other groups and ours that support the initial idea of the importance of IRRDR in the prediction of treatment outcome[52,53,123]. In a pilot study conducted on patients who underwent liver transplantation, the value of viral genetic factors, including sequence polymorphisms in the core protein, ISDR and IRRDR, in prediction of PEG-IFN/RBV treatment outcome after transplantation was investigated[124]. Interestingly, IRRDR $\geqslant$ 6 was the strongest viral genetic factor associated with SVR. Moreover, in a well executed viral genome wide associated study that scanned the whole HCV genome for polymorphisms at certain amino acids or in genomic regions that are significantly associated with PEG-IFN/RBV treatment outcome in HCV-1b infection, a high degree of IRRDR sequence heterogeneity (IRRDR $\geqslant$ 4) was selected in multivariate analysis as the strongest factor to predict SVR among various host and viral genetic factors and baseline demographic parameters[125].

Also, it is important to point out that the cutoff number of mutations in IRRDR that is associated with treatment outcome might possibly vary with different geographical regions: In certain geographical regions where HCV isolates with a high degree of sequence heterogeneity are predominant, a higher cutoff number of IRRDR mutations (such as 6 mutations) is applicable[51,53] whereas a lower cutoff number of IRRDR mutations (such as 4 mutations) is better applicable in regions where HCV isolates with a low degree of sequence heterogeneity are predominant[52,125].

While the predictive value of the IRRDR was initially identified in Japanese patients infected with HCV-1b, its predictive value was also confirmed in HCV-2a infection, the second most prevalent HCV genotype in Japan[74]. Furthermore, we investigated for the first time the impact of viral genetics, including the core protein and NS5A polymorphisms, on PEG-IFN/RBV treatment outcome in Egyptian patients with HCV genotype 4 infection. The result clearly demonstrated that the degree of sequence heterogeneity in the IRRDR was the only viral factor that was significantly associated with PEG-IFN/RBV treatment outcome[73]. Therefore, we proposed that the IRRDR would be a useful positive and negative predictive marker for treatment outcome in Egyptian patients infected with HCV genotype 4. Collectively, a series of our studies and others have suggested the significance of IRRDR sequence heterogeneity predicting treatment outcome in different ethnic populations infected with different HCV genotypes.

The clinical correlation between IRRDR sequence heterogeneity and virological responses to IFN-based therapy in HCV infection can be linked to an experimental observation by Tsai *et al*[126] that an HCV subgenomic RNA replicon containing NS5A of HCV-1b exerted more profound inhibitory effects on IFN activities than the original HCV-2a replicon, and that domain swapping between NS5A sequences of HCV-1b and -2a in the V3 and/or a C-terminal region including the IRRDR resulted in a transfer of their anti-IFN activities. Consistent with this observation, Kumthip *et al*[127] found that the overexpression of either HCV genotype 1 or genotype 3 NS5A proteins significantly inhibited IFN-induced signaling of IFN-stimulated response element, STAT1 phosphorylation and IFN-stimulated gene expression compared to the respective controls. NS5A of HCV genotype 1 exhibited stronger inhibitory effects on IFN signaling than did that of genotype 3. Furthermore, NS5A of HCV genotype 1 bound to STAT1 with a higher affinity compared to genotype 3. Interestingly, domain mapping revealed that the C-terminal region of NS5A, including ISDR and IRRDR, conferred these inhibitory effects on IFN signaling. Whereas IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV[87], its upstream and downstream sequences show a higher degree of sequence conservation (Figure 4). We speculate that whereas the upstream and downstream sequences are conserved to maintain the capacity of NS5A to participate in RNA replication and virion production across all the HCV genotypes, IRRDR sequences have a genotype-dependent or even a strain-dependent modulatory function(s)[128-130]. Therefore, the sequence heterogeneity of the IRRDR and its significant correlation with IFN responsiveness suggest the possibility that the IRRDR is involved, at least partly, in the viral strategy to evade IFN-mediated antiviral host defense mechanisms. The IRRDR sequence heterogeneity also suggests genetic flexibility of this region and, indeed, a short stretch of sequence in a C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions. This means that the short stretch of sequence is not essential for virus replication in cultured cells. It does not exclude the possibility, however, that the same region might play an important role in modulating the interaction with various host systems, including IFN responsiveness. It is also possible that the genetic flexibility of this region, especially the IRRDR, is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN therapy. We hypothesize that the IRRDR functions as evolution-adaptation machinery for HCV to cope with changes in the surrounding environment. For example, when sequence evolution of NS5A was investigated during IFN treatment, most of the evolutionary mutations were accumulated in the C-terminus, including the IRRDR[119,131]. Furthermore, in a recent retrospective study we investigated sequence evolution of the core protein, NS3 and NS5A (IRRDR and ISDR) during the follow-up period from chronic hepatitis to HCC development by comparing the sequences between pre- and post-HCC isolates[69]. The results showed that IRRDR sequences tended to be more polymorphic at the time of HCC occurrence. The frequency of HCV isolates with IRRDR $\geqslant$ 6 was significantly higher in patients with HCC than in those without HCC, and also higher in post-HCC isolates than in pre-HCC isolates. This might imply the possibility that HCV utilizes IRRDR evolution to accommodate certain selective pressures encountered during the course of HCC development. Further studies are needed to elucidate the issue.

E2 AND NS3

E2, one of the two envelope proteins, is the viral component that is required for direct contact with the cell-surface receptors[132]. The first 27 amino acids (aa) of E2 were identified as hypervariable region 1 (HVR1) because of its significant sequence variability and have been reported to be an immunodominant target for neutralization antibodies. Sequence variations in this region might contribute to immune evasion and thereby the persistence of viral infection[133-135]. Also, a region of 12 residues between aa 659-670 of E2, designated as PKR/eIF-2α phosphorylation homology domain (PePHD), has been reported to interact and inhibit the antiviral activity of PKR[136,137]. Accordingly, sequence variations within the PePHD were suspected to influence responses to IFN-based therapy. However, data obtained from clinical studies investigating this issue were controversial[103,138-142].

The NS3 region of the HCV genome is less variable compared to E2 and NS5A, but still displays significant sequence diversity[143]. In a previous study, we demonstrated that polymorphisms in the secondary structure of an N-terminal region of NS3 of HCV-1b were associated with different virological responses to PEG-IFN/RBV therapy and proposed that the viral grouping based on the NS3 polymorphism could be used to predict the outcome of the therapy[144]. In addition, we have recently found that a particular combination of point mutations in aa 1082 and 1112 of NS3 (NS3-Tyr^{1082}/Gln^{1112}) is closely associated with HCC development in HCV-1b infection. We have also noticed that a combination of NS3-Tyr^{1082}/Gln^{1112} and core-Gln^{70} is more strongly associated with HCC development than is the mutations of NS3 alone or the core protein alone[69]. Therefore, we propose that NS3-Tyr^{1082}/Gln^{1112} and core-Gln^{70} would be independent predictive markers for development of HCV-1b-associated HCC.

Apart from the IFN responsiveness, NS3 has been an intense focus of attention since the introduction of NS3 protease inhibitors as DAAs for treatment of HCV infection. Mutations in four positions in the NS3 protease domain are known to be associated with resistance or reduced sensitivity to telaprevir; R155K/T/S/M, A156T/V/S, V36A/M and T54A[145-148]. Three mutations, T54A, V170A and A156S, conferred low to moderate levels of resistance to boceprevir while variants with the A156T mutation are highly resistant[145-149]. Deep sequencing analysis using "next-generation" sequencers revealed that those DAA-resistant mutations were present even before the initiation of treatment in patients who did not achieve SVR. Thus, the prevalence of the DAA-resistant variants is determined by their replicative fitness and selective pressure of the DAAs[150,151]. In this connection, deep sequencing analysis is also useful to study the possible importance of a viral factor(s) in disease manifestations, including IFN resistance, especially when the target variant(s) constitutes a minor population in the sample and, therefore, undetectable by ordinary direct sequencing.

CONCLUSION

HCV is an interesting virus to study because of its ability to evade host defense mechanisms, including both innate and acquired immune responses, so as to establish persistent infection, which causes a wide spectrum of pathogenicity, such as lipid and glucose metabolism disorders and HCC development. The HCV genome is characterized by a high degree of genetic diversity that can be associated with the viral sensitivity or resistance (reflected by different virological responses) to IFN-based therapy. In addition to the IL28B SNP as the most important host factor that governs the IFN responsiveness of the patients, a point-mutation at position 70 of the core protein and sequence heterogeneity of the ISDR and IRRDR in NS5A of HCV have significant impact on the outcome of a standard regimen of PEG-IFN/RBV combination therapy. Currently, the HCV therapeutic field is heading towards IFN-free treatment where there are several ongoing clinical trials testing new specific DAAs against HCV. Whether these DAAs can overcome the HCV genetic diversity barrier without the emergence of resistant variants should be carefully monitored and properly assessed. New technologies, such as second and third generations of deep sequencing technologies that are currently available, will open up new doors to further understand the impact of HCV genetics on HCV pathogenesis and treatment responsiveness in more detail.

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Inhibition of hepatitis C virus replication by chalepin and pseudane IX isolated from *Ruta angustifolia* leaves

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ABSTRACT

Hepatitis C virus (HCV) infection is highly prevalent among global populations, with an estimated number of infected patients being 170 million. Approximately 70–80% of patients acutely infected with HCV will progress to chronic liver disease, such as liver cirrhosis and hepatocellular carcinoma, which is a substantial cause of morbidity and mortality worldwide. New therapies for HCV infection have been developed, however, the therapeutic efficacies still need to be improved. Medicinal plants are promising sources for antivirals against HCV. A variety of plants have been tested and proven to be beneficial as antiviral drug candidates against HCV. In this study, we examined extracts, their subfractions and isolated compounds of *Ruta angustifolia* leaves for antiviral activities against HCV in cell culture. We isolated six compounds, chalepin, scopoletin, γ-fagarine, arborinine, kokusaginine and pseudane IX. Among them, chalepin and pseudane IX showed strong anti-HCV activities with 50% inhibitory concentration (IC_{50}) of 1.7 ± 0.5 and 1.4 ± 0.2 μg/ml, respectively, without apparent cytotoxicity. Their anti-HCV activities were stronger than that of ribavirin (2.8 ± 0.4 μg/ml), which has been widely used for the treatment of HCV infection. Mode-of-action analyses revealed that chalepin and pseudane IX inhibited HCV at the post-entry step and decreased the levels of HCV RNA replication and viral protein synthesis. We also observed that arborinine, kokusaginine and γ-fagarine possessed moderate levels of anti-HCV activities with IC_{50} values being 6.4 ± 0.7, 6.4 ± 1.6 and 20.4 ± 0.4 μg/ml, respectively, whereas scopoletin did not exert significant anti-HCV activities at 30 μg/ml.

1. Background

Hepatitis C virus (HCV) is an enveloped virus that belongs to the *Hepacivirus* genus within the *Flaviviridae* family. The viral genome is a single-stranded, positive-sense RNA of 9.6 kb with highly structured 5′- and 3′-untranslated regions [1,2]. It encodes a polyprotein precursor consisting of about 3000 amino acid residues, which is cleaved by the host and viral proteases to generate 10 mature proteins, such as core, E1, E2, a putative ion channel p7, and nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. The core, E1 and E2 are cleaved off by the signal peptidase and signal peptide peptidase of the host cell and, together with the viral genome, form the virus particles. The E1 and E2 glycoproteins are responsible for binding to a number of different virus receptor molecules on the cell surface, such as scavenger receptor class B type I, CD81, claudin 1 and occludin. On the other hand, nonstructural proteins play crucial roles in virus replication. NS2 possesses a metalloprotease activity that mediates cleavage between NS2

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and NS3. After this cleavage takes place, NS3 exerts a serine protease activity that is responsible for the cleavage at the remaining cleavage sites of the polyprotein. NS3 also possesses a helicase activity in its C-terminal domain, which is required for viral RNA replication. NS4A stabilizes NS3 by forming a complex with it and also acts as an inducer of membrane alterations. NS4B is a hydrophobic protein and is involved in the membranous web formation, a characteristic feature of HCV-infected cells. NS5A is a phosphoprotein with versatile functions and is required for viral RNA replication and particle assembly [1,3]. NS5B possesses an RNA-dependent RNA polymerase activity. It is known that the HCV replication cycle is linked to the lipid metabolism of the host cells. It should also be noted that the HCV genome exhibits a considerable degree of sequence heterogeneity, based on which HCV is currently classified into 7 genotypes (1 to 7) and more than 70 subtypes (1a, 1b, 2a, 2b, etc.) [4].

HCV infection is highly prevalent among global populations, especially in Africa and Asia, with an estimated number of infected patients being 170 million worldwide. Approximately 3 million people are newly infected with HCV worldwide every year [3,5]. Seventy to 80% of newly infected patients progress to chronic infection. Patients with chronic HCV infection have a high risk to develop severe liver diseases such as cirrhosis and hepatocellular carcinoma, and also to develop extra hepatic manifestations, including glucose and lipid metabolic disorders [6,7]. A standard care of HCV infection using pegylated interferon (Peg-IFN)-α and ribavirin can achieve sustained virological response (SVR) in ca. 50% of patients infected with HCV genotype 1 or 4 [5]. Triple combination therapy using Peg-IFN-α, ribavirin and an NS3 protease inhibitor increased the SVR rate to 70 to 80%. Moreover, recent approval of other direct-acting antivirals (DAA), including NS5A inhibitors, can further improve the SVR rate. However, they are not equally effective for all of the seven HCV genotypes and, more importantly, serious adverse effects are observed with some patients [5,8]. This highlights the need for a new alternative and/or complementary agent(s) for treatment of HCV.

A wide variety of medicinal plants and their phytochemical constituents have been reported to inhibit HCV infection. For example, an extract of *Phyllantus amarus* root significantly inhibited HCV NS3 protease with a 50% inhibitory concentration (IC_{50}) of 5 μg/ml whereas *P. amarus* leaves inhibited HCV NS5B polymerase with the same IC_{50} value [9]. We tested ethanol extracts of Indonesia plants for their anti-HCV activities and reported that *Toona sureni* leaves, *Melicope latifolia* leaves, *Melanolepis multiglandulosa* stem and *Ficus fistulosa* leaves possessed anti-HCV activities [10]. We also reported that extracts of *Glycyrrhiza uralensis* root and isolated compounds, such as glycycoumarin, glycerin, glycyrol, and liquiritigenin, and extracts of *Morinda citrifolia* leaves, an isolated compound, pheophorbide a, and its related compound, pyropheophorbide a, exhibited anti-HCV activities [11,12]. Likewise, silymarin, iridoid, epigallocatechin-3-gallate were reported to inhibit HCV infection at the entry step while diosgenin, luteolin, quercetin, 3-hydroxy caruilignan C, excoecariphenol D and apigenin at the post-entry step [13,14]. Although a number of novel antivirals against HCV are being developed, further studies are still needed to identify a safer, more effective and cheaper anti-HCV substance(s). Medicinal plants are a good target for the study.

Ruta angustifolia belongs to the Rutaceae family. Plants in the *Ruta* genus have been used as traditional remedy, such as antiseptics, antihelminthics and anti-inflammatory, wound-healing and pain-relief drugs, to cure malconditions during pregnancy and disorders in the gastrointestinal, respiratory, nervous, skin and musculoskeletal systems [15]. In Indonesia, *R. angustifolia* has been known as traditional medicine for liver disease and jaundice. It contains coumarin, alkaloid and flavonoid compounds. Angustifolin and four aromatic derivatives, moskachan A, B, C and D, have been identified as constituents of *R. angustifolia* [16,17]. In this study, we examined the anti-HCV activities of extracts from *R. angustifolia* and its constituents.

2. Materials and methods

2.1. Cells and viruses

Huh7.5 cells and the plasmid pFL-J6/JFH1 to produce the J6/JFH1 strain of HCV genotype 2a [18] were kindly provided by Dr. C. M. Rice, The Rockefeller University, New York, NY, USA. Huh7.5 cells were cultivated in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with fetal bovine serum (Biowest, Nuaille, France), non-essential amino acids (Invitrogen, Carlsbad, CA), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Invitrogen). Cells were grown at 37 °C in a 5% CO_2 incubator.

2.2. Collection, extraction, fractionation and compound isolation of R. angustifolia leaves

R. angustifolia leaves were collected at Lembang, a mountain area of the West Java region, Indonesia. The collected samples were verified by botanical researchers at the Purwadadi Botanical Garden, Purwadadi, Indonesia. Leaves of the plants were dried at room temperature, pulverized and extracted by means of two different extraction procedures; (i) 96% ethanol and (ii) *n*-hexane, dichloromethane and methanol, successively. Maceration process was repeated over 3 days. The obtained filtrates were concentrated under reduced pressure to yield ethanol, *n*-hexane, dichloromethane and methanol extracts. The dichloromethane extract was subjected to the open column chromatography with silica gel (development solvent: gradient of chloroform–methanol system). A bioactivity-positive fraction(s) was further fractionated under open column chromatography with silica gel and mobile phase gradient of hexane–ethyl acetate system. Based on thin layer chromatography (TLC) profiles, several fractions were combined and passed through an activated charcoal column and eluted by each 2 l of methanol (100%), 30% of chloroform–methanol, and chloroform (100%) [19]. Each fraction was concentrated in vacuo and further subfractionated by recycling high-performance liquid chromatography (HPLC) (solvent system: 100% methanol, column: GS-320 + GS-310, 21.5 mm ID × 1000 mm, flow rate: 5.0 ml/min, detection: UV 210 nm) and preparative HPLC (column: Waters XBridge C18 10 × 250 mm, solvent system: gradient solvent of 0.1% trifluoroacetic acid (TFA) — acetonitrile, flow rate: 2.5 ml/min, column temperature: 30 °C). Preparative HPLC was run on JASCO LC-2000 plus series. Recycling preparative HPLC was performed on a Japan Analytical Industry LC-908W.

To determine the structure of the isolated compounds, liquid chromatography–mass spectrometry (LC–MS), ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C NMR analyses were performed. ^{1}H and ^{13}C NMR spectra were measured with BRUKER Ascend 600 spectrometer (600 MHz). LC–MS was performed with a Thermofischer Scientific Orbitrap Elite equipped with an electrospray ionization (ESI) source [20].

2.3. Analysis of anti-HCV activities

R. angustifolia extracts and isolated compounds were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions at a concentration of 100 mg/ml. The stock solutions were stored at −20 °C until used. Ribavirin was purchased (Sigma-Aldrich, Steinheim, Germany) and used as a positive control.

Huh7.5 cells were seeded in 24-well plates (1.9×10^5 cells/well). A fixed amount of the J6/JFH1-P47 strain of HCV genotype 2a [10,21], with a multiplication of infection (MOI) of 0.5 focus-forming units (ffu)/cell, was mixed with serial dilutions of the extracts (100, 30, 10, 1 and 0.1 μg/ml) and compounds (30, 10, 3, 1 and 0.1 μg/ml), and inoculated to the cells. After 2 h, the cells were washed with medium to remove the residual virus and further incubated in the medium containing the same concentrations of the test samples as those during virus inoculation.

Time-of-addition experiments were performed to assess the mode of action of the samples, as described previously [10–12]. In brief, three sets of experiments were done in parallel. (i) To assess the antiviral effect at the entry step, the mixture of HCV and a sample was inoculated to the cells. After virus adsorption for 2 h, the residual virus and the sample were removed, and cells were refed with fresh medium without the sample for 46 h. (ii) To assess the antiviral effect at the post-entry step, HCV was inoculated to the cells in the absence of the sample. After virus adsorption for 2 h, the residual virus was removed and cells were refed with fresh medium containing the sample for 46 h. (iii) As a positive control, HCV mixed with the sample was inoculated to the cells. After virus adsorption for 2 h, the residual virus and the sample were removed, and cells were refed with fresh medium containing the sample for 46 h. Culture supernatants were obtained at 1 and 2 days post-infection (dpi) and titrated for virus infectivity [21]. Virus and cells treated with medium containing 0.1% DMSO served as a control. The percent inhibition of virus infectivity by the samples was calculated by comparing to the control using SPSS probit analysis, and IC_{50} values were determined.

2.4. Real-time quantitative RT-PCR

Total RNA was extracted from the cells using a ReliaPrep RNA cell miniprep system (Promega, Madison, WI) according to the manufacturer's instructions. One μg of total RNA was reverse transcribed using a GoScript Reverse Transcription system (Promega) with random primers and subjected to quantitative real-time PCR analysis using SYBR Premix Ex Taq (TaKaRa, Kyoto, Japan) in a MicroAmp 96-well reaction plate and an ABI PRISM 7500 system (Applied Biosystems, Foster City, CA). The primers used to amplify an NS5A region of the HCV genome were 5′-AGACGTATTGAGGTCCATGC-3′ (sense) and 5′-CCGCAGCGACGGTGCTGATAG-3′ (antisense). As an internal control, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression levels were measured using primers 5′-GCCATCAATGACCCCTTCATT-3′ (sense) and 5′-TCTCGCTCCT GGAAGATGG-3′ (antisense).

2.5. Immunoblotting

Cells were lysed and separated with SDS-polyacrylamide gel electrophoresis as described previously [10,11]. The samples were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), which was then incubated with the respective primary antibodies. The primary antibodies used were mouse monoclonal antibodies against HCV NS3 and GAPDH (Millipore). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Invitrogen) was used to visualize the respective proteins by means of an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK). The relative band intensity was quantified using densitometry analysis with ImageJ software. The NS3 protein expression levels were normalized to their respective GAPDH protein levels.

2.6. WST-1 assay for cytotoxicity

WST-1 assay was performed as described previously [10]. In brief, Huh7.5 cells in 96-well plates were treated with serial dilutions of the test samples or 0.1% DMSO as a control for 48 h. After the treatment, 10 μl of WST-1 reagent (Roche, Mannheim, Germany) was added to each well and cells were cultured for 4 h. The WST-1 reagent is absorbed by the cells and converted to formazan by mitochondrial dehydrogenases. The amount of formazan, which correlates with the number of living cells, was determined by measuring the absorbance of each well using a microplate reader at 450 nm and 630 nm. Percent cell viability compared to the control was calculated for each dilution of substances and 50% cytotoxic concentration (CC_{50}) values were determined by SPSS probit analysis.

3. Results

3.1. Bioactivity-guided fractionation and purification of extracts from R. angustifolia leaves and isolation of compounds

Dried and pulverized *R. angustifolia* leaves were extracted with ethanol, *n*-hexane, dichloromethane and methanol as described in the Materials and methods section, and examined for antiviral activities against the J6/JFH1-P47 strain of HCV genotype 2a [10,21]. The results revealed that the dichloromethane extract of *R. angustifolia* leaves had potent anti-HCV activity with IC_{50} of 1.6 ± 0.3 μg/ml (Table 1). The dichloromethane extract was further purified by open column chromatography to

Table 1
Anti-HCV activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity index (SI) of *R. angustifolia* leaves extracts.

Sample	IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
Ethanol extract	3.0 ± 1.4[a]	>100	>30.3
n-Hexane extract	15.6 ± 5.2	>100	>6.4
Dichloromethane extract	1.6 ± 0.3	49.2 ± 3.6	30.8
Methanol extract	8.1 ± 2.0	>100	>12.3

[a] Mean ± SEM of data from two independent experiments.

obtain 6 fractions, of which fraction 4 showed potent anti-HCV activity with IC_{50} of 0.7 μg/ml (Table 2). Fraction 4 was further fractionated under open column chromatography with silica gel and mobile phase gradient of hexane–ethyl acetate system to obtain 29 fractions. Based on the TLC profile, some of the subfractions were combined and 4 groups of subfractions were passed through an activated charcoal column, which were eluted by each 2 l of 100% methanol, 30% chloroform–methanol and 100% chloroform to give 3 fractions (a, 100% methanol; b, 30% chloroform–methanol; c, 100% chloroform) [19]. Each fraction was concentrated in vacuo. Fraction 1a was subjected to recycling HPLC to afford F1a-1 (3.1 mg) and F1a-2 (96.7 mg), which were determined as an identical compound (compound 1). Fraction 2b was separated using preparative HPLC to obtain F2b-1 (compound 2), F2b-2 and F2b-3 (compound 3). Fraction 3c was separated by filtration method with methanol to yield F3c-1 (compound 4) and F3c-2, the latter of which was subjected to preparative HPLC to obtain 3c-2A (compound 5) and 3c-2B (compound 4). Fraction 4a was subjected to preparative HPLC to obtain F4a-3 (compound 6). The structures of the isolated compounds were determined by LC–MS and NMR analyses (Supplementary Information).

Compound 1 (100.7 mg) was isolated as colorless amorphous powder with a molecular formula of $C_{19}H_{22}O_4$ by orbitrap MS, *m/z* 315.15719 $[M + H]^+$ and was identified as chalepin by comparison with NMR literature data [22]. Compound 2 (5.0 mg) was a yellow amorphous powder with a molecular formula of $C_{10}H_9O_4$, *m/z* 193.04833 $[M + H]^+$ and was identified as scopoletin [23]. Compound 3 (3.0 mg) was identified as γ-fagarine [24], an alkaloid compound, which has a molecular formula of $C_{13}H_{12}O_3N$, *m/z* 230.07985 $[M + H]^+$. Compound 4 (28.7 mg) was identified as arborinine [23], another alkaloid compound with a molecular formula of $C_{16}H_{16}O_4N$, *m/z* 286.10952 $[M + H]^+$. Compound 5 (6.5 mg) with a molecular formula of $C_{14}H_{14}O_4N$ was identified as kokusaginine [23,25], an alkaloid compound. Compound 6 (3.7 mg) with a molecular formula of $C_{18}H_{26}ON$, *m/z* 272.20089 was identified as pseudane IX [26]. The structures of the compounds are shown in Fig. 1.

3.2. Anti-HCV activities of the isolated compounds

Anti-HCV activities of the isolated compounds were tested. Ribavirin, which has been widely used for the treatment of HCV infection, was used as a positive control. The results obtained revealed that chalepin (compound 1) and pseudane IX (compound 6) possessed strong anti-HCV activities, with IC_{50} being 1.7 ± 0.5 and 1.4 ± 0.2 μg/ml, respectively (Table 3).

Table 2
Anti-HCV activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity index (SI) of fractions from a dichloromethane extract of *R. angustifolia* leaves.

Sample	IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
Fraction 1	>100[a]	>100	n.a.[b]
Fraction 2	>100	>100	n.a.[b]
Fraction 3	>100	>100	n.a.[b]
Fraction 4	0.7	42.1	64.8
Fraction 5	>100	>100	n.a.[b]
Fraction 6	7.4	>100	>13.5

[a] Not detectable at the concentration of 100 μg/ml.
[b] Not applicable.

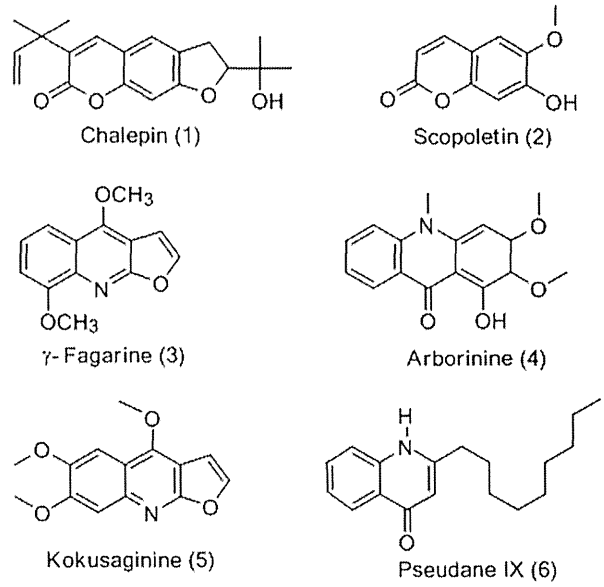

Fig. 1. Molecular structures of the compounds isolated from *R. angustifolia* leaves. Chalepin (compound 1); scopoletin (compound 2); γ-fagarine (compound 3); arborinine (compound 4); kokusaginine (compound 5); pseudane IX (compound 6).

Their anti-HCV activities were stronger than that of ribavirin (2.8 ± 0.4 μg/ml). Also, γ-fagarine (compound 3), arborinine (compound 4) and kokusaginine (compound 5) showed weaker but significant anti-HCV activities, with IC_{50} being 20.4 ± 0.4, 6.4 ± 0.7 and 6.4 ± 1.6 μg/ml, respectively. On the other hand, scopoletin (compound 2) did not show any significant inhibitory effect at the concentration of 30 μg/ml. Dose-dependent profiles of anti-HCV activities and cytotoxicity of those compounds are shown in Fig. 2.

3.3. Mode-of-action of anti-HCV activities of chalepin and pseudane IX

Time-of-addition experiments were performed to determine whether the compounds inhibit HCV at the entry or post-entry step [10]. Percent HCV inhibitions by chalepin and pseudane IX at the concentrations of 30, 10, 3, 1 and 0.1 μg/ml were measured in the experiments where the treatment was done either during (at the entry step), after virus inoculation (at the post-entry step) or both (Fig. 3). The IC_{50} values of chalepin for treatment at the entry step, post-entry step and both were

Table 3
Anti-HCV activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity index (SI) of the isolated compounds.

Isolate code	Compound	IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
Compound 1	Chalepin	1.7 ± 0.5[a]	14.0 ± 2.4	8.2
Compound 2	Scopoletin	>30[b]	>30[b]	n.a.[c]
Compound 3	γ-Fagarine	20.4 ± 0.4	>30[b]	>1.5
Compound 4	Arborinine	6.4 ± 0.7	16.3 ± 6.2	2.5
Compound 5	Kokusaginine	6.4 ± 1.6	>30[b]	>4.7
Compound 6	Pseudane IX	1.4 ± 0.2	26 ± 0.9	18.6
Positive control	Ribavirin	2.8 ± 0.4	>30[b]	>10.7

[a] Mean ± SEM of data from two independent experiments.
[b] Not detectable at the concentration of 30 μg/ml.
[c] Not applicable.

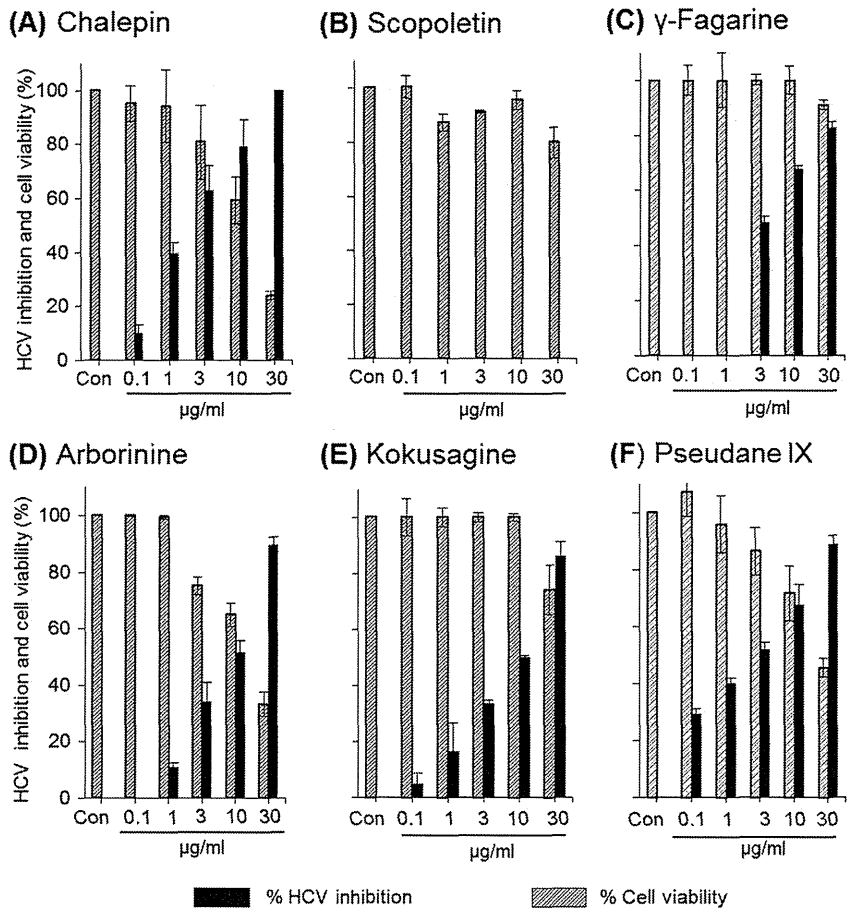

Fig. 2. Dose-dependent inhibition of HCV infection by isolated compounds and their cytotoxicity. Various concentrations of isolated compounds, chalepin (A), scopoletin (B), γ-fagarine (C), arborinine (D), kokusaginine (E) and pseudane IX (F), were mixed with an equal amount of HCV to obtain a final concentration of 30 to 0.1 μg/ml and inoculated to Huh 7.5 cells (MOI = 0.5). After virus adsorption, the cells were cultured with the same concentrations of compounds for 46 h. The culture supernatants were harvested and titrated for virus infectivity. Percent inhibitions of HCV infectivity by each compound are shown. In parallel, cytotoxicity of the compounds was measured by WST-1 assay. Con, control. The data represent means ± SEM of data from two independent experiments.

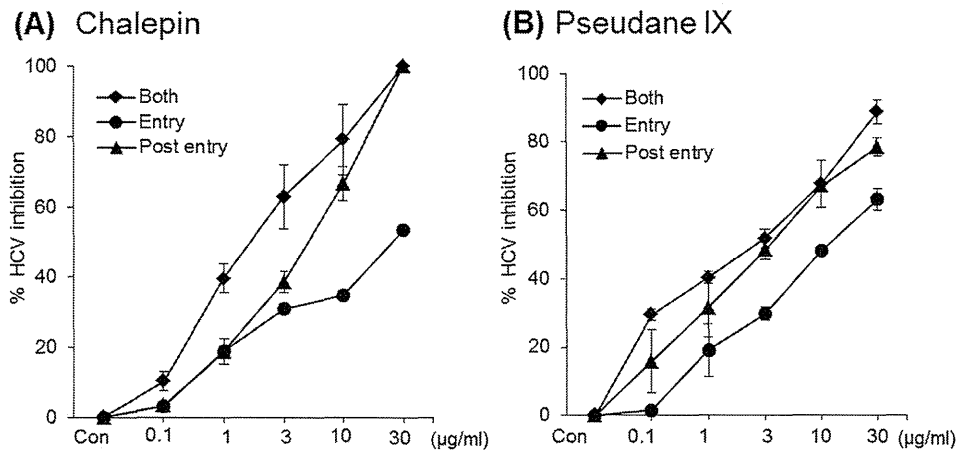

Fig. 3. Mode-of-action analysis of chalepin and pseudane IX. Huh7.5 cells infected with HCV were treated with various concentrations of chalepin (A) and pseudane IX (B) at different timings; (i) only during virus inoculation (entry), (ii) only after virus inoculation (post entry) and (iii) both during and after virus inoculation (both), with a total incubation time of 48 h. Con, control. Percent inhibitions at each concentration and IC_{50} values are shown. The data represent mean ± SEM of data from two independent experiments.

26.7 ± 1.3, 5.2 ± 0.7 and 1.7 ± 0.5 μg/ml, respectively. Also, those for pseudane IX were 11.5 ± 0.2, 3.0 ± 0.9 and 1.4 ± 0.9 μg/ml, respectively. These results suggested that chalepin and pseudane IX inhibited HCV predominantly at the post-entry step.

3.4. Inhibition of HCV RNA replication and HCV protein synthesis by chalepin and pseudane IX

To further confirm that chalepin and pseudane IX inhibit HCV infection at the post-entry step of HCV life cycle, we investigated the effect of those compounds on HCV RNA replication, viral protein synthesis and infectious virus production. Real-time quantitative RT-PCR analysis revealed that chalepin and pseudane IX at 3 and 10 μg/ml inhibited HCV RNA replication (Fig. 4A). Consistently, immunoblotting analysis demonstrated that both compounds inhibited HCV protein synthesis (Fig. 4B). We confirmed in the same experiment that they inhibited HCV production in the culture (Fig. 4C).

4. Discussion

Medicinal plants are good resources to search a novel drug candidate(s). A wide variety of phytochemicals that inhibit virus infections have been isolated and reported. In the present study, we examined the possible anti-HCV activity of *R. angustifolia* extracts and its constituents. *R. angustifolia* is widely distributed throughout the world and has been used as folk medicine for treatment of certain diseases. Plants of the genus *Ruta* have been commonly used

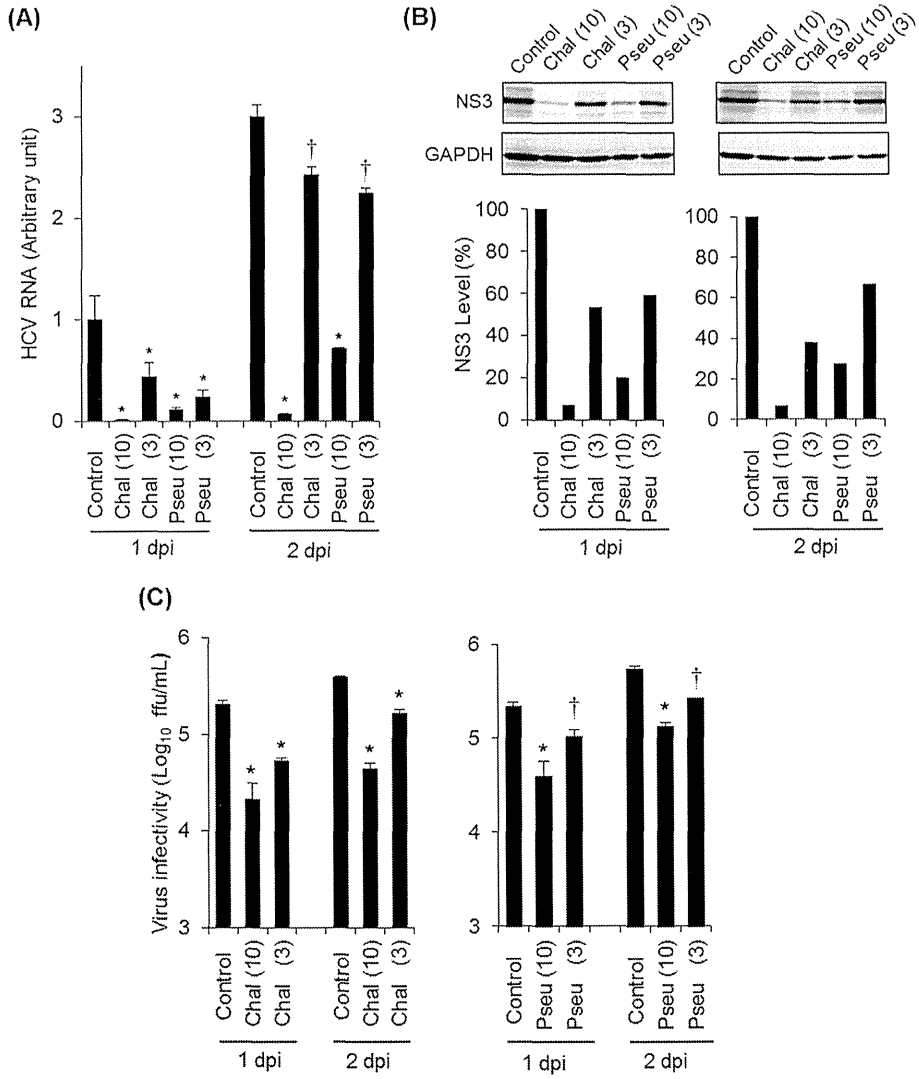

Fig. 4. Inhibition of HCV RNA replication, HCV protein synthesis and infectious virus production by chalepin and pseudane IX. (A) Huh7.5 cells infected with HCV (MOI = 2) and treated with chalepin or pseudane IX (10 and 3 μg/ml) and the untreated control were subjected to real-time quantitative RT-PCR analysis. HCV RNA levels were normalized to GAPDH mRNA expression levels. Data represent mean ± SEM of data from two independent experiments, and the value for the untreated control at 1 dpi was arbitrarily expressed as 1. Chal, chalepin; Pseu, pseudane. *, $p < 0.001$; †, $p < 0.05$. (B) Levels of HCV NS3 protein synthesis in the cells described in (A) were measured by immunoblotting analysis using anti-NS3 monoclonal antibody at 1 and 2 dpi. GAPDH served as an internal control to verify equal amounts of sample loading. Signal intensities of NS3 were normalized to the corresponding GAPDH signal. (C) Virus infectivity in the culture supernatants of the cells described in (A) was measured. *, $p < 0.001$; †, $p < 0.05$.

in both ancient and modern medicine practices in the Mediterranean region to cure pulmonary diseases, rheumatic diseases and helminthic infections [15,27]. A large number of chemical constituents have been isolated from the genus *Ruta*, such as coumarins, alkaloids, benzoquinones, flavone glycosides, sterols, triterpenoids, acridone alkaloids, stigmasterol, lupeol, 5-methoxyarborinine, 5-hydroxyarborinine, ostruthin, bergapten, psoralen, xanthotoxin, limonoid obacunone, isopimpinellin, integriquinolone, kokusaginine, dictamnine, furoquinolin alkaloid, xanthyletin and xanthoxyletin [28,29]. It was also reported that coumarin compounds are the major constituents of the plants in the Rutaceae family, with about two hundred different coumarins being identified [30,31]. Specifically, four aromatic compounds, such as moskachans A, B, C and D have been identified from *R. angustifolia* [17]. Another study on a chloroform extract of aerial part of *R. angustifolia* identified angustifolin, alkaloid graveolin, scoparone and 6,7,8-trimethoxycoumarin [16].

In this study, *R. angustifolia* leaves were subjected to extraction in different polarities of solvents and antiviral activities of the extracts were examined against the J6/JFH1-P47 strain of HCV. The results revealed that a dichloromethane extract of *R. angustifolia* leaves possessed the most potent activity (Table 1), suggesting that a semi-polar compound(s) extracted by dichloromethane was involved in the anti-HCV activity. We further fractionated the dichloromethane extract to obtain 6 fractions. Anti-HCV assay showed that fraction 4 inhibited HCV with IC_{50} of 0.7 μg/ml without apparent cytotoxicity (Table 2). As the TLC profile of the positive fractions suggested the presence of chlorophyll or its related substance(s) in this fraction (data not show), we used activated-charcoal in column chromatography to remove them. Finally, we isolated six compounds and determined their structures by combination of HPLC, LC–MS and NMR; they were chalepin, scopoletin, γ-fagarine, arborinine, kokusaginine, and pseudane IX. Chalepin and scopoletin are classified as coumarins while the remaining four (γ-fagarine, arborinine, kokusaginine and pseudane IX) are alkaloids.

Chalepin, which has been isolated from *R. chalepensis* [32], *Stauranthus perforates* [33], *Clausena anisata* [34] and *Clausena lansium* [35], belongs to furocoumarin compounds with furan ring fused to the coumarin structure. It was reported to possess antimicrobial activities against *Pseudomonas aeruginosa* and *Trichomonas* as well as anti-coagulant activities [34,35]. However, there has been no report so far regarding its antiviral activity against HCV. To the best of our knowledge, the present study is the first to demonstrate anti-HCV activities of chalepin with IC_{50} being 1.7 ± 0.5 μg/ml (5.4 ± 0.5 μM) (Table 3). It was reported that chalepin inhibited respiration of isolated rat liver mitochondria by 40% at the concentration of 16 μM (5.0 μg/ml) [36]. Under our experimental conditions using cultured cells, chalepin exerted only marginal cytotoxicity, if any, at 30 μg/ml (Fig. 2).

We have reported that other coumarin compounds, such as glycycoumarin, glycyrin and glycyrol, possess anti-HCV activities, with IC_{50} of 8.8, 7.2, and 4.6 μg/ml, respectively [11]. The basic structure (1,2-benzopyron) of coumarin appears to be important for binding to HCV [37]. Fourteen compounds out of 24 coumarin derivatives were reported to inhibit HCV NS5B RNA polymerase activities with IC_{50} values between 17 and 63 μM. The activities of those compounds were influenced by the position of methylation or hydroxylation groups in the benzopyron ring [37]. Recently, benzimidazole derivatives of coumarins have been reported to possess increased inhibitory effect on RNA polymerase of HCV NS5B [38,39]. On the other hand, scopoletin, which is the other coumarin isolated in the present study, did not inhibit HCV at the concentration of 30 μg/ml (155 μM). Scopoletin isolated from several plants, such as *Erycibe obtusifolia* Benth, *Aster tataricus* and *Foeniculum vulgare*, and its synthetic derivatives have been extensively studied [40]. Further detailed analyses of derivatives of chalepin and scopoletin would help us understand the structural basis of anti-HCV activity of chalepin and generate a more potent anti-HCV compound(s).

γ-Fagarine, arborinine and kokusaginine, which are alkaloid compounds, showed moderate inhibition with IC_{50} of 20.4 ± 0.4, 6.4 ± 0.7 and 6.4 ± 1.6 μg/ml, respectively (Table 3). These compounds have been isolated from several plants, including the Rutaceae family [41–44] and arborinine was previously reported to inhibit human rhinovirus with IC_{50} of 3.19 μM by virtual model [45]. Pseudane IX, another alkaloid, also known as 2-nonyl-4 (1H)-quinolone, 2-nonyl-4-hydroxyquinoline (NHQ) or 4-hydroxy-2-nonylquinolone [46], showed potent anti-HCV activities with IC_{50} of 1.4 ± 0.2 μg/ml (5.1 ± 0.2 μM) (Table 3). A wide variety of quinolones have been used as antimicrobial, anticancer and antiallergenic agents. Quinolones are known as broad-spectrum antibacterial agents with the main structure of 1,4 dihydro-4-oxo-quinolinyl moiety. Quinolones inhibit prokaryotic type II topoisomerases through direct binding to bacterial chromosome, and likewise, it may bind to viral nucleic acids and/or nucleoprotein complexes to act as antivirals [47]. Quinolones consist of heterobicyclic aromatic compounds and the moiety of C_9H_{19} at carbon number 2 of pseudane IX may play an important role in its activities. Quinolones have been reported to act as inhibitors of HCV NS5B RNA polymerase by binding to the allosteric site II (non-nucleoside inhibitor-site 2) of this protein [48]. Twelve fluoroquinolone derivatives were reported to inhibit HCV replication and HCV NS3 helicase activity in cultured cells. Among them, fleroxacin, difloxacin, ofloxacin, 8-quinolinol, enoxacin, lumifloxacin and flumiquine were reported to inhibit HCV with IC_{50} of 3.8, 2.5, 2.4, 2.2, 1.0, 1.9 and 1.7 μM, respectively [49].

The key steps in HCV life cycle include entry into the host cells, uncoating and replication of the viral genome, translation of virus proteins, and assembly and release of the virion [8,18]. To determine the impact of active compounds on HCV life cycle, we conducted time-of-addition experiments. We observed that chalepin and pseudane IX exhibited their anti-HCV activities at the post-entry step, inhibiting HCV RNA replication and NS3 protein synthesis.

In conclusion, we have identified chalepin and pseudane IX as anti-HCV compounds. These compounds could be good candidates to develop novel anti-HCV drugs.

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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.fitote.2014.10.011.

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Prominent Steatosis with Hypermetabolism of the Cell Line Permissive for Years of Infection with Hepatitis C Virus

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Abstract

Most of experiments for HCV infection have been done using lytic infection systems, in which HCV-infected cells inevitably die. Here, to elucidate metabolic alteration in HCV-infected cells in a more stable condition, we established an HCV-persistently-infected cell line, designated as HPI cells. This cell line has displayed prominent steatosis and supported HCV infection for more than 2 years, which is the longest ever reported. It enabled us to analyze metabolism in the HCV-infected cells integrally combining metabolomics and expression arrays. It revealed that rate-limiting enzymes for biosynthesis of cholesterol and fatty acids were up-regulated with actual increase in cholesterol, desmosterol (cholesterol precursor) and pool of fatty acids. Notably, the pentose phosphate pathway was facilitated with marked up-regulation of glucose-6-phosphate dehydrogenase, a rete-limiting enzyme, with actual increase in NADPH. In its downstream, enzymes for purine synthesis were also up-regulated resulting in increase of purine. Contrary to common cancers, the TCA cycle was preferentially facilitated comparing to glycolysis pathway with a marked increase of most of amino acids. Interestingly, some genes controlled by nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a master regulator of antioxidation and metabolism, were constitutively up-regulated in HPI cells. Knockdown of Nrf2 markedly reduced steatosis and HCV infection, indicating that Nrf2 and its target genes play important roles in metabolic alteration and HCV infection. In conclusion, HPI cell is a *bona fide* HCV-persistently-infected cell line supporting HCV infection for years. This cell line sustained prominent steatosis in a hypermetabolic status producing various metabolites. Therefore, HPI cell is a potent research tool not only for persistent HCV infection but also for liver metabolism, overcoming drawbacks of the lytic infection systems.

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Introduction

Chronic persistent infection in liver is one of the clinical characteristics of hepatitis C virus (HCV), frequently causing liver cirrhosis and hepatocellular carcinoma (HCC) [1]. Recently, in addition to the therapy of pegylated interferon plus ribavirin, emerging anti-HCV drugs are bringing about dramatic improvement for chronic hepatitis C. However, for extermination of HCV, the development of other anti-HCV drugs targeting its persistent HCV infection and a vaccine are needed.

HCV is an enveloped, positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae* family, and its genome encodes a large polyprotein precursor of approximately 3,000 amino acid residues, which is cleaved by host and viral proteases into ten individual proteins, *i.e.* core, envelope 1 and 2 (E1, E2), p7, and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [2,3]. Since an infectious strain of genotype 2a HCV (JFH-1) has been established [4], *in vitro* research for HCV infection has been accelerated. We also generated an infectious strain of chimeric HCV consisting of genotypes 1b and 2a, designated as TNS2J1 strain, whose infectivity is comparable to that of JFH-1 [5] [6].

On the other hand, a hepatoma cell line, Huh7, and its subclone such as Huh7.5 are susceptible to infection with these HCV strains and have been used for *in vitro* experiments. However, the infected cells are unstable and eventually undergo cell death, so-called lytic infection. Although some cell lines persistently infected with HCV were reported, the periods of persistency were months [7–9].

Thus, strictly speaking, they cannot be called persistent infection systems. Here, to study HCV-infected cells in a more stable condition, we firstly established a cell line persistently infected with TNS2J1. We have maintained this cell line for more than 2 years, the longest ever reported, since the initial transfection with RNA of TNS2J.

It was noteworthy that this cell line displayed prominent steatosis, accumulation of lipid droplet (LD). Clinically, chronic hepatitis C are frequently associated with steatosis [10]. Thus, secondary, to elucidate alterations in the metabolism and gene expression underlying such steatosis, we performed integrated analyses with metabolomics and expression arrays taking advantage of the cell line established here.

Recently, it has been reported that nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a master transcriptional activator of an array of genes for metabolisms as well as genes for cytoprotection, detoxification and antioxidation [11], in complex with v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (Maf) [12–14]. Thus, finally, we investigated involvement of the Nrf2/Maf complex in the metabolic alteration in the HCV-persistently-infected cells.

Results

Establishment of an HCV-persistently-infected Cell Line, HPI Cell

We transfected Huh7.5 cells with synthetic HCV RNA of TNS2J1, where the structural region of JFH-1 (2a) was replaced with that of genotype 1b (Figure 1A) [5]. The vast majority of the infected cells with TNS2J underwent cell death, so-called 'lytic infection', displaying maximum of HCV core concentration in the medium (389 fmol/ml). Yet, we noticed that a tiny population of the infected cells survived this lytic phase. We maintained them for around 500 days monitoring HCV core protein concentration in the medium (Figure 1B) and checking immunofluorescence for intracellular HCV protein (Figure 1C). Even early after the transfection, at day 25 (passage 6), HCV core production was not so robust (60 fmol/ml) (i: Figure 1B), probably because the ratio of HCV-positive cells was reduced by the repeated passages and actually became undetectable at day 216 (passage 73) (i–iv: Figure 1C).

Nonetheless, we observed two minor surges of core production with slight increase in the ratio of HCV-positive cells (v and vii: Figure 1B, C) and hypothesized that the cells at the surges contain cells that are resistant to death and permissive for HCV persistent infection. In order to isolate such a cell clone, we attempted limiting dilutions using the cells at the 2nd surge (day 396). We performed this procedure three times consecutively to purify a clone, C3, C3-6 and finally C3-6-15 cell (ix, x and xi: Figure 1B, C). We considered the C3-6-15 cell as an HCV-persistently-infected cell line and designated it as 'HPI cell' because 100% of the cells were infected with HCV and they has supported HCV for 609 days (396 and 213 days before and during the consecutive limiting dilutions, respectively).

HPI Cells Supported HCV Infection for More than a Year after Establishment

To confirm persistence of HCV, we maintained HPI cells for about 500 days after the establishment. Core protein production was sustained all through the culture course, albeit with fluctuation from 27 to 275 fmol/ml, highest of which was comparable to that of the lytic infection (389 fmol/ml) (Figure 2A). Infectivity of HCV in cell culture medium (HCVcc) was also confirmed at passage 5, 72, 103, and 161 after the establishment, and intracellular HCV has been detected immunocytochemically at least until day 479 (Figure 2B). To ensure the existence of HCV in HPI cells, we performed RT-PCR and western blotting for HCV. PCR product covering full length of HCV, the regions from 5′-untransated region to NS2 and from NS3 to NS5A, was amplified (Figure S1A), and HCV proteins were detected in HPI cells (Figure S1B). NS5A protein of the HPI cell at passage 176 exhibited a slightly lower molecule weight than that of lytic infection and the HPI cell at passage 8. It is likely that the lower molecular weight was attributed to reduction of serine phosphorylation because deduced amino acid sequences of NS5A at passage 176 diverged remarkably and some serine residues changed to non-serine residues (manuscript in preparation). These results indicate that HPI cells have supported infectious HCV for more 479 days even after the establishment, totally for more than 2 years (1088 days) after the initial RNA transfection and that the duration is the longest ever reported to the best of our knowledge.

Characterization of HCVcc from HPI Cells

It was shown that HCVcc from lytic infection with JFH-1 contains two types of HCV particles: low-density particles with high infectivity and high-density particles with low infectivity [15]. A similar result was obtained by sedimentation analysis of HCVcc from HPI cells (Figure S2A), suggesting that infectious HCVcc might be associated with the lipoproteins, similar to lytic infection. Then, to explore the HCV genomic variations that might have occurred in the process of the establishment, we sequenced the RT-PCR products for HCV in the culture medium of HPI cells at passage 7 and found that deduced amino acid substitutions were frequent in the E1, E2, and NS5A regions (Figure S2B).

Since the supernatant from the cultured HPI cells induced cell lysis when used to inoculate naïve Huh7.5 cells (Figure S2C), we speculated that the persistence of HCV depended not on such HCV genomic variations, but on cellular factor(s) of HPI cells. To verify this, we cured HPI cells with cyclosporine, and designated the resulting cells as 'CuHPI'. Expectedly, these cells were susceptible to HCVcc but permissive for HCV persistency for at least 120 days (Figure S2D). Therefore, cellular factor(s), such as genetic alteration occurred during the establishment, might have conferred resistance to apoptosis and permissiveness for HCV persistent infection.

Remarkable Accumulation of Lipid Droplets in HPI Cells

It was noteworthy that prominent steatosis has sustained in HPI cells for long-term, from passage 8 to 166 as long as we observed. The core proteins were almost localized with the LDs, while the NS5A proteins were widely distributed in the cytoplasm but partly surrounding the LDs (Figure 3A, Figure S3). The distributions were similar to those of lytic infection, but the amount of LD was much more [15]. Actually, quantification of cellular lipid contents showed that major components of LDs, free cholesterol, cholesterol esters, and triacylglycerol, increased significantly in the HPI cells, whereas minor components, phospholipids, did not increase so much (Figure 3B). These result indicated HPI cell displayed prominent steatosis microscopically and biochemically.

Integrated Analysis of HPI Cells with Metabolomics and Expression Arrays

To clarify the metabolic alteration underlying in the remarkable steatosis of HPI cells, we performed global metabolomics profiling comparing with Huh7.5 cells. For hydrophobic metabolites, liquid chromatography (LC)-time-of-flight mass spectrometry (TOFMS) was performed, and 45 metabolites were detected. Of them, the

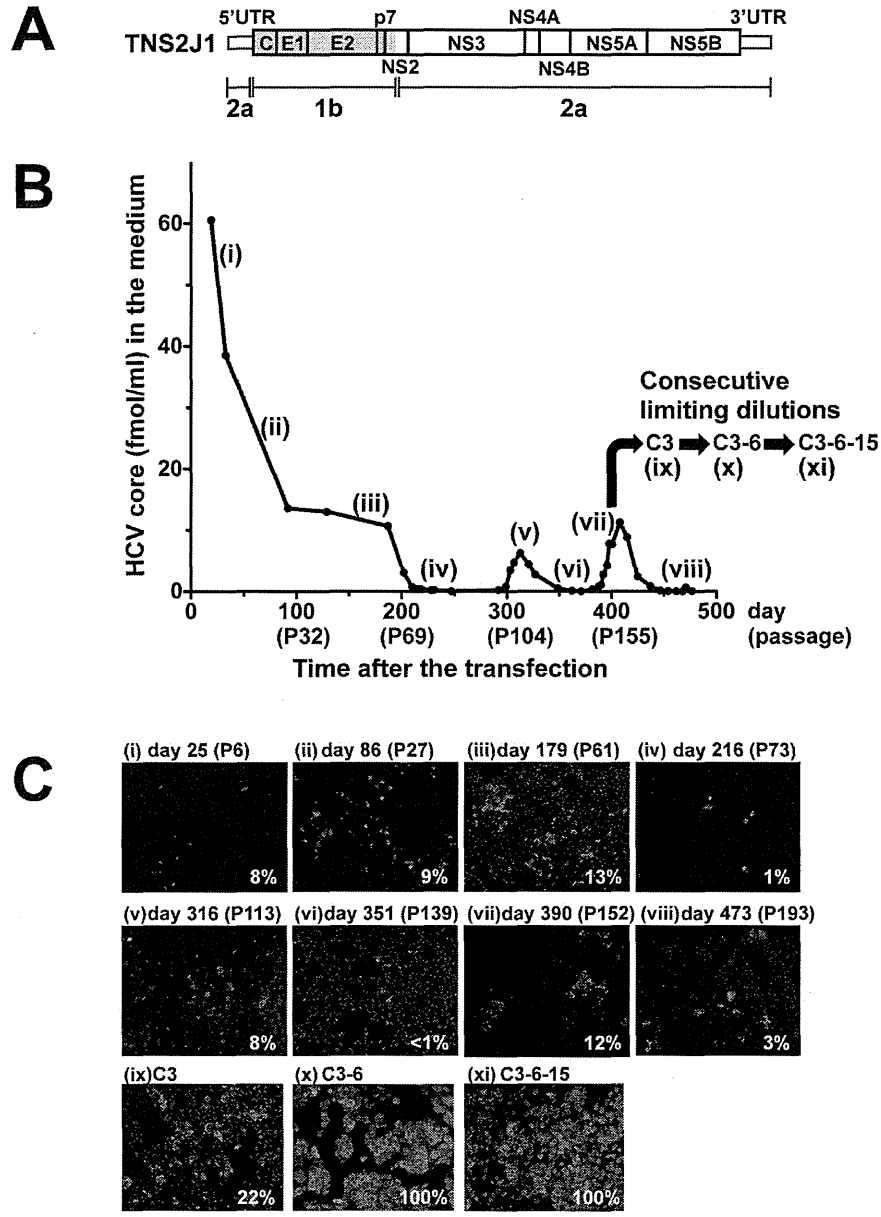

Figure 1. Establishment of the HCV-persistently-infected clonal cell line, HPI, monitoring HCV proteins in the culture medium and cells. (**A**) Structure of the infectious strain of a chimeric HCV (TNS2J1). Blank and shaded regions indicate genotypes 2a and 1b, respectively. (**B**) HCV core protein concentration in the medium was determined after the transfection. At time points indicated in Roman numerals, immunofluorescence staining for HCV was performed (C). Using the cells at day 396, limiting dilutions were performed three times consecutively to isolate cell clones C3 (ix), C3-6 (x), and C3-6-15 (xi). P-numbers in parentheses represent passage numbers after transfection. (**C**) Immunofluorescence staining for HCV NS5A protein in the cells was performed. Percentages indicate ration of HCV-positive cells.
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levels of 29 metabolites increased more than 1.4-fold, and five decreased to less than 0.7-fold in HPI cells (Table S1). For hydrophilic metabolites, capillary electrophoresis (CE)-TOFMS was performed, and 172 metabolites were detected. Of them, the levels of 99 metabolites increased more than 1.4-fold, and 16 decreased to less than 0.7-fold in HPI cells (Table S2). For integration of metabolomics and expression arrays, expression arrays (approximately 25,000 transcripts/array) were performed simultaneously. The expression data of genes encoding enzymes for a corresponding reaction appearing in the metabolomics profiling were selected for following pathway analyses (Table S3). Differential expression was confirmed with immunoblot analysis, when corresponding antibody was available.

Increased Cholesterol and Desmosterol

Cholesterol and cholesterol ester are major constituents in LD and HCV replication complex is fractionated in lipid raft, which is rich in cholesterol [16,17]. Thus, at first, we analyzed the biosynthetic pathway of cholesterol. Its first step is translocation of citrate from mitochondria to cytosol, where citrate is converted to acetyl CoA. This conversion is catalyzed by ATP-citrate lyase (ACLY), whose expression was moderately up-regulated (Figure 4A, B). Increase of citrate (Figure 4A) and increase of