this study, we established a reference panel with HCV-positive samples and evaluated the correlation among multiple assays for HCV RNA and core Ag quantification.

Using this reference panel, we found that the results from two HCV RNA assay kits, CAP/CTM-RNA and ART-RNA, correlated with excellent agreement ( $r = 0.978$ ,  $P < 0.0001$  [Fig. 1]), although discrepancies for values generated by these two assays have been reported for strains of genotypes 1, 2, and 4 (5, 6, 34). In Japan, the prevalent genotypes are 1b, 2a, and 2b (11); no genotype 4 sample was included in our reference panel (Table 1). In quantification with CAP/CTM-RNA, underestimation of HCV RNA titer has been reported for French genotype 2 samples (5). In our panel, no underestimation was observed for data from genotype 2 samples compared to values obtained using ART-RNA. Therefore, underestimation in quantification with CAP/CTM-RNA is expected to be rare in Japanese samples, and the two assays for HCV RNA quantification should be considered accurate and reliable, at least for Japanese samples. Additionally, the prepared reference panel appears to be suitable for the evaluation of HCV quantification assays, because genotypes of samples in this panel are representative of those found in Japan and viral loads are distributed evenly across the range of expected titers.

The quantification of HCV core Ag is an alternative test for HCV infection and viral load. However, in this study, several core Ag quantitative assays failed to provide accurate results for all of the samples in the reference panel (Fig. 2). Some quantified values were below the kits' detection limits. This shortcoming was mainly attributable to the lower sensitivity of the core Ag assay kits; increased sensitivity is urged in the future development of HCV core Ag kits. Among the kits tested here, Architect-Ag assay exhibited the highest sensitivity and was sufficient for quantifying the viral load in all samples. However, even in the case of Architect-Ag, theoretical lower limits of detection, calculated by correlation formula using CAP/CTM-RNA and ART-RNA, were 3.2 and 3.4 log IU/ml, respectively; these detection limits still exceeded the lower limits of the HCV RNA quantification assays. Therefore, the sensitivity of the available HCV core Ag assays is still insufficient to detect low-titer HCV infections. Core Ag kits therefore may be unsuitable for the detection of breakthrough hepatitis during antiviral therapy or for the detection of HCV infection in a window period.

Comparison between HCV RNA and core Ag assays revealed good correlations, with  $r$  coefficients ranging from 0.8877 to 0.9666 and  $P$  values being less than 0.0001 (Fig. 2; also, see Fig. S1 in the supplemental material). Therefore, the HCV core Ag levels may serve as an alternative to HCV RNA levels when titers remain within the detection ranges of the core Ag kits. However, several discordances were detected when core Ag levels were compared with those of HCV RNA. For one sample in our panel (sample C-01), core Ag levels were lower than expected when quantified using any of the three core Ag kits (Architect-Ag, Lumipulse-Ag, and Lumispot-Ag) (Fig. 3; also, see Fig. S2 in the supplemental material). Another sample (C-73) also yielded lower-than-expected levels when assayed with Architect-Ag and Lumipulse-Ag kits. Sequence analysis of the core region revealed that polymorphisms at aa 47 and 48 correlated with these underestimates by core Ag kits (see Fig. S4 in the supplemental material). These results are consistent with our previous study, which suggested that core Ag levels of HCV strain JFH-1 were underestimated by the Lumipulse-Ag kit in comparison to the ELISA-Ag assay (28). Strain JFH-1 harbors an Ala-to-Thr substitution at aa 48; conversion of Thr to Ala at this position in JFH-1 was sufficient to overcome this underestimation. This region of the core Ag presumably corresponds to one of the epitopes recognized by the monoclonal antibodies used in the Lumipulse-Ag kit, such that polymorphisms at this position affected the antigenicity of the core protein. In this study, we found that the presence of other polymorphisms in this region (aa 47 to 49) correlated with reduced core Ag levels as detected by Lumipulse-Ag, as well as by other assays (Architect-Ag and Lumispot-Ag). Sample C-01 demonstrated a drastic deviation from expected core Ag levels in these assays (Fig. 3; also, see Fig. S2 in the supplemental material). The HCV strain in this sample contains two polymorphisms (Arg to Gly at aa 47 and Ala to Thr at aa 48); the multiple polymorphisms may impair antibody binding more severely and therefore result in underestimation of core Ag levels. Interestingly, this sample exhibited reasonable core Ag levels when assayed using ELISA-Ag. Thus, the underestimation of core protein levels in this sample was kit dependent, suggesting the targeting of distinct epitopes by the antibodies used in each of these kits. This hypothesis could not be confirmed, because the identity of the epitopes targeted by each kit is proprietary.

Of 12 samples with amino acid polymorphisms in this region, 2 (5.7%) were of genotype 1b, 8 (30.8%) were of genotype 2a, and

TABLE 2 Number of strains in the sequence database<sup>a</sup> with polymorphisms at amino acid residues 47 to 49 of the HCV core region

Genotype	No. (%) of strains				Total
	Tested	With polymorphism			
		At aa 47 (R/C, G)	At aa 48 (A/T, P)	At aa 49 (T/A, P, L)	
1b	543	2 (0.36)	4 (0.74)	16 (2.96)	22 (4.1)
2a	24	0	6 (25.0)	1 (4.2)	7 (29.2)
2b	39	0	0	2 (6.9)	2 (6.9)

<sup>a</sup> <http://s2as02.genes.nig.ac.jp/>.

2 (11.8%) were of genotype 2b (Table 1). Searches of the Hepatitis Virus Database (<http://s2as02.genes.nig.ac.jp/>) revealed that corresponding amino acid polymorphisms were observed in 22 of 543 strains (4.1%) of genotype 1b, 7 of 24 strains (29.2%) of genotype 2a, and 2 of 39 strains (6.9%) of genotype 2b (Table 2). These percentages were consistent with our observations in the proposed reference panel. These data (our results and those from the database) clearly indicate that genotype 2a strains are the most frequent source of underestimation of core Ag levels. Notably, our search of the sequence database did not yield any HCV strain with multiple polymorphisms in the region from aa 47 to 49, as we saw in our sample C-01. Therefore, strains with such multiple polymorphisms are rare so far, but detection of this isolate among donated blood specimens suggests that such HCV strains could be emerging in clinical samples. For patients harboring such strains, HCV viral load may be underestimated if measurement of HCV viral load is performed by core Ag assay. Such underestimates may result in erroneous selection of therapy, adversely affecting patient outcome. Thus, this shortcoming in HCV core Ag assay kits needs to be addressed.

There is a growing need for evaluation of clinical assay kits with domestic specimen reference panels, since the performance of these kits may be affected by the genotypes or polymorphisms of predominant strains in different geographic regions. To our knowledge, such an investigation of HCV clinical assay kits with domestic specimens has not previously been conducted in Japan. The Japanese HCV reference panel described here was generated with plasma samples collected from Japanese volunteers. Each sample was divided into small aliquots, and the panel was prepared in multiple sets. The samples in our HCV reference panel represent the predominant strains and genotypes seen in Japan. We expect that this reference panel will be of use for the development, evaluation, and optimization of HCV assay kits for the Japanese clinical market.

In conclusion, we have established a Japanese reference panel for evaluation of HCV quantification assays. Using this reference panel, we found that two assay kits for HCV RNA could quantify HCV titers concordantly. We also found that the data generated by HCV core Ag assay kits correlated with the results of HCV RNA assays. However, the nominal core Ag levels measured by several kits underestimated actual levels for HCV samples with polymorphisms at aa 47 to 49 of the core Ag. The panel established in this study is expected to be useful for estimating the accuracy of currently available and upcoming HCV assay kits; such quality control is essential for clinical usage of these kits.

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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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**Competing interests:** K. Wake is employed by Minophagen Pharmaceutical Co., Ltd. GL (20<math>\beta</math>-carboxyl-11-oxo-30-norolean-12-en-<math>\beta</math>-yl-2-O-<math>\beta</math>-D-glucopyranuronosyl-<math>\beta</math>-D-glucopyranosiduronic acid) was kindly provided by the Minophagen Pharmaceutical Co., Ltd. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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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- $\alpha$ ) 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 $\beta$ -carboxyl-11-oxo-30-norolean-12-en-3 $\beta$ -yl-2-O- $\beta$ -D-glucopyranuronosyl- $\beta$ -D-glucopyranosiduronic acid) and IFN- $\alpha$  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% OsO<sub>4</sub> 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  $\mu$ M GL (Figure S1). As shown in Figure 1A, infectivity of supernatant following GL treatment at 3, 30, or 500  $\mu$ M was reduced by 12, 62, or 71% of the control levels, respectively. The calculated 50% effective concentration (EC<sub>50</sub>) was 16.5  $\mu$ 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 EC<sub>50</sub> value of 728  $\mu$ 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 EC<sub>50</sub> value of 738  $\mu$ 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).

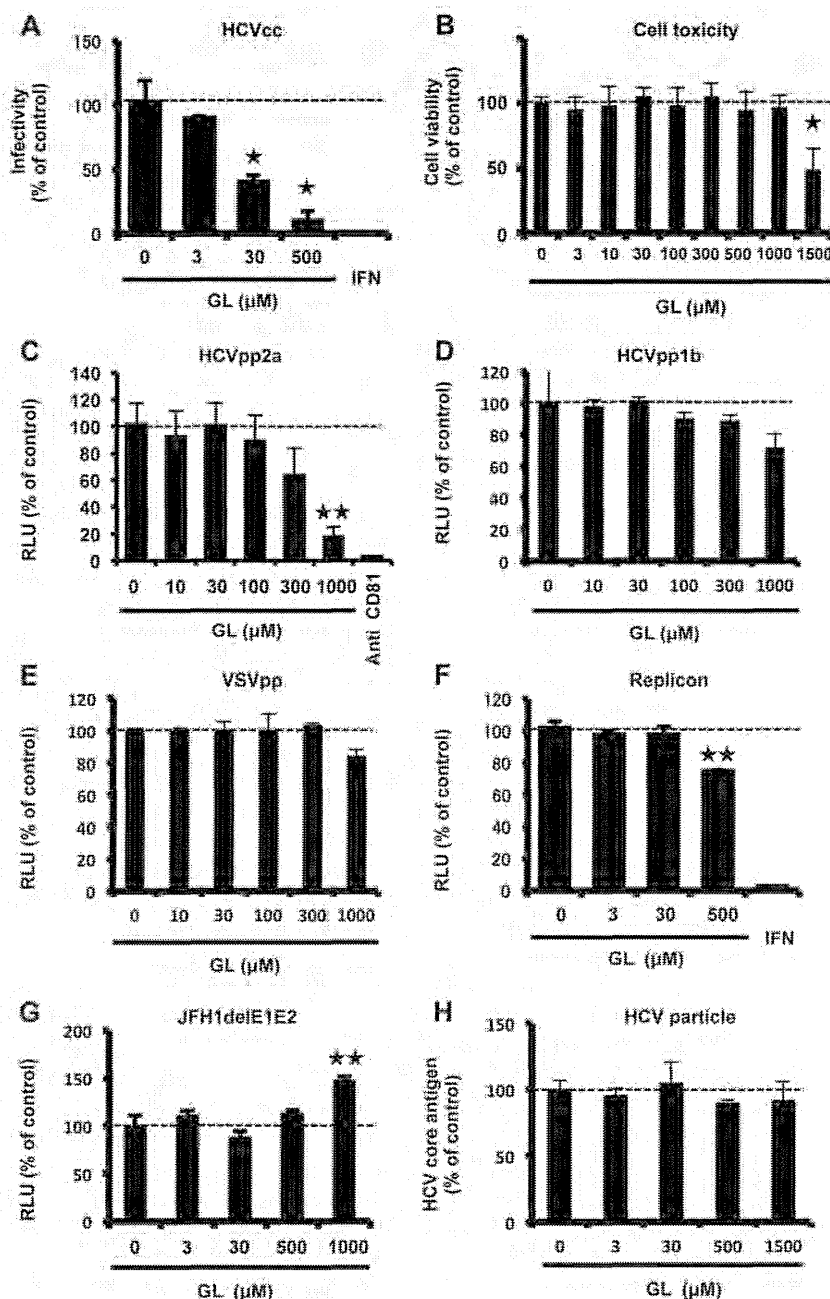
To investigate the effect of GL on entry, HCV particles were treated with increasing concentrations (0 to 1500  $\mu$ M) of GL. The viral samples were then used to inoculate Huh7 cells cultured in GL-containing medium. Several hours post-infection, medium was replaced with DMEM without GL. The levels 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  $\mu$ 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).

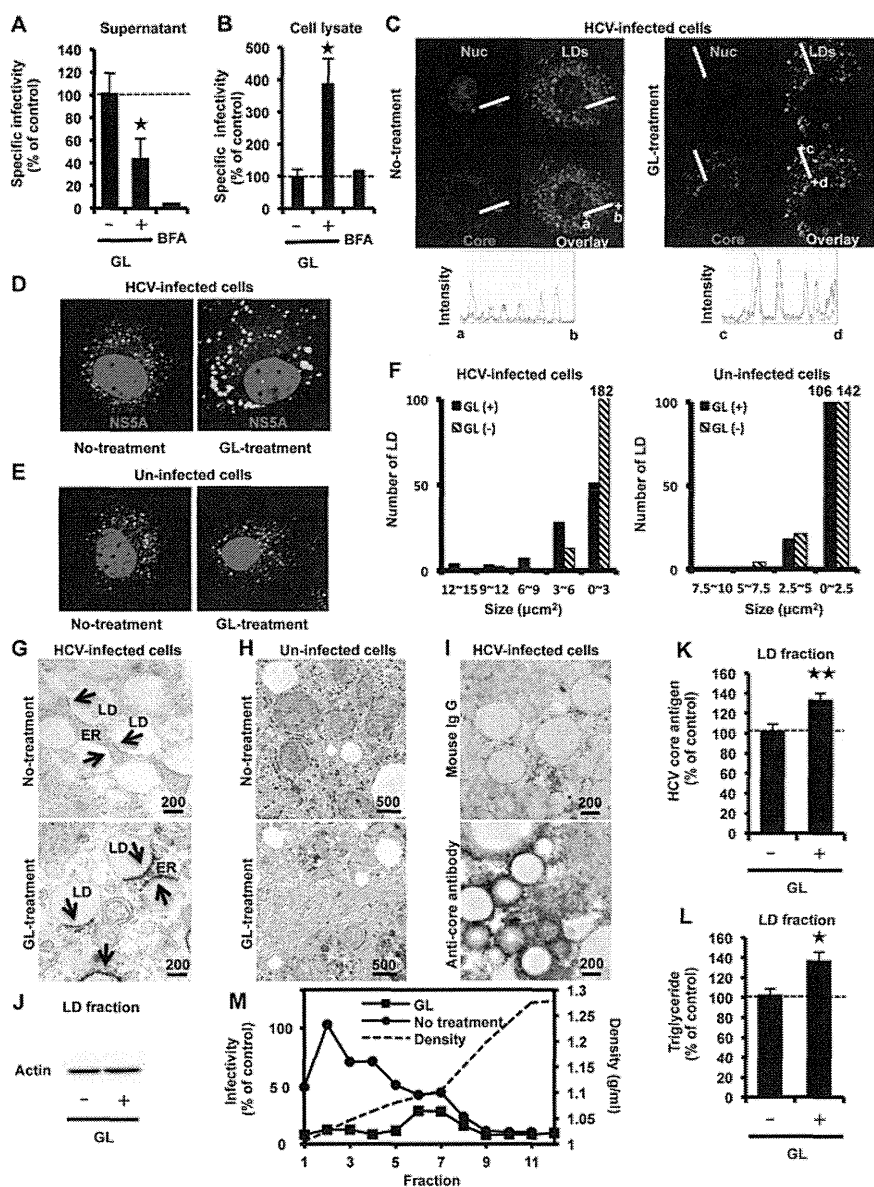
It has been previously reported that virus assembly takes place around lipid droplets (LDs) [29]. By immunofluorescence staining, we examined the subcellular co-localization of HCV core (Figure 2C) or NS5A (Figure 2D) with LDs in HCVcc-infected cells with or without GL treatment. Un-infected cells were 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 (JFH1ΔE1E2). (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 in release of infectious HCV particles.** HCVcc-infected cells were treated with GL at a concentration of 500  $\mu\text{M}$  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 un-infected cells (right panel) and HCV-infected cells (left panel) were quantified. Transmission EM of LDs in infected cells (G) and un-infected cells (H) treated with GL at 500  $\mu\text{M}$ . 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  $\mu\text{M}$ . 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 to that in total cell lysate was determined. (M) HCVcc-infected cells were treated with GL at 500  $\mu\text{M}$  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  $\mu\text{M}$  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  $\mu\text{M}$  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 to 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.

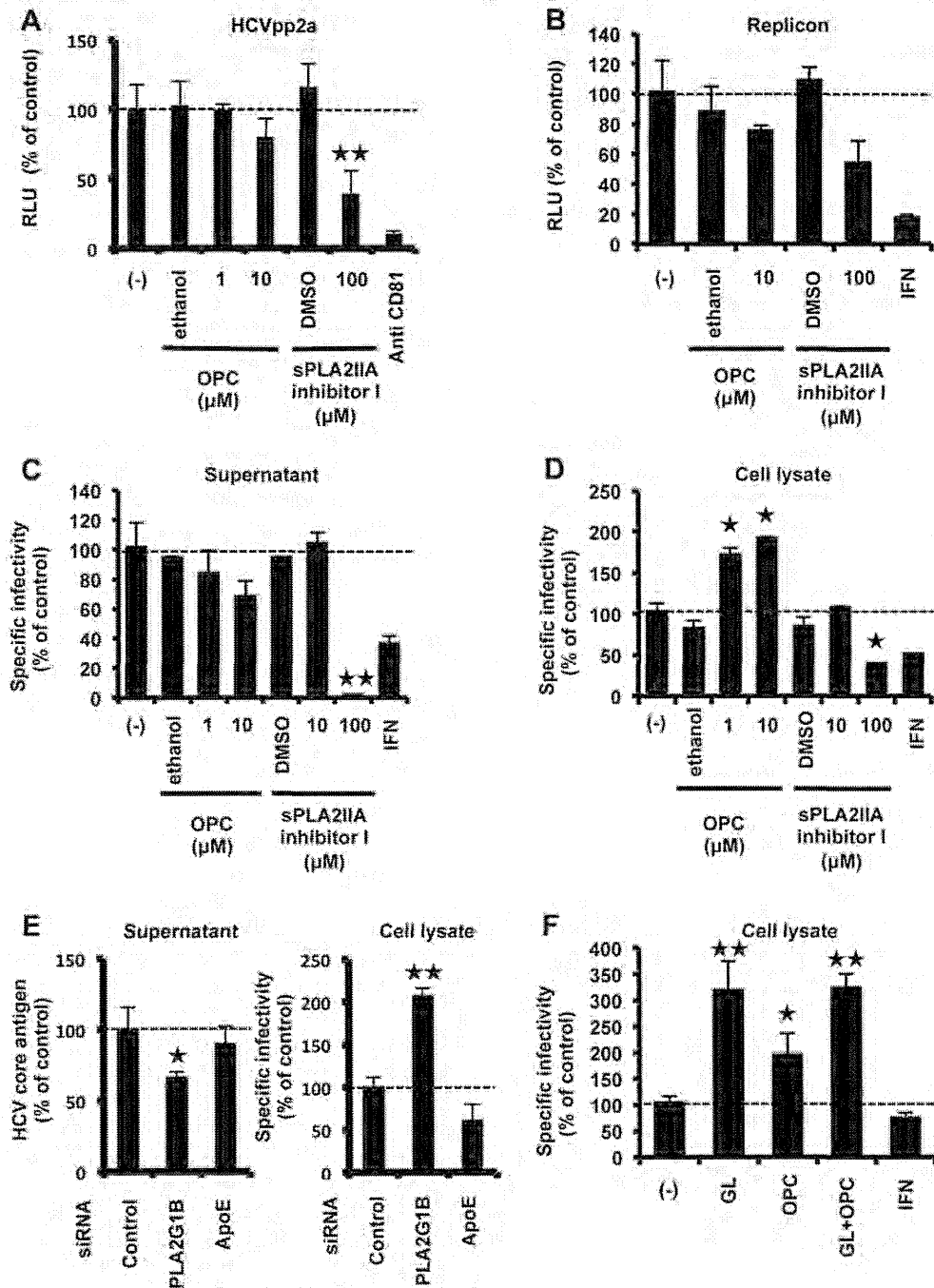
### Effect of GL on IFN induction and secretion proteins

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.

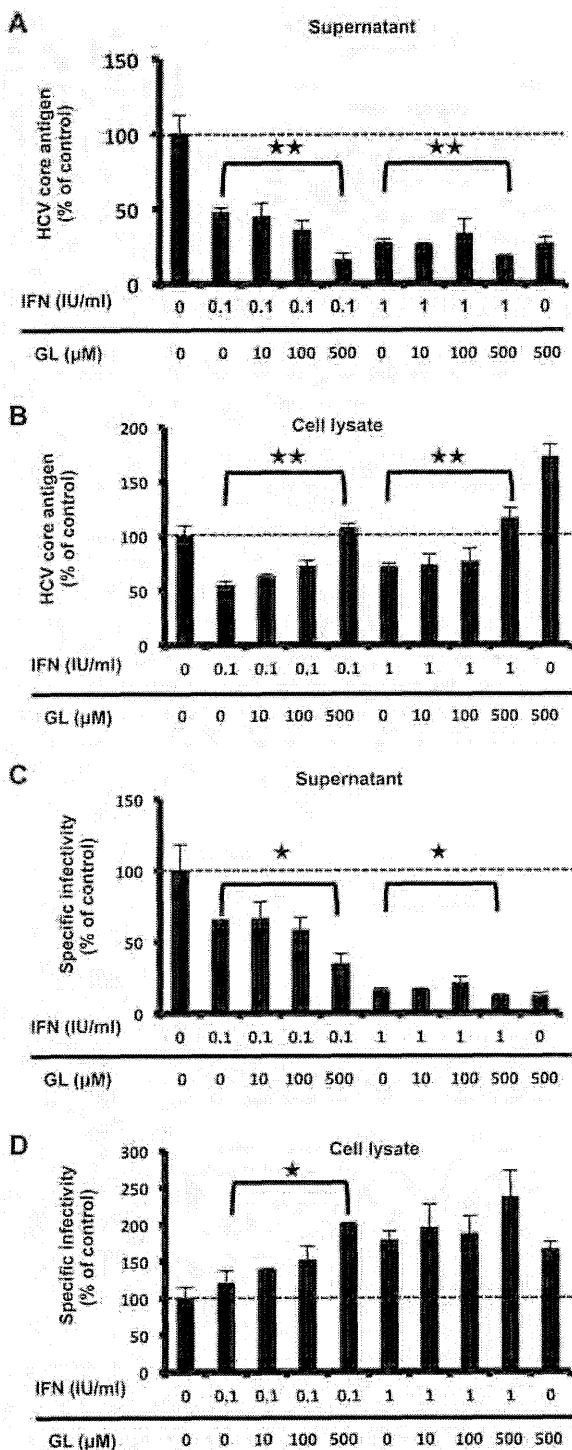
We found core protein accumulation on LDs in GL-treated cell (Figure 2C, 2I and 2K). This inverse correlation between



**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  $\mu$ g/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  $\mu$ M) with or without OPC (10  $\mu$ M), and intracellular specific infectivity was measured. IFN (10 IU/ml) was used as a positive control. 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  $\mu$ M 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  $\mu$ M (Figure 1A). It has been reported that the maximum peripheral concentration of GL in normal patients is 145  $\mu$ 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, Ladanin, 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  $\mu$ 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  $\mu$ M) for 72 hours and lysed in lysis buffer, followed by immunoblotting with anti-PLA2G1B and anti-actin antibodies. OPC (10  $\mu$ 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 pISRE-Luc vector contains the firefly luciferase reporter gene, downstream

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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 pISRE-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  $\mu$ 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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