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Supplementary material

The following supplementary material is available online:

Fig. S1. NS3-4A blocked the Cardif-mediated signaling pathway, but not the TRIF-mediated signaling pathway in HeLa cells.

Fig. S2. NS3-4A is capable of cleaving Cardif, but not TRIF in HeLa cells.

Table S1. Quantitative RT-PCR analysis of mRNA expression of several factors involving in innate immune response in the various cell lines.

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Forum Minireview

Life Style-Related Diseases of the Digestive System: Cell Culture System for the Screening of Anti-Hepatitis C Virus (HCV) Reagents: Suppression of HCV Replication by Statins and Synergistic Action With Interferon

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Abstract. Hepatitis C virus (HCV) infection causes chronic hepatitis and leads to liver fibrosis and hepatocellular carcinoma. Pegylated-interferon and ribavirin is the current standard therapy for chronic hepatitis C. However, the therapy is only effective in 50% of the patients. To overcome this problem, we recently developed the HCV cell culture system (OR6 system) for the screening of anti-HCV reagents. In this OR6 system, the luciferase gene was introduced into the upstream portion of the HCV genome to facilitate the monitoring of HCV RNA replication. Recently lipid metabolism is reported to be involved in HCV RNA replication. Cholesterol and sphingolipid are the major components in lipid rafts, which seem to be the scaffold for HCV RNA replication. Statins inhibit cholesterol biosynthesis and also have the pleiotropic effects by the inhibition of prenylation. We demonstrated different anti-HCV effects of statins (atorvastatin, simvastatin, fluvastatin, lovastatin, and pitavastatin) using the OR6 system. Surprisingly, in contrast to the other statins, pravastatin exhibited no anti-HCV effect. Furthermore, statins enhanced the anti-HCV effect of interferon in combination. Statins may be a promising candidate for the adjuvant in interferon therapy and may improve the efficiency of the current interferon and ribavirin therapy.

Keywords: life style-related disease, hepatitis C virus (HCV), statin, interferon, cell culture system

Introduction

Approximately 170 million people worldwide are infected with the hepatitis C virus (HCV). HCV infection causes chronic hepatitis C (CH-C) and leads to liver-related death by liver cirrhosis and/or hepatocellular carcinoma. To prevent the progress of fatal liver disease after HCV infection, the elimination of the virus seems to be the most effective strategy. However, the current pegylated-interferon (PEG-IFN) and ribavirin therapy was only effective in 50% of the patients (1). Therefore, the development of more effective anti-HCV reagents is an urgent concern. When HCV replicates in hepatocytes, some of the cellular factors are essential for HCV RNA replication. These cellular factors are the targets for antiviral as well as viral proteins such as NS3 protease or NS5B RNA-dependent RNA polymerase. Inhibition of cellular factors may cause side effects by the inhibition of their primary roles. However, one of the advantages of this strategy is that it could overcome the viral mutation leading to the resistance to the reagent against the viral proteins. Lipid metabolism is one of the candidates in the context of this strategy. To explore the best partner of IFN, we examined different six statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, using our recently developed OR6 system (2). In the OR6 system, genome-length HCV RNAs (HCV-O strain of genotype 1b) replicate efficiently and the HCV RNA level can be monitored by luciferase activities (3, 4). Statins exhibited various anti-HCV activities except for pravastatin that was not active against HCV (2). We also
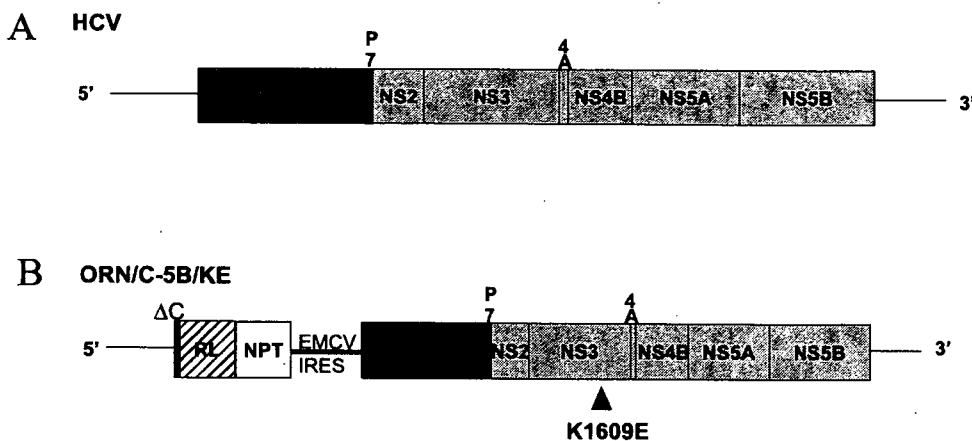
Fig. 1. HCV RNA with reporter gene. Schematic gene organization of genome-length HCV RNA. A: The authentic HCV RNA was composed of the N-terminal part of the structural region and C-terminal part of the nonstructural region. B: The genome-length HCV RNA with reporter gene was constructed based on the authentic HCV RNA. EMCV IRES was introduced for the translation of HCV proteins. Renilla luciferase was expressed as a fusion protein with NPT. The position of the adaptive mutation, K1609E, is indicated by a black triangle.

investigated whether or not statins could enhance the inhibitory effect of IFN on HCV RNA replication. In this review, we would like to summarize our recent findings and the literature regarding lipid metabolism as the target of anti-HCV with a focus on statins.

Cell culture system for HCV RNA replication

Cell culture systems for HCV have been developed since the first breakthrough of the establishment of the subgenomic replicon by Lohmann et al. (5). The replicon system has provided the information concerning the mechanism of the replication machinery of HCV and has revealed the cellular factors essential for HCV RNA replication. After the development of the subgenomic replicon, genome-length HCV RNA replication systems using different HCV strains (H, N, Con1, and O) were developed by several groups since the subgenomic replicon did not possess the structural region in the genome (4, 6 – 8). For the screening of anti-HCV reagents, the replicon system has also been improved by the introduction of reporter genes (9). The introduction of the reporter gene into the HCV genome facilitated the monitoring of HCV RNA replication. For this purpose, we developed a cell culture system (OR6 system) in which genome-length HCV RNA containing renilla luciferase (RL) replicate efficiently under the selection by G418 (4). As shown in Fig. 1, RL, neomycin phosphotransferase (NPT), and encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) genes were introduced between the 5' untranslated region and Core (C) of HCV. This genome-length HCV RNA robustly replicated in the hepatoma cell line HuH-7 after the electroporation and one of the colonies designated OR6 was selected by G418 and used for the studies including determining the anti-HCV effect of statins. A recent milestone was the development of an HCV infection system using a genotype 2a HCV strain, JFH-1

(10 – 12). This system could reconstruct the HCV life cycle in cell culture. The future issue of the cell culture system is the development of a robust genotype 1 HCV virus production system because the efficiency of PEG-IFN and ribavirin therapy in patients with genotype 1 HCV remained lower than that in patients with genotype 2 HCV: the sustained virological responses were approximately 50% versus 80% – 90%, respectively (13). More recently, pioneering studies have been reported by several groups using genotype 1 HCV strains for virus production (14, 15). However, the genotype 1 HCV virus production systems could not allow re-infection with the supernatant from the HCV-infected cells. These ongoing studies will lead to the development of a robust genotype 1 HCV infection system like genotype 2a HCV in the near future.

HCV and lipid metabolism

Lipid metabolism is involved in the life cycle of many viruses. The resulting metabolites work as physiologically active molecules such as eicosanoids and so on, and some of them are incorporated into the lipid raft membrane. A lipid raft is distinct from other lipid membranes. It is enriched in cholesterols and sphingolipids and is detergent-resistant. Lipid rafts play an important role in virus entry, replication, and assembly. HCV also forms a replication complex on the lipid raft membrane structure (16). Therefore, the depletion of the cholesterol and sphingolipid from the lipid raft leads to the inhibition of HCV RNA replication. Aizaki et al. (17) reported that lovastatin inhibited HCV RNA replication in HCV replicon-harboring cells. Statins are inhibitors for HMG-CoA reductase in the cholesterol biosynthesis pathway (Fig. 2). Statins also possess the cholesterol-independent action (pleiotropic effect) (18). Many of these pleiotropic effects are mediated by the isoprenoid. Farnesyl pyrophosphate (FPP) and gera-

Acetyl-CoA + Acetoacetyl-CoA

HMG-CoA synthase ↓

HMG-CoA reductase   HMG-CoA

↓ ├——— Statins

Mevalonate

↓

Isopentenyl-PP

↓ \

Geranyl-PP   GGPP→ Geranylgeranylation

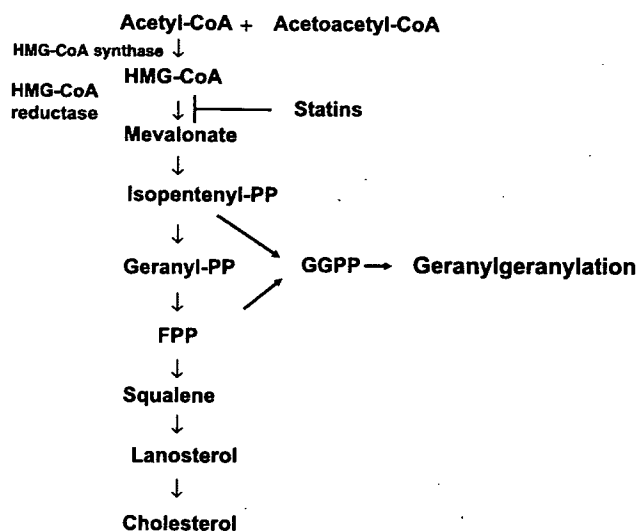↓ /

FPP

↓

Squalene

↓

Lanosterol

↓

Cholesterol

Fig. 2. Cholesterol biosynthesis pathway and statins. In the cholesterol biosynthesis pathway, the production of mevalonate by HMG-CoA reductase is the rate-limiting step. Statins inhibit HMG-CoA reductase, resulting in the inhibition of the production of isoprenoids as well as cholesterol. Geranyl-PP: geranylpyrophosphate and GGPP: geranylgeranylpyrophosphate.

nylgeranyl pyrophosphate (GGPP) are mevalonate-derived isoprenoids (Fig. 2). The attachment of iso-prenoid to the cellular proteins is called prenylation. Prenylation regulates a variety of cellular functions, including growth, differentiation, and oncogenesis. From the aspect of the pleiotropic effect of the statins, Wang et al. (19) recently identified FBL2 as geranyl-geranylated cellular protein required for HCV RNA replication. FBL2 belongs to the FBL family of proteins, all of which contain an F box and a multiple leucine-rich repeat. These two possible inhibitory mechanisms are proposed for the anti-HCV effect of statins. The low-density lipoprotein receptor (LDLR) is reported as one of the potential HCV receptors (20). However, the pre-cise role of LDLR for HCV is still controversial (21). It will be worth trying to examine the effect of statins in the JFH-1 infection system since statins enhance the expression of LDLR.

Sphingolipid is another major component of lipid rafts and thereby is also the 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. Sakamoto et al. (22) and Umehara et al. (23) reported that myriocin, a selective inhibitor of SPT, inhibited the HCV RNA replication in replicon-harboring cells and in HCV-infected chimeric mice with humanized livers, respectively. These results further support the significance of lipid metabolism in HCV RNA replication.

Other than cholesterol and sphingolipid biosynthesis, fatty acids are reported to be metabolites that affect HCV RNA replication. Leu et al. (24) reported that polyunsaturated fatty acids (PUFAs) possessed an anti-HCV effect using HCV-replicon harboring cells. Arachidonic acid, docosahexaenoic acid, linoleic acid, and eicosapentaenoic acid belonging to PUFAs pos-sessed anti-HCV activity. On the other hand, saturated fatty acids enhanced HCV RNA replication. The precise mechanisms of fatty acids regarding HCV RNA replica-tion have remained unclear. Very recently, we examined the effect of ordinary nutrients on HCV RNA replication using the OR6 system (25). Interestingly, we found that vitamin E negated the anti-HCV effect of linoleic acid (25). Given that linoleic acid and vitamin E are an oxidant and antioxidant, respectively, oxidative stress may be involved in HCV RNA replication. Further study in this field will provide clues for developing anti-HCV reagents.

Different anti-HCV effects of statins

Statins are one of the most worldwide used reagents for the treatment of hypercholesterolemia and they are beneficial in the prevention of coronary heart disease. In the cholesterol biosynthesis pathway, the production of mevalonate by HMG-CoA reductase is the rate-limiting step. Statins inhibit mevalonate synthesis by inhibiting HMG-CoA reductase, resulting in decreased production of isoprenoids as well as cholesterol. The activities of some cellular proteins are regulated by the attachment of isoprenoids (prenylation). For example, statins inhibited the function of small G proteins, Ras and Rho. Ras and Rho are major substrates for prenylation with FPP and GGPP, respectively. So far, among the statins, lovastatin is the only one with a well-characterized inhibitory effect against HCV RNA replication in cell culture (17, 26, 27). Recently, FBL2 was identified as one of the of geranylgeranylated cellular proteins required for HCV RNA replication (19). Geranylgeranyrated FBL2 binds to NS5A of HCV and the resulting complex is required for HCV RNA replication (19).

The anti-HCV effect of the statins other than lovastatin remains to be clarified. Therefore, we used the OR6 system to test anti-HCV effect of five statins: lovastatin, simvastatin, atorvastatin, fluvastatin, and pravastatin (2). More recently, we also added pitava-statin to this list, so that finally six statins were tested for their effects on HCV RNA replication. None of the statins exhibited cytotoxicity at the concentrations tested. The 50% effective concentrations (EC_{50}) of statins are summarized in Table 1. The anti-HCV effects of simvastatin, atorvastatin, fluvastatin, and pitavastatin

Table 1. EC$_{50}$ of statins on HCV RNA replication

Statins	EC$_{50}$ (μM)
Lovastatin	2.16
Simvastatin	1.57
Atorvastatin	1.39
Fluvastatin	0.90
Pitavastatin	0.45

were stronger than that previously reported for lovastatin. The EC$_{50}$ of lovastatin, simvastatin, atorvastatin, fluvastatin, and pitavastatin were 2.16, 1.57, 1.39, 0.90, and 0.45 μM, respectively. Pitavastatin possessed the strongest anti-HCV activity among the statins tested and its EC$_{90}$ was calculated as 1.25 μM (Fig. 3A). In contrast, pravastatin exhibited no anti-HCV effect. Pravastatin is the only hydrophilic statin among the statins tested and does not cross the cellular membrane passively. It has been reported that a human liver-specific organic anion transporter, LST-1, mediates the uptake of pravastatin in human hepatocytes (28). Therefore, we examined the expression levels of LST-1 in OR6 cells. OR6 cells expressed the mRNA of LST-1 at levels equivalent to that in normal human liver (2). We ruled out the possibility that pravastatin didn't actually work as the inhibitor for HMG-CoA reductase in the cells. We confirmed that pravastatin induced HMG-CoA reductase by a positive feedback mechanism in response to the

decrease of cholesterol by the inhibition of HMG-CoA reductase by pravastatin (2). These results suggest that there may be another mechanism underlying the depletion of GGPP and cholesterol by statins. One of the clues for resolving this puzzle is that pravastatin has a different effect on P450 induction compared with the other statins (29). However, further study will be needed to clarify this issue.

Statins in combination with IFN

The combination therapy of PEG-IFN and ribavirin is a current standard therapy for patients with CH-C. Ribavirin by itself possessed no anti-HCV effect for the patients. However, ribavirin alone exhibited an anti-HCV effect in the OR6 cell culture system when it was used at a concentration higher than that in the serum of patients undergoing ribavirin treatment. The EC$_{50}$ of ribavirin is calculated as 76 μM in the OR6 system and this is approximately 5 – 7 times higher concentration than that in serum from the patients with ribavirin treatment (3). Furthermore, the synergistic effect of ribavirin at the low concentration with IFN was also confirmed in different cell culture systems, including the OR6 system (3, 30, 31). These results suggest that ribavirin works as a kind of the adjuvant for IFN at the low concentration.

To test the effect of statins in combination with IFN-α on HCV RNA replication, we treated the OR6 cells with
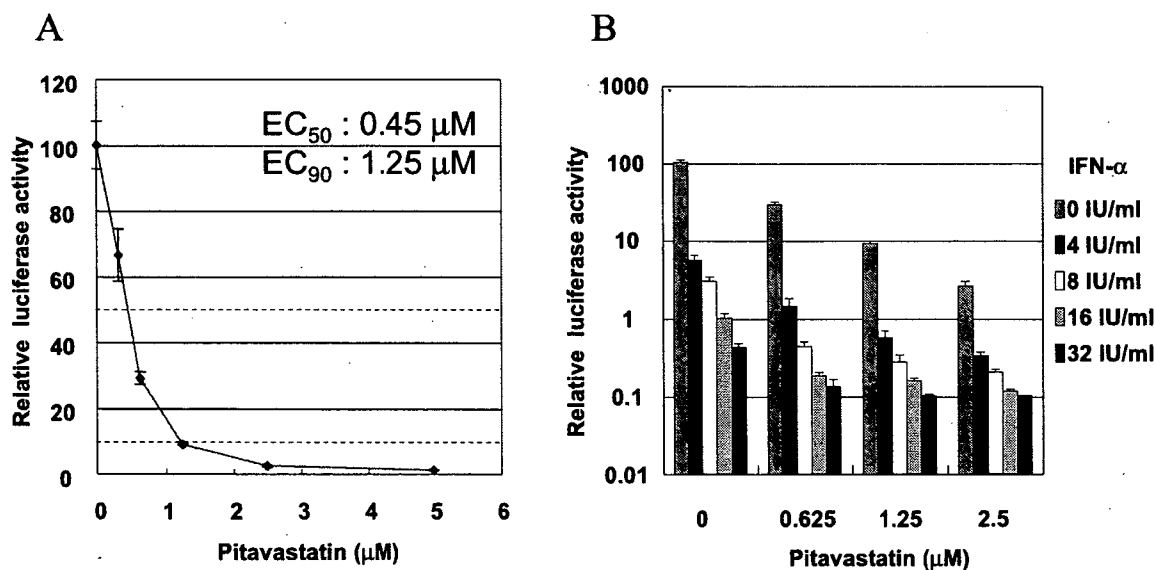

Fig. 3. Anti-HCV effect of pitavastatin in combination with IFN-α. A: OR6 cells were treated with pitavastatin at concentrations of 0, 0.625, 1.25, 2.5, and 5 μM for 72 h. The EC$_{50}$ and EC$_{90}$ were calculated from the result. Shown here is the relative luciferase activity (%) calculated when the luciferase activity of untreated cells was assigned as 100%. B: The effect of pitavastatin in combination with IFN-α. OR6 cells were treated with pitavastatin (0, 0.625, 1.25, and 2.5 μM) and IFN-α (0, 4, 8, 16, and 32 IU/ml) for 72 h. The relative luciferase activity was calculated as shown above.

pitavastatin (0, 0.625, 1.25, and 2.0 μM) and IFN-α (0, 4, 8, 16, and 32 IU/ml) (Fig. 3B). Pitavastatin enhanced the anti-HCV effect of IFN-α in a dose-dependent manner for a fixed concentration of IFN-α, 0, 4, 8, 16, or 32 IU/ml (Fig. 3B). Furthermore, we observed the decrease of luciferase activity to almost the background level in the OR6 reporter assay when OR6 cells were co-treated with 32 IU/ml of IFN-α and pitavastatin at the concentration of 1.25 or 2.5 μM (Fig. 3B). The concentrations of the statins tested in the cell culture were higher than that in the sera from patients with statin administration. However, the statins may enhance the anti-HCV effect of IFN for patients with CH-C at a lower concentration than the EC_{50} in cell culture. Recently O'Leary et al. (32) reported that the mono-therapy of atorvastatin does not exhibit anti-HCV activity in a pilot clinical trial. Although the mono-therapy of statin seems to be insufficient for patients with CH-C, statin may be a candidate for the adjuvant of IFN therapy like ribavirin.

Conclusions

The OR6 system was developed for the precise and quantitative assay of HCV RNA replication in cell culture. The statins were compared for their anti-HCV effects using the OR6 system and were found to possess different effects on HCV RNA replication. Lovastatin, simvastatin, atorvastatin, fluvastatin, and pitavastatin had different anti-HCV profiles in cell culture. However, pravastatin had no anti-HCV effect, although it worked as inhibitor for HMG-CoA reductase. Pitavastatin exhibited the strongest anti-HCV effect (EC_{50}: 0.45 μM) among the statins tested and enhanced the effect of IFN-α. It may be difficult to achieve the cell culture based EC_{50} of statins in patients with CH-C. However, statins at lower concentration than the EC_{50} in cell culture may enhance the anti-HCV effect of IFN-α in patients with CH-C. Therefore, statins may be suitable as an adjuvant of IFN-α like ribavirin rather than for monotherapy. Lipid metabolism including cholesterol, sphingolipid, and fatty acid biosynthesis seems to be an attractive field for the development of antiviral reagents for HCV.

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Modulation of host metabolism as a target of new antivirals [☆]

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Abstract

The therapy for chronic hepatitis C (CH–C) started with interferon (IFN) monotherapy in the early 1990s and this therapy was considered effective in about 10% of cases. The present standard therapy of pegylated IFN with ribavirin achieves a sustained virologic response in about 50% of patients. However, about half of the CH–C patients are still at risk of fatal liver cirrhosis and hepatocellular carcinoma. The other significant event in hepatitis C virus (HCV) research has been the development of a cell culture system. The subgenomic replicon system enables robust HCV RNA replication in hepatoma cells. And recently, the complete life cycle of HCV has been achieved using a genotype 2a strain, JFH1. These hallmarks have provided much information about the mechanisms of HCV replication, including information on the host molecules required for the replication. Anti-HCV reagents targeting HCV proteins have been developed, and some of them are now in clinical trials. However, the RNA-dependent RNA polymerase frequently causes mutations in the HCV genome, which lead to the emergence of drug-resistant HCV mutants. Some of the cellular proteins essential for HCV RNA replication have already been discovered using the HCV cell culture system. These host molecules are also candidate targets for antivirals. Here, we describe the recent progress regarding the anti-HCV reagents targeting host metabolism.

Contents

1. Introduction

Hepatitis C virus (HCV) was discovered in 1989 [1] as the causative agent of chronic hepatitis C (CH–C), liver cirrhosis and hepatocellular carcinoma (HCC) [2]. It is estimated that 170 million people worldwide are infected with HCV [3]. The ultimate goal of both clinical and basic HCV studies is the suppression of liver-related death caused by HCV infection. With respect to clinical studies, interferon (IFN) has played a major role in the treatment of patients with CH–C. IFN therapy started with IFN monotherapy in the early 1990s, and a sustained virologic response (SVR) was obtained in about 10% of patients [4]. IFN therapy was developed by the hepatologists, and the current therapy of pegylated IFN (PEG-IFN) with ribavirin has improved the SVR to about 50% [4]. Therefore, the next stage of the therapy for CH–C is to develop new anti-HCV reagents to improve the SVR.

During the development of IFN therapy, the most striking discovery in the basic research was the development of a cell culture system for robust HCV RNA replication. In 1999, Lohmann et al. [5] achieved subgenomic HCV RNA replication in a human hepatoma cell line, HuH-7. The advantages of this novel system (known as the replicon system) were that it provided not only a way to screen for anti-HCV reagents but also information about the mechanism of HCV RNA replication. This cell culture system has been further improved, and recently the complete life cycle of HCV was achieved using a genotype 2a HCV strain, JFH1 [6–8]. This newest system has extended the targets of the anti-HCV therapy to the virus infection and release.

The effects of anti-HCV reagents selected from the cell culture-based screening should be evaluated using an animal model system for HCV infection before they can be released to clinical trial. Chimpanzees were the only animal model in the early HCV studies [9]. However, the use of chimpanzees is limited for ethical and financial reasons. In addition to chimpanzees, a study using tree shrews (Tupaia belangeri chinensis) has been reported [10]. A different approach to the study of HCV using animal models was achieved using the related GB virus B (GBV-B). GBV-B belongs to the Flaviviridae family and can be transmitted to tamarins and marmosets

[11,12]. These animal models may be valuable surrogate models for HCV study. Another approach was demonstrated in a study using urokinase plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice transplanted with human hepatocytes [13]. This chimeric mouse model can support chronic HCV viremia under the circumstance without immune system. Mass screening for anti-HCV reagents using cell culture systems will become a more powerful tool when combined with small animal model systems to evaluate the antiviral effects of selected reagents before clinical trial.

In considering a new strategy for CH–C to be used in place of or in combination with IFN, the main targets are HCV proteins and HCV RNA. With respect to the HCV proteins, two of these, nonstructural (NS) 3-4A and NS5B, have been well-characterized as protease and RNA-dependent RNA polymerase (RdRp), respectively [14,15]. Several reagents have been reported to be inhibitors of NS3-4A serine protease, including SCH6 [16,17], SCH503034 [18], VX-950 [19,20], and BILN-2061 [21]. Valopicitabine (NM283) was reported to inhibit NS5B RdRp [22]. HCV RNA itself is also a target of antivirals, and recent RNA interference technologies using siRNA or shRNA have targeted HCV RNA [23–25]. As RdRp lacks proofreading activity, the high mutation rate of RdRp allows the virus to escape from the reagents targeting HCV proteins and HCV RNA. These anti-HCV reagent-targeting viral proteins and genome will be reviewed in another section.

Other targets are the cellular proteins essential for HCV RNA replication and infection. The expression of HCV proteins is thought to affect the host cells' gene expression profiles and vise versa [26]. The interaction of the specific cellular proteins with HCV proteins is essential for HCV replication (Table 1). Cyclosporine A (CsA) is one of the best characterized inhibitors targeting the cellular proteins required for HCV replication [27–36]. The interaction of cyclophilin B (CyPB) with NS5B is required for HCV RNA replication [28]. CsA inhibits HCV RNA replication by interrupting the interaction between NS5B and CyPB. Heat shock protein 90 (HSP90) has also been reported to be an essential cellular protein for HCV RNA replication [37–39]. Knockdown or inhibition of HSP90 has been shown to result in the anti-HCV activity in cell culture and in uPA-SCID mouse systems [37].

Table 1
Host molecules as targets of anti-HCV

Target molecules	Reagents	References
HMG–CoA reductase	Statin	[68–71]
Serine palmitoyltransferase	NA255	[81]
	Myriocin	[82]
IMP dehydrogenase	VX-497	[98]
	Ribavirin	[74,86]
	Mizoribin	[74]
	MPA	[97]
Protein arginin methyltransferase	AdoMet, Betaine	[102]
α-Glucosidase	Deoxynojirimycin	[101]
Cyclophilins	CsA	[27–36]
	NIM811	[27,33]
	DEBIO-025	[34]
HSP90	Geldanamycin, Radicicol	[37–39]
FKBP8		[38]
Unknown	PUFAs	[70,103,108]

FKBP8, a member of the FK506-binding protein family, specifically interacts with NS5A and forms a complex with HSP90 [38]. The La autoantigen (La) and polypyrimidine tract-binding protein (PTB) are also candidate cellular proteins for the inhibition of HCV RNA replication [40], although no inhibitors for these proteins have been reported to date. Thus, inhibition of the metabolism has recently been reported as a target of the new antivirals. Here, we survey the recent progress on enzyme inhibitors of the cholesterol, sphingolipid, and guanosine triphosphate (GTP) synthesis pathways, as well as other metabolic pathways.

2. Current standard therapy for chronic hepatitis C

HCV was discovered to be the causative agent of non-A, non-B hepatitis by the Chiron Corporation in 1989 [1]. However, a treatment for patients with non-A, non-B hepatitis was established before the discovery of HCV. In 1986, Hoofnagle et al. reported that IFN-α treatment normalized the serum alanine aminotransferase (ALT) levels in patients with non-A, non-B hepatitis [41]. Since the initial discovery of its anti-HCV activity, IFN-α has become the major reagent for CH–C treatment [4]. The replication of HCV RNA itself seems to stimulate IFN production signaling, and our recent results have suggested that core and/or NS5B induce IFN-stimulated genes [42–44]. However, viral NS3-4A protease inhibits the IFN production, although it does not completely shut it off. Therefore, exogenous IFN administration is needed for patients with CH–C. The SVR is affected by multiple factors, such as genotype, viral load and duration of therapy. IFN-α mono-therapy was begun in the early 1990s, but an SVR was achieved in only about 10% of patients. In the early 2000s, IFN-α and ribavirin combination therapy was developed and the SVR was improved to about 30-40%. Furthermore, IFN itself has been modified by the attachment of PEG, thereby enhancing its stability in the blood. The SVR of the current standard therapy by PEG-IFN and ribavirin is as high as 50% [4]. In the current PEG-IFN and ribavirin combination therapy, the genotype of HCV is one of the major determinants of the

SVR. HCV genotypes are classified into 6 groups, and genotype 1 is currently considered a problem due to its IFN resistance [45]. For example, in genotype 1 HCV, 12 months of treatment resulted in an SVR in 50% of patients, while in genotype 2, 6 months of treatment achieved an SVR of 80-90% [46]. The precise mechanisms of the IFN resistance remain unclear. However, the recently developed IFN-resistant HCV replicon-harboring cells will be useful for studies examining ways to improve the SVR [47–49]. Therefore, the focus in the treatment of patients with CH–C has shifted to increasing the SVR in genotype 1 HCV.

3. Cell culture-based HCV RNA-replication system

Before the development of an HCV replicon system, screening of anti-HCV reagents was rather difficult. The HCV replicon system developed by Lohmann et al. [5] was the first milestone in HCV study using a cell culture system. The replicon system has provided a wealth of information concerning the replication machinery of HCV. We can make strategies for the Achilles' heel of HCV based on the information regarding HCV RNA repli-cation. The HCV replicon has been improved to be a suitable system for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase [50]. However, this system does not contain a structural region. Therefore, selectable genome-length HCV RNA-replicating cell culture systems have been developed [51–54]. The second milestone was the infectious virus production system established by the three groups using a genotype 2a HCV strain, JFH1 [6–8]. This system has extended the range of the HCV study to the viral entry and release. Therefore, the life cycle of HCV in the cells has been reconstructed in vitro. Since the development of the HCV replicon and infectious HCV production systems, many cellular proteins have been identified as essential host molecules for HCV RNA replication.

3.1. From HCV replicon to infectious HCV production

The HCV replicon reported by Lohmann et al. contained neomycin phosphotransferase (Neo) and encephalomyocarditis virus (EMCV) internal ribosome entry sites (IRES) instead of the HCV structural regions (Fig. 1) [5]. This HCV replicon consists of 2 cistrons. In the first cistron, Neo is translated by HCV-IRES and in the second cistron NS3-NS5B is translated by EMCV-IRES introduced in the region upstream of the NS region (Fig. 1). After the development of the HCV replicon system [52,53,55–58], genome-length HCV RNA replication systems using different HCV strains (H, N, Con1, and O) were developed by several groups [51–54]. In these genome-length HCV RNA replication systems, a complete open reading frame (ORF) of HCV was introduced into the second cistron instead of the NS region (Fig. 1).

For the mass screening for anti-HCV reagents, evaluation of the levels of HCV RNA or HCV proteins requires time and complicated procedures. To facilitate the monitoring of the replication level of HCV RNA, the reporter gene (Renilla luciferase) was fused to the Neo gene. In this system, anti-HCV activity was evaluated by the value of the reporter instead of the

HCV

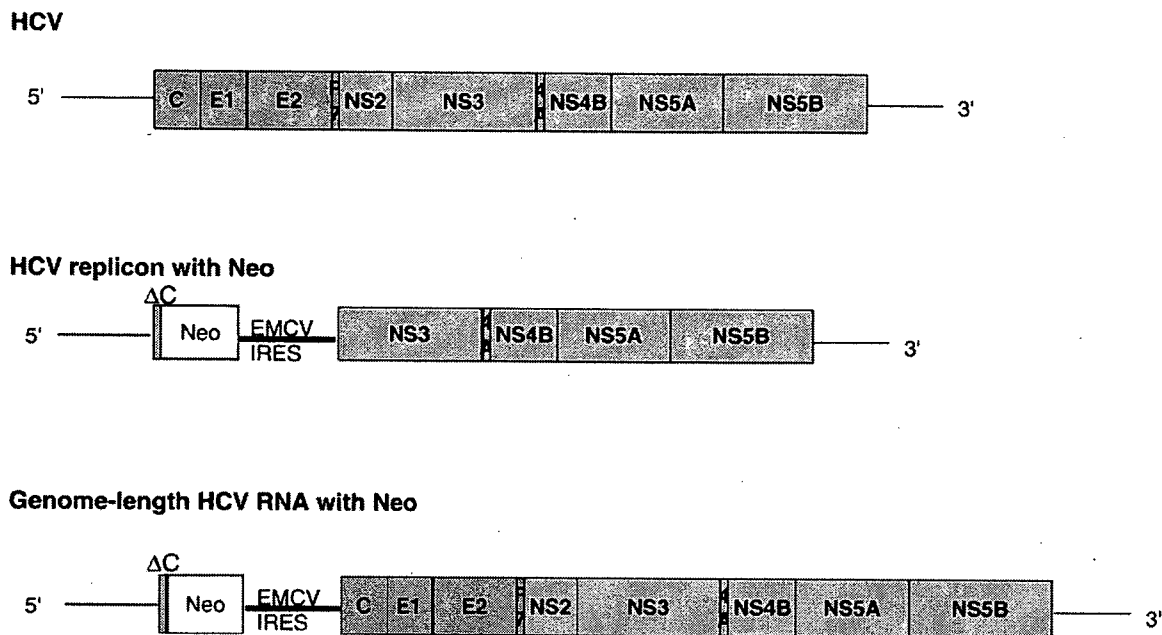**HCV replicon with Neo**

Genome-length HCV RNA with Neo

Fig. 1. Gene organizations of HCV and selectable HCVs. HCV ORF, untranslated regions, EMCV IRES, and Neo are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES.

quantification of HCV RNA or HCV proteins. As shown in Fig. 2A, ORN/C-5B/KE contains the fused Renilla luciferase and Neo genes in the first cistron [51]. One of the cloned cell lines, OR6, was established by the G418 selection after introduction of ORN/C-5B/KE RNA into HuH-7 cells. HCV

RNA and HCV proteins were stably expressed in the OR6 cells, and the Renilla luciferase activity was correlated well with the level of HCV RNA [51]. Therefore, the antiviral effect of the reagents on HCV RNA replication could be monitored by the activity of Renilla luciferase. The OR6 assay system
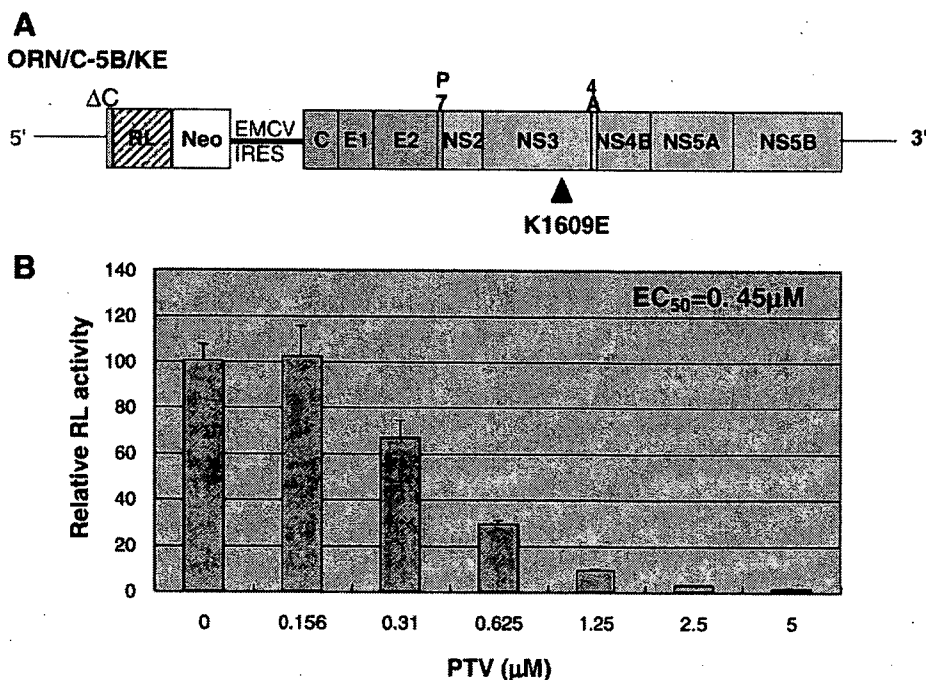
Fig. 2. Inhibitory effect of statin on HCV RNA replication in OR6 cells. (A) Schematic gene organization of genome-length HCV RNA (ORN/C-5B/KE) derived from genotype 1b, strain O. The Renilla luciferase gene, which is symbolized as RL, is depicted as a striped box and is expressed as a fusion protein with Neo. The adaptive mutation from lysine (K) to glutamine (E) at amino acids position 1609 was previously reported [51] and introduced into the genome-length HCV RNA. (B) Inhibition of HCV RNA by PTV. OR6 cells were cloned cell line selected by G418 [51]. OR6 cells were treated with PTV at a concentration of 0, 0.156, 0.31, 0.625, 1.25, 2.5, or 5 μM. After 72 hours of treatment the RL activities were determined. Shown here is the relative RL activity (%) calculated when the RL activity of untreated cells was assigned as 100%. The data indicate the means±standard deviation from three independent experiments. The EC_{50} of PTV was determined as 0.45 μM.

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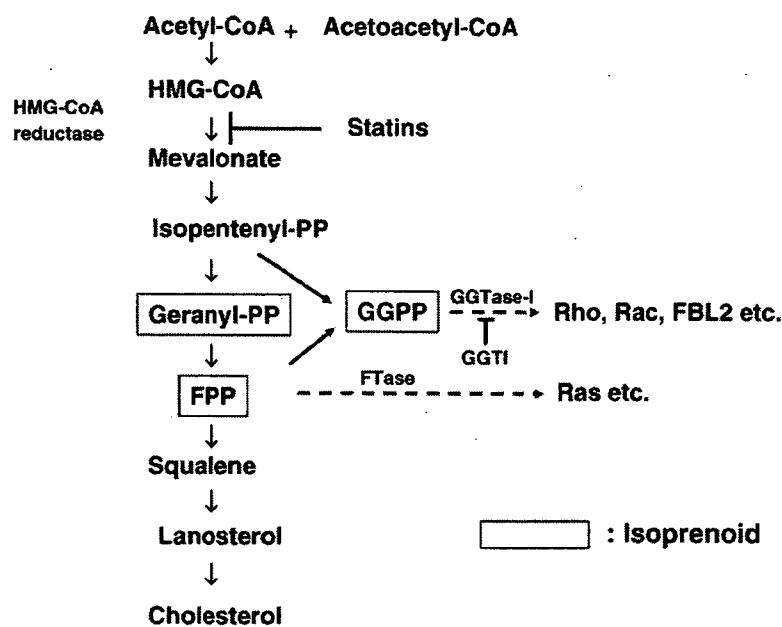
Fig. 3. Cholesterol-biosynthesis pathway. The inhibition of HMG–CoA reductase by statins leads to the suppression of mevalonate and of the production of its downstream metabolites. Decreased prenylation on the GTP-binding proteins had a significant effect on the signal transduction.

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

Plamitoyl-CoA + L-Serine

SPT ↓ |——— Myriocin, NA255

3-Ketodihydrosphingosine

↓

Dihydrosphingosine

↓ |——— Fumonisin B1

Dihydroceramide

↓

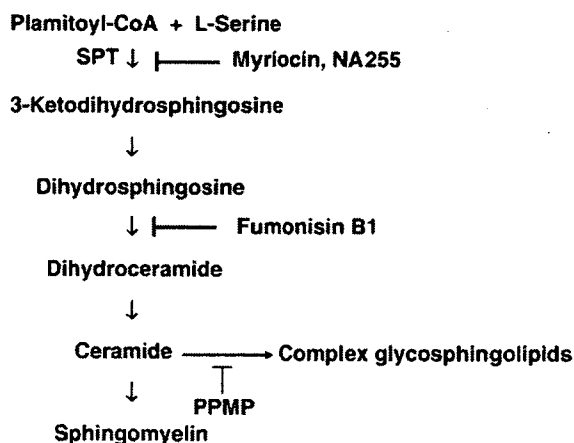**Ceramide** ———→ Complex glycosphingolipids

↓ ⊤ PPMP

Sphingomyelin

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].

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.

Ribose-5P
↓
↓
↓
IMP **Ribavirin**
IMPDH ↓ |—— **Mizoribine**
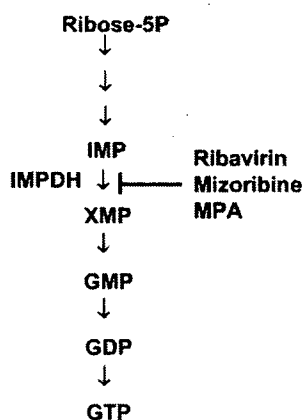**XMP** **MPA**
↓
GMP
↓
GDP
↓
GTP

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

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]
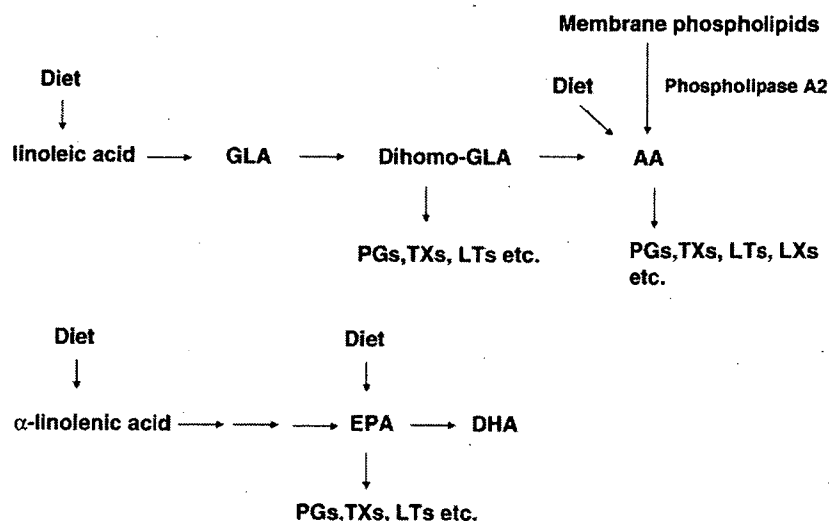

Fig. 6. Fatty acid-biosynthesis pathway. The PUFA metabolism from diet or membrane phospholipids.

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.

Acknowledgements

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