facilitates the mass screening for anti-HCV reagents. HCV RNA replicating in OR6 cells contained an adaptive mutation, K1609E, in the NS3 region. Adaptive mutations have been reported to enhance the replication level of HCV RNA in cell culture [59–61]. In the case of HCV-O, two adaptive mutations were required for robust replication of the genome-length HCV RNA replication [60]. For example, authentic HCV-O RNA with the adaptive mutations of E1202G and K1609E can robustly replicate in HuH-7 cells for 9 months or more (Ikeda et al., unpublished data).

In 2005, three groups reported infectious HCV production systems using the JFH1 strain in cell culture [6–8]. These reports showed that the life cycle of HCV could be reconstructed in HuH-7 cells, and thus became landmarks in the search for an ideal HCV cell culture system. The unique features of these systems were the origin of this strain and the cell lines. JFH1 was a genotype 2a strain derived from a patient with fulminant hepatitis and did not require any adaptive mutations for robust replication, unlike other HCV strains. The unique feature of this system was that it employed HuH-7 cells such as Huh-7.5 or Huh-lunet cells, since the parental HuH-7 cells could not support robust production of infectious HCV [6–8,62]. Recently, the genotype 1a H77-S strain was reported to produce infectious HCV in cell culture, although the production level of infectious H77-S was lower compared with that by JFH1 [63]. Interestingly, five adaptive mutations were introduced into the H77-S genome in order to enhance the efficiency of infectious virus production. The presence of these adaptive mutations is the most striking and controversial characteristic regarding the production of infectious HCV described above. Further study will be needed to understand the role of adaptive mutations on infectious virus production.

### *3.2. HCV life cycle*

The establishment of an infectious HCV production system gradually led to clarification of the life cycle of HCV. Information regarding the HCV RNA replication has been accumulated since the development of the HCV replicon system, and the infectious HCV production system [6–8] has further provided information about the step of virus entry and release. The life cycle of HCV includes the (1) receptor binding and cell entry, (2) cytoplasmic release and uncoating, (3) IRES-mediated translation, (4) processing, (5) RNA replication, (6) packaging and assembly, (7) virion maturation, and (8) virion release. Although some of the mechanisms are still unclear, each of these steps is a target for antivirals. Among the proteins involved in these steps, the protease in step (4) and polymerase in step (5) have been especially well characterized. Specific inhibitors for these proteins have been developed and some of them are now in clinical trials for patients with CH–C [21,64].

### *3.3. Cellular proteins required for HCV RNA replication*

Cellular proteins are required for HCV RNA replication and may determine the cell tropism of HCV. As HCV is a parasite, it utilizes the cellular proteins for its replication machinery. Therefore, cellular proteins essential for HCV RNA replication are the targets for antivirals. Using cell culture systems, several cellular proteins have been identified as effective molecules for HCV RNA replication (Table 1). La and PTB were representative molecules reported as essential host factors for HCV RNA replication [40]. Recently, an immunosuppressant, CsA, has been reported to inhibit HCV RNA replication by blocking the binding of CyPB to NS5B [28]. HSP90 and the FK-506-binding protein 8 (FKBP8) form a complex with NS5A and geldanamycin, an inhibitor of HSP90, suppressed HCV RNA replication by blocking the formation of these complex [38]. The advantage of the inhibitors targeting cellular factor is that these reagents do not affect the viral escape achieved through mutations. The high mutation rate caused by RdRp frequently produced escape mutants toward the antiviral reagents for HCV proteins. A disadvantage of the inhibitors targeting cellular factors may be that they induce side effects by inhibiting the primary roles of the cellular factors.

## 4. Host metabolism as anti-HCV targets

The cellular factors are the targets of the antivirals independent of the viral escape via the genetic mutations caused by RdRp. The cellular factors were synthesized in their metabolic pathways and modified by the enzymes. These enzymes are also targets in the antiviral strategy (Table 1). Furthermore, some of the reagents have already been used in the clinical treatment of the respective diseases. One of the advantages of using existing reagents is that their characterizations—including safety and side effects—have already been performed. Therefore, screening of the existing reagents for anti-HCV will be a new field of antivirals. The development of a cell culture system for HCV led to the revelation that HCV incorporates many cellular factors into the replication machinery of the virus. Now we have both the information of the HCV life cycle and the cell culture assay system—the input and output—that we need to develop a pool of antiviral reagents. Below, we will discuss the particular host cell metabolic pathways that are currently being targeted by anti-HCV reagents including more recently found pitavastatin (PTV) (Fig. 2B).

### *4.1. Cholesterol-biosynthesis pathway and geranylgeranylation*

In the cholesterol-biosynthesis pathway, the region downstream of mevalonate branches into separate pathways for cholesterol and isoprenoid synthesis (Fig. 3). The attachment of the isoprenoid is called prenylation of the protein. Prenylation regulates a variety of cellular functions, such as growth, differentiation, and oncogenesis. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are mevalonate-derived isoprenoids and are attached to the target proteins by farnesyltransferase (FTase) and geranylgeranyl transferase type I (GGTase-I), respectively. FTase and GGTase-I recognize protein substrates with a C-terminal tetrapeptide recognition motif called the CaaX box: in the case of GGTase-I, C is cysteine, a is an aliphatic amino acid, and X is leucine, isoleucine, valine, or phenylalanine. Production of mevalonate by 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase is the rate-

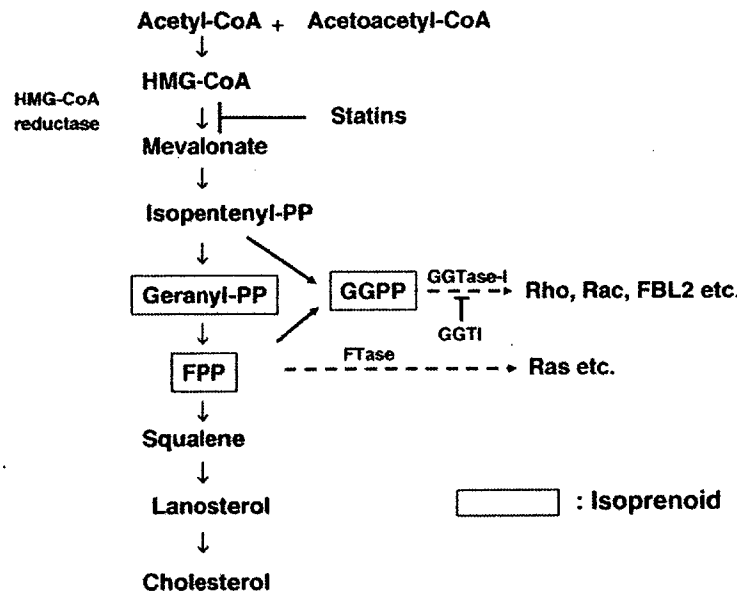


Fig. 3. Cholesterol-biosynthesis pathway. The inhibition of HMG–CoA reductase by statins leads to the suppression of mevalonate and of the production of its downstream metabolites. Decreased prenylation on the GTP-binding proteins had a significant effect on the signal transduction.

limiting step in the cholesterol biosynthesis. Statins are potent HMG–CoA reductase inhibitors and are beneficial in the prevention of coronary heart disease. Statins also inhibit the prenylation of the proteins.

Lipid metabolism is essential for the life cycle of many viruses. The cholesterol-rich lipid raft plays an important role in virus entry, replication, and assembly. HCV also forms a replication complex on the lipid raft membrane structure [65]. HCV RNA replication occurs in the lipid raft and the cholesterol supply is crucial to maintain the structure of the lipid raft [65]. Aizaki et al. [66] reported that lovastatin (LOV), one of the HMG–CoA reductase inhibitors, inhibited HCV RNA replication in HCV replicon-harboring cells.

Statins also possess the cholesterol-independent action (pleiotropic effect) [67]. Many of these pleiotropic effects are mediated by the isoprenoid. For example, inhibition of small GTP-binding proteins, Ras and Rho, whose proper membrane localization and function are dependent on prenylation, may play a significant role in the pleiotropic effect of statins. Ras and Rho are major substrates for prenylation with FPP and GGPP, respectively. GDP-bound Ras and Rho are localized in the cytoplasm. When FPP or GGPP is bound to the inactive Ras or Rho, they are translocated to the cell membrane and converted to GTP-bound active forms. Recently, Wang et al. [68] identified FBL2 as one of the geranylgeranylated cellular proteins required for HCV RNA replication. FBL2 belongs to the FBL family of proteins, all of which contains an F box and a multiple leucine-rich repeat, with the F box binding to a multicomponent ubiquitin ligase complex. Geranylgeranylated FBL2 binds to NS5A, and the resulting complex seems to be required for HCV RNA replication. In HCV replicon-harboring cells, knockdown of FBL2 by siRNA has been shown to reduce HCV RNA by 65% [68]. Depletion of the GGPP by statins may inhibit the geranylgeranylation of cellular proteins such as FBL2 and cause the anti-HCV effect in the cells.

Statins are among the most widely used reagents to lower cholesterol. One of the statins used clinically, LOV, has been well characterized and shown anti-HCV activity in cell culture. [66,69,70]. However, the anti-HCV activities of other statins remain to be clarified. Recently the anti-HCV activities of several statins were characterized using an OR6 assay system [71]. The anti-HCV activities were tested for five statins: atorvastatin (ATV), fluvastatin (FLV), pravastatin (PRV), simvastatin (SMV), and LOV. FLV exhibited the strongest anti-HCV activity (50% effective concentration to inhibit HCV RNA replication ($EC_{50}$): 0. 9 μM), while ATV and SMV showed moderate inhibitory effects ($EC_{50}$: 1.39 and 1.57 μM, respectively). However, LOV, which has been reported to inhibit HCV replication, was shown to possess the weakest anti-HCV activity ($EC_{50}$: 2.16 μM). More recently, we found that PTV possessed stronger anti-HCV activity than FLV (Fig. 2B). The $EC_{50}$ of PTV was calculated as 0.45 μM. The anti-HCV activities of statins were reversed by supplying mevalonate or geranylgeraniol. However, surprisingly, PRV exhibited no anti-HCV activity, although it worked as an inhibitor for HMG–CoA reductase. Although PRV is a water-soluble reagent (the others are lipophilic), PRV induced the expression of HMG–CoA reductase by a positive feedback mechanism. There may be another mechanism underlying the depletion of GGPP by the statins. Interestingly, it has been reported that only PRV has a different effect on the induction of P450 compared with the other statins [72].

Ribavirin is the only reagent currently used with IFN-α to treat patients with CH–C [73]. In the previous study on anti-HCV activity using the OR6 assay system, the $EC_{50}$ of ribavirin was 76 μM [74]. This concentration is much higher than the clinically achievable ribavirin concentration (10–14 μM) reported previously [75,76]. Since FLV exhibited strong anti-HCV activity, FLV was examined for its anti-HCV activity in combination with IFN-

α in OR6 cells [71]. Co-treatment of IFN-α and FLV exhibited synergistic inhibitory effects on HCV RNA replication. For example, when administered in combination with IFN-α (2 IU/ml) and FLV (5 μM), the level of HCV RNA replication was remarkably reduced to approximately 3%, compared with the effects of treatment with IFN-α alone. The combination therapy of FLV may be effective for the treatment of patients with CH–C.

It is not appropriate to further reduce the cholesterol level of CH–C patients who already have a normal cholesterol level. For these patients, statin-related anti-HCV reagents possessing no cholesterol-lowering activity would be good candidates for future clinical use. The specific inhibition of GGPP synthesis and prenylation will be worth testing, and GGTase-I inhibitor (GGTI) is one of the candidates for this purpose. Furthermore, specific inhibition of the proteins modified by GGTase-I may be more effective. FBL2 may be one of the target proteins, because its formation of a complex with NS5A is required for HCV RNA replication. Therefore, the reagents blocking the association of FBL2 with NS5A will be able to inhibit the HCV RNA replication with fewer side effects. Prenyltransferase recognizes a broad range of protein substrates with a CaaX motif. Reid et al. [77] reported a list of hypothetical prenyltransferase substrates within the human genome. Other than FBL2, the host molecules involved in HCV RNA replication may be exist in this list.

Antiviral activity of statins has also been reported in other viruses. In the respiratory syncytial virus (RSV), LOV exhibited antiviral activity via the inhibition of RhoA [78]. RhoA is activated by geranylgeranylation, and activated RhoA interacts with the F glycoprotein of RSV. FLV inhibited cytomegalovirus (CMV) replication by abolishing CMV-induced NF-κB activity, which is involved in a pathway that is crucial for CMV replication [79]. In human immunodeficiency virus (HIV), LOV and SIV reduced HIV replication via suppression of the binding between the integrin intercellular adhesion molecule 1(ICAM1) and lymphocyte function associated antigen-1 (LFA-1) [80]. Statins were recently shown to bind to LFA-1, and ICAM1-bearing viruses were reduced by statins in a dose-dependent manner. It is noteworthy that the inhibition of LFA-1 binding to ICAM-1 by statins is independent of the inhibition of HMG CoA reductase. Statins inhibited the cholesterol-biosynthesis pathway and branched prenylation pathways by depletion of mevalonate. The latter caused pleiotropic effects in growth, differentiation, and antivirals. However, an unknown function of statins may exist—for example, the binding of LFA-1 is likely independent of the cholesterol-lowering and the inhibition of prenylation. Furthermore, the finding that PRV has a different effect on the induction of P450 than the other statins has not been clearly explained by the characterization of these mechanisms of statins. A better understanding of this finding may lead to the discovery of statin-related anti-HCV reagents that do not have exhibit any cholesterol-lowering activity or inhibition of prenylation.

### 4.2. Sphingolipid synthesis pathway

Lipid rafts are detergent resistant membranes (DRM) and are enriched in cholesterol and sphingolipids. The active replication complex of HCV is present in lipid rafts [65]. Therefore, sphingolipid metabolism is also an antiviral target for HCV. Serine palmitoyltransferase (SPT) is the enzyme responsible for the condensation of L-serine with palmitoyl–CoA to produce 3-ketodihydrosphingosine in the first step of sphingolipid biosynthesis (Fig. 4). Myriocin, a selective inhibitor of SPT, inhibited the replication of HCV replicon [81,82].

Sakamoto et al. [81] reported that the compound NA255, which is structurally similar to myriocin, also inhibited the replication of the HCV replicon. NA255 has been identified as the secondary fungal metabolite derived from *Fusarium* sp. NA255 suppressed HCV replicon in a dose-dependent manner, and its $EC_{50}$ was 2 nM. They further examined the involvement of the sphingolipid synthetic pathway in HCV RNA replication. Fumonisin B1, an inhibitor of dihydroceramide synthase, also suppressed the replication of HCV replicon. In mammalian cells, ceramide is synthesized in the endoplasmic reticulum (ER) and translocates to the Golgi compartment for conversion to sphingomyelin. HPA-12, an inhibitor of ceramide trafficking from the ER to the Golgi apparatus, also inhibited the replication of HCV replicon. Glycosphingolipids (GSLs) are also a component of lipid rafts, and PPMP, an inhibitor of GSL biosynthesis, also suppressed the replication of HCV replicon. Furthermore, they demonstrated that after treatment with NA255, the NS5B ratio in the DRM was markedly decreased. Interestingly, however, the DRM fraction of NS3 and NS5A were not affected. Inhibition of sphingolipid biosynthesis by NA255 disrupted the association of lipid rafts with NS5B, but not with NS3 or NS5A. They identified a helix-turn-helix motif (Glu230-Gly263) in NS5B as a sphingolipid-binding domain (SBD), which was similar in structure to the SBD of the V3 loop of HIV-1.

Umehara et al. [82] reported that myriocin suppressed HCV RNA replication *in vivo*, using HCV-infected chimeric mice with humanized livers. Myriocin reduced the HCV RNA levels in both serum and liver to 1/10–1/100 of the levels prior to the 8 day treatment. They also demonstrated that the combined treatment of myriocin with PEG-IFN reduced the HCV RNA level to less than 1/1000 of the control levels. These results

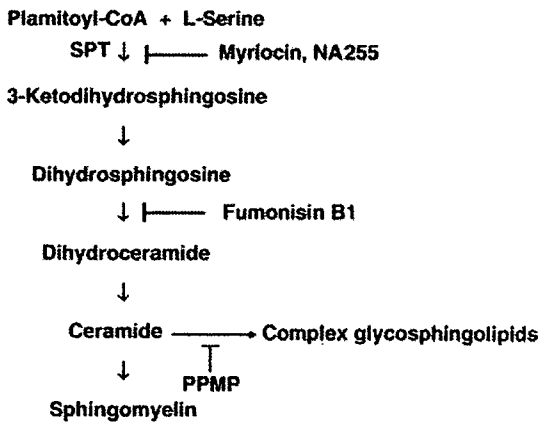


Fig. 4. Sphingolipid-biosynthesis pathway. The spingolipid-biosynthesis pathway. Myriocin and NA255 inhibited the SPT and caused the depletion of spingomyelin and glycosphingolipids.

suggest that the sphingolipid biosynthetic pathway is also a suitable target for the development of HCV therapies.

### 4.3. GTP-biosynthesis pathway

At the beginning of GTP-biosynthesis pathway, inosine monophosphate dehydrogenase (IMPDH) is the enzyme responsible for the conversion of inosine 5′ monophosphate (IMP) into xanthosine 5′ monophosphate (XMP) (Fig. 5). Ribavirin, mizoribine, mycophenolic acid (MPA), and VX-497 are IMPDH inhibitors and inhibit HCV RNA replication.

Ribavirin enhanced the SVR of PEG-IFN therapy from 29% to 56% compared to the PEG-IFN monotherapy [83]. However, the antiviral mechanisms of ribavirin remain to be clarified. Four possible mechanisms have been proposed [73,84]: (1) direct inhibition of RNA replication; (2) inhibition of IMPDH; (3) immunomodulation; (4) mutagenesis. Ribavirin is phosphorylated to mono-, di-, and triphosphate (RMP, RDP, and RTP, respectively). (1) RTP, an analog of GTP, is incorporated into replicating RNA by RdRp and caused termination of the RNA synthesis. (2) RMP competitively inhibits the host enzyme IMPDH, which is essential for the synthesis of GTP, and causes a depletion of the GTP pool. (3) Rivavirin has been suggested to cause immunomodulatory effects, such as the shift of Th2 to Th1 in immune response, and to induce an HCV-specific T cell response. (4) Ribavirin acts as an RNA mutagen and causes error catastrophe. In poliovirus replication, 100 μM of ribavirin increased the mutation rate from about 1.5 mutations/genome (wild type) to about 1.9 mutations/genome and resulted in a decrease of infectivity of 70% [85]. The mutation rate increased in a ribavirin dose-dependent manner: 6.9 mutations/genome and 15.5 mutations/genome at 400 μM and 1000 μM, respectively [85].

In the clinical study of CH–C, the enhancement of SVR has been observed only in combination therapy of ribavirin with IFN, but not in ribavirin monotherapy. It may be difficult to test the effect of ribavirin monotherapy, since the clinically achievable concentration of ribavirin without severe side effects such as anemia is too low (10–14 μM). However, in the cell culture model [74,86], a higher concentration of ribavirin suppressed HCV RNA replication ($EC_{50}$: 76 μM) [74].

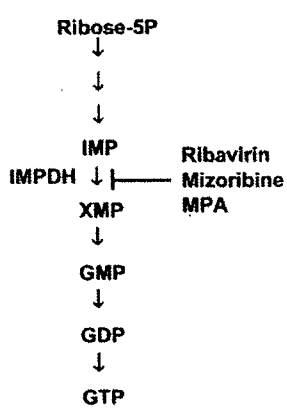


Fig. 5. GTP-biosynthesis pathway. The de novo GTP-biosynthesis pathway. Ribavirin, mizoribine, and MPA suppressed the XMP synthesis by the inhibition of IMPDH.

Mizoribine is an imidazole nucleoside that is isolated from culture medium of the mold *Eupenicillium brefeldianum* M-2166 and is structurally similar to ribavirin. Mizoribine was authorized by the Japanese Government as an immunosuppressive drug for renal transplantation; thereafter, lupus nephritis, rheumatoid arthritis, and nephritic syndrome were also added to the list of diseases for which this agent is indicated [87,88]. Based on the similarity of mizoribine to ribavirin, the anti-HCV activity of mizoribine has been tested using an OR6 assay system. The anti-HCV activity of mizoribine ($EC_{50}$: 99 μM) was similar to that of ribavirin [74]. Furthermore, a low dose (at least 5 μM) of mizoribine was able to enhance the antiviral activity of IFN [74]. Mizoribine was reported to exhibit antiviral activity on influenza virus types A and B [87] and recently on bovine viral diarrhea virus [89] and severe acute respiratory syndrome (SARS)-associated coronavirus [90]. The precise antiviral mechanism of mizoribine remains unclear. However, any of the four hypothesized mechanisms of ribavirin mentioned above may be possible. Since mizoribine has not been associated with severe side effects, it will be an alternative reagent for combination therapy with IFN.

Like mizoribine, MPA is used as an immunosuppressant and is known to inhibit IMPDH. It has been reported to show *in vitro* antiviral activity against dengue virus [91,92], hepatitis B virus (HBV) [93], avian reovirus [94], yellow fever virus [95], and West Nile virus [96]. The anti-HCV activity of MPA was reported by Henry et al. [97]. At clinically relevant concentrations (1.0–6.0 μg/ml), MPA inhibited HCV RNA replication to approximately 75% in a study using HCV replicon-harboring cells. Furthermore, combination treatment of MPA with CsA or IFN showed synergistic inhibition of HCV RNA replication. We also recently confirmed that the combination of CsA and mizoribine had a synergistic effect on the inhibition of HCV RNA replication (Yano et al., unpublished data). These data suggest that immunosuppressive drugs possessing anti-HCV activity, such as CsA, MPA, and mizoribine, may prevent not only the rejection of the graft but also the recurrence of HCV infection after liver transplantation, and that a combination of these drugs may be of additional benefit for such patients.

VX-497 is a reversible uncompetitive IMPDH inhibitor that is structurally unrelated to other known IMPDH inhibitors. Markland et al. [98] reported the broad-spectrum antiviral activity of VX-497.VX-497 exhibited 10- to 100-fold more potency than ribavirin against HBV, human CMV, RSV, herpes simplex virus type 1, parainfluenza 4 virus, EMCV, and Venezuelan equine encephalomyelitis virus in cell culture [98]. Zhou et al. [99] reported that VX-497 alone had only marginal effect on HCV replicon, although combination treatment with ribavirin and VX-497 enhanced anti-HCV activity. They also reported that in their HCV replicon assay system, MPA showed only a marginal anti-HCV effect [99]. This result is different from the anti-HCV effect of MPA reported by Henry et al. [97]. Further study will be needed to clarify these controversial results.

### 4.4. N-glycosylation pathway

HCV morphogenesis is a target of antivirals in the life cycle of the virus. The HCV envelope glycoproteins E1 and E2 are highly N-glycosylated [100]. The consensus sequence for N-glycosylation is Asn-X-Ser/Thr, where X is any amino acid except for Pro, and E1 and E2 contain 5-6 and 11 glycosylation sites, respectively. From the previous study using bovine viral diarrhea virus, inhibition of α-glucosidase is expected to prevent the proper folding and assembly of HCV. Therefore, the N-glycosylation pathway may be a novel molecular target for antivirals. Chapel et al. [101] reported an anti-HCV effect of the α-glucosidase inhibitor in the binding step using HCV virus-like particles (VLPs) derived from baculovirus. The glucose analogue deoxynojirimycin derivatives, which are α-glucosidase inhibitors, caused the retention of unprocessed, hyperglycosylated N-linked glycans on HCV glycoproteins and led to the reduction in binding of VLP to the cells [101]. These results will be examined using a recently developed infectious HCV production cell culture system. α-glucosidase inhibitor may be one of the candidates for an effective combination therapy.

### 4.5. STAT1 methylation

It is crucial that the SVR for patients with CH–C receiving the current standard therapy of PEG-IFN plus ribavirin is improved from the current value of about 50%. The anti-HCV effect of IFN-α is caused through the Jak-STAT signaling pathway. Duong et al. [102] proposed that hypomethylation of STAT1 by HCV protein caused the resistance to IFN therapy. Unmethylated STAT1 is less active because it can be bound and inactivated by its inhibitor, the protein inhibitor of activated STAT1 (PIAS1). Protein arginine methyltransferase 1 (PRMT1) is the enzyme responsible for the methylation of STAT1. HCV proteins induced the expression of the catalytic subunit of protein phosphatase 2A (PP2Ac), and overexpression of PP2Ac induced STAT1 hypomethylation via the inhibition of PRMT1. Finally, PIAS1 interacted with and inhibited hypomethylated STAT1 and resulted in the suppression of IFN signaling [102].

S-adenosyl-L-methionine (AdoMet) is a methyl group donor for STAT1 methylation by PRMT1. AdoMet is used for the treatment of alcoholic liver disease and is available in many countries as a nonprescription drug. Betaine has been known to raise the intracellular concentration of AdoMet and plays the central role in the recycling of AdoMet. When PP2Ac was overexpressed in HuH-7 and UHVH 57.3 cells, IFN-α signaling was suppressed [102]. However, the co-treatment of AdoMet and betaine restored the IFN-α signaling. These results suggest that the addition of AdoMet and betaine to the current standard therapy with PEG-IFN and ribavirin may enhance the SVR for patients with CH–C.

### 4.6. Fatty acid-biosynthesis pathway

Lipid metabolism is one of the most important pathways for HCV RNA replication. Other than cholesterol and sphingolipid synthesis, fatty acids are reported to be metabolites involved in HCV RNA replication [70,103]. However, the precise mechanisms of fatty acids on HCV RNA replication have remained unclear.

Leu et al. [103] reported that polyunsaturated fatty acids (PUFAs) inhibited HCV replicon replication. Arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) belong to PUFAs (Fig. 6) and possessed anti-HCV activity. The $EC_{50}$ of AA was 4 μM. However, at 100 μM, α-linolenic acid, γ-linolenic acid (GLA), and linoleic acid reduced HCV RNA levels slightly, and saturated fatty acids, including oleic acid, myristic acid, palmitic acid, and steric acid, slightly enhanced HCV RNA levels. Similar results were also reported by Kapadia et al. [70] using a genome-length HCV RNA-replicating cell line.

AA produces lipid mediators such as prostaglandins (PGs), thromboxanes (Txs), leukotriens (LTs), and lipoxins (LXs) (Fig. 6). However, the antiviral activity of these eicosanoids remains unclear. In their clinical study, Hyman et al. [104]

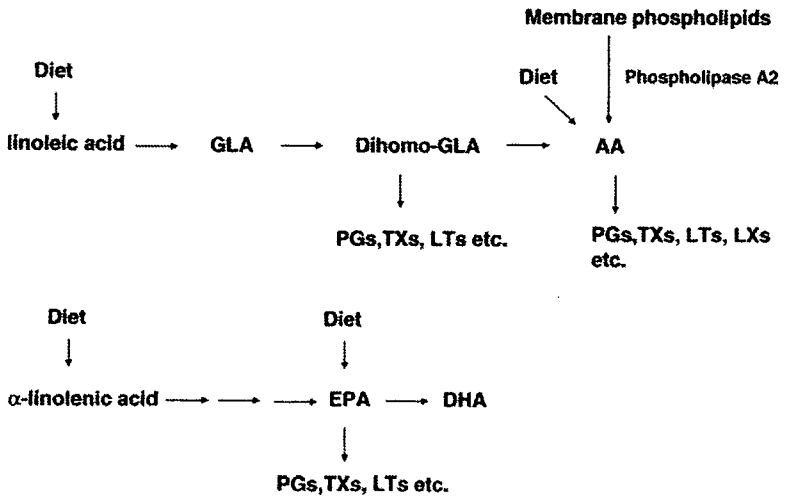


Fig. 6. Fatty acid-biosynthesis pathway. The PUFA metabolism from diet or membrane phospholipids.

reported that oral prostaglandin E2 therapy resulted in no beneficial effect on patients with CH–C. Investigation of the anti-HCV effects of the metabolites of PUFAs will lead to a new field of antivirals based on the host metabolism.

## 5. Conclusions

Ever since HCV was discovered to be the causative agent of non-A, non-B hepatitis virus, IFN has played the central role in treating the disease. Currently IFN has been modified by PEG and accompanied by the powerful partner, ribavirin, which boosts the anti-HCV activity of IFN. During the development of IFN therapy for patients with CH–C, the lack of a robust method of HCV RNA replication in cell culture has hampered research into the HCV life cycle and the discovery of potent new anti-HCV reagents. It is difficult to attack the Achilles' heel of HCV without information on the replication machinery of the virus. However, the development of a subgenomic replicon system by Lohmann et al. [5] partially revealed the HCV life cycle. The information about HCV RNA replication in the virus life cycle provided clues to the development of antivirals both from the standpoint of the virus and the host. A representative example is the discovery that NS3-4A inhibits innate immunity [105]. HCV runs through the cellular first defenses of the IFN-production system. NS3-4A, a serine protease, cleaved the unexpected cellular target Cardif and disrupted RIG-I signaling [106]. HCV replicon contributed to the discovery of the viral serine protease inhibitor. Surprisingly, a serine protease inhibitor, SCH6, inhibited HCV RNA replication not only by the inhibition of NS3-4A activity but also by the inhibition of the RIG-I signaling [105]. This serine protease inhibitor possesses dual functions, inhibiting both viral (NS3-4A) and cellular (Cardif) proteins involved in IFN production.

Viral and cellular molecules are the targets of antivirals. HCV RdRp caused a high mutation rate and the mutations accumulated in virus genome [107]. The high mutation rate enhances the viral evolution. As for the reagents targeting viral proteins, such as NS3-4A or NS5B, resistance to the therapy happens by the frequent mutations caused by RdRp. In fact, in the clinical trial of the NS3-4A protease inhibitor, VX-950, HCV RNA rapidly decreased within 3 days after treatment [20]. However, HCV RNA increased again at around 14 days after treatment [20]. HCV mutants may not be the problem in the anti-HCV reagent against cellular proteins, although the inhibition of the primary functions of the cellular proteins may cause side effects. In this review, host metabolic pathways are overviewed. One of the advantages of targeting host metabolism as antivirals is that multiple enzymes involved in the metabolism could become candidates for antivirals. In the strategy targeting host metabolism, we should be careful in regard to the side effects caused by inhibition of the primary function of the metabolite. To minimize these undesirable effects, pinpoint inhibition of the enzyme should be done.

Lipid metabolism is one of the important targets for antivirals among cellular factors. Very recently, we examined the effect of ordinary nutrients on HCV RNA replication [108]. Using an OR6 assay system, we found that linoleic acid possessed an anti-HCV effect and its combination with CsA exerted synergistic inhibitory effect on HCV RNA replication [108]. However, the anti-HCV mechanism of PUFAs remains unclear. An improved understanding of the anti-HCV effect of PUFAs will extend the field of host metabolism as a target of antivirals in the future.

One recent striking advance is the development of a method for infectious HCV production in cell culture. This system provides information regarding the complete life cycle of HCV and will extend our understanding of the antivirals to virus entry, assembly and release. The discovery of anti-HCV reagents targeting host metabolism in the HCV life cycle will improve the SVR in combination with IFN. Or, the development of new anti-HCV reagents could lead to the retirement of IFN in the near future.

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# Identification of Novel Epoxide Inhibitors of Hepatitis C Virus Replication Using a High-Throughput Screen▽†§

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**Using our high-throughput hepatitis C replicon assay to screen a library of over 8,000 novel diversity-oriented synthesis (DOS) compounds, we identified several novel compounds that regulate hepatitis C virus (HCV) replication, including two libraries of epoxides that inhibit HCV replication (best 50% effective concentration, < 0.5 μM). We then synthesized an analog of these compounds with optimized activity.**

Hepatitis C virus (HCV) infects over 170 million people worldwide and frequently leads to cirrhosis, liver failure, and hepatocellular carcinoma (1). Currently, the best therapy for the treatment of chronic hepatitis C is a combination of pegylated interferon and ribavirin, which has suboptimal efficacy and has an unfavorable side effect profile (14). The identification of more-effective and better-tolerated agents is therefore a high priority.

We have recently reported the successful adaptation of the Huh7/Rep-Feo replicon cell line (18) to a high-throughput screening assay system (8). Using this system, we previously screened a library of 2,568 well-known compounds whose biological activity is fully characterized (8). In order to discover novel regulators of HCV replication, we then screened a library of 8,064 diversity-oriented synthesis (DOS) compounds (15, 16). This library, known as the DOS set, is a

TABLE 1. Hits by library from the primary high-throughput screening with the DOS set[a]

| Library | Increased luciferase signal hit libraries | | | Antiviral hit libraries | | |
|---|---|---|---|---|---|---|
| | Hits | Members | Reference(s) | Hits | Members | Reference(s) or sources |
| FPA | 11 | 319 | 5 | | | |
| BUCMLD | 4 | 880 | 10, 17 | 4 | 880 | 10, 17, Fig. 1, Table 2 |
| JMM | 4 | 544 | 13 | | | |
| UGISS | 1 | 319 | 2 | | | |
| BUCMLD epoxyquinol | | | | 12 | 34 | 10, 17, Fig. 1 and 2, Table 2 |
| SM | | | | 9 | 27 | Fig. 1 and 2, Table 2 |
| SpOx | | | | 6 | 612 | 6, 12 |
| BEA | | | | 3 | 238 | 3 |
| ICCB6 | | | | 3 | 352 | 4 |
| YKK | | | | 2 | 281 | 9 |
| RTE | | | | 2 | 159 | 19 |

[a] The total number of compounds which comprise each library is listed in the "Members" columns.

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§ Supplemental material for this article may be found at http://aac.asm.org/.

‡ L.F.P. and S.S.K. contributed equally to this project.

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TABLE 2. Results of secondary screening with antiviral hit compounds from the SM and BUCMLD libraries[a]

| Compound name | $EC_{50}$ | $CC_{50}$ |
|---|---|---|
| BUCMLD-B10A11 | <0.5 (<0.5–0.5) | 19.5 (19.4–22.4) |
| BUCMLD-B10A3 | 0.7 (<0.5–5.2) | 9.0 (7.1–10.0) |
| BUCMLD-XL-184 | 1.4 (0.8–3.9) | >50 |
| BUCMLD-B10A5 | 1.5 (<0.5–5.4) | 39.3 (28.6–>50) |
| BUCMLD-XL-190 | 2.5 (<0.5–10) | >50 |
| BUCMLD-B10A1 | 2.6 (1.0–5.0) | 18.0 (15.9–19.5) |
| SM_A14B5 | 3.5 (2.7–4.4) | 27.1 (18.0–44.6) |
| BUCMLD-XL-189 | 3.8 (2.2–7.0) | >50 |
| SM_A6B5_2P100 | 6.6 (4.0–15.3) | >50 |
| BUCMLD-B10A8 | 7.0 (0.9–30.0) | >50 |
| BUCMLD-B10A10 | 7.0 (5.4–30.0) | >50 |
| BUCMLD-B10A14 | 7.6 (1.0–23.0) | >50 |
| BUCMLD-B10A7 | 7.75 (1.0–30.0) | 35.3 (33.6–36.7) |
| SM_A4B6_2P123 | 8.0 (6.3–10.0) | >50 |
| SM_A5B5_2P118 | 9.1 (2.5–16.7) | >50 |
| SM_A7C2_2P155 | 12.7 (7.0–24.0) | >50 |
| BUCMLD-B13A2 | 14.2 (6.4–45.0) | >50 |
| SM_A1B2_1P32 | 19.6 (11.7–28.2) | >50 |
| SM_A1B5_2P24 | 19.7 (6.25–50.0) | >50 |
| BUCMLD-B13A1 | 21.1 (7.5–36.7) | >50 |
| SM_A5B3_2P141 | 25.7 (18.3–39.8) | >50 |
| SM_A5B2_2P142 | 26.7 (9.1–50.0) | >50 |
| BUCMLD-NTM-EN2-67A | 30.0 (0.7–46.1) | >50 |
| SM_A12B3 | >30 | >50 |
| BUCMLD-B10A13 | 42.9 (25.2–59.4) | >50 |
| BUCMLD-XL-130 | >100 | >50 |

[a] Note that structure-activity relationship SM library compounds are also included. The $EC_{50}$ and 50% cytotoxic concentration ($CC_{50}$) are reported in μM with 95% confidence intervals in parentheses. A value of <0.5 indicates a concentration of less than 0.5 μM; >30 indicates a concentration of greater than 30 μM; >50 indicates a concentration of greater than 50 μM; and >100 indicates a concentration of greater than 100 μM.

meta-library comprised of DOS libraries from chemists throughout the United States and Canada. Information about the DOS set is available at http://www.broad.harvard.edu/chembio/platform/screening/compound_libraries/index.htm.

The high-throughput primary screen and the secondary validation assays were performed as described in our previous publication (8).

Computational data analysis of the primary screening results was performed as previously described (8) except for the hit criteria. As the characteristics of this data set are different from those generated by our previous screen (8), different threshold values were chosen to assure optimal hit selection. Compounds were considered hits for inhibiting replication if they had a composite Z score of $<-2.57$ in the reporter gene screen, a reproducibility of >0.9 or $<-0.9$ in that screen, and a composite Z score of $>-2.00$ in the cell viability screen. Compounds were considered hits for stimulating luciferase production if they had a composite Z score of >2.50 in the reporter gene screen, a reproducibility of >0.9 or $<-0.9$ in that screen, and a composite Z score of <1.00 in the cell viability screen.

Full synthetic experimental procedures and spectroscopic data for the SM library compounds discussed in this publication are provided in the supplemental material. The synthesis of the full SM library, including compounds not discussed here, will be the subject of an upcoming report.

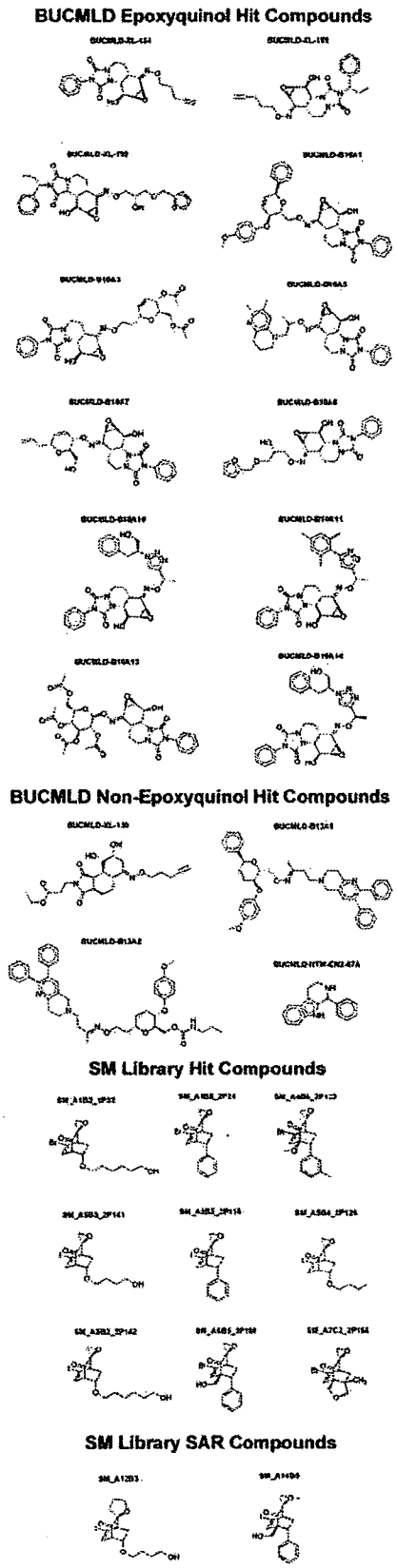


FIG. 1. Structures of antiviral hit compounds from the BUCMLD and SM libraries. SAR, structure-activity relationship.

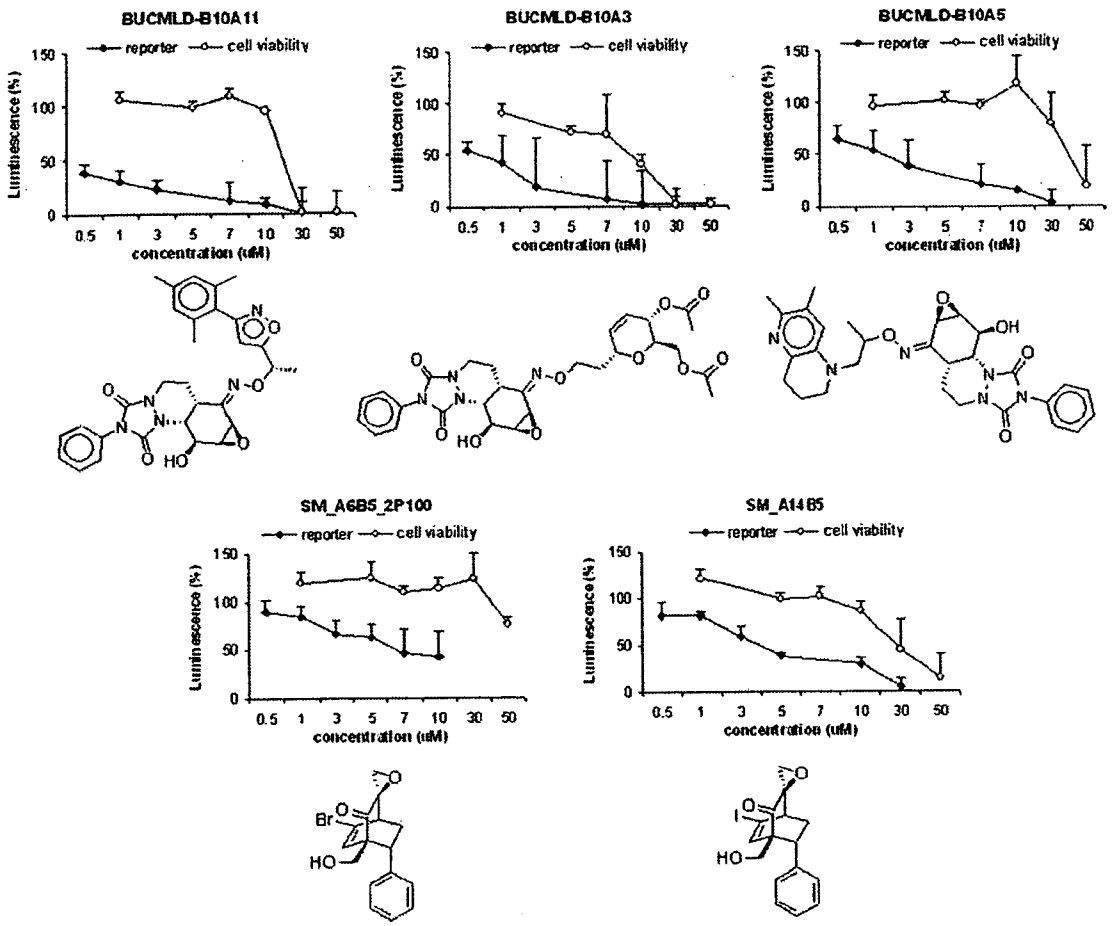


FIG. 2. Selected graphical results of secondary screening with antiviral hit compounds from the SM and BUCMLD epoxyquinol libraries. Luciferase activity for HCV RNA replication levels is shown as a percentage of control. Cell viability is also shown as a percentage of control. Each point represents the average of triplicate data points with standard deviation represented as the error bar.

The synthesis of the BUCMLD epoxyquinol library has been previously described (10, 17).

Full experimental details regarding the JFH1 HCVcc system (11) are provided in the supplemental material. We identified 41 antiviral compounds that inhibited HCV replication and 20 proviral compounds that increased luciferase production (Table 1). In our analysis of the antiviral hit compounds from the DOS set, a striking finding was that 21 of the 41 compounds contained an epoxide moiety. Moreover, the most potent of these compounds were epoxides. Further analysis revealed that these epoxides came from only two DOS libraries, SM and BUCMLD epoxyquinol (10, 17), with very high sublibrary hit rates of 35% and 33%, respectively (Table 1). Of note, the non-hit members of these two libraries did exhibit antiviral activity but failed to meet the formal hit criteria.

As we were especially intrigued by these epoxide-bearing compounds, we restricted our hit validation to these compounds (Table 2 and Fig. 1). SM_A6B5_2P100 was the most active member of the SM library, while BUCMLD-B10A11 was the most potent member of the BUCMLD epoxyquinol library (Table 2 and Fig. 2).

Structure-activity relationship analysis of the SM library reveals the structural elements most important for antiviral activity (Table 2 and Fig. 1). Comparing SM_A5B5_2P118 to SM_A1B5_2P24, iodinated compounds are more active than brominated ones. Comparing SM_A5B5_2P118 to SM_A5B3_2P141 and SM_A5B2_2P142, compounds with a phenyl substituent are more active than those with aliphatic chains. Finally, the most active compounds, SM_A4B6_2P123 and SM_A6B5_2P100, have a bridgehead substituent. Thus, we hypothesized that the most active compound should bear an iodine, a phenyl substituent, and a bridgehead substituent.

SM_A14B5, which incorporates all of these elements, was therefore synthesized, as it was reasoned to be the most active SM library compound. Indeed, SM_A14B5 had a 50% effective concentration ($EC_{50}$) of approximately 3.5 μM, which is about half that of SM_A6B5_2P100 (Table 2 and Fig. 2).

The most potent compounds from each library, SM_A14B5 and BUCMLD-B10A11, underwent further validation in the infectious JFH1 HCVcc system (11). They were tested at concentrations of 5 μM and 1 μM, respectively, and inhibited HCV replication 48.4% ± 5.9% and 45.1% ± 5.2%, respectively, relative to the level of inhibition achieved by interferon at a concentration of 1 ng/ml. These data roughly approximate the $EC_{50}$ validation data derived from the OR6 system (7) in

which inhibition was also measured relative to that of interferon at a concentration of 1 ng/ml.

Our observations suggest that the epoxide moiety is essential for potent antiviral activity. Analyzing the BUCMLD compounds, those compounds that bear an epoxide moiety are, in general, more-potent antivirals than those that do not (Table 2 and Fig. 1). Furthermore, all of the compounds from the SM library bear epoxides. SM_A12B3, an analog of SM_A5B3_2P141, which bears a tetrahydrofuran moiety in place of an epoxide, was therefore synthesized to further test this hypothesis. SM_A12B3 had negligible antiviral activity (Table 2), while SM_A5B3_2P141 displayed modest antiviral activity. Other analogs of SM compounds bearing tetrahydrofuran rings in place of epoxides showed similar attenuation of antiviral activity relative to their parent compounds. Unfortunately, attempts to synthesize the tetrahydrofuran analog of the most potent SM compound, SM_A14B5, have so far been unsuccessful.

It is interesting to note that it is the urazole-containing epoxyquinol constituents of the BUCMLD epoxyquinol library, rather than the maleimide-derived ones, that demonstrated anti-HCV activity in the primary screen. It is therefore likely that the combination of a urazole with the epoxide is necessary for the activity of the BUCMLD epoxyquinol compounds.

Although none of our most potent antiviral DOS compounds showed significant cytotoxicity at their $EC_{50}$s, all of them ultimately proved to be cytotoxic at higher concentrations (Table 2 and Fig. 2). Therefore, future modifications should not only aim to improve anti-HCV activity but should also attempt to decrease cytotoxicity, in order to widen the therapeutic window.

It is tempting to hypothesize that these epoxides exert their antiviral effects through a common pathway. Presumably, they act as electrophiles, with the nucleophilic target making a covalent bond by attacking and opening the epoxides. Studies to elucidate their mechanism of action are under way.

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# DDX3 DEAD-Box RNA Helicase Is Required for Hepatitis C Virus RNA Replication▽

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**DDX3, a DEAD-box RNA helicase, binds to the hepatitis C virus (HCV) core protein. However, the role(s) of DDX3 in HCV replication is still not understood. Here we demonstrate that the accumulation of both genome-length HCV RNA (HCV-O, genotype 1b) and its replicon RNA were significantly suppressed in HuH-7-derived cells expressing short hairpin RNA targeted to DDX3 by lentivirus vector transduction. As well, RNA replication of JFH1 (genotype 2a) and release of the core into the culture supernatants were suppressed in DDX3 knockdown cells after inoculation of the cell culture-generated HCVcc. Thus, DDX3 is required for HCV RNA replication.**

DEAD-box RNA helicases are involved in various RNA metabolic processes, including transcription, translation, RNA splicing, RNA transport, and RNA degradation (9, 20). DDX1 and DDX3, DEAD-box RNA helicases, have been implicated in the replication of human immunodeficiency virus type 1 (HIV-1). Both DDX1 and DDX3 interact with HIV-1 Rev and enhance Rev-dependent HIV-1 RNA nuclear export (10, 24).

On the other hand, DDX3 binds to the hepatitis C virus (HCV) core protein (17, 19, 25), and DDX3 expression is deregulated in HCV-associated hepatocellular carcinoma (HCC) (7, 8). However, the biological function of DDX3 in HCV replication is still not understood. To address this issue, we first used lentivirus vector-mediated RNA interference to stably knock down DDX3 in three HuH-7-derived cell lines: O cells, harboring a replicative genome-length HCV RNA (HCV-O, genotype 1b) (13); sO cells, harboring its subgenomic replicon of HCV RNA (14); or RSc cured cells, which cell culture-generated HCV (HCVcc) (JFH1, genotype 2a) (23) could infect and effectively replicate in (M. Ikeda et al., unpublished data). Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences against DDX3 in the lentivirus vector: for DDX3i#3, 5′-GATCCCCGGAGGA AATTATAACTCCCTTCAAGAGAGGGAGTTATAATTT CCTCCTTTTTGGAAA-3′ (sense) and 5′-AGCTTTTCCAA AAAGGAGGAAATTATAACTCCCTCTCTTGAAGGGA GTTATAATTTCCTCCGGG-3′ (antisense); for DDX3i#7, 5′-GATCCCCGGTCACCCTGCCAAACAAGTTCAAGAG ACTTGTTTGGCAGGGTGACCTTTTTGGAAA-3′ (sense) and 5′-AGCTTTTCCAAAAAGGTCACCCTGCCAAACAA GTCTCTTGAACTTGTTTGGCAGGGTGACCGGG-3′ (antisense). These oligonucleotides were annealed and subcloned into the BglII-HindIII site, downstream from an RNA polymerase III promoter of pSUPER (6). To construct pLV-DDX3i#3 and pLV-DDX3i#7, the BamHI-SalI fragments of the corresponding pSUPER plasmids were subcloned into the BamHI-SalI site of pRDI292 (5), an HIV-1-derived self-inactivating lentivirus vector containing a puromycin resistance marker allowing for the selection of transduced cells. The vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1-based vector system has been described previously (18). We used the second-generation packaging construct pCMV-ΔR8.91 (26) and the VSV-G-envelope plasmid pMDG2. The lentivirus vector particles were produced by transient transfection of 293FT cells with FuGene 6 (Roche).

Western blot analysis of the lysates demonstrated the only trace of DDX3 protein in DDX3 knockdown O cells (DDX3i#3) (Fig. 1A). In this context, the HCV core expression level was significantly decreased in the DDX3 knockdown O cells (Fig. 1A). To further confirm this finding, we examined the level of HCV RNA in these cells. We found that accumulation of genome-length HCV-O RNA was notably suppressed in DDX3 knockdown O cells (Fig. 1B). Furthermore, the efficiency of colony formation in DDX3 knockdown Oc cells (created by eliminating genome-length HCV RNA from O cells by interferon treatment) transfected with the genome-length HCV-O RNA with an adapted mutation at amino acid (aa) position 1609 in the NS3 helicase region (K1609E) (13) was also notably reduced compared with that in control cells (Fig. 1C). In contrast, highly efficient knockdown of an unrelated host factor, poly(ADP-ribose) polymerase 1 (PARP-1) (4), had no observable effects on HCV RNA replication, the efficiency of colony formation, or the core expression level (data not shown), suggesting that our finding was not due to a nonspecific event. Interestingly, accumulation of the subgenomic replicon RNA (HCV-sO) was also suppressed in DDX3 knockdown sO cells (Fig. 1D). Moreover, we examined the potential role of DDX3 in an HCV infection and produc-

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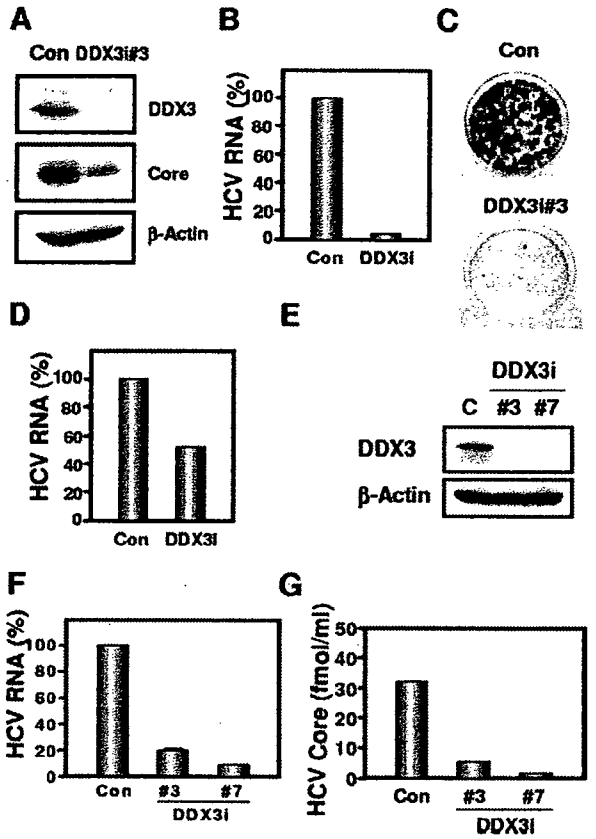


FIG. 1. Requirement of DDX3 for HCV replication. (A to D) Effect of DDX3 knockdown on HCV RNA replication. (A) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 (ProSci), anti-HCV core (CP-9; Institute of Immunology), or an anti-β-actin antibody (Sigma) in O cells expressing shRNA against DDX3 (DDX3i#3) as well as in O cells transduced with a control lentivirus vector (Con) are shown. (B) The level of genome-length HCV RNA was monitored by real-time LightCycler PCR (Roche). Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (C) Efficiency of colony formation in DDX3 knockdown cells. In vitro-transcribed ON/C-5B K1609E RNA (2 μg) was transfected into the DDX3 knockdown Oc cells (DDX3i#3) or the Oc cells transduced with a control lentivirus vector (Con). G418-resistant colonies were stained with Coomassie brilliant blue at 3 weeks after electroporation of RNA. Experiments were done in duplicate, and representative results are shown. (D) The level of subgenomic replicon RNA was monitored by real-time LightCycler PCR. Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (E to G) Effect of DDX3 knockdown on HCV infection. (E) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 or an anti-β-actin antibody for RSc cells expressing the shRNA DDX3i#3 or DDX3i#7 and for RSc cells transduced with a control lentivirus vector (Con) are shown. (F) The level of genome-length HCV (JFH1) RNA was monitored by real-time LightCycler PCR after inoculation of the cell culture-generated HCVcc. Results from three independent experiments are shown. (G) The levels of the HCV core in the culture supernatants were determined by an enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories). Experiments were done in duplicate, and bars represent the mean HCV core protein levels.

tion system (23). We found 80 to 90% reductions in the accumulation of JFH1 RNA and 82 to 94% reductions in the release of the core into the culture supernatants in DDX3 knockdown HuH-7-derived RSc cured cells at 4 days after inoculation of HCVcc (Fig. 1E to G). Thus, DDX3 seems to be required for HCV RNA replication.

Previously, DDX3 was identified as an HCV core-interacting protein by yeast two-hybrid screening. This interaction required the N-terminal domain of the core (aa 1 to 59) and the C-terminal domain of DDX3 (aa 553 to 622) (17, 19, 25). To determine whether the core can interact with DDX3 regardless of the HCV genotype, we used the HCV-O core (genotype 1b) and the JFH1 core (genotype 2a) (Table 1). We first examined their subcellular localization by confocal laser scanning microscopy as previously described (3). Consistent with previous reports (17, 19, 25), both the HCV-O core and JFH1 core mostly colocalized with DDX3 in the perinuclear region (Fig. 2A). Then we immunoprecipitated lysates from 293FT cells in which hemagglutinin (HA)-tagged DDX3 and HCV-O core, JFH1 core, or their 40-aa N-truncated mutants were overexpressed with an anti-HA antibody. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 0.5% NP-40, 10 mM NaF, 0.1 mM $Na_3VO_4$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysates were precleared with 30 μl of protein G-Sepharose (GE Healthcare Bio-Sciences). Precleared supernatants were incubated with 1 μg of anti-HA antibody (3F10; Roche) at 4°C for 1 h. Following absorption of the precipitates

TABLE 1. Primers used for construction of the HCV core-expressing plasmids[a]

| Plasmid name | Direction | Primer sequence |
|---|---|---|
| pCXbsr/core(HCV-O) | Forward | 5′-GGAATTCCCACCATGAG CACGAATCCTAAACCTC-3 |
| | Reverse | 5′-ATAAGAATGCGGCCGCC TATCAAGCGGAAGCTGG GATGGT-3′ |
| pcDNA3/core(HCV-O) | Forward | 5′-CGGGATCCAAGATGAGC ACGAATCCTAAACCTCAA AGA-3′ |
| pcDNA3/FLAG-core(HCV-O) | Reverse | 5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′ |
| pcDNA3/Δcore(HCV-O) | Forward | 5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′ |
| pcDNA3/FLAG-Δcore(HCV-O) | Reverse | 5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′ |
| pcDNA3/core(JFH1) | Forward | 5′-CGGGATCCAAGATGAGC ACAAATCCTAAACCTCAA AGA-3′ |
| pcDNA3/FLAG-core(JFH1) | Reverse | 5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′ |
| pcDNA3/Δcore(JFH1) | Forward | 5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′ |
| pcDNA3/FLAG-Δcore(JFH1) | Reverse | 5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′ |

[a] To construct pCXbsr/core(HCV-O), a DNA fragment encoding the core was amplified by PCR from pON/C-5B (13) with the indicated primers. The PCR product was digested with EcoRI-NotI and subcloned into the same site of pCX4bsr (1). To construct pcDNA3/core(HCV-O), pcDNA3/FLAG-core(HCV-O), pcDNA3/Δcore(HCV-O), and pcDNA3/FLAG-Δcore(HCV-O), DNA fragments encoding the core were amplified by PCR from pON/C-5B (13) with the indicated primer sets. To construct pcDNA3/core(JFH1), pcDNA3/FLAG-core(JFH1), pcDNA3/Δcore(JFH1), and pcDNA3/FLAG-Δcore(JFH1), DNA fragments encoding the core were amplified by PCR from pJFH1 (23) with the indicated primer sets. The PCR products were digested with BamHI and XhoI and then subcloned into the same site of pcDNA3 (Invitrogen) or pcDNA3-FLAG (2).

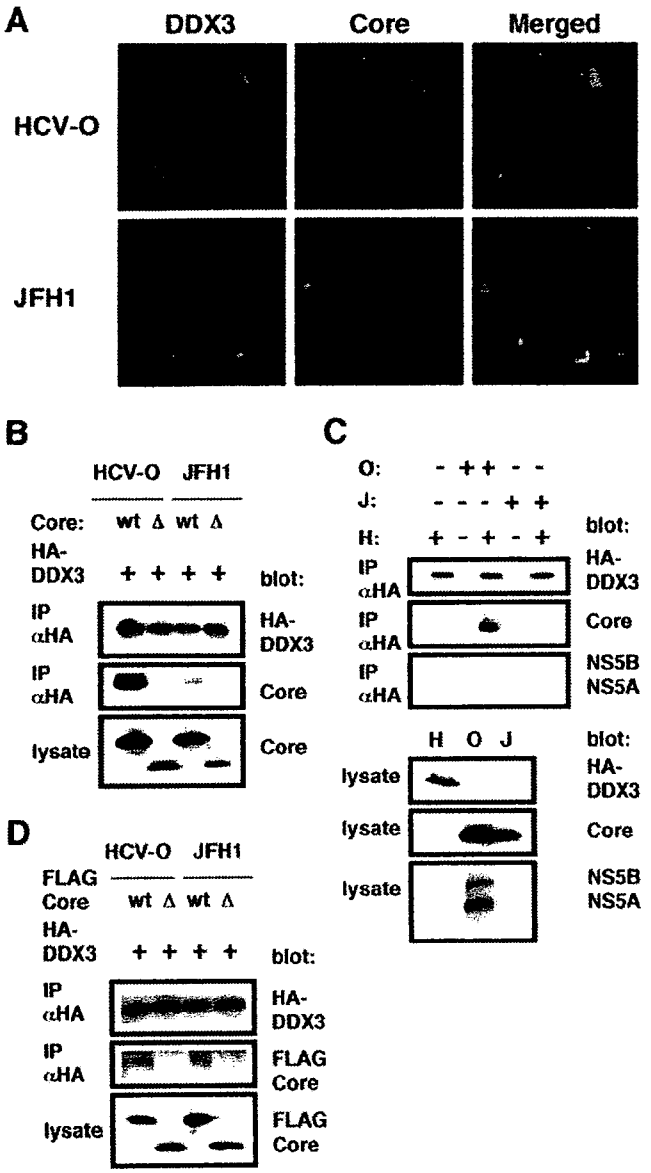


FIG. 2. Interaction of the HCV core with DDX3. (A) The HCV core colocalizes with DDX3. 293FT cells cotransfected with 100 ng of pCXbsr/core(HCV-O) or pcDNA3/core(JFH1) and 200 ng of pHA-DDX3 were examined by confocal laser scanning microscopy. Cells were stained with anti-HCV core (CP-9 and CP-11 mixture) and anti-DDX3 antibodies and were then visualized with fluorescein isothiocyanate (DDX3) or Cy3 (core). Images were visualized using confocal laser scanning microscopy (LSM510; Carl Zeiss). The right panels exhibit the two-color overlay images (Merged). Colocalization is shown in yellow. (B) The core binds to DDX3. 293FT cells were cotransfected with 4 μg of pHA-DDX3 and 4 μg of pCXbsr/core(HCV-O) (wt), pcDNA3/Δcore(HCV-O) (Δ), pcDNA3/core(JFH1) (wt), or pcDNA3/Δcore(JFH1) (Δ). The cell lysates were immunoprecipitated with an anti-HA antibody (3F10; Roche), followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). (C) 293FT cells transfected with 4 μg of pHA-DDX3 (H), O cells (O), or RSc cells 3 days after inoculation of HCVcc (JFH1) (J) were lysed and immunoprecipitated (IP) with 1 μg of anti-HA antibody (3F10), followed by immunoblotting with anti-HA (HA-7), anti-core (CP-9 and CP-11 mixture), or anti-HCV NS5A (no. 8926) and anti-HCV NS5B. (D) 293FT cells transfected with 4 μg of pHA-DDX3 and 4 μg of pcDNA3/FLAG-core(HCV-O) (wt), pcDNA3/FLAG-Δcore(HCV-O) (Δ), pcDNA3/FLAG-core(JFH1) (wt), or pcDNA3/FLAG-Δcore(JFH1) (Δ) were lysed and immunoprecipitated with 1 μg of an anti-HA antibody (3F10), followed by immunoblotting with an anti-HA (HA-7) or anti-core (CP-9 and CP-11 mixture) antibody.

on 30 μl of protein G-Sepharose resin for 1 h, the resin was washed four times with 700 μl lysis buffer. Proteins were eluted by boiling the resin for 5 min in 1× Laemmli sample buffer. The proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). We observed that the HCV-O core but not its N-truncated mutant could strongly bind to HA-tagged DDX3 (Fig. 2B). In contrast, the binding activity of the JFH1 core to HA-tagged DDX3 seemed to be fairly weak (Fig. 2B). As well, immunoprecipitation of lysates of 293FT cells expressing HA-tagged DDX3, O cells, or JFH1-infected RSc cells, or mixtures of these lysates, with an anti-HA antibody revealed that HCV-O core but not JFH1 core could bind strongly to DDX3 (Fig. 2C). The anti-HCV core antibody we used could detect both HCV-O core and JFH1 core (Fig. 2), while both anti-HCV NS5A and anti-NS5B antibodies failed to detect JFH1 NS5A and NS5B (Fig. 2C). At least, we failed to detect an interaction between DDX3 and either HCV-O NS5A or NS5B under experimental conditions that permitted the core to interact with DDX3 by immunoprecipitation (Fig. 2C). In contrast, the FLAG-tagged JFH1 core could bind to HA-tagged DDX3 just as efficiently as the FLAG-tagged HCV-O core could (Fig. 2D). Thus, the binding affinity or stability of the complex formed between the JFH1 core and DDX3 might be weaker than that between the HCV-O core and DDX3.

Since DDX3 is required for HIV-1 and HCV replication, we hypothesized that the HCV core might affect the function of HIV-1 Rev when both proteins were coexpressed. To test this hypothesis, we used the Rev-dependent luciferase-based reporter plasmid pDM628, harboring a single intron that includes both the Rev-responsive element (RRE) and the luciferase coding sequences (Fig. 3A) (10). In this system, Rev binds to RRE on the unspliced reporter mRNA, allowing its export from the nucleus for luciferase reporter gene expression, while the intron containing the luciferase gene is excised during RNA splicing when cells are transiently transfected with pDM628 alone. As previously reported (10), the luciferase activity in 293FT cells transfected with this reporter plasmid was stimulated by Rev, which induced a fourfold increase in the reporter signal (Fig. 3B). Luciferase activity was increased eightfold by the combination of Rev and DDX3, whereas neither the HCV-O core nor the JFH1 core had any effect on this Rev function (Fig. 3B). Since the Rev-binding domain (the N-terminal domain) and the core-binding domain (the C-terminal domain) do not overlap in DDX3, the HCV core might not compete with HIV-1 Rev for binding with DDX3. However, the development of a novel DDX3 inhibitor might provide a powerful antiviral agent against both HIV-1 and HCV (15).

Taking these results together, this study has shown for the first time that DDX3 is required for HCV RNA replication.

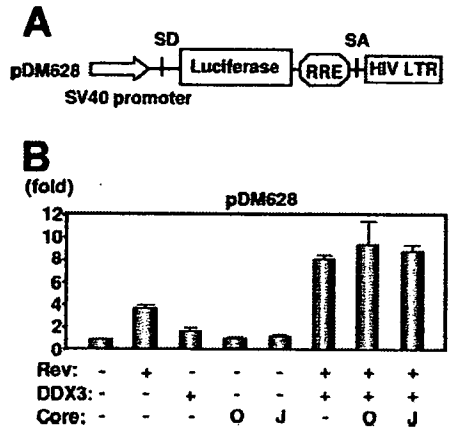


FIG. 3. HCV core does not affect the DDX3-mediated synergistic activation of Rev function. (A) Schematic representation of HIV-1 Rev-dependent luciferase-based reporter plasmid pDM628 harboring a splicing donor (SD), splicing acceptor (SA), and RRE. (B) 293FT cells were cotransfected with 100 ng of pDM628, 200 ng of pcRev, 200 ng of pHA-DDX3, and/or 100 ng of either pcDNA3/core(HCV-O) (O) or pcDNA3/core(JFH1) (J). A luciferase assay was performed 24 h later. All transfections utilized equal total amounts of plasmid DNA owing to the addition of the empty vector pcDNA3 to the transfection mixture. The relative stimulation of luciferase activity (*n*-fold) is shown. The results shown are means from three independent experiments.

Since helicases are motor enzymes that use energy derived from nucleoside triphosphate hydrolysis to unwind double-stranded nucleic acids, the DDX3–core complex might unwind the HCV double-stranded RNA and separate the RNA strands or might contribute to the function of HCV NS3 helicase. Since the replication of subgenomic replicon RNA was also partially suppressed in DDX3 knockdown cells (Fig. 1D), DDX3 might be associated with an HCV nonstructural protein(s) or HCV RNA itself. Indeed, Tingting et al. recently reported that DDX1 bound to both the HCV 3′ untranslated region (3′ UTR) and the HCV 5′ UTR and that short interfering RNA-mediated knockdown of DDX1 caused a marked reduction in the replication of subgenomic replicon RNA (22). Furthermore, Goh et al. demonstrated that DDX5/p68 associated with HCV NS5B and that depletion of endogenous DDX5 correlated with a reduction in the transcription of negative-strand HCV RNA (11). However, we failed to observe an interaction between DDX3 and NS5A or NS5B by immunoprecipitation under our experimental conditions in which the core could interact with DDX3 (Fig. 2C). Importantly, our DDX3 knockdown study demonstrated a more significant reduction in the accumulation of genome-length HCV RNA (95% reduction) than in the accumulation of subgenomic replicon RNA (52% reduction) (Fig. 1B and D). To date, it has been demonstrated that the 5′ UTR, the 3′ UTR, and the NS3-to-NS5B coding region are sufficient for HCV RNA replication (16); however, the core might be partly involved in the replication of genome-length HCV RNA. Importantly, DDX1 and DDX3 were specifically detected in the lipid droplets of core-expressing Hep39 cells by proteomic analysis (21), suggesting that DDX3 might be associated with HCV assembly or might incorporate into the HCV virion through interaction with the core to act as an RNA chaperone.

Recent studies have suggested a potential role of DDX3 and DDX5 in the pathogenesis of HCV-related liver diseases. DDX3 expression is deregulated in HCV-associated HCC (7, 8), and Huang et al. identified single-nucleotide polymorphisms in the DDX5 gene that were significantly associated with an increased risk of advanced fibrosis in patients with chronic hepatitis C (12). Interestingly, DDX3 might be a candidate tumor suppressor. DDX3 inhibits colony formation in various cell lines, including HuH-7, and up-regulates the $p21^{waf1/cip1}$ promoter (8). If DDX3 could in fact suppress tumor growth, then the core might overcome DDX3-mediated cell growth arrest and down-regulate $p21^{waf1/cip1}$ through interaction with DDX3, and it might also be involved in HCC development.

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# Comprehensive Analysis of the Effects of Ordinary Nutrients on Hepatitis C Virus RNA Replication in Cell Culture[▽]

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**To date, only a limited number of studies have reported finding an influence of ordinary nutrients on hepatitis C virus (HCV) RNA replication. However, the effects of other nutrients on HCV RNA replication remain largely unknown. We recently developed a reporter assay system for genome-length HCV RNA replication in hepatoma-derived HuH-7 cells (OR6). Here, using this OR6 assay system, we comprehensively examined 46 nutrients from four nutrient groups: vitamins, amino acids, fatty acids, and salts. We found that three nutrients—β-carotene, vitamin $D_2$, and linoleic acid—inhibited HCV RNA replication and that their combination caused additive and/or synergistic effects on HCV RNA replication. In addition, combined treatment with each of the three nutrients and interferon alpha or beta or fluvastatin inhibited HCV RNA replication in an additive manner, while combined treatment with cyclosporine synergistically inhibited HCV RNA replication. In contrast, we found that vitamin E enhanced HCV RNA replication and negated the effects of the three anti-HCV nutrients and cyclosporine but not those of interferon or fluvastatin. These results will provide useful information for the treatment of chronic hepatitis C patients who also take anti-HCV nutrients as an adjunctive therapy in combination with interferon. In conclusion, among the ordinary nutrients tested, β-carotene, vitamin $D_2$, and linoleic acid possessed anti-HCV activity in a cell culture system, and these nutrients are therefore considered to be potential candidates for enhancing the effects of interferon therapy.**

Hepatitis C virus (HCV) is a major pathogen of chronic hepatitis (CH) and leads to fatal liver diseases, such as liver cirrhosis and hepatocellular carcinoma (1, 23, 38). Approximately 170 million people worldwide are infected with HCV (40). Therefore, HCV infection is a global health problem. The combination of pegylated interferon (IFN) with ribavirin is currently the most effective therapy for CH C (11, 28), and long-term treatment has been shown to improve the sustained virological response (SVR) rate (37). However, the SVR rate still remains at approximately 55% (11). Combination therapy occasionally also causes adverse effects, such as severe anemia (10) and cerebral vascular lesions (7), and reduction of the dosage leads to insufficient treatment. These adverse effects are more serious in older patients. Therefore, it remains necessary to identify alternative agents that have fewer side effects to couple with IFN. In developing countries, it is difficult to administer expensive IFN therapy. Hence, in such countries, inexpensive anti-HCV reagents are especially desirable (36).

The lack of a small-animal model and a cell culture system to support efficient HCV RNA replication hampered the development of anti-HCV reagents. Since an HCV subgenomic replicon system was developed in 1999 (25), the mechanism of HCV RNA replication has been gradually elucidated by a number of groups (2). However, this subgenomic replicon system may not necessarily reflect actual HCV RNA replication in hepatocyte cell lines, due to the absence of structural proteins. To overcome this problem, a cell culture system for genome-length HCV RNA replication was developed by several groups (4, 15, 33). We also developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as a reporter, which facilitated the prompt and precise monitoring of HCV RNA replication in hepatocyte cell lines (13, 30). In the OR6 assay system, the luciferase activity was well correlated with HCV RNA levels when cells were treated with IFN-α (13). Therefore, we quantified the luciferase activity instead of HCV RNA for the indirect evaluation of HCV RNA replication, although the OR6 assay system doesn't strictly quantify the HCV RNA replication. More recently, several groups have developed cell culture systems that produce infectious viral particles (genotype 2a), which can be used to reconstruct the life cycle of HCV infection in hepatocyte cell lines (24, 39, 45).

Using this OR6 assay system, we demonstrated that mizoribine (30), as an immunosuppressant, and fluvastatin (FLV) (14), as the reagent for hypercholesterolemia, inhibited HCV RNA replication in hepatocyte cell lines. Another immunosuppressant, cyclosporine (CsA), was also identified as an anti-HCV reagent in a subgenomic replicon system (41). These results suggested the expansion of the primary application of the existing therapeutic drugs to new anti-HCV therapy.

Other studies have also revealed that certain ordinary nutrients contained in common foods can influence HCV RNA replication (5, 8, 9, 17, 21, 26). However, in these studies, only a limited number of nutrients were tested. To date, there has

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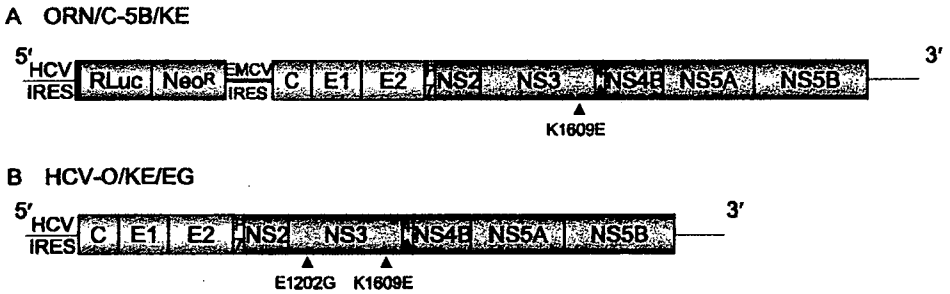


FIG. 1. Schematic gene organization of genome-length HCV RNA. (A) ORN/C-5B/KE RNA replicated in OR6 cells. RL (RLuc) is expressed as a fusion protein with neomycin phosphotransferase (NeoR). The position of an adaptive mutation, K1609E, is indicated by a black triangle. (B) Authentic HCV RNA, HCV-O/KE/EG, was introduced into the OR6c cells by electroporation as previously described (13). The positions of two adaptive mutations, E1202G and K1609E, are indicated by black triangles. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus.

been no comprehensive analysis of the effects of nutrients on HCV RNA replication. Thus, we inclusively investigated the effects of nutrients on genome-length HCV RNA replication using the OR6 assay system. Here, we report finding that three nutrients, namely, β-carotene (BC), vitamin $D_2$ (VD2), and linoleic acid (LA), inhibited HCV RNA replication and that combination treatments including each of these nutrients and CsA synergistically inhibited HCV RNA replication. Furthermore, we found that vitamin E (VE) enhanced HCV RNA replication, and that the anti-HCV activities of each of the three nutrients and CsA were abrogated by VE.

## MATERIALS AND METHODS

**Reagents.** Vitamin $B_{12}$, vitamin $K_1$ (VK1), vitamin $K_3$, elaidic acid, and vaccenic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BC, vitamin A (VA), vitamin $B_1$, vitamin $B_2$, vitamin $B_3$, vitamin $B_6$, vitamin C (VC), VD2, vitamin $D_3$ (VD3), VE, vitamin $K_2$ (VK2), pantothenic acid, biotin, folic acid, inositol, leucine, isoleucine, valine, tryptophan, phenylalanine, tyrosine, lauric acid, palmitic acid, stearic acid, behenic acid, oleic acid, elaidic acid, LA, arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), $Fe(II)SO_4$, $Na_2SeO_4$, $Fe(III)(NO_3)_3$, $ZnCl_2$, NaCl, KCl, $CaCl_2$, $PCl_3$, $MgCl_2$, $CuCl_2$, $MnCl_2$, and IFN-α were purchased from Sigma (St. Louis, MO), and CsA and FLV were purchased from Calbiochem (Los Angeles, CA). IFN-β was kindly provided by Toray Industries, Inc. Fatty acids, except for LA-albumin and oleic acid-albumin compounds (Sigma), were compounded with fatty acid-free bovine serum albumin (Sigma) as described in a previous report (6). We treated the HCV RNA-harboring cells at various concentrations of salts [$Fe(II)SO_4$ at 5, 25, and 50 μM, $Fe(III)(NO_3)_3$ at 10, 100, and 200 μM, $ZnCl_2$ at 20, 50, and 100 μM, $Na_2SeO_4$ at 1, 2.5, and 5 μM, NaCl at 100, 150, and 300 μM, KCl at 5, 10, and 20 μM, $CaCl_2$ at 2, 4, and 8 μM, $PCl_3$ at 1, 2.5, and 5 μM, $MgCl_2$ at 0.5, 2.5, and 5 μM, $CuCl_2$ at 20, 50, and 100 μM, and $MnCl_2$ at 30, 60, and 120 μM]. Dimethyl sulfoxide or ethanol was used for the dissolution of the liposoluble nutrients, and the final concentrations (0.1%) of each were equal for the cells with nutrients and those without.

**Cell cultures.** OR6 cells, polyclonal genome-length HCV RNA (ORN/C-5B/KE)-replicating cells, and subgenomic replicon (ORN/3-5B/KE) cells, which were derived from hepatoma cell line, HuH-7, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin (designated as the control medium in this study), and G418 (300 μg/ml) (Geneticin; Invitrogen, Carlsbad, CA), and the cells were passaged twice a week at a 5:1 split ratio. ORN/C-5B/KE and ORN/3-5B/KE are derived from HCV-O (strain O of genotype 1b). OR6c cells are cured OR6 cells from which

TABLE 1. Summary of anti-HCV activities of various nutrients

| Nutrient type | Nutrient(s) with the indicated characteristic for HCV[a] | | |
|---|---|---|---|
| | Inhibitory | Enhancing | Ineffective |
| Vitamins | | | |
| Liposoluble | BC, VD2 | VA (retinol), VE, VK1, VK2, VK3 | VD3 |
| Water soluble | | VC | *VB1*, *VB2*, *VB3* (*niacin*), *VB6*, VB12, *pantothenic acid*, biotin, *folic acid*, *inositol* |
| Amino acids | | | |
| Branched-chain | | | *Leucine*, *isoleucine*, *valine* |
| Aromatic | | *Tryptophan* | *Phenylalanine*, *tyrosine* |
| Fatty acids | | | |
| Saturated | | | Lauric acid ($C_{12}$), palmitic acid ($C_{16}$), stearic acid ($C_{18}$), behenic acid ($C_{22}$) |
| Mono-unsaturated | | | Oleic acid ($C_{18}$; 9-unsaturated), elaidic acid ($C_{18}$; *trans*-form of oleic acid), vaccenic acid ($C_{18}$; 11-unsaturated) |
| Polyunsaturated | LA ($C_{18:2}$, n-6), AA ($C_{20:4}$, n-6), EPA ($C_{20:5}$, n-3), DHA ($C_{22:6}$, n-3) | | |
| Salts | $Fe(II)SO_4$, *$Fe(III)(NO_3)_3$*, $ZnCl_2$ | $Na_2SeO_4$ | *NaCl*, *KCl*, *$CaCl_2$*, *$PCl_3$*, *$MgCl_2$*, $CuCl_2$, $MnCl_2$ |

[a] Nutrients already contained in the medium are indicated in italics. VB1, vitamin $B_1$; VB2, vitamin $B_2$; VB3, vitamin $B_3$; VB6, vitamin $B_6$; VB12, vitamin $B_{12}$; VK3, vitamin $K_3$.